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Grape and Wine Research and Development Corporation

Better quality wine and lower production costs from new processing technologies for protein-haze removal

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Chief Investigator: Dr Chris Colby

Research Organisation: Chemical Engineering Department
The University of Adelaide

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1 Abstract

Investigations were performed into issues relevant to improving the performance of bentonite fining in the Australian wine industry. Alternative (non-bentonite) technologies, e.g. combined heat/enzyme treatment, to prevent protein haze in white wines were also considered. Several key findings were as follows. Commercial bentonites vary widely in performance, in particular, the volume of wine occluded in lees. Selection decisions should be based on potential wine loss, not price. Bentonite performance could be improved by optimising bentonite slurry preparation. In-line dosing combined with centrifugal clarification is a feasible alternative to batch-wise addition, which could virtually eliminate wine and value losses.

2 Executive summary

Batch-wise addition of bentonite, which non-selectively adsorbs wine protein, is a method universally used by the Australian wine industry [and worldwide] for protein haze prevention. However, up to 10 % of wine can be occluded in bentonites lees. This results in significant wine and value losses, estimated at up to $45 million per annum for the Australian wine industry. Other problems involved with bentonite fining include; long settling [or processing times]; perceptions of reduced wine quality that may arise from stripping of colour and flavour compounds; manual handling requirements; and the increasing environmental cost for disposal of bentonite waste. Because of these issues, the Australian wine industry would welcome a more cost effective, environmentally friendly, better performing haze prevention technology that could overcome some or all of these disadvantages.

The objectives of this project were to reduce costs of wine production and enhance wine quality and value for the Australian wine industry by finding solutions to current disadvantages of bentonite use for haze prevention:

(i) Make current forms of bentonite more efficient and effective, and hence less costly, for batch-wise addition, by understanding and improving its adsorption and settling characteristics.

(ii) Develop and demonstrate more economical methods of utilising bentonite, better suited to large-scale commercial application, by using continuous addition / contacting [as opposed to batch-wise] systems.

(iii) Establish the commercial and engineering feasibility of “non-bentonite” haze-prevention technologies as alternatives to bentonite for haze prevention.

A diverse range of investigations were undertaken in conjunction with the Australian Wine Research Institute and industrial partner, Hardy Wine Company. These included; experimental and desk-top investigations of commercial bentonites to evaluate and improve their performance characteristics; field testing an alternative method of using bentonite: in-line dosing with centrifugal clarification; and investigating non-bentonite technologies such as combined heat/enzyme treatment and alternative adsorption media.

The major outcomes from the project are summarised below.

- The project produced valuable data and knowledge regarding the properties and performance of different commercial bentonites. This information will allow industry to make better decisions when selecting bentonites to improve fining efficiency and reduce wine losses.
- It was found that the most practical approach to bentonite slurry preparation is to use pure water and vigorous mixing. Furthermore, bentonite performance may be

improved by re-suspending bentonite lees in wine after they have settled. This could further reduce the bentonite dose necessary to achieve stability, and thus, decrease wine losses.

- The most important factors affecting protein adsorption during bentonite fining appear to be slurry age, wine pH and potassium concentration.
- It was found that some heat treated bentonites can reduce the volume of lees three-fold but a higher dose rate may be required.
- In-line dosing of bentonite combined with centrifugal clarification can reduce contact-time requirements to < 3 min; decrease wine losses by up to 80 %; and potentially avoid subsequent lees processing by rotary drum vacuum filtration.
- The project has developed an alternative HPLC method for quantifying protein content in wine and identifying those proteins responsible for haze formation.
- The project has verified that combined/heat enzyme treatment has potential as an alternative technology to bentonite fining.

Whilst the work in this project was wide-ranging there are many areas and issues that deserve further attention:

- Current industry decision making practices often appear to be based on bentonite price not on wine loss, which is the key cost driver in bentonite fining.
- Bentonite suppliers should be encouraged by the wine industry to develop improved forms of bentonite with performance characteristics that optimise bentonite fining.
- The industry should develop best practice methods for preparation of bentonite slurry to ensure optimal fining performance.
- Several industrial case studies should be undertaken to further evaluate in-line dosing of bentonite with centrifugal clarification. This should that include calculation of wine losses and cost savings.
- Further research should investigate non-bentonite haze prevention alternatives. This includes enzyme addition with or without heat treatment.
- A more convenient method for measuring the heat stability of juice and wine would be invaluable for the industry.

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3 Background

3.1 Introduction

An essential processing step for white wine production is prevention of protein haze or "heat instability". Protein haze is caused by the slow denaturing of wine proteins, which can occur before or after bottling and results in the accumulation of unattractive suspensions and precipitates. Currently, batch-wise addition of bentonite, which non-selectively adsorbs wine protein, is used universally by the Australian wine industry [and worldwide] for haze prevention.

The current costs of haze prevention by bentonite fining to the Australian wine industry are estimated at up to $45 million per annum (Høj PB et al. 2000). This cost estimate is based on the loss or diminished value of wine occluded in bentonite lees. However, other problems involved with bentonite fining include; long settling [or processing times]; perceptions of reduced wine quality that may arise from stripping of colour and flavour compounds; manual handling requirements; and the increasing environmental cost for disposal of bentonite waste.

It is widely accepted that, because of these issues, the Australian wine industry would welcome a more cost effective, environmentally friendly, better performing haze prevention technology that could overcome some or all of these disadvantages.

Recent wine science research by The Australian Wine Research Institute (AWRI) has been invaluable in identifying the haze-forming proteins and defining their characteristics. This has revealed some potentially promising non-bentonite methods for haze prevention, but these have not been commercially evaluated. Current methods of bentonite use are also mostly based on relatively outdated historical practices, and thus, there is considerable scope for implementing new and more efficient ways to use bentonite.

In 2002 the School of Chemical Engineering at The University of Adelaide initiated a PhD project in conjunction with Hardy Wine Company to investigate new processing technologies for protein-haze removal. The project was undertaken in collaboration with AWRI. Following a thorough literature review by the PhD student, significant gaps were discovered in the industry's current knowledge of bentonite fining and heat stabilisation of wines. These gaps in knowledge are summarised briefly below and are divided into two sections. The first addresses current knowledge regarding existing methods of bentonite use, and potential ideas for finding new and more efficient methods of utilising bentonite in the same way. The second section considers completely new technologies for protein haze prevention that could be employed to replace bentonite use in the wine industry.

3.2 Existing & New Methods of Bentonite Use

The adsorption of wine proteins by bentonite is a cation-exchange process (Boulton et al. 1996). The cation-exchange [or adsorption] sites are negative charges located on exposed surfaces of alumino-silicate mineral sheets that make up a bentonite particle (van Olphen 1963). Each bentonite particle contains tens, if not hundreds, of these individual sheets. In aqueous media, bentonite particles swell as water hydrates and separates the sheets (Bradley & Grim 1948). It is widely believed that swelling opens gaps between the sheets that wine proteins may diffuse into. Therefore, swelling can potentially increase surface area available for wine protein adsorption (Boulton et al. 1996). Sodium [exchanged] bentonites swell more than calcium [exchanged] bentonites. The sheets separate completely and have gaps measured in hundreds of angstroms [Å] (Rodriguez et al. 1977). The gaps between sheets in calcium [exchanged] bentonites are much smaller, in the order of 10 Å (Boulton et al. 1996). A typical wine protein has a diameter estimated at about 50 Å. Previously, it has been assumed wine proteins access and adsorb to internal surfaces inside sodium bentonite particles, while they only adsorb to external surfaces of calcium bentonite particles (Boulton et al. 1996). Intuitively, this would imply that the adsorption capacity of a sodium bentonite should be many times that of a calcium bentonite [when internal surface area is compared with external surface area]. Consequently, sodium bentonites are usually favoured over calcium bentonites, even though increased swelling produces poorer settling characteristics and more lees (Rankine 1989).

However, experimental measurements suggest that the protein adsorption capacity of sodium and calcium bentonites is actually very similar. Typically, sodium bentonites only have twice the adsorption capacity of calcium bentonites (Boulton et al. 1996). There may be several plausible explanations for this. One example is that not all intra-particle surface area in sodium bentonites is available for protein adsorption. Another reason could be that adsorption of wine proteins on external surface areas blocks deeper penetration further into a particle.

Presently, no published research exists that validates where wine proteins adsorb on bentonite particles. This knowledge could decide which types of commercial bentonite are better for haze prevention. It may also reveal opportunities for improving bentonite performance. For example, grinding to roughen bentonite particles could be used to increase external surface areas. It may be possible to modify commercial bentonites to make available or increase intra-particle surface areas for protein adsorption. Non-wine-industry-related research has discovered that swelling properties of bentonite particles were diminished following heat treatment (Grim 1968). Whilst the same research also indicated that heat treatment reduced total ion-exchange capacity of bentonites with mono-valent cations (i.e. Na+), protein adsorption capacity might remain unaffected if adsorption only

occurs on external surface areas. Reducing the swelling of bentonite particles in this way could make them more rigid, denser and faster settling. It may be possible to fuse bentonite particles together. The goal could be to create macro-particles, retaining high adsorption capacity, but with improved settling properties. [Similar approaches elsewhere lead to development of perfusion chromatography resins, now widely used in other industries.]

A more rigid particle and/or larger macro-particle might make bentonite more suitable for a continuous contacting process. A continuous process has the potential to reduce wine losses, decrease waste lees, and markedly lower the processing time compared to batch-wise addition. Recovery, regeneration and reuse of bentonite might also be possible, virtually eliminating waste lees. The idea of continuous processes has been previously proposed but do not appear to have been seriously considered because of bentonite's unsuitable properties (i.e. small size, compressibility) (Boulton et al. 1996). Examples of possible configurations include in-line dosing with a centrifuge, or a packed or fluidised bed. In these systems contact times [between bentonite and wine] would be significantly reduced, in the order of seconds to minutes. These contact times correspond more closely to the time required for protein adsorption that has been observed during batch-wise addition of bentonite (Boulton et al. 1996, Lee 1985). To accurately design a continuous system, knowledge of wine protein mass transfer rates, diffusion coefficients and adsorption equilibrium for bentonite particles would need to known. Currently, there is no published research providing this information. Mass transfer rates and diffusion coefficients may be estimated using pre-existing correlations in conjunction with relatively standard chemical engineering experiments. [The methods are identical to those used elsewhere (e.g. modelling protein adsorption in packed beds of chromatographic resins).] Protein adsorption equilibrium on bentonite has been previously studied, but only with model proteins, not actual wine proteins. This previous work should be repeated using wine proteins in real wine environments (i.e. alcohol present). AWRI has recently developed techniques which would be used for recovery and purification of haze-forming wine proteins from grape juice, which could be used for this purpose.

Several studies have also investigated regeneration of bentonite under batch conditions (Churchman 1999, Armstrong 1964). Regenerating agents included salts and alkalis. A single batch experiment was always performed. Protein desorption was observed but concluded to be ineffective because complete desorption could not be achieved in a single batch-wise step. In a continuous-type process this problem would not eventuate: the bentonite would subject to the equivalent of multiple batch-wise steps, which would continue to progressively desorb protein until none was left. Protein desorption during regeneration may be measured using the same methods mentioned earlier for protein adsorption. Using this information, the behaviour and performance of continuous contacting systems could be

predicted by process engineering techniques involving mathematical modelling and computer simulation.

3.3 Alternative Protein Removal Technologies

Alternative techniques have been investigated for the removal of proteins from wine, but with limited success to date. They include: ultrafiltration; proteolytic enzymes, flash pasteurisation; and adsorption by immobilised phenolic compounds.

Ultrafiltration could be a suitable bentonite substitute for protein stabilisation. Stability of some wines has been achieved by ultrafiltration with membranes of 10 to 30 kDa cut-off (Hsu & Heatherbell 1987c). Where stability was not obtained, ultrafiltration enabled a substantial reduction in bentonite addition. However, residual protein in filtrate, reportedly high costs, and possible loss of organoleptic compounds has made ultrafiltration unattractive (Høj et al. 2000).

Batch addition of proteolytic enzymes has proven ineffective under normal winemaking temperatures (Waters EJ et al. 1992). Many wine proteins originate in the grape berry and are believed to be pathogenesis related: produced as a defence against fungal pathogens – consequently they are inherently proteolytically resistant (Høj et al. 2000).

Flash pasteurisation may be used to cause precipitation of wine proteins, so they may be removed during wine processing (Ferenczy 1966). Historically, heating of wines has been previously assumed too detrimental for wine quality (Tattersall et al. 2001). However, short periods of heating at 90ºC have been found to reduce bentonite requirements by 50% to 70% without affecting sensory profile of wines (Francis et al. 1994). Short periods of heating have also been combined with addition of proteolytic enzymes (Pocock et al. 2002). Enzyme addition produced similar results at lower heating temperatures. Further investigation could enable optimisation of this process and eliminate the need for subsequent addition of bentonite.

Adsorption of proteins by phenolics immobilised on agarose chromatography resins has been used (Powers et al. 1988) to successfully to stabilise wines. However, protein-binding capacity was lost during regeneration. This problem currently prevents commercial applications. With additional study this might be overcome.

Other protein separation processes used elsewhere include affinity chromatography and ion-exchange. Affinity chromatography is an established industrial process. It would use biological molecules [the ligand] with natural, bio-specific interactions for wine protein to adsorb and remove them from wine (Asenjo & Chaudhuri 1996). Recently, AWRI has discovered compounds: Haze Protective Factors (discussed below), that may be suitable as the ligand for an affinity chromatography process.

Ion-exchange would work in the same way as bentonites: adsorption by cation exchange. Ion-exchange has been used successfully for protein purification in the food and

pharmaceutical industries. It reportedly compares favourably with other protein-removal technologies in terms of cost and efficiency (Deshpande 1995), although such comparisons did not include bentonite. From an oenological perspective, ion exchange could also be employed to prevent potassium bitartrate precipitation, adjust wine pH, as well as remove protein. However, ion-exchange is currently not an accepted oenological process under the Agreement between Australia and the European Community on Trade in Wine (Commonwealth of Australia 1994). Therefore it cannot presently be used for wine exported to member countries of the European Community. The use of ion-exchange is being re-negotiated by Australia.

AWRI has recently discovered that certain natural polysaccharide fractions isolated from wines, when added back to wines [in higher concentrations], will reduce haze formation. These polysaccharide-rich fractions are referred to as Haze Protective Factors (HPF) (Waters et al. 1993, Waters et al. 1994). They reduce haze by interacting with proteins and decreasing the particle size of the haze, rather than preventing protein precipitation. However, methods for cost-effective manufacture and industrial application of HPF are yet to be considered.

3.4 GWRDC Project

To address knowledge gaps and investigate alternative methods/technologies for protein haze prevention in the Australian wine industry an application for GWRDC funding was made. This application was successful and funding was provided for a 3 year project commencing July 2003. The funding included provision to

- Allow the existing PhD student, Dr Richard Muhlack, to continue investigations of new processing technologies for protein-haze removal but to focus on more efficient methods of using bentonite;
- Appoint a new PhD student (for 3 years) to consider alternative technologies to bentonite fining for protein-haze removal; and
- Appoint a research engineer (or postdoctoral researcher) in Year 2 of the project (for 2 years) to assist the PhD students to, and/or undertake, field testing of new technologies for protein-haze removal.

4 Project Aims and Performance targets

4.1 Project objectives

The objectives of this project, as stated in the GWRDC Application, were as follows.

To reduce costs of wine production and enhance wine quality and value for the Australian wine industry by finding solutions to current disadvantages of bentonite use for haze prevention:

(i) Make current forms of bentonite more efficient and effective, and hence less costly, for batch-wise addition, by understanding and improving its adsorption and settling characteristics.

(ii) Develop and demonstrate more economical methods of utilising bentonite, better suited to large-scale commercial application, by using continuous addition / contacting [as opposed to batch-wise] systems.

(iii) Establish the commercial and engineering feasibility of "non-bentonite" haze-prevention technologies as alternatives to bentonite for haze prevention.

4.2 Performance targets

The performance targets for the project, as given in the GWDRC project application, were as follows.

Table 4.1 GWRDC Project Application outputs and performance targets

Outputs	***Performance Targets***
2003-2004	
Information on the absorption properties of commercially available bentonites and modified bentonites. [A prerequisite to establishing new bentonite technologies for haze prevention.]	• ***December 2003:*** *Development of at least one modified and enhanced form of bentonite(s).* • ***June: 2004:*** *Characterisation of the physical and protein absorption properties of at least two different types of commercially available bentonite and at least one experimentally modified bentonite.*
2004-2005	
Information on the likely commercial feasibility of a least two new haze prevention technologies.	• ***August 2004:*** *Completion of feasibility analysis of different haze-prevention (including non-bentonite) technologies* • ***January 2005:*** *Construction of bench-scale units for at least two different haze-prevention technologies.* • ***June 2005:*** *Completion and analysis of bench-scale trials.*
2005-2006	
Information, from field trials in a commercial setting, on the performance of new technologies to prevent haze formation in wine and their likely adoption by the wine industry.	• ***August 2005:*** *Selection of no more than two preferred technologies for field evaluation.* • ***November 2005:*** *Design and construction of pilot plant(s).* • ***April 2006:*** *Completion of field trials.* • ***April 2006:*** *Completion of data analysis.* • ***June 2006:*** *Completion of report on business case for technology transfer and commercialisation.*

4.3 Summary of R&D Program

Consistent with the project objectives (see section 4.1) the project was carried out in three stages as follows.

4.3.1 Stage I: Characterisation of bentonite properties

This stage involved laboratory investigations at the School of Chemical Engineering and AWRI. The investigations were designed to better understand

- Protein adsorption by bentonite, and
- Bentonite swelling and settling characteristics during fining applications.

The majority of this work was undertaken by the original PhD student, Dr Richard Muhlack, and/or the Research Engineer, Mr Simon Nordestgaard, appointed in Year 2 of the project, under the direction of the Chief Investigator, Dr Chris Colby, who was also Dr Muhlack's Principal Supervisor.

The outcomes from this work are documented in three sections of this report:

- Section 5: Characterization of natural and heat-modified bentonites
- Section 6: Factors affecting bentonite performance
- Section 7: Bentonite preparation

In addition, an alternative HPLC method for quantifying heat unstable proteins in wine was developed and evaluated as part of this stage. This work is reported in

- Section 11: Improved quantification of wine proteins by HPLC

4.3.2 Stage II: New methods of bentonite use

This stage included both laboratory investigations and field testing:

- Laboratory investigations were conducted to determine if heat treatment could improve the properties of bentonite to make it more suitable for industrial application.
- Process simulation and field testing was performed to evaluate in-line dosing of bentonite, a continuous contacting paradigm, for improving the efficiency of bentonite fining.
- Desk-top studies were undertaken to consider and interpret current knowledge and research and how it might be exploited improve the efficiency of bentonite fining by industry.

Again the majority of this work was undertaken by the original PhD student and/or the Research Engineer for the project under the direction of the Chief Investigator. The outcomes from this work are included within four sections of this report:

- Section 5: Characterization of natural and heat-modified bentonites
- Section 8: In-line dosing of bentonite: Contact-time requirement?
- Section 9: In-line dosing of bentonite: Field testing
- Section 10: In-line dosing: Multi-component adsorption
- Section 12: Bentonite fining: Can we improve efficiency and decrease wine losses?

4.3.3 Stage III: Non-bentonite technologies

This stage of the project was initially undertaken by the newly appointed second PhD student, Ms Felicity Lloyd, under the direction of the Chief Investigator, Dr Chris Colby, who was also her Principal Supervisor, and Associate Professor Elizabeth Waters from AWRI.

At the commencement of this stage it was decided that the early focus should be to evaluate combined enzyme/heat treatment for protein-haze removal. This decision was made because

- Laboratory studies by AWRI already appeared to have successfully demonstrated proof-of-concept, thus there was a superior probability that successful commercialisation could be achieved; and
- The technological concepts and processing equipment involved were already being used by, or were familiar to, the industry, hence this would enable rapid commercialisation to occur once optimal design and operating parameters were identified (assuming processing costs were deemed favourable).

Unfortunately, the PhD student elected to discontinue her studies after twelve months. As a consequence, the only work undertaken was a preliminary study to verify earlier research by AWRI and compare performance of combined enzyme/heat treatment with that of bentonite fining. The outcomes of this preliminary study are reported in

- Section 13: Combined protein/enzyme treatment

The loss of the PhD student did not allow further significant research of non-bentonite technologies during the project. However, some additional work was subsequently performed in conjunction with Professor Peter Majewski at the Ian Wark Research Institute. Professor Majewski had developed SAM-coated sand particles which were believed to be suitable for adsorption of wine proteins. The particles were provided to the GWRDC project team for evaluation. The outcomes of this work are contained in

- Section 14: SAM coated nanoparticles

The GWRDC project team also contributed to research by AWRI to investigate the use of Haze Protective Factors as an alternative or adjunct to bentonite fining. This contribution

included provision of a PhD student from chemical engineering and supervision of this student. The outcomes from this work are not included in this report as this was a distinct project undertaken separately to the GWRDC project by AWRI.

4.4 Overview of project achievements

The principal goal of the project was to achieve the project objectives and performance targets presented in Sections 4.1 and 4.2. As can be observed from Section 4.3, which documents the R&D program actually undertaken, these project objectives and performance targets were fulfilled except that

- There was some slippage in timelines in achieving performance targets arising from a delay in commencing the project. This delay was caused by an initial six-month hold-up in execution of the Research Agreement. Thus, the Research Agreement between the GWRDC and the Research Agencies was not executed until January 2004. Hence, funding was not available and the project did not commence until this time.
- The loss of the second PhD student did not permit the completion of studies to investigate non-bentonite haze-prevention technologies.

The project achievements can be summarised as follows.

- The project produced new data and knowledge regarding the properties and performance of different commercial bentonites. This will allow industry to make better decisions when selecting bentonites to improve fining efficiency and reduce wine losses (Section 5).
- The project has provided new data and knowledge that will allow industry to identify the optimal conditions for bentonite slurry preparation (Section 7).
- The project has provided a better understanding of what factors affect protein adsorption during bentonite fining (Sections 5 and 6).
- The project developed an alternative method for purification of gram quantities of heat unstable proteins from juice for R&D applications (Section 6).
- The project has identified that modification of commercial bentonites by heat treatment has the potential to improve the performance of bentonite fining by reducing the volume of lees and achieving more rapid settling (Section 5).
- The project has demonstrated in-line dosing of bentonite as a more efficient method for bentonite fining, which can reduce contact-time requirements, decrease wine losses and potentially avoid subsequent lees process by rotary drum vacuum filtration (Section 9).

- The project has demonstrated that mathematical modelling tools used widely by other industries can also be applied for process simulation, analysis and optimisation of wine production operations (Sections 5, 6, 8 and 10).
- The project has developed an alternative HPLC method for quantifying protein content in wine and identifying those proteins responsible for haze formation (Section 11).
- The project has verified that combined/heat enzyme treatment has potential as an alternative technology to bentonite fining (Section 12).

4.5 Note on report presentation & content

The majority of the project work was undertaken by the two PhD students and/or research engineer under the direction of the Chief Investigator. Most of the project work has already been (or will be published), either in Dr Muhlack's PhD thesis or in journal or conference publications (refer Appendix 1: Communication). In this respect, these documents have formed the sources for the majority of information presented in this report, which has been prepared by the Chief Investigator. Where relevant, the Chief Investigator has included additional analysis, interpretation and comments.

As can be observed from Section 4.3, the amount of work was significant and the project activities were diverse. As a consequence, the presentation of the work in this report does not necessarily reflect the actual chronological sequence of project activities. To present all of the work in a sensible and rational manner it was necessary to combine and/or separate components of various project activities. Thus, each section of the report has been designed to be stand-alone so it can be read, interpreted and understood as a self-contained discourse of research. Many of these demarcations were decided prior to preparation of the report to aid publication or earlier project reporting. Hence, the organisation and content of sections in the report also closely reflects that of various project documents that were the primary sources of information.

5 Characterization of Natural and Heat-Modified Bentonites

5.1 Introduction

In this part of the project, a comparison of the performance of different bentonites was undertaken. In particular, the relationship between the physical and chemical properties, and adsorption performance and settling behaviour was examined. At the same time, the effect of heat treatment on the protein adsorption performance and settling behaviour of bentonites was also investigated. The adsorption experiments were performed using a model white wine containing bovine serum albumin (BSA) as a surrogate for heat unstable proteins.

5.2 Materials and Methods

5.2.1 Bentonite preparation

Six natural bentonites were used for this work: Volclay KWK (Volclay Pty Ltd, North Geelong, Australia), Vitiben (BPM Minerals, Denver, USA), Microcol CL-G (Laffort Œnologie, Burgundy, France), SiHA-Aktiv-Bentonit G ("SiHA Aktiv") (E. Begerow GmbH & Co., Langenlonsheim, Germany), Nacalit (ERBSLÖH Geisenheim Getränketechnologie GmbH & Co. KG, Germany) and Arumpo (size fraction < 1mm) (Arumpo Bentonite Pty Ltd, Australia). Twenty four heat-modified bentonites were prepared as follows: samples of Vitiben and Microcol CL-G bentonites (approximately 200 g and 170 g, respectively) were each heated to 200^{o}C, 300^{o}C, 400^{o}C and 500^{o}C for 3, 6, and 12 hours (24 samples in total) in a high temperature furnace (Model AFT1 ELP, Carbolite Co. Ltd., Sheffield, England), and then cooled down to room temperature.

Bentonite slurries were prepared by dispersing 8 g of bentonite into approximately 180 mL of Nanopure® (Apple Scientific, Chesterland, Ohio) water at room temperature and stirring for 24 hours to allow for hydration to take place. The slurry volume was brought up to 200 mL with Nanopure® water, to produce a final slurry concentration of 4 % w/v.

5.2.2 pH and electical conductivity

The pH and electrical conductivity (EC) of 1:50 w/v slurries of each of the bentonites was measured using an Orion model 520A pH meter, and Orion model 180 conductivity meter with Orion model 018014 conductivity probe (K = 0.097), respectively.

5.2.3 Particle size analysis

Particle size analysis of each of the 24 hour old bentonite slurries was performed by laser light diffraction using a Microtrac S3500 (64 channels) over a range of 0.0215-1408 μm with Nanopure® water as the carrier fluid. Measurements were conducted at room temperature with a run time of 10 s and final values were averaged over 10 runs. The particle shape and particle refractive index instrument settings were “Irregular” and 1.54, respectively.

5.2.4 Exchangeable cations and cation exchange capacity

The extraction of the exchangeable cations was performed by shaking 20 mg of the clay with 11 mL of 1 M NH_4Cl solution in a centrifuge tube for 24 hours, followed by centrifugation and collection of the supernatant solution (10 mL). 10 mL of 1 M NH_4Cl solution was then added to the centrifuge tube and the bentonite resuspended. The centrifuge tube was shaken for 2 hours before centrifugation and collection of the supernatant solution (10 mL). 10 mL of 1M NH_4Cl solution was again added to the centrifuge tube, which was shaken for 2 hours before centrifugation and collection of the supernatant solution (10 mL). These extractions were combined and the cations (Na, K, Mg, Al, Ca, Cr, Mn, Fe, Sr, Se and Ba) displaced by NH_4Cl were measured using an Inductively Coupled Plasma Mass Spectrometer (ICPMS) (Agilent 7500 series, Waldbronn, Germany). The total negative charge (or CEC) of the clays was taken to be the sum of the charges on cations displaced by NH_4Cl.

5.2.5 Protein adsorption analysis

5.2.5.1 White wine model solution

The model wine consisted of the following components: Nanopure® water; 11.5% (v/v) ethanol, 1.0 g/L potassium hydrogen tartrate (KHT), 650 mg/L potassium (by KHT and KCl addition), 72 mg/L sodium (by NaCl addition) and 78 mg/L calcium (by $CaCl_2$ addition). The pH of the model wine was adjusted to 3.2 by addition of tartaric acid. The level of each of these factors is typical of Australian white wine (Gishen and Coulter, 2003), and provides the required conditions for the protein adsorption process by bentonite, namely pH, dielectric constant and ionic strength (Sarmento *et al.*, 2000). Bovine serum albumin (BSA) was used as the protein in this model wine rather than an authentic wine protein (such as VVTL1), due the prohibitively large quantity of protein (in excess of 7 g) which would otherwise need to be purified from grape juice or wine. BSA is a commercially available protein with an isoelectric point of 4.3 - 4.6 (Sarmento *et al.*, 2000) which is in the range of most wine proteins. A protein concentration of 200 mg/L was used, which is typical of the

wine protein concentration of Australian white wines such as Sauvignon Blanc and Muscat of Alexandria (Peng *et al.,* 1997; Pocock *et al.,* 2000, 2003).

5.2.5.2 Adsorption Tests

Aliquots (ranging from 0.1 – 8 mL) of the bentonite slurry were added to 40 mL of model wine solution in a polypropylene sample tube. All tubes were well mixed and then placed on an orbital shaker table for 2 hours. After this, 1 mL aliquots from each sample tube were centrifuged at 14000 rpm for 5 minutes. Following centrifugation, 800 μL of supernatant was drawn from each micro-centrifuge tube and placed into a glass HPLC vial for analysis.

5.2.5.3 Protein assay

The equilibrium concentration of BSA remaining in solution after adsorption was evaluated using reverse phase high pressure liquid chromatography (HPLC). Samples (100 μL) were loaded at 0.8 mL/min onto a C4 column (Brownlee Aquapore, 2.1 x 100mm, 7 μm, 300Å) equilibrated in a mixture of 75 % solvent A [10 % v/v acetonitrile (ACN), 0.1 % v/vtrifluoroacetic acid (TFA)] and 25 % solvent B [90 % v/v ACN, 0.1 % v/v TFA]. The initial solvent mixture (25 % solvent B) was held constant for 1 minute after loading. Protein was then eluted by a gradient of 25 to 70 % solvent B from 1 to 2 min, and then held at 70 % solvent B for a further 2.5 min. Peaks were detected at 220 nm using a UV detector (Agilent 1100 series, Waldbronn, Germany) and compared to a BSA standard curve to establish the concentration of BSA in the sample.

5.2.6 Bentonite settling analysis

Batch settling tests of the unmodified and heat-modified bentonites using a range of bentonite doses (1.0 - 2.5 g/L) were conducted in 250 mL graduated volumetric glass cylinders filled with the model white wine solution used during adsorption tests.

For each test, the required dose of bentonite was added to the model wine solution in a glass bottle. The bottle was shaken to produce homogenous slurry and then quickly poured into the graduated volumetric cylinder. Rear lighting directly behind the volumetric cylinder (in addition to overhead lighting) was employed to improve contrast at the interface. The height of the interface was recorded at regular intervals starting from when the agitated slurry was poured into the cylinder. This data was used to calculate the hindered settling velocity, v_p, as a function of time.

5.2.7 Partial Least Squares Analysis

Partial Least Squares (PLS) regression analysis (conducted using the CAMO™ "The Unscrambler v9.2" software package) was used to determine relationships between

physical and chemical properties of bentonite and the corresponding adsorption and settling behaviour.

PLS regression utilises a form of factor analysis called Principal Component Analysis (PCA). PCA is a technique which transforms the original set of variables into a smaller set of linear combinations that account for most of the variance of the original set (Dillon *et al.,* 1984; Kline, 1994). Principal components are extracted so that the first principal component, PC_1, is a linear combination of the observed variables, X_j, $j = 1,2,...p$:

$$PC_1 = w_{1,1}X_1 + w_{1,2}X_2 + ... + w_{1,p}X_p \quad (5.1)$$

where the weights (or loadings) $w_{1,1}, w_{1,2}, ..., w_{1,p}$ have been chosen to maximise the ratio of the variance of PC_1 to the total variation, subject to the constraint that $\sum_{j=1}^{p} w_{1,j}^2 = 1$ (Dillon *et al.,* 1984). The variance is defined as the mean square of the residuals corrected for the degrees of freedom (CAMO, 1994). In most cases, the *first* principal component explains far more variance than the other components (Kline, 1994). The second principal component, PC_2, is the weighted combination of the observed variable which is uncorrelated with (i.e orthogonal to) the first linear combination and which accounts for the maximum amount of the remaining variation not already accounted for by PC_1. Thus, the *n*th principal component is the weighted linear combination of the X's which has the largest variance of all linear combinations that are uncorrelated with all of the previously extracted principal components:

$$PC_m = w_{m,1}X_1 + w_{m,2}X_2 + ... + w_{m,p}X_p \quad (5.2)$$

PLS regression performs a simultaneous and interdependent PCA decomposition in both observed (X) and measured (Y) matrices, in such a way that the information in the Y-matrix is used directly as a guide for optimal decomposition of the X-matrix, and then performs regression of Y (CAMO, 1994). PLS analysis is well suited to systems where variables are to some degree co-linear and where a predictive model is required. In comparison, conventional multiple linear regression (MLR) requires that the number of X-variables must be smaller than the number of samples. In case of co-linearity among X-variables, the MLR b-coefficients are not reliable and the model may be unstable. Also, MLR tends to overfit when noisy data is used (CAMO, 2005).

The PLS analysis conducted using the CAMO™ "The Unscrambler v9.2" software package involved both *calibration* and *validation* stages. During the calibration stage, a calibration model is established based on a representative set of corresponding measurements of both X- and Y- variables. The validation stage determines the accuracy of data prediction using the calibration model. Validation can be either performed with a separate test data set, or using the original data set by full cross-validation. The *explained calibration variance* is the

total (or cumulative) variance in the original data which is explained by the calibration model. The *explained validation variance* is the total variance in the validation data set (either a separate test set, or by full cross-validation) which is explained by the calibration model. (CAMO, 1994). The CAMO^{TM} “The Unscrambler v9.2” software package can also be used to estimate regression coefficients (B-coefficients) which describe the relations between X and Y in a linear form similar to that given by standard MLR methods. The resulting predicted Y-values obtained using these regression coefficients are *exactly the same* as the predicted Y-values obtained using the original principle component model for prediction (CAMO, 1994). This allows inspection of which predictors or factors have the dominant effect on response variables, and provides a more convenient regression equation for predictive purposes.

5.3 Results

5.3.1 Physical and chemical properties

Physical and chemical properties of unmodified and heat-modified bentonites are shown in Table 5.1. For the heat-modified bentonites, the naming convention used was: [bentonite][heating temperature]-[heating time]. For example, “V200-3h” refers Vitiben heated at 200^{o}C for three hours, “M300-6h” refers to Microcol CL-G heated at 300^{o}C for six hours, etc.

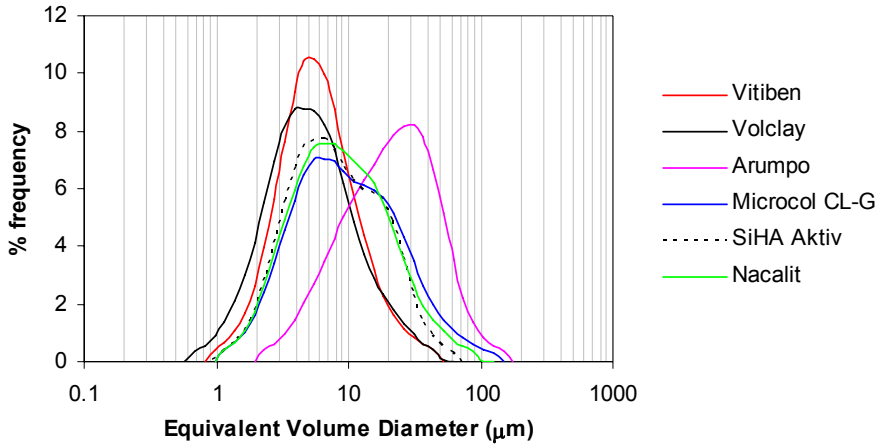
The pH of 1:50 slurries of the unmodified bentonite slurries were all in alkaline range of 8.2 - 9.4, with the exception of Arumpo, which had an acidic pH of 5.24. In general, pH was found to decrease with increasing heating temperature for both Vitiben and Microcol CL-G bentonites, which is consistent with the observations made by Bojemueller *et al.* (2000) of heat treatment of a Wyoming bentonite at temperatures from 350 - 550^{o}C.

Analysis by ICPMS of the exchangeable ions present in each bentonite showed both Volclay and Vitiben to be predominantly sodium exchanged bentonites. Microcol CL-G, SiHA Aktiv and Nacalit bentonites were shown to be calcium and magnesium rich, with high sodium content. The Arumpo bentonite was predominantly sodium and magnesium exchanged, with no calcium detected. In general, heat treatment was found to reduce the concentration of exchangeable ions detected by ICPMS, which is consistent with the observations of Chorom *et al.* (1996) of thermal treatment of Wyoming bentonites.

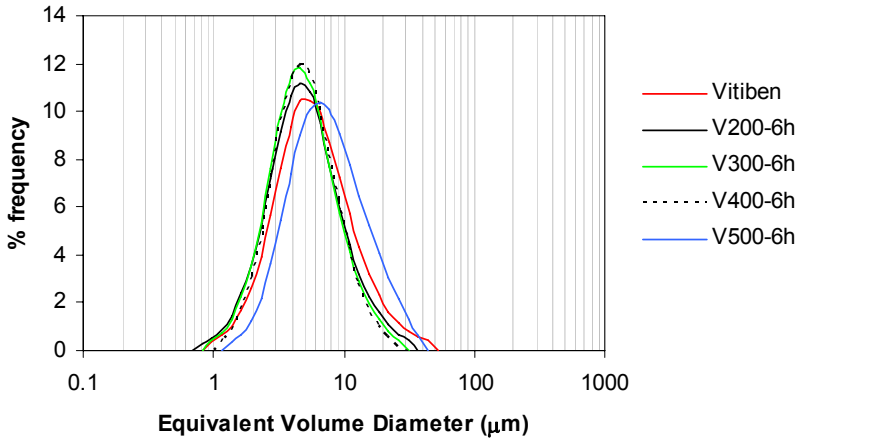
Particle size analysis by laser diffraction showed that the particle size distribution ranged from less than 2 μm to greater than 60 μm for the unmodified bentonites, as shown in Figure 5.1(a). Three distinct bentonite groupings were also observed: (1) Volclay, unmodified Vitiben and heat-modified Vitiben bentonites; (2) SiHA Aktiv, Nacalit, unmodified Microcol CL-G and heat-modified Microcol CL-G bentonites; and (3) Arumpo bentonite. In general, both Microcol CL-G and Vitiben bentonites were found to decrease in mean volume diameter, d_v, and mean surface area diameter, d_A, with increasing heating temperature, up to 400^{o}C (Figure 5.1(b) and Figure 5.1(c)). Heating at 500^{o}C resulted in a dramatic increase in mean particle size. The mean volume diameter of all 30 bentonites was well correlated (by PLS regression) with ICPMS results (concentration of the eleven exchangeable ions assayed plus total CEC) and pH (explained calibration and validation variance of 91.3% and 77.8%, with 4 principal components).

Table 5.1: pH, EC, particle size and cation exchangeability of natural and heat-modified bentonites

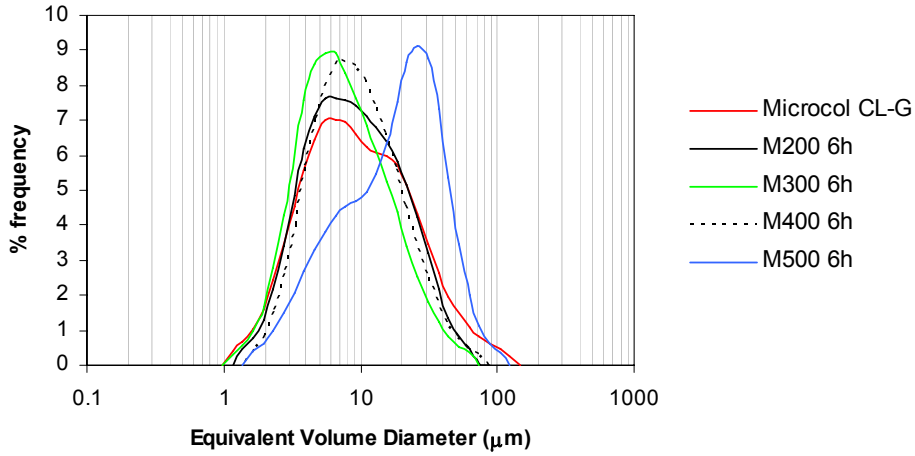
Bentonite	*pH*	*EC (mS/cm)*	*Particle Size Analysis*		*Exchangeable cations (meq/100g)*			
			d_v (μm)	d_A (μm)	*Na*	*Mg*	*Ca*	*CEC*
Volclay	9.26	323	6.47	3.53	44.2	11.0	20.3	76.6
Vitiben	9.16	326	6.83	4.26	62.9	13.9	10.5	90.6
Microcol CL-G	8.93	121.3	13.66	6.05	12.8	24.5	23.6	62.4
SiHA Aktiv	9.37	147.2	10.06	5.30	12.4	23.3	24.7	62.5
Nacalit	8.24	174.4	11.48	5.69	19.2	33.3	14.1	69.9
Arumpo	5.24	244.6	25.11	13.17	33.6	40.8	0.0	79.4
V200-3h	9.73	375	4.10	3.06	65.6	13.6	13.2	95.4
V300-3h	9.74	369	5.95	3.83	64.5	12.6	13.9	94.6
V400-3h	9.53	382	5.11	3.34	64.0	10.6	12.8	90.8
V500-3h	8.9	401	8.90	5.22	57.2	7.3	12.3	81.7
V200-6h	9.79	387	5.63	3.70	68.3	13.4	12.7	99.6
V300-6h	9.69	393	5.32	3.70	60.2	12.9	12.4	88.2
V400-6h	9.47	390	5.35	3.88	61.6	10.2	12.1	86.6
V500-6h	8.83	400	8.29	5.48	58.7	5.6	11.5	80.6
V200-12h	9.86	368	5.04	3.83	62.2	13.0	11.8	90.0
V300-12h	9.74	382	4.99	3.83	61.8	11.3	12.2	87.6
V400-12h	9.5	390	6.77	4.24	61.5	8.8	18.2	91.3
V500-12h	8.9	408	12.38	6.39	58.1	5.7	11.3	78.0
M200-3h	8.95	125.4	12.73	6.27	13.3	25.9	24.7	67.1
M300-3h	8.83	136.2	9.04	5.36	12.9	24.7	25.9	67.2
M400-3h	8.45	120.9	17.11	8.57	11.4	19.5	22.7	57.0
M500-3h	7.94	123.7	23.41	10.92	5.9	13.1	16.6	38.8
M200-6h	8.47	146.8	11.22	6.10	13.0	26.1	24.7	68.1
M300-6h	8.47	129.2	9.20	5.25	11.5	23.1	23.1	61.0
M400-6h	8.34	128.5	11.24	6.54	9.9	17.8	21.7	53.2
M500-6h	7.99	129.1	20.38	10.03	5.3	11.7	14.6	35.5
M200-12h	8.91	111.3	10.78	5.94	13.0	26.3	24.3	67.8
M300-12h	8.82	119.1	12.12	6.65	12.1	24.2	22.9	64.2
M400-12h	8.37	138.9	11.30	6.23	11.8	18.8	22.2	57.1
M500-12h	7.97	119.3	19.15	8.82	1.5	10.6	11.9	27.3



(a)



(b)



(c)

Figure 5.1: PSD of (a) Natural , (b) Heat-modified Vitiben and (c) Heat-modified Microcol CL-G bentonites

5.3.2 Adsorption tests

Adsorption was characterised using the Langmuir adsorption isotherm:

$$q = q_{\max} \frac{K_L c_e}{1 + K_L c_e} \tag{5.3}$$

where q is the mass of protein adsorbed per mass of bentonite (mg/mg), q_{max} is the maximum amount of protein that can be adsorbed per mass of bentonite (mg/mg), c_e is the concentration of protein in solution at equilibrium (mg/L) and K_L is the Langmuir equilibrium constant.

Adsorption test results were regressed against a modified version of Equation 5.3:

$$\frac{1}{q} = \frac{1}{q_{\max} K_L} \cdot \frac{1}{c_e} + \frac{1}{q_{\max}} \tag{5.4}$$

Equation 5.4 is analogous to the Lineweaver-Burk transformation of the Michaelis-Menton equation used in enzyme kinetics (Bailey et al., 1986).

The results of the adsorption tests are summarised in Table 5.2. The results of adsorption tests for the unmodified bentonites and the Langmuir adsorption curves obtained from linear regression with Equation 5.4 are illustrated in Figure 5.2.

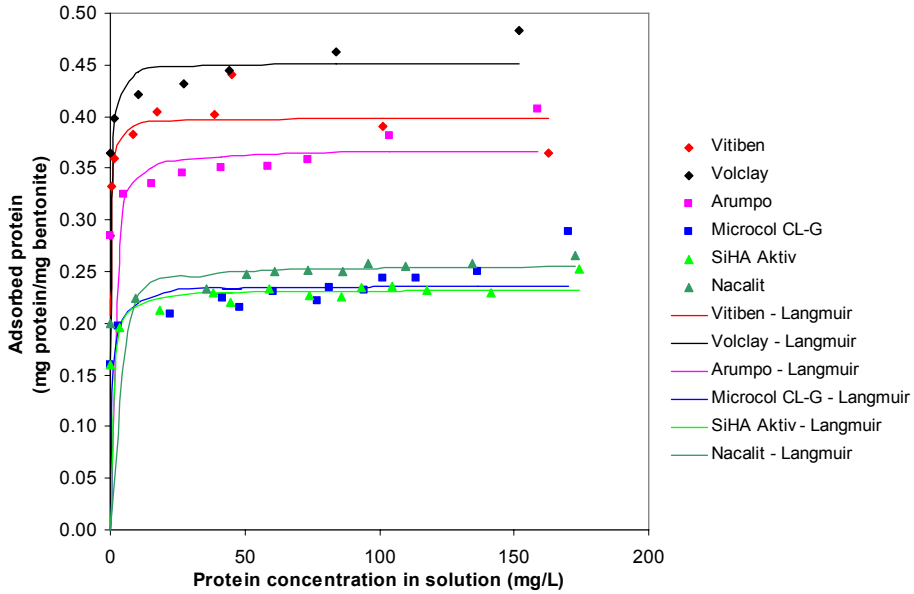


Figure 5.2: Adsorption Isotherms for Unmodified Bentonites

Table 5.2: Adsorption performance and settling behaviour of natural and heat-modified bentonites

Bentonite	*Langmuir parameters*		*Dose = 1.0 g/L*		*Dose = 1.5 g/L*		*Dose = 2.0 g/L*		*Dose = 2.5 g/L*	
	q_{max}	K_L	*Initial Settling Velocity (cm/min)*	*Lees Volume (mL)*	*Initial Settling Velocity (cm/min)*	*Lees Volume (mL)*	*Initial Settling Velocity (cm/min)*	*Lees Volume (mL)*	*Initial Settling Velocity (cm/min)*	*Lees Volume (mL)*
Volclay	0.451	5.39	8.6	20	7.5	31.5	4.8	40	-	-
Vitiben	0.399	5.62	11.9	20	8.7	29.5	4.6	40.5	-	-
Microcol CL-G	0.237	1.55	28.5	9	21.4	16.5	11.6	18	18.0	23
SiHA Aktiv	0.233	1.41	25.6	6.5	24.7	12.5	15.1	16	18.5	18.5
Nacalit	0.257	0.68	17.4	11.5	16.8	19.0	13.1	24	9.9	30
Arumpo	0.368	1.27	12.4	12.8	10.9	22.0	9.0	25	-	-
V200-3h	0.436	9.98	12.1	17.5	7.7	31.0	7.2	36	-	-
V300-3h	0.418	7.02	10.9	20	8.2	31.5	5.4	37	-	-
V400-3h	0.361	5.80	11.6	15.5	11.8	24.0	9.5	29.5	-	-
V500-3h	0.224	0.19	14.0	8	21.4	11.0	11.0	16.5	-	-
V200-6h	0.451	2.86	11.4	18	8.1	32.0	6.9	36	-	-
V300-6h	0.399	7.64	9.1	18	9.0	31.0	7.0	37	-	-
V400-6h	0.336	6.64	11.4	16	13.4	22.0	10.9	31	-	-
V500-6h	0.233	0.05	19.1	3	19.0	9.0	19.0	12	-	-
V200-12h	0.418	4.33	11.2	15.8	8.4	30.0	6.7	34	-	-
V300-12h	0.399	5.78	10.3	17.5	9.0	30.0	7.0	36.3	-	-
V400-12h	0.332	3.82	11.5	12.5	13.4	23.0	11.8	25	-	-
V500-12h	0.171	0.09	18.7	3	29.6	6.0	14.8	7.5	-	-
M200-3h	0.220	8.30	27.5	7	32.4	13.0	11.9	20	14.9	25.2
M300-3h	0.189	4.89	-	-	36.0	11.0	-	-	-	-
M400-3h	0.122	0.42	-	-	38.9	5.0	-	-	-	-
M500-3h	0.068	0.39	-	-	41.6	5.0	-	-	-	-
M200-6h	0.233	0.34	29.1	5.6	34.1	11.5	16.4	19	21.3	18.5
M300-6h	0.177	3.32	-	-	36.4	6.5	-	-	-	-
M400-6h	0.110	0.46	-	-	40.6	1.0	-	-	-	-
M500-6h	0.040	8.30	-	-	63.8	2.0	-	-	-	-
M200-12h	0.202	13.63	14.1	6.5	23.9	12.0	21.9	16.8	20.4	19.5
M300-12h	0.173	2.66	-	-	32.6	7.0	-	-	-	-
M400-12h	0.108	0.08	-	-	34.8	4.0	-	-	-	-
M500-12h	0.073	0.02	-	-	36.9	8.0	-	-	-	-

The results in Table 5.2 show two distinct groups: (1) Vitiben, Volclay and Arumpo; and (2) Microcol CL-G, SiHA Aktiv and Nacalit. Group (1) has approximately twice the adsorption capacity (q_{max}) of Group (2).

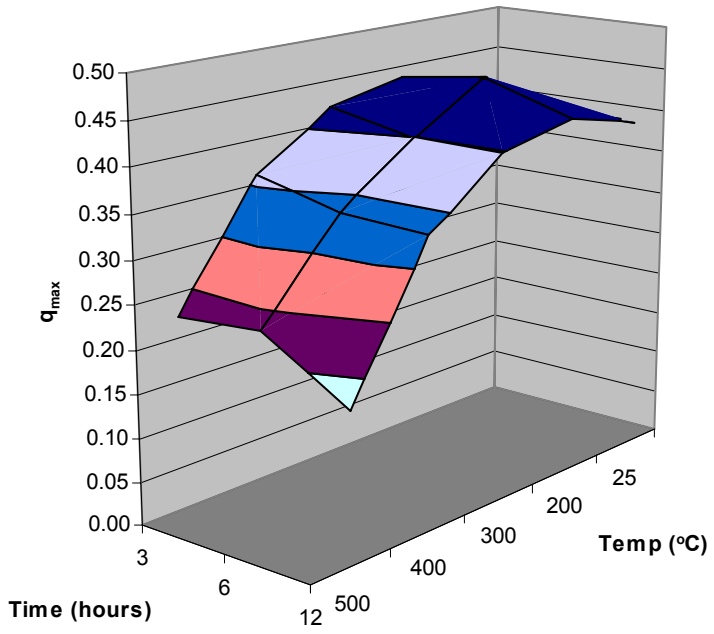
The effect of heating time and temperature on q_{max} for the heat-modified Vitiben and Microcol CL-G bentonites is shown in Figure 5.3. Generally, a decrease in q_{max} with heating temperature was observed. However, q_{max} was found to increase at 200^{o}C for heating times of 3, 6, and 12 hours for Vitiben, and at 200^{o}C for 6 hours for Microcol CL-G.

5.3.3 Settling tests

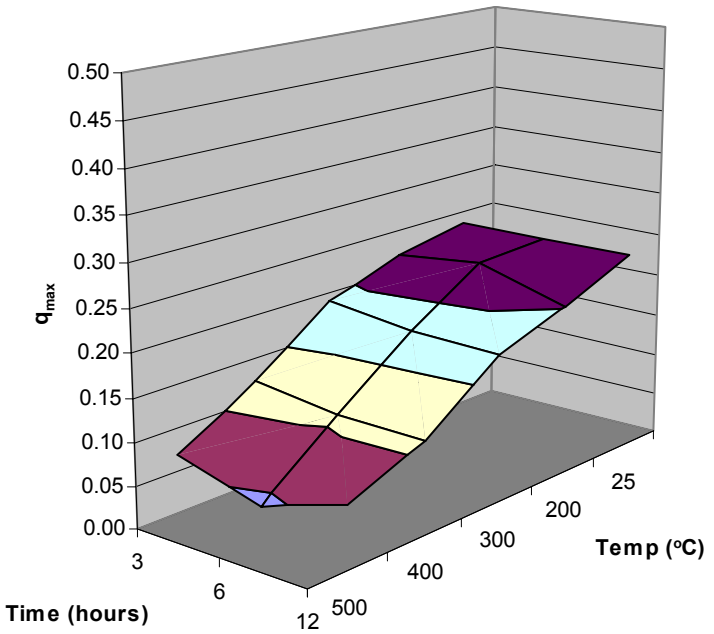
Settling test results for all 30 bentonites in wine are included in Table 5.2: initial settling velocity and final lees volume. Figure 5.5(a) illustrates the settling test results of the six unmodified bentonites for a bentonite dose of 1.5 g/L.

Three distinct types of settling behaviour were observed, with each group displaying different initial settling velocities and final lees volumes. These groups were: (1) Volclay, unmodified Vitiben and heat-modified Vitiben bentonites; (2) SiHA Aktiv, Nacalit, unmodified Microcol CL-G and heat-modified Microcol CL-G bentonites; and (3) Arumpo bentonite. The final lees volume of Group (1) was usually more than twice that of Group (2), with the Arumpo bentonite in between.

The effect of heat treatment on settling behaviour is demonstrated in Figure 5.4(b). In general, the initial hindered settling velocity was found to increase with increasing heating temperature. The final lees volume was dramatically reduced at heating temperatures of 400^{o}C and 500^{o}C, as shown in the surface plot for heat-modified Vitiben in Figure 5.5.

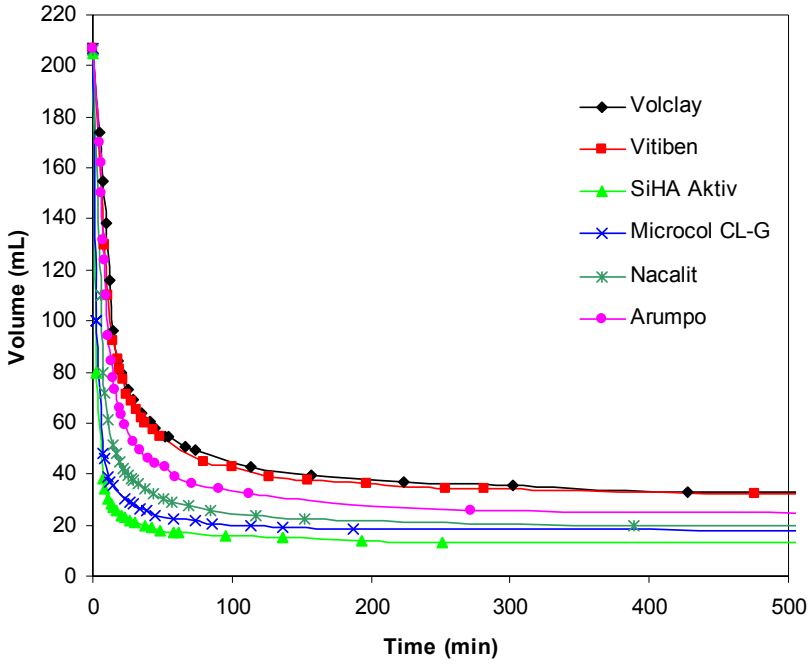


(a)

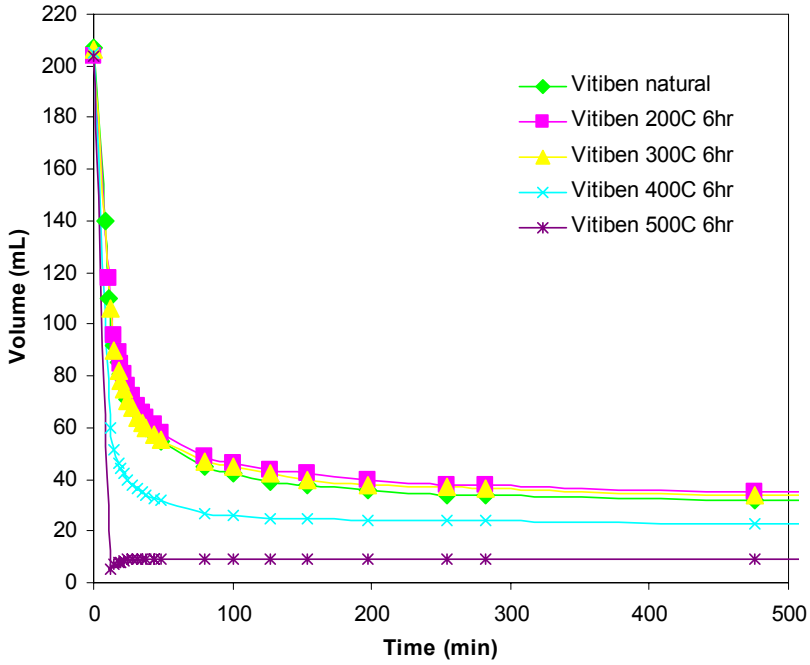


(b)

Figure 5.3: Effect of Heating Time and Temperature on q_{max} for (a) Heat-modified Vitiben and (b) Heat-modified Microcol CL-G bentonites



(a)



(b)

Figure 5.4: Sedimentation of (a) Natural and (b) Heat-modified Vitiben Bentonites, Dose = 1.5 g/L

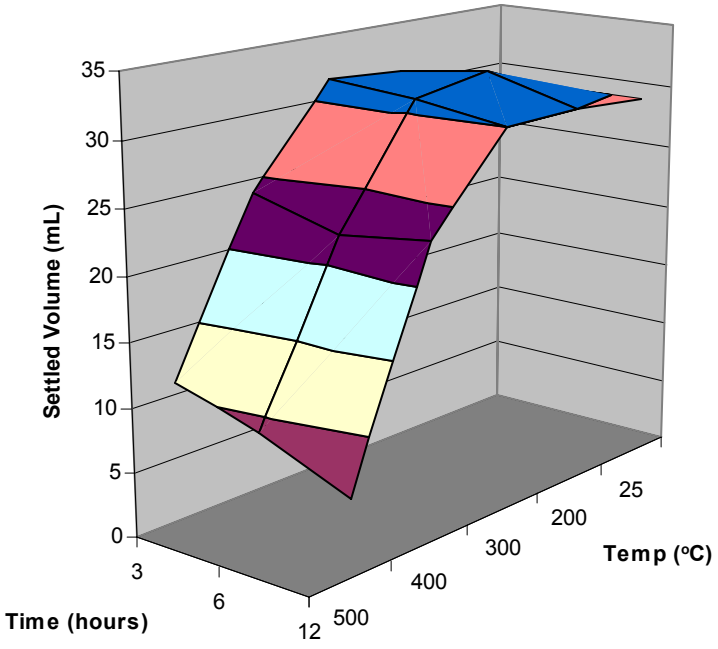


Figure 5.5: Effect of Heating Time and Temperature on Final Lees Volume for Heat-modified Vitiben, Dose = 1.5 g/L

5.3.4 Correlation of bentonite properties with adsorption and settling performance

The PLS regression results, including the regression coefficients (which have been normalized to give a regression slope and intercept of 1 and 0 respectively) are summarised in Table 5.3.

The observed variance in protein adsorption capacity, q_{max}, was found to be well correlated with the variance in total CEC and exchangeable Na, K, Mg, Ca, Cr, Fe, Sr, and Se, for all thirty bentonites (i.e. inclusion of the other measured variables e.g. d_v, d_A, EC etc did not improve the ECV and EVV). The observed variance in initial hindered settling velocity and final lees volume for all thirty bentonites was shown to be well correlated with the variance in d_v, Total CEC and exchangeable Na, K, Mg, Ca, Cr, Fe, Sr, and Se. The observed variance in the equilibrium constant, K_L, was found to be well correlated with the variance in total CEC and exchangeable Na, K, Mg, Ca, Cr, Sr, and Se, for the six unmodified bentonites only. The linear form of the PLS models for q_{max}, K_L, v_p and V_{lees} estimated by the CAMO^{TM} "The Unscrambler v9.2" software package were as follows.

$$q_{\max} = \beta_{0q} + \sum_{i=1}^{10} \beta_{iq} x_{iq} \quad (5.5)$$

$$K_L = \beta_{0K} + \sum_{i=1}^{10} \beta_{iK} x_{iK} \quad (5.6)$$

$$v_p = \beta_{0v_p} + \sum_{i=1}^{10} \beta_{iv_p} x_{iv_p} \quad (5.7)$$

$$V_{lees} = \beta_{0V_{lees}} + \sum_{i=1}^{10} \beta_{iV_{lees}} x_{iV_{lees}} \quad (5.8)$$

where β_{0q}, β_{iq}, β_{0K}, β_{iK}, β_{0v_p}, β_{iv_p}, $\beta_{0V_{lees}}$ and $\beta_{iV_{lees}}$ are regression constants; and x_i the level of the corresponding factor.

Table 5.3: PLS regression results for q_{max}, K_L, Settling Velocity and Final Lees Volume

Parameter (x_i)	Units	i	q_{max} (β_{iq})	K_L (β_{iK})	Settling Velocity (β_{iv_p})	Final Lees Volume ($\beta_{iV_{lees}}$)
Constant		0	-0.0621	-0.363	35.1	12.1
D_v	μm	1	0	0	0.537	-0.430
Na	meq/100g	2	1.55E-03	3.19E-02	-1.36E-01	1.02E-01
K	meq/100g	3	-1.83E-02	-3.10E-02	3.17E+00	-3.25E+00
Mg	meq/100g	4	4.80E-03	-6.40E-02	-4.79E-01	3.09E-01
Ca	meq/100g	5	-2.09E-03	1.15E-02	4.72E-01	-3.05E-01
Cr	meq/100g	6	-1.55E-01	-1.26E+01	2.97E+01	-2.40E+01
Fe	meq/100g	7	3.86E-01	0	-3.51E+01	2.84E+01
Sr	meq/100g	8	3.42E-01	2.44E+00	-1.26E+01	3.07E+01
Se	meq/100g	9	1.26E-01	3.39E+00	-9.57E+00	8.07E+00
Total CEC	meq/100g	10	3.64E-03	4.52E-02	-2.98E-01	2.14E-01
n			30	6	30	30
ECV (%)			89.62	99.73	85.24	81.20
EVV (%)			83.04	96.44	75.73	75.06
R			0.9467	0.9929	0.9233	0.9011
PCs			3	3	2	2

n = number of samples

ECV = Explained Calibration Variance

EVV = Explained Validation Variance

R = Regression Coefficient

PCs = number of Principal Components

5.3.5 Occlusion of model wine by bentonite

The volume fraction of model wine occluded by bentonite after batch settling was regressed against the corresponding bentonite dose using linear-regression through the origin (as a zero dose will produce zero lees):

$$F = b\frac{m}{V} \tag{5.9}$$

where F is the fraction of the model wine volume occluded, m is mass of bentonite added (mg), V is the volume of wine (L), and b is the slope of the regression line.

By combining the Langmuir isotherm (Equation 5.3) with a mass balance on the protein, the mass of bentonite per unit volume of wine (i.e. the dose) required to achieve a specified final protein concentration for a given initial concentration of protein may be calculated, as shown in Equation 5.10,

$$\frac{m}{V} = \left(\frac{1 + K_L c_e}{q_{\max} K_L c_e}\right)(c_i - c_e) \tag{5.10}$$

where m is the mass of bentonite added (mg), V is the volume of wine (L), c_i is the initial concentration of protein in solution (mg/L).

Thus, the theoretical volume fraction of model wine which would be occluded by bentonite, when fining to achieve a specified final protein concentration for a given initial concentration, may be calculated by combining Equations 5.9 and 5.10, as shown in Equation 5.11.

$$F = b\left(\frac{1 + K_L c_e}{q_{\max} K_L c_e}\right)(c_i - c_e) \tag{5.11}$$

Anecdotal evidence suggests that a wine with a protein concentration of 10 mg/L of less will be ‘heat stable’. Figure 5.6 shows, for each of the thirty bentonites, the theoretical volume fraction of model wine which would be occluded if that bentonite was added to a model wine to reduce its protein concentration from 200 mg/L to 10 mg/L. These results are the *combined effect* of protein adsorption capacity *and* settling performance. For bentonites where more than one batch fining test was conducted, the standard deviation of F as calculated from data processed by Equation 5.11 is also shown. For bentonites where only one batch fining test was conducted, results should be regarded as indicative only.

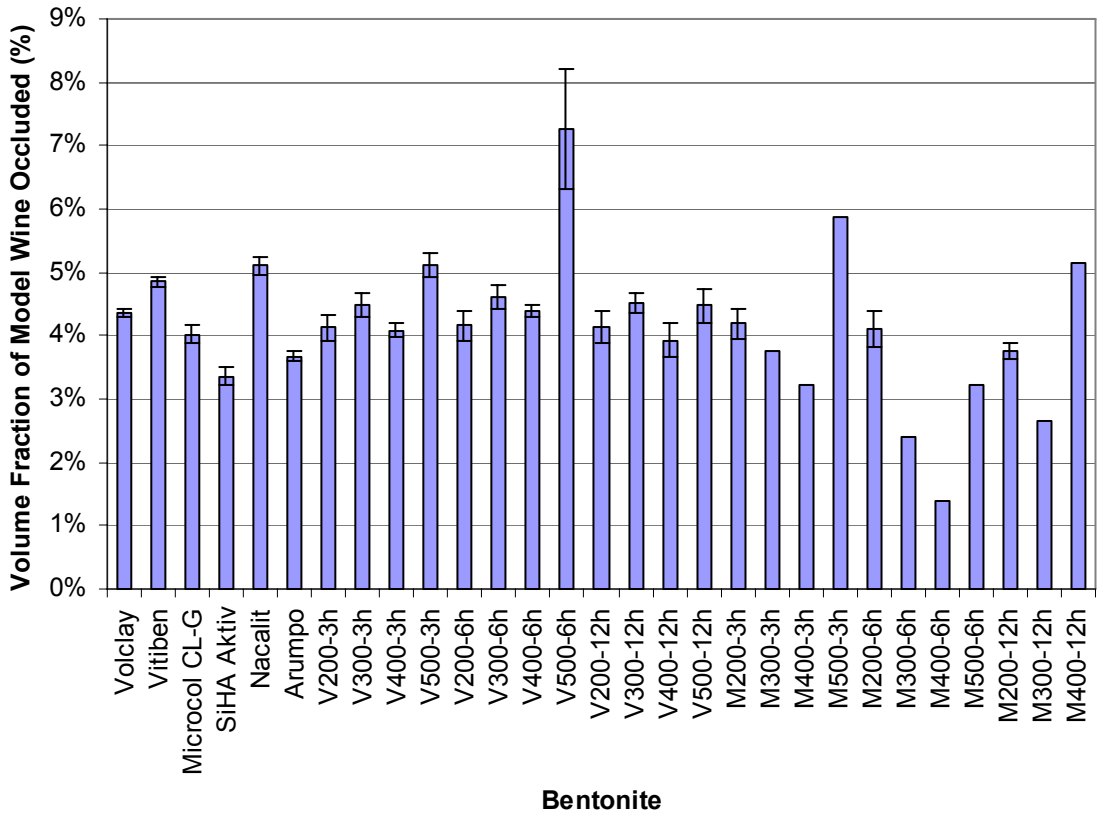


Figure 5.6: Volume Fraction occluded by bentonite to reduce BSA concentration of model wine from 200 mg/L to 10 mg/L

5.4 Discussion

5.4.1 Physical and chemical properties

The pH of the 1:50 bentonite dispersions were found to increase on heating to 200°C for both Vitiben and Microcol CL-G. It is postulated that heating at 200°C increases bond strength between H^+ and the clay surface –O and –OH, which in turn reduces the exchangeability of H^+ (similar to the process described by Chorom *et al,.* (1996)) and increases the pH. Altin *et al.* (1999) asserts that an increase in pH is associated with an increase in interlayer repulsive forces which creates a more porous structure. This would in turn lead to greater accessibility and mobility of cations within the sheet layers (in particular, Na and Ca for Vitiben and Na, Mg and Ca for Microcol CL-G) and is consistent with an increase in exchangeable Na, Ca, Mg, total CEC and q_{max} observed in the present study on heating to 200°C.

On heating from 200°C to 500°C, the pH of heat-modified Vitiben and Microcol CL-G slurries (1:50) was observed to decrease, which is consistent with the change in pH observed on heating of bentonite by Bojemueller *et al.* (2001). A lowering of the pH is associated with a reduction in particle swelling, due to reduction in short-range repulsive forces by replacement of adsorbed H^+ by surface ions (Pashley, 1981), which occurs on

heating. This may in part be responsible for the reduction observed in mean volume diameter (d_v) and mean area diameter (d_A), on heating from 200°C to 300°C.

A study by Chorom *et al.* (1996) showed that for unheated clays, particle diameter is influenced by the nature of the exchangeable cations present, with diameters increasing with exchange cation in the following order: $Li^+ < Na^+ < K^+ < Mg^{2+} < Ca^{2+} < Al^{3+}$. In this present study, Microcol CL-G was found to have the largest particle size of the unmodified bentonites followed by Nacalit, SiHA Aktiv, Arumpo, Vitiben and then Volclay. This is consistent with the findings of Chorom *et al.* (1996) in that Ca and Mg account for over 70% of the exchange sites for Microcol CL-G, Nacalit and SiHA Aktiv. For Volclay and Vitiben, Na accounts for approximately 60 - 70% of the exchange sites. The exchange sites on Arumpo bentonite are occupied primarily by Na and Mg.

The mean volume diameter, d_v, and mean surface area diameter, d_A, were found to decrease moderately from 200°C to 300°C (200°C – 400°C for V300-3h), and then increase considerably at 500°C. A significant increase in surface area for bentonites heated above 400°C for 12 h was also observed by Bojemueller *et al.* (2001), who attributed this phenomenon to increased attraction and agglomeration of particles as a result of changes in clay-surface charge. Bojemueller *et al.* (2001) postulated that this change in surface charge was a result of the liberation of Al^{3+} ions or oligomeric hydroxoaluminium cations. These cationic aluminium species do not remain in the diffuse ionic layer around the particle or in solution but enrich at the surface. The increased positive charge density influences colloidal behaviour due to the increase in face-edge contacts formed. This in turn leads to increased attraction and agglomeration between bentonite particles and also to accelerated sedimentation. Research conducted by Chorom *et al.* (1995), which found net particle charge to be “the most important factor in controlling clay dispersion” supports this hypothesis. Therefore, the results of this study suggest that a degree of particle agglomeration occurs in slurries prepared using Vitiben or Microcol CL-G bentonite heated above 400°C.

In a study by Chorom *et al.* (1996), thermal treatment was shown to affect the nature of the bonding between exchangeable cations and the clay surface. On heating, smaller cations (ionic radius < 0.7Å) can migrate to octahedral vacant sites (Calvet *et al.* 1971) and form strong covalent-like bonds, which reduces the overall negative charge of the clay. Charge reduction and increased bond strength inhibit swelling and reduce dispersion. Larger cations (Na^+, K^+, Ca^{2+}) do not migrate into lattice sites on thermal treatment (Calvet *et al.* 1971), but there is an increase in the degree of covalent-like character of the bonding between cations and clay surface -O and -OH surfaces due to thermal effects (Chorom *et al.* 1996). Not only are these cations less exchangeable (as shown by ICPMS results), but stronger bonding and charge reduction also results in increased agglomeration with a corresponding increase in particle size, as observed.

5.4.2 Protein adsorption by bentonite

Adsorption test results were shown to be well characterised by the Langmuir isotherm. The Langmuir equation is commonly used to model adsorption behaviour in various systems. Despite the fact that the assumptions involved in Langmuir adsorption (namely that: one molecule adsorbs on one site, and the number of sites is limited; adsorption is reversible; there are no lateral interactions between adsorbed solute molecules) are seldom satisfied in the case of protein adsorption (Norde, 1986) it nevertheless provides a good fit for many systems (Fargues *et al.*, 1998). It has been used previously to describe the adsorption of proteins such as BSA onto charged surfaces (Fargues *et al.*, 1998; Skidmore *et al.*, 1990a; Skidmore *et al.*, 1990b, Graham *et al.*, 1987; Suen, 1996; Whitley *et al.*, 1989; Sarmento *et al.*, 2000), and more specifically to characterize the adsorption of (non-wine) proteins such as BSA, lysozyme and ovalbumin onto sodium-exchanged bentonite in model wine systems (Blade *et al.*, 1988; Achaerandio *et al.* 2001).

Vitiben, Volclay and Arumpo possessed approximately twice the adsorption capacity (q_{max}) of Microcol CL-G, SiHA Aktiv and Nacalit. This is consistent with the findings of Blade *et al.* (1988) and others who have compared the adsorption capacity of sodium and calcium exchanged bentonites. Adsorption capacity increased on heating to 200^{o}C, and then decreased on heating from 200^{o}C to 500^{o}C. This effect is consistent with both the corresponding change in total CEC that was observed with heating, and the previous discussion on the effect of heating on particle size and cation exchangeability.

PLS analysis shows that the protein adsorption capacity (q_{max}) was well correlated for all 30 (six unmodified and 24 heat-modified) bentonites with total CEC and exchangeable Na, K, Mg, Ca, Cr, Fe, Sr, and Se with an explained calibration and (full cross) validation variance of 89.6% and 83.0% respectively for three principal components. Total CEC is largely co-linear with exchangeable ions, but will also reflect changes to surface charge brought about by migration of Al^{3+} to the clay surface. Inclusion of particle diameter, particle volume and surface area, pH, and EC did not improve the fit. This is not surprising given that changes in total CEC and exchangeable cations are largely responsible for changes in particle size and charge, as previously discussed (i.e particle size, pH and EC are co-linear with total CEC and exchangeable cations). It is hypothesised that the remaining unexplained variance is a result of changes to other physical attributes on heating such as specific lattice substitutions, pore size and zeta potential which were not measured in this experiment. Further work would be required to investigate this.

PLS analysis shows that the variance in adsorption equilibrium constant, K_L, was well correlated for the natural bentonites with the variance in total CEC and exchangeable Na, K, Mg, Ca, Cr, Fe, Sr, and Se with an explained calibration and (full cross) validation variance of 99.7% and 96.4% respectively for three principal components (n = 6). This

agrees with research by Bosma *et al.*, (1998), who state that protein-ion (as well as protein-protein and protein surface) *interactions* are important in protein ion-exchange. In fact the Steric Mass-Action (SMA) model discussed by Bosma *et al.* (1998) explicitly states that the interactions between local protein charge and ions in solution are a crucial part of the mechanism of protein ion-exchange. When PLS analysis of the adsorption equilibrium constant, K_L, was extended to include the heat-treated bentonites, the variance in K_L could not be explained with any of the physical and chemical properties measured. As for q_{max}, it is hypothesised that the remaining unexplained variance is a result of changes to other physical attributes that occurred on heating (such as specific lattice substitutions, pore size and zeta potential) which were not measured.

5.4.3 Bentonite settling performance

The settling velocity of a single particle in a fluid is determined from the equilibrium of the drag force per unit volume and the weight per unit volume, as shown by Equation 5.12,

$$v_o = \left(\frac{4 d_{sv} \left(\rho_p - \rho_f \right) g}{3 \rho_f C_{D_o}} \right)^{\frac{1}{2}} \tag{5.12}$$

where the single particle drag coefficient, C_{D_o} is a function of the particle Reynolds Number, Re_p.

Thus, the terminal velocity, v_o, of a single particle is related to the surface-volume particle diameter, d_{sv}, as well as particle (ρ_s) and fluid density (ρ) and fluid viscosity. When many particles are present, surrounding particles interfere with the motion of other particles. For this hindered flow, the settling velocity (referred to as the *hindered settling velocity*) is considerably less than calculated with Equation 5.12. This is a consequence of the particle settling through a suspension, rather than through the fluid itself. For instance, Richardson and Zaki (1954) reported the following correlation relating the initial hindered settling velocity of a suspension of inert spheres to the single particle terminal velocity, v_o, the solids volume fraction, c and an exponent, n:

$$v_p = v_o \left(1 - c\right)^n \tag{5.13}$$

For very small particles, n = 4.65.

Of the unmodified bentonites, Microcol CL-G, SiHA Aktiv and Nacalit exhibited the highest initial hindered settling velocity for all doses, followed by Arumpo, Vitiben and Volclay. This is consistent with the larger particle diameters of Microcol CL-G, SiHA Aktiv and Nacalit compared with Arumpo, Vitiben and Volclay. As predicted, the initial hindered settling velocity was reduced as the bentonite dose was increased. Some particle flocs

were observed during the batch settling tests. These flocs settled faster than individual bentonite particles.

The initial hindered settling velocity increased with heating as temperature rose from 200^{o}C to 500^{o}C. As discussed, changes in clay charge (as a consequence of migration of Al^{3+} to the surface and increased bonding strength on heating) result in increased agglomeration and reduced swelling. This combination results in increased particle size and density, with a corresponding increase in particle settling velocity (according to Equation 5.12). The results of the present study are consistent with this behaviour.

The degree of swelling and particle agglomeration is related to the cations present on the clay, and the extent to which they are exchangeable. Clays that exhibit a high degree of agglomeration and reduced swelling will produce a smaller volume of lees compared to high swelling clays with a reduced tendency to agglomerate. Of the unmodified bentonites, Microcol CL-G, SiHA Aktiv and Nacalit produced the smallest lees volume for all doses tested, followed by Arumpo, Vitiben and Volclay. This is consistent with the exchangeable cations present in these clays. On heating from 200^{o}C to 500^{o}C, the lees volume reduced, which is in agreement with the previous discussion of the changes in charge density and bonding strength induced by heating.

PLS analysis showed that the initial hindered settling velocity and final lees volume were highly correlated for all thirty (6 unmodified and 24 heat-modified) bentonites with d_v, total CEC and exchangeable Na, K, Mg, Ca, Cr, Fe, Sr, and Se. As for q_{max}, inclusion of particle volume and surface area, pH, and EC did not improve the fit of the regression, as these factors are (at least partially) co-linear with cation exchangeability. Again, it is hypothesised that the remaining unexplained variance results from changes in other physical attributes on heating such as specific lattice substitutions, pore size and zeta potential which were not measured.

5.4.4 Occlusion of model wine by bentonite

From a commercial winemaking perspective, a key performance attribute of bentonite is the volume fraction of wine that is occluded by the bentonite lees after fining. Recovery of this fraction by rotary drum vacuum filtration typically results in a significant reduction in wine quality due to oxidation, to the point where the wine cannot be blended with the original product. It is estimated that the cost of bentonite treatment to the Australian Wine Industry is in the order of $A45 million per annum. Therefore, a reduction in the volume fraction of wine occluded will result in significant cost savings.

Figure 5.6 illustrates the *combined effect* of protein adsorption capacity and settling performance. Of the unmodified bentonites, SiHA Aktiv theoretically occludes the smallest volume fraction (3.4 %) of model wine, followed (in order) by Arumpo, Microcol CL-G, Volclay, Vitiben and Nacalit. Although SiHA Aktiv has lowest protein adsorption capacity, it

also produces the smallest volume of lees. In comparison, Volclay, with the greatest protein adsorption capacity (nearly twice that of SiHA Aktiv), produced approximately 2.5 times more lees (on average) for the *same* dose. Therefore, even though less Volclay (mass basis) is required to reduce the protein concentration of the model wine from 200 mg/L to 10 mg/L, it theoretically produces a larger volume of lees (4.4 %) than the SiHA Aktiv. The analysis also suggests that the following five heat-modified bentonites may occlude a smaller volume fraction of lees than SiHA Aktiv: M400-3h, M300-6h, M400-6h, M500-6h and M300-12h. Of these five, M400-6h would theoretically occlude only 1.9 % of the model wine volume, which is 57 % less than Volclay and 44 % less than SiHA Aktiv. It is important to note that only one batch fining test was conducted for each of these five bentonites, and so these results should be regarded as indicative only. The volume of wine that would theoretically be occluded by bentonites heated at 500^{o}C is substantially greater than the volume occluded by any of the other heat-treated bentonites. Even though the bentonites which were heat-treated at 500^{o}C generally produced the smallest volume of lees for a given dose, the adsorption capacity of these bentonites is extremely poor. Thus the *combined effect* of protein adsorption capacity and settling performance results in these bentonites occluding a larger volume of lees when used to reduce the protein concentration of the model wine from 200 mg/L to 10 mg/L.

If the results of the present study are representative of the bentonite-protein interactions occurring in a ‘real’ wine system, then potential exists for significant reduction in the volume of wine occluded by bentonite. However, this study was conducted using a single model wine solution with BSA as the target protein rather than in an authentic wine. The effect of different wine characteristics (eg pH, temperature, ethanol concentration, metal ion concentration etc) was not investigated. Therefore, further work to validate the performance of these different bentonites in ‘real’ wine conditions would be desirable.

5.5 Concluding remarks

Particle size, pH and cation exchangeability of bentonites and the changes to these properties that occur on heating are related to the nature of bonding between cations and the clay surface, as are protein adsorption performance and settling behaviour. Heating reduced both protein adsorption capacity of bentonite and the final lees volume produced on fining. PLS analysis shows that the variance in cation exchangeability and the total cation exchange capacity is primarily responsible for the variance in protein adsorption performance and settling behaviour. Further, PLS analysis was used to develop correlations for the prediction of protein adsorption capacity (q_{max}), the adsorption equilibrium constant (K_L), the initial hindered settling velocity (v_p) and the final lees volume (V_{lees}), based on the physical and chemical properties of the bentonites tested. These correlations could potentially be used to screen additional bentonites for protein adsorption efficacy and settling behaviour.

Qualitative comparison of the volume fraction of model wine occluded by bentonite for each of the bentonites tested indicates that certain heat treatments may result in a combination of protein adsorption performance and settling behaviour which would produce a reduction in wine loss.

6 Factors affecting bentonite performance

6.1 Introduction

In this part of the project, the effect of different wine attributes (or factors) on the adsorption behaviour of an authentic wine protein (VVTL1) was investigated. The wine attributes included temperature, pH, ethanol concentration, potassium ion concentration, concentration of simple monomeric phenolic compounds, and sugar concentration. Experimental data were regressed against the Langmuir equation, and used to identify significant factors and interactions.

To undertake this work, a new laboratory-based method for purifying gram quantities of VVTL1 was developed. This allowed separation and purification to be achieved simultaneously in a single step directly from grape juice by cation-exchange, followed by ultrafiltration for desalting and concentration. This new method is more rapid and compact than current techniques and can produce gram quantities rather than milligram quantities. It could also be used to purify other heat-unstable proteins in wine.

6.2 Materials and Methods

6.2.1 Purification of VVTL1 from Muscat of Alexandria grapes

6.2.1.1 Purification of VVTL1 by cation-exchange chromatography

Concentrated grape juice (pH 2.8) from *V. vinifera* cv. Muscat of Alexandria grapes was obtained from the Hardy Wine Company. Batches of 25 L of juice were filtered using a 0.45 μm cross-flow microfiltration cartridge. The filtrate (24 L) from each batch of juice was loaded onto a SP Sepharose Fast Flow cation-exchange column equilibrated with 20 mM Tri-Na citrate buffer, pH 3.0. Four different column and gradient configurations were trialled, as follows.

Configuration 1 - Column dimensions: 5 x 24 cm; Gradient: Step to 120 mM NaCl for 10 Column Volumes (CV), step to 250 mM NaCl for 10 CV, linear gradient to 500 mM NaCl over 2 CV, linear gradient to 1000 mM NaCl over 2 CV, held at 1000 mM NaCl for 2 CV; Flow rate: 5 mL/min.

Configuration 2 - Column dimensions: 5 x 22 cm; Linear gradient: 0 – 200 mM over 40 CV, 200 – 500 mM over 4 CV, 500 – 1000 mM over 2 CV, held at 1000 mM NaCl for 5 CV; Flow rate: 10 mL/min.

Configuration 3 - Column dimensions: 5 x 14.5 cm; Linear gradient: 0 – 200 mM over 40 CV, 200 – 500 mM over 4 CV, 500 – 1000 mM over 4 CV, held at 1000 mM NaCl for 5 CV; Flow rate: 10 mL/min.

Configuration 4 - Column dimensions: 5 x 14.5 cm; Linear gradient: 0 – 200 mM over 40 CV, 200 – 500 mM over 4 CV, 500 – 1000 mM over 2 CV, held at 1000 mM NaCl for 5 CV; Flow rate: 10 mL/min.

In total, four batches of juice were processed, one batch per configuration.

6.2.1.2 Identification of chromatography fractions containing VVTL1

Fractions containing the major grape protein, VVTL1 were identified by reverse phase HPLC, SDS-PAGE and mass spectrometry as follows.

Reverse Phase HPLC - Samples (100μL) were loaded at 1.6 mL/min onto a C4 column (Brownlee Aquapore, 2.1 x 100mm, 7 μm, 300Å) equilibrated in solvent A [10 % v/v acetonitrile (ACN), 0.1% trifluoroacetic acid (TFA)] and held at 40^0C. The initial solvent mixture was held for 2 minutes after loading. Protein was then eluted by a linear gradient of 0 to 28 % solvent B [90 % v/v ACN, 0.1 % TFA] from 2 to 4 min, 28 to 48 % solvent B from 4 to 6 min, 48 to 55 % solvent B from 6 to 8 min, 55 to 58 % solvent B from 8 to 9 min, 58 to 60 % solvent B from 9 to 14 min, 60 to 65 % solvent B from 14 to 15 min, and then 65 to 80 % solvent B from 15 to 16 min. Peaks were detected at 220 nm using a UV detector.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) - The composition of proteins in original juice and ion exchange fractions were assessed by SDS-PAGE in 4-20% Tris-glycine gels as described by Tattersall *et al.* (1997). Proteins were stained with Coomassie brilliant blue R-250.

Protein Trap Electrospray Mass Spectrometry (Trap-MS) - Samples were subjected to mass spectrometric analysis using a PE Sciex API 300 with ion spray ionization (Perkin-Elmer, Thornhill, Ontario, Canada) at the Mass Spectrometry Facility of the Waite Campus, University of Adelaide, as described by Hayasaka *et al.* (2001).

6.2.1.3 Desalting and concentration of VVTL1 rich fractions

Fractions rich in VVTL1 from batches processed using Methods 1, 3 and 4 (ie 3 x 25L lots, or 75L, in total) were combined, concentrated and desalted using a 3000 molecular weight (MW) cutoff cross-flow ultrafiltration cartridge and then freeze dried.

Gel Permeation Chromatography - The composition of final purified VVTL1 rich sample was assessed by gel permeation chromatography (GPC) essentially as described by Vidal *et al.* (2003) with the following modifications: The size-exclusion columns were connected to an Agilent 1100 series HPLC system (Agilent, Waldbronn, Germany) fitted in line with an Agilent G1362A refractive detector. The mobile phase consisted of 0.1 M $NaNO_3$.

Samples were concentrated 50 fold and desalted using 3kDa MW ultrafilters prior to analysis.

High Sensitivity Amino-acid Analysis - Protein quantitation by amino-acid analysis was performed by the Australian Proteome Analysis Facility (APAF) at Macquarie University. Samples underwent 24 hr gas-phase hydrolysis at 110^{o}C. Cysteine determination was performed using performic acid oxidation followed by 24 hr acid hydrolysis at 110^{o}C. All analysis was performed using the Waters AccQTag chemistry.

6.2.2 Experimental design of adsorption experiments

A two level (one quarter) fractional factorial experimental design was used to investigate the main effects of the factors: pH, temperature, ethanol concentration, potassium ion concentration, concentration of simple monomeric phenolic compounds, and sugar concentration on adsorption behaviour. The factors, and their associated levels (which are typical of the levels found in Australian white wines) (Gishen and Coulter, 2003), are shown in the Table 6.1.

Table 6.1: Experimental Factors – Fractional Factorial Screening Study

Factor	Description	Low Level	High Level
A	pH	3.0	3.4
B	temperature	5.2^{o}C	23.6^{o}C
C	% ethanol	10 %	13 %
D	[K]	400 mg/L	900 mg/L
E	[“phenolics”]	100 mg/L	200 mg/L
F	[“sugar”]	1.0 g/L	8.0 g/L

I=ABCE and I=ACDF were chosen as the generating relations. The experiment was performed in four blocks on four separate days; the treatment combinations in each block, are shown in Table 6.2 (a high level of any factor in the treatment combination is denoted by the corresponding lowercase letter; a low level of a factor is denoted by the absence of the corresponding letter; (1) denotes all factors at the low level).

Table 6.2: Block treatment combinations and slurry age– Fractional Factorial Screening Study

	Block 1	Block 2	Block 3	Block 4
Treatments	df, bdef, ac, abce	cef, be, acdf, abd	(1), bcf, ade, abcdef	cde, bcd, aef, abf

One of the benefits of the fractional factorial design employed is that if two factors are shown to be insignificant, the statistical design of the experiment permits them to be excluded from the analysis. Therefore the factorial design can be consolidated, or collapsed, into a full factorial design for the remaining factors. If three factors can be excluded, then the design can be consolidated into a duplicated factorial experiment of the remaining three factors, and so on. Even though certain factors may be eliminated, this type of experimental design does not bias the main effect for any of the factors, so it is *not* necessary to know in advance which factors are likely to be important. Thus a fractional factorial experimental design provides an efficient method of screening for significant factors and interactions.

6.2.3 White wine model solutions

Model wine consisted of the following components: Milli Q water; 10 or 13 % (v/v) ethanol (*Factor C*); 1.0 g/L potassium hydrogen tartrate (KHT), 400 or 900 mg/L potassium (by KHT and KCl addition) (*Factor D*); 72 mg/L sodium (by NaCl addition); 78 mg/L calcium (by $CaCl_2$ addition); 100 or 200 mg/L “phenolics” (15 % w/w catechin, 85 % w/w caffeic acid) (*Factor E*); 1.0 or 8.0 g/L “sugar” (25 % w/w glucose, 75 % w/w fructose) (*Factor F*). The concentration of each of these components is typical of Australian white wine. For each model wine, pH (*Factor* A) was adjusted to the required level (pH 3.0 or 3.4) and buffered by addition of tartaric acid. Model wine solutions were placed in a temperature controlled room (5.2 or 23.6^{o}C) (*Factor B*) 24 hours prior to conducting the adsorption experiments.

6.2.4 Bentonite slurry

A sodium bentonite, Volclay WG (Volclay Pty Ltd, North Geelong, Australia) was used for all adsorption experiments. The 0.2 % (w/v) slurry was prepared using Milli Q water at room temperature and stirred for 8 hours. The slurry was allowed to stand for at least 24 hours prior to use.

6.2.5 Adsorption tests

A stock solution of purified VVTL1 was prepared. Aliquots of this protein stock solution were put into 10 mL sample tubes, together with 4 mL aliquots of model wine solution. Aliquots (ranging from 330 – 500 μL) of the bentonite slurry were then added to the sample tubes to achieve different bentonite doses. Finally, Milli Q water was added to bring the total volume in each sample tube to 5 mL. This resulted in an initial concentration of VVTL1 of 74 mg/L. Three different controls were also prepared for each run: protein in model wine without bentonite; model wine and bentonite without protein; and model wine without bentonite or protein. The total volume for each control was also adjusted to 5 mL using Milli Q water.

All vials were vortex mixed and then placed on an orbital shaker table for two hours. After this, 1 mL aliquots from each sample tube were centrifuged (11500 g, five minutes). Following centrifugation, 800 μL of supernatant was drawn from each centrifuge tube and placed into a glass HPLC vial for analysis.

All experiments were conducted in temperature controlled rooms (5.2 or 23.6^{o}C) and performed in triplicate.

6.2.6 Protein assay

The equilibrium concentration of VVTL1 remaining in solution after adsorption was evaluated using reverse-phase high-pressure liquid chromatography (HPLC). Samples (100 μL) were loaded at 0.8 mL/min onto a C4 column (Brownlee Aquapore, 2.1 x 100mm, 7 μm, 300Å) equilibrated in a mixture of 75 % solvent A [10 % v/v acetonitrile (ACN), 0.1 % trifluoroacetic acid (TFA)] and 25 % solvent B [90 % v/v ACN, 0.1 % TFA] and held at 40^{0}C. The initial solvent mixture was held for 1 minute after loading. Elution was by a gradient of 25 to 70 % solvent B from 1 to 2 min, and then isocratically at 70 % solvent B for a further 2.5 min. Peaks were detected at 220 nm using a UV detector (Agilent 1100 series, Waldbronn, Germany) and compared to a VVTL1 standard curve to establish the concentration of VVTL1 in the sample.

6.2.7 Mathematical and Statistical Analysis

A Langmuir isotherm was regressed to the experimental adsorption data:

$$q(\hat{x}) = q_{\max}(\hat{x})\frac{K_L(\hat{x})c_e}{1+K_L(\hat{x})c_e} \tag{6.1}$$

where $q(\hat{x})$ is the mass of protein adsorbed per mass of bentonite (mg/mg), which depends on the factor levels represented by the vector $\hat{x}: x_1,...,x_9$ where $x_1,...,x_6$ were the value of the six factors (x_i = -1 for “low” and 1 for “high”) and $x_7,...,x_9$ were dummy

variables to describe block effects; $q_{\max}(\hat{x})$ was the maximum amount of protein adsorbed per mass bentonite (mg/mg), which was a function of $\hat{x}$:

$$q_{\max}(\hat{x}) = \beta_{0q} + \sum_{i=1}^{9} \beta_{iq} x_i \quad (6.2)$$

where β_{0q} and β_{iq} were the coefficients obtained by regression; $K_L(\hat{x})$ was the Langmuir equilibrium constant, also a function of $\hat{x}$:

$$K_L(\hat{x}) = \beta_{0K} + \sum_{i=1}^{9} \beta_{iK} x_i \quad (6.3)$$

β_{0K} and β_{iK} were the coefficients obtained by regression and c_e is the concentration of protein in solution at equilibrium (mg/L).

The Langmuir isotherm is non-linear. Therefore, regression was performed using the Microsoft Excel Solver tool (Frontline Systems, Inc. Nevada, USA) to find the values of regression coefficients (i.e. β_{iq} and β_{iK}) in Equations 6.2 and 6.3 which minimised the sum of the square of the errors between Equation 6.1 and the experimental data. The coefficient variances were estimated from a linearised version of Equation 6.1 to allow approximation of the asymptotic covariance matrix (Bates and Watts, 1988). This was used to estimate standard errors and hence the significance of regression coefficients according to the test statistic (Montgomery, 2001):

$$t_o = \frac{\beta_j}{se(\beta_j)} \quad (6.4)$$

where β_j was the value of the regression coefficient; and $se(\beta_j)$ was the standard error of the regression coefficient β_j.

6.3 Results

6.3.1 Purification of VVTL1

The chromatographic elution profile of *V. vinifera* cv. Muscat of Alexandria grape juice on SP Sepharose Fast Flow cation-exchange media is shown in Figure 6.1. The majority of juice proteins bound to the media was eluted with less than 200mM NaCl. Several peaks were resolved, many containing two or more different proteins (as determined by RP HPLC, data not shown).

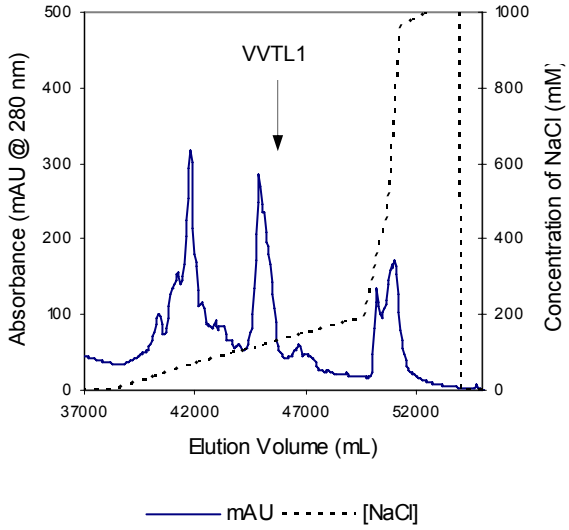


Figure 6.1: Cation-exchange chromatogram for elution of cv. Muscat of Alexandria grape juice (Configuration 4)

Cation-exchange fractions rich in VVTL1 were identified by reverse phase HPLC (Figure 6.2) and SDS-PAGE (data not shown). The fractions from the cation exchange chromatography containing the major grape protein VVTL1 are indicated (Figure 6.1). The estimated molecular mass of VVTL1 according to the SDS-PAGE analysis was approximately 22-23 kilo-Daltons (kDa) (± 2 kDa), which is consistent with molecular mass observed by others (Waters *et al.,* 1991, Tattersall *et al.,* 1997 and Pocock *et al.,* 2000) when using SDS-PAGE.

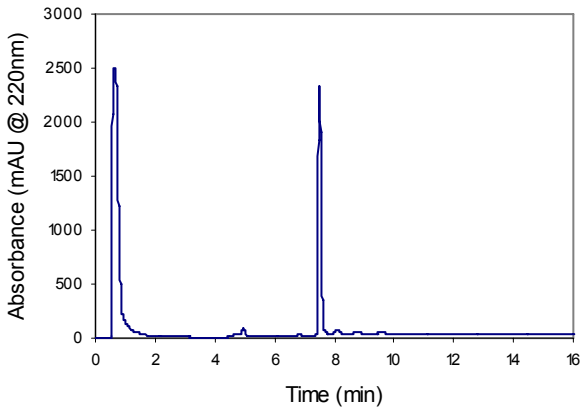


Figure 6.2: Reverse Phase HPLC chromatogram for elution of VVTL1 rich cation exchange fraction

Selected fractions from the cation-exchange method were also subjected to electrospray mass spectrometry. The molecular mass of 21272 obtained for VVTL1 is consistent with values reported by others (Tattersall *et al.,* 1997, Pocock *et al.,* 2000 and Hayasaka *et al.,* 2001) using this method. The anomalous migration during SDS-PAGE of PR proteins to positions corresponding to proteins that are several thousands of Daltons higher than molecular weight of the protein as determined by mass spectrometry (which has a typical resolution of ±5 Da) has been noted by several authors (Cusack *et al.,* 1988; Fils-Lycaon *et al.,* 1996; Pocock *et al.,* 2000).

Following ultrafiltration, purity of the concentrated VVTL1 fraction was confirmed using RP HPLC (Figure 6.3), SDS-PAGE (Figure 6.4), and GPC (data not shown). The amino-acid composition of the sample is shown in Table 6.3. These data are consistent with the amino-acid composition of VVTL1 as reported on the ExPASy website: (http://au.expasy.org). These data are also included in Table 6.3. Tryptophan was not detected using the acid hydrolysis technique employed in this study. The purity of the sample according to amino-acid analysis was 71 %. These results indicated that VVTL1 had been isolated from the other Muscat of Alexandria grape juice proteins. It is likely that the remaining impurities were cation- exchange buffer salts not entirely removed during desalting by ultrafiltration. A summary of the purification procedure is presented in Table 6.4. A high purification was achieved (221-fold after stirred cell ultrafiltration), with a final recovery of 5.6 % following freeze drying.

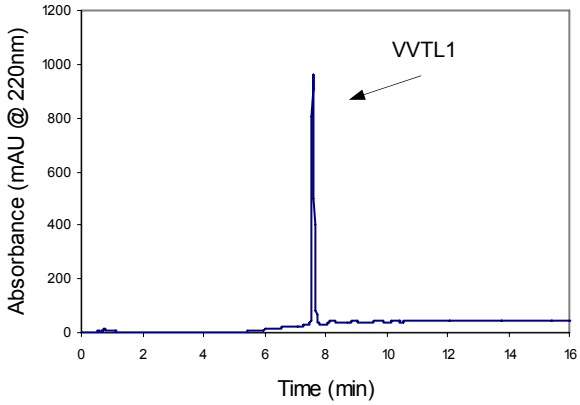
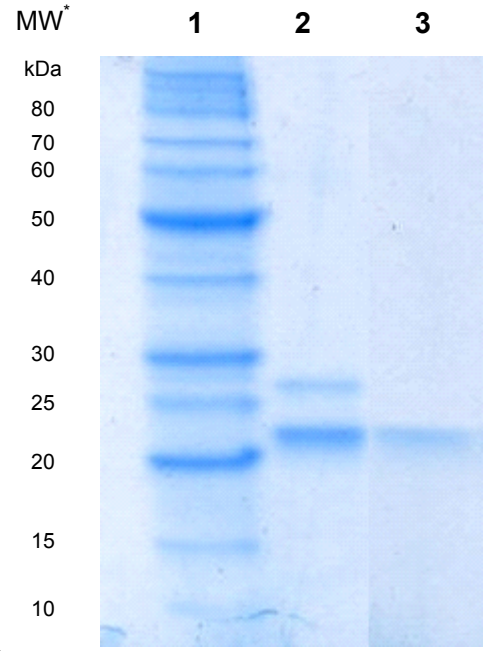


Figure 6.3: Reverse Phase HPLC chromatogram for elution of pooled VVTL1 fraction after ultrafiltration



*Invitrogen "Benchmark" Protein Latter Cat 10747-012

Figure 6.4: SDS-PAGE. Lane 1: Molecular Weight Markers; Lane 2: Juice; Lane 3: pooled VVTL1 fraction after ultrafiltration

Table 6.3: Amino-acid Analysis

Amino-acid	% Mass (μg/mg)	% mole of sample	% mole VVTL1*
Alanine	25.4	5.6	5.1
Arginine	34.3	3.5	3.5
Aspartic Acid + Asparagine	128.6	17.1	14.7
Cysteine	66.6	6.4	8.1
Glutamic Acid + Glutamine	55	6.6	5.5
Glycine	39	10.7	11.1
Histidine	2.6	0.3	0
Isoleicine	28.1	3.9	4
Leucine	36.7	5.1	5.1
Lysine	44.5	5.4	4
Methionine	3.9	0.5	0.5
Phenylalanine	41.5	4.4	5.1
Proline	46.5	7.5	7.6
Serine	36.1	6.5	7.1
Threonine	60.7	9.4	10.1
Tryptophan			1.5
Tyrosine	40.3	3.9	4
Valine	20.5	3.3	3
Total	710.3	100	100

*Source: http://au.expasy.org

Table 6.4: Purification of VVTL1 from *V. vinifera* cv. Muscat of Alexandria grape juice

Purification Step	*Mass of VVTL1 (mg)*	*Volume (mL)*	*Purification (fold)*	*Recovery (%)*
Grape juice	9911	75000		100
Cation-exchange	2695	5871	3	27.2
Cross-flow Ultrafiltration	766*	400*	14*	7.7*
Stirred Cell Ultrafiltration	702	24	221	7.1
Freeze Drying	554			5.6

*approximate

There were losses which were observed during the cation exchange and cross-flow ultrafiltration steps. The loss that occurred during cation-exchange may be due to breakdown of protein during the chromatography process or alternatively may be the result of protein remaining adsorbed on the chromatography column. The cross-flow ultrafiltration step was employed to quickly reduce the volume of the sample to a size suitable for processing in the stirred cell ultrafiltration apparatus in our laboratory (400 mL capacity). It is uncertain how protein loss occurred during this step but it may have been caused by protein denaturation or adsorption onto the membrane surface. Further work is required to optimise these steps, and so achieve the maximum benefit from direct loading of grape juice onto the cation-exchange column without the need for pre-treatment by ammonium sulfate precipitation and dialysis.

6.3.2 Adsorption tests

Table 6.5 shows the regression statistics for the fractional factorial design. The regression coefficients and their respective standard error and p–values obtained by fitting Equation 6.1 to the experimental data are shown in Tables 6.6 and 6.7. Factors deemed to be statistically significant ($p < 0.05$) are indicated by **.

The R^2, adjusted R^2 and p-values for the regression were 0.852, 0.802 and 4.68×10^{-17} respectively, indicating that the VVTL1 adsorption data were well characterised by the Langmuir isotherm. The regression analysis suggested that the main effects for pH, temperature and [K] were significant for $q_{\max}(\hat{x})$ but none of the factors were significant for $K_L(\hat{x})$. Table 6.6 also indicates that the block effects were important for $q_{\max}(\hat{x})$. As experiments were blocked and prepared on separate days subsequent to preparation of the bentonite slurry, bentonite age might have been an explanation for these effects on $q_{\max}(\hat{x})$. This was investigated by re-performing the regression by substituting slurry age (24, 120, 168 and 192 hours for blocks 1, 2, 3 and 4, respectively) for block dummy variables in Equations 3.2 and 3.3. The regression analysis for $q_{\max}(\hat{x})$ is given in Table 6.8.

Table 6.5: Analysis of Variance for Significance of Regression – Fractional Factorial Design

Source of Variation	Sum of Squares	Degrees of Freedom	Mean Square	F_0	p-value
Regression (incl. Blocks)	0.0753	19	0.00396	17.2	4.68E-17
Blocks	0.0261	3	0.00870	37.8	1.42E-13
Error	0.0131	57	0.00023		
Total	0.0884	76	0.00116		

Table 6.6: β_{iq} regression coefficients for the Fractional Factorial Design with Block Effects

Coefficient	i	Value	se(β_{iq})	p-value	Significance
constant	0	0.461	0.0055	1.83E-61	**
pH	1	-1.14E-02	2.42E-03	1.50E-05	**
temperature	2	1.55E-02	2.33E-03	1.30E-08	**
% ethanol	3	-9.78E-04	2.44E-03	0.69	ns
[K]	4	1.18E-02	2.43E-03	9.18E-06	**
[phenolics]	5	-1.89E-03	2.32E-03	0.42	ns
[sugar]	6	-1.70E-03	2.45E-03	0.49	ns
BDV 1	7	-6.71E-02	7.11E-03	2.96E-13	**
BDV 2	8	-4.14E-02	7.16E-03	3.32E-07	**
BDV 3	9	-2.17E-02	7.42E-03	4.90E-03	**

BDV = Block Dummy Variable

Table 6.7: β_{iK} regression coefficients for the Fractional Factorial Design with Block Effects

Coefficient	i	Value	se(β_{iq})	p-value	Significance
constant	0	14.5	1.87	1.56E-10	**
pH	1	0.003	3.51	1.00	ns
temperature	2	-0.321	1.49	0.83	ns
% ethanol	3	-0.007	3.53	1.00	ns
[K]	4	-1.329	3.53	0.71	ns
[phenolics]	5	-0.414	1.45	0.78	ns
[sugar]	6	-0.622	3.55	0.86	ns
BDV 1	7	8.021	13.7660	0.56	ns
BDV 2	8	4.287	3.9475	0.28	ns
BDV 3	9	0.244	2.8379	0.93	ns

BDV = Block Dummy Variable

Table 6.8: β_{iq} regression coefficients for the Fractional Factorial Design with Bentonite Slurry Age substituted for Block Effects

Coefficient	i	Value	se(β_{iq})	p-value	Significance
constant	0	0.385	4.97E-03	1.23E-62	**
pH	1	-1.03E-02	2.48E-03	1.04E-04	**
temperature	2	1.57E-02	2.47E-03	2.98E-08	**
% ethanol	3	-1.29E-03	2.53E-03	0.61	ns
[K]	4	1.11E-02	2.51E-03	3.95E-05	**
[phenolics]	5	-1.26E-03	2.43E-03	0.60	ns
[sugar]	6	-9.46E-04	2.50E-03	0.71	ns
slurry age	7	3.49E-04	3.71E-05	1.73E-13	**

The block effects appear to correctly correlate with slurry age for this parameter. pH, temperature and [K] were again the only significant factors; no factors or slurry age were found significant for $K_L(\hat{x})$ (data not presented).

As three of the factors (ethanol, phenolics and sugar) were statistically insignificant for both $q_{\max}(\hat{x})$ and $K_L(\hat{x})$, the fractional factorial design was consolidated into a duplicated full factorial design of the remaining significant factors. This allowed analysis of the two and three factor interactions between pH, temperature and [K]. The new regression analysis results for $q_{\max}(\hat{x})$ obtained for the full factorial design are shown in Table 6.9.

Table 6.9: β_{iq} regression coefficients the *Full* Factorial Design with Bentonite Slurry Age

Coefficient	i	Value	se(β_{iq})	p-value	Significance
constant	0	0.376	4.95E-03	1.50E-60	**
A	1	-1.02E-02	2.27E-03	3.49E-05	**
B	2	1.53E-02	2.27E-03	8.46E-09	**
D	3	1.17E-02	2.28E-03	3.36E-06	**
AB	4	1.92E-04	2.26E-03	0.933	ns
AD	5	-6.46E-03	2.59E-03	0.015	**
BD	6	2.56E-03	2.27E-03	0.263	ns
ABC	7	8.91E-04	2.26E-03	0.696	ns
slurry age	8	4.16E-04	3.88E-05	1.68E-15	**

The R^2, adjusted R^2 and p-values for the regression were 0.848, 0.804 and 4.46 x 10^{-18} respectively. In addition to the main effects for each factor, the interaction between pH and [K] was found to be significant for $q_{\max}(\hat{x})$. No main effects or interactions were significant for $K_L(\hat{x})$ (data not shown). Based on regression coefficients in Table 6.9, Figure 6.5 shows adsorption behaviour predicted by Equation 6.1 versus experimental data for two of the factor combinations which were investigated. Excellent agreement between experimental data and the Langmuir adsorption isotherms is observed. This is also evident in Figure 6.6, which presents a parity plot of VVTL1 adsorption predicted by Equation 6.1 versus all experimental data. The majority of predictions lie within ± 10 % of experimental values.

The significant interaction between pH and [K] is visualised in Figure 6.7, which shows a three dimensional response surface for $q_{\max}(\hat{x})$ as a function of these two factors.

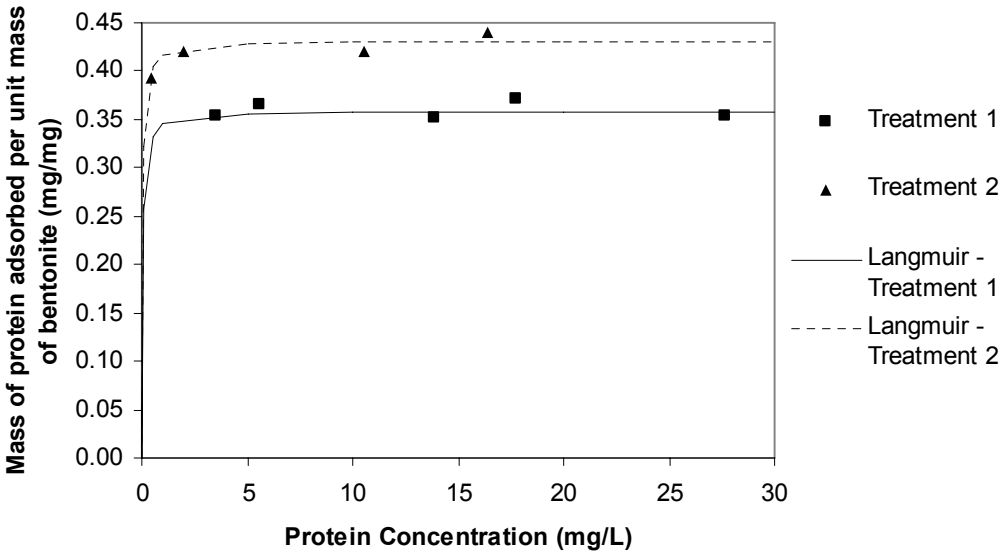


Figure 6.5: Typical Protein Adsorption Isotherms (R^2 = 0.848, p-value = 4.46 x 10^{-18})

Treatment 1 = high pH, low temperature, low [K], slurry age = 24 hours

Treatment 2 = low pH, high temperature, high [K], slurry age = 24 hours

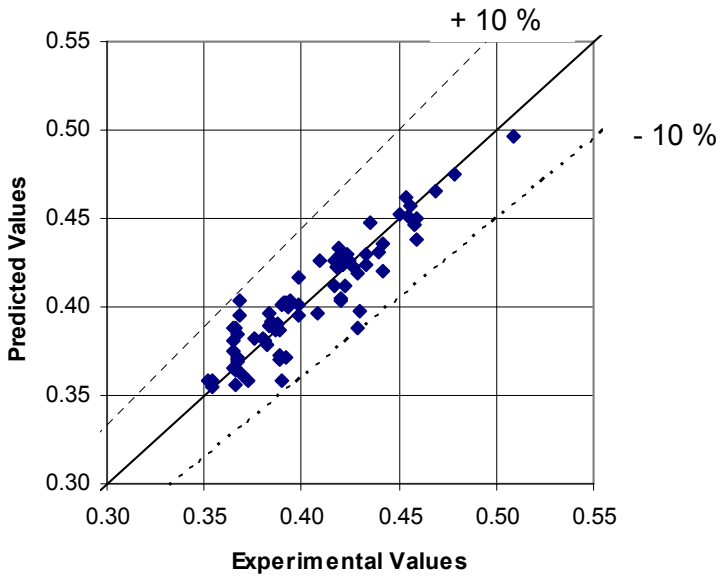
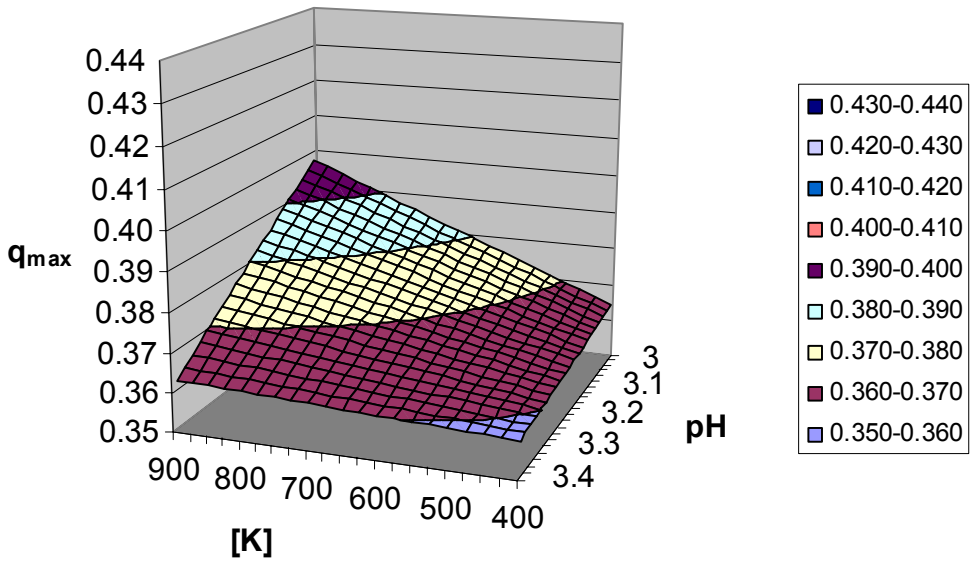
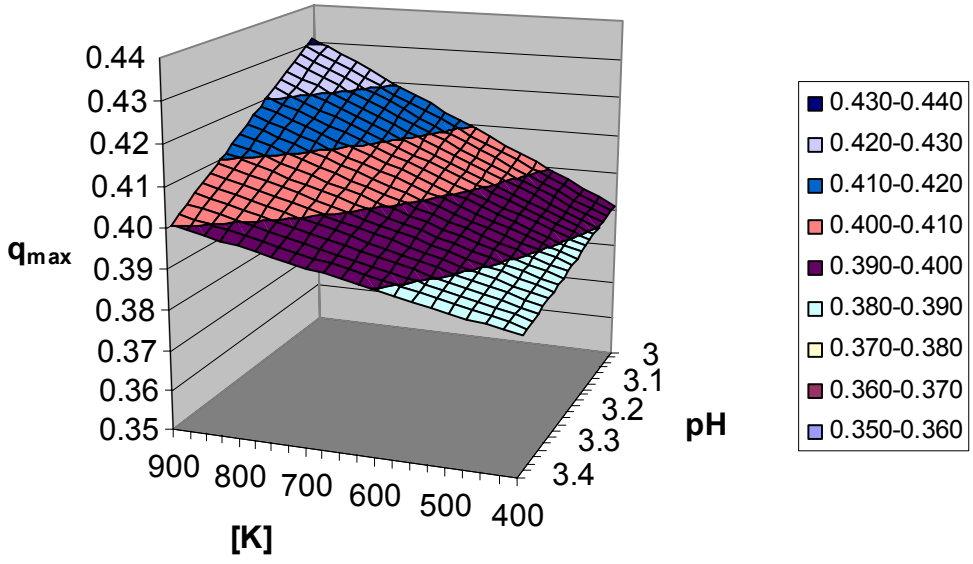


Figure 6.6: Parity Plot of q for the *Full* Factorial Design with Bentonite Slurry Age



(a)



(b)

Figure 6.7: Response surface showing q_{max} as a function of the level of Factor A (pH) and Factor D ([K]) for (a) "low" and (b) "high" temperature and a slurry age of 24 hours

6.4 Discussion

The Langmuir equation is commonly used to model adsorption behaviour in various systems. In this project, the Langmuir isotherm was also found to provide an excellent fit to experimental adsorption data for VVTL1. It also provided a mechanistic basis for more systematically investigating the effect of wine conditions on adsorption behaviour.

The statistical analysis of the *full* factorial study indicated that the main effects A (pH), B (Temperature) and D ([K]) and the interaction AD had a significant effect ($p < 0.05$) on the value of $q_{\max}(\hat{x})$. None of the main effects or interactions were found to have a statistically significant effect on the value of the equilibrium constant, $K_L(\hat{x})$.

Adsorption capacity, $q_{\max}(\hat{x})$, was found to be increased at "low" pH, and at "high" temperature. This is consistent with the observations of Rankine (1989), which state that "the more acid the wine, the more effective is the removal of protein" and "removal is more effective in warm than cold juice or wine". This differs however from the findings of Blade and Boulton (1988), in which adsorption capacity was found to be independent of temperature, and greater at pH 3.52 than 3.20. Fargues *et al.* (1998) report that although changes in pH will result in changes to the global or net charge of the protein, the *global* charge of a protein is rarely the "main parameter of retention" but rather it is the interaction of *local* exposed areas of charge which interact with the charged surface. This is consistent with the hypothesis of Gougeon *et al.* (2003) which states that (for their model system) it is polypeptide side chains only which adsorb within the interlayer spaces at the edge of the bentonite particle. A change in pH and/or temperature will result in a corresponding change in protein conformation (Norde *et al.*, 1978; Norde 1986; Fargues *et al.*, 1998) and therefore, the exposure of different charged groups. This will affect protein-ion, protein-protein and protein surface interactions, all of which are important in protein ion-exchange (Bosma and Wesselingh, 1998) and are specific to the protein-solution system (Norde *et al.*, 1978). Therefore, it should be noted that in the Blade and Boulton study, BSA was used as the model protein (rather than the authentic wine protein VVTL1). Due to differences in the size, conformation and presence of charged groups between BSA and VVTL1, these two proteins cannot be expected to necessarily display similar adsorption behaviour.

Achaerandio *et al.* (2001) studied the effect of ethanol concentration on protein adsorption. Increasing ethanol over the range of 0-8 % v/v was found to slightly increase the adsorption capacity of BSA. However, the adsorption capacity of BSA did not increase as ethanol was increased from 8 to 13 % v/v. In comparison, the adsorption capacity of ovalbumin was unaffected as ethanol was increased from 0 to 13 % v/v. In this project, the range of ethanol concentration studied was 10-13 % v/v and no effect was found.

Adsorption capacity was found to increase with [K]. One reason for this could be that the higher concentration of K^+ ions interferes with the charge potential within the boundary layer surrounding the bentonite particle. Related to this is the possibility of rapid exchange of K^+ ions for Na^+ ions on the bentonite surface. In a study by Chorom and Rengasamy (1996) investigating the physical and chemical properties of bentonite exchanged with different cations, samples of a natural bentonite were washed with different metal chloride solutions to change the exchangeable ion on the bentonite. They observed that potassium exchanged bentonite had a mean volume diameter over twice that of the sodium exchanged bentonite using the same starting material. Clearly the exchangeable ion affects the surface charge potential of the bentonite as well as swelling, which may increase the external surface area available for adsorption. This is significant given the study by Gougean *et al.* (2003), which hypothesised that proteins are adsorbed at the periphery of the bentonite particle, through interactions between side chains and the silicate sheets. Gougean *et al.* (2003) describe this as a 'plugging process', in that adsorption of side chains within the interlayer spaces but at the periphery of the particle block the interlayer space and prevent additional protein from accessing the interlayer surfaces for adsorption. Particle swelling and the resultant widening of sheet spacing would produce an increase in the surface area available at the particle edge, which should in turn provide a greater surface area for protein adsorption. Particle swelling may also be the explanation for the apparent correlation between $q_{\max}(\hat{x})$ and slurry age associated with block effects. Swelling occurs with slurry age, producing greater sheet separation and thus larger surface area.

Protein-ion, protein-protein and protein surface interactions are all important in protein ion-exchange (Bosma and Wesselingh, 1998), as is the role of pH in protein conformation and charge exposure. In fact the Steric Mass-Action Model (Bosma and Wesselingh, 1998) explicitly states that the interactions between local protein charge and ions in solution are a crucial part of the mechanism of protein ion-exchange. It is also possible that hydrogen ions may be exchanging with sodium ions to influence swelling and bentonite sheet spacing, as discussed above in the case of potassium. A "low" pH corresponds to an increase in the concentration of hydrogen ions in solution, which in turn may not just alter protein charge and the transport of proteins to the bentonite surface, but interact *together with* potassium ions to effect a change in bentonite swelling. This would support the observation of a significant AD (pH*[K]) two-factor interaction.

6.5 Concluding remarks

The adsorption of VVTL1 by sodium bentonite was well characterised by the Langmuir adsorption isotherm. pH, temperature, [K] and the two-factor interaction pH*[K] were found to have a highly significant effect on $q_{\max}(\hat{x})$. The equilibrium constant $K_L(\hat{x})$, was shown to be independent of all factors investigated.

Changes in pH and temperature affect protein conformation and the exposure of locally charged groups within the protein. It is these local areas of charge which interact with the bentonite surface. pH and potassium ion concentration affect protein-protein, protein-ion and protein-surface interactions, all of which are important in protein-ion exchange. Changes in pH and potassium ion concentration, as well as bentonite slurry age may also affect bentonite swelling and interlayer sheet spacing, with increased swelling resulting in an increase in the external surface area available for adsorption.

7 Bentonite preparation

7.1 Introduction

Preparation of bentonite greatly impacts on its protein adsorption capacity, and thus, its ability to prevent haze formation. Bentonite is a complex hydrated aluminium silicate ($Al_2O_3 \cdot 4SiO_2$-H_2O) with exchangeable cationic components (Zoecklein et al. 1999). In solution, bentonite can swell to a volume twelve to fifteen times its dry weight (Rankine 2004), vastly increasing its surface area. The structure of bentonite particles consist of mineral layers or platelets. During hydration, the charged platelets repel each other and separate, resulting in swelling of the bentonite (Zoecklein et al. 1999). The extent of swelling dictates the exposed surface area for protein binding. Protein removal is principally by cation exchange action (Blade and Boulton 1988).

Bentonite is available in both powder and granulated forms, however, the latter is more popular in Australia. Pre-swelling in water is a necessity for granulated bentonites but is also advisable for powdered bentonites (Ribéreau-Gayon et al. 2000).

Typically, bentonite slurries are prepared at concentrations of 50-150 g/L (Ribéreau-Gayon et al. 2000). Boulton et al. (1996) suggest that bentonite slurry preparations have optimum protein adsorption after two days soaking while Weiss et al. (2001) found no significant difference in fining performance between a 1 day old and a 30 day old bentonite slurry preparation.

Swelling of bentonite is faster in hot water than in cold water (Ribéreau-Gayon et al. 2000, Peynaud 1984, Rankine 2004), however, some agglomerated bentonites can be prepared in cold water (Weiss et al. 2001). Mixing of the bentonite is also important. Bentonite should be slowly poured onto the surface of the liquid as it is agitated to avoid lumps (Ribéreau-Gayon et al. 2000, Peynaud 1984). Vigorous mechanical mixing should be used (Rankine 2004).

Zoecklein et al. (1999) suggests that the water used in the hydration phase should have a low mineral content as dissolved metal cations present in slurry water preferentially replace sodium ions on the clay surface and detrimentally affect the hydration, viscosity and binding capacity of the bentonite. Rankine (2004) also advises that as pure water as possible should be used.

Swelling of bentonites in wine or direct addition of powdered bentonites is sometimes practiced. Marchal et al. (2002) compared the use of dry bentonite and bentonite swelled in tap water for 24 hours, on a Sauvignon white wine. They observed that the clarifying kinetics were identical for 0.1 g/L swelled bentonite and 1 g/L dry bentonite. For heat stabilisation, 0.3 g/L of swelled bentonite was required, compared with 0.6 g/L of dry bentonite. Based on these results, they concluded that the use of swelled bentonites was more promising. However, in the second part of their study (Marchal et al. 2003), they also

considered lees volumes after treatment and conducted an economic analysis. From these estimates, the dry bentonite was more promising.

Zoecklein et al. (1999) suggested that if bentonite is hydrated in the wine to be treated, rather than water, the lees volume should be about half that obtained by conventional hydration protocol. However, they advise that this may significantly reduce the binding capacity due to premature flocculation. Peynaud (1984) and Ribéreau-Gayon et al. (2000) also caution that preparation of bentonite in wine makes it coagulate and causes it to lose some of its clarifying properties. Swelling is limited in solutions containing ethanol because of the reduced dielectric constant, leading to a reduced binding capacity for proteins (Weiss et al. 2001). Rankine (2004) advises that bentonite suspensions should never be prepared in wine.

The above information suggests that the critical factors in bentonite hydration include time, temperature, agitation and the solution ethanol and ion content. Furthermore, optimal preparation would ideally be achieved with long periods of vigorous mixing in pure hot water. However, anecdotal reports from industry suggest preparation is often performed in cold impure water for periods of 5 to 8 hours with less than vigorous agitation.

In addition to the preparation of bentonite slurry, the method of addition and mixing with the wine has also been found to be important. In laboratory scale experiments, Weiss et al. (2001) found that the level of mixing following bentonite addition considerably influenced protein removal. Rankine (2004) states that bentonite treatment is a mixing problem and that to ensure rapid mixing, it is best to add the bentonite suspension on the suction side of a pump circulating wine in a vessel.

The purpose of this study during the project was to investigate how bentonite preparation affected protein adsorption and lees volume. The goal was a preparation method that minimised bentonite lees. A granulated sodium bentonite was chosen for this study as these are common in the Australian wine industry.

7.2 Materials & methods

7.2.1 Wine

A commercially produced 2006 Sauvignon Blanc wine was used for these experiments. It was sampled after it had been centrifuged from the yeast lees at the winery. The sample was collected and transported to the University of Adelaide in a 25 L high density polyethyelene drum. Some analyses of the wine are presented in Table 7.1.

Table 7.1: Analysis of the Sauvignon Blanc wine used in this trial

Characteristic	2006 Sauvignon Blanc
Alcohol	11.3 % v/v
pH	3.16
Turbidity	22 NTU
Heat-unstable protein	270 mg/L

7.2.2 Apparatus for bentonite preparation

Vitiben bentonite (BPM Minerals, Houston, TX, USA), a granulated sodium bentonite, was used for all experiments. Bentonite was prepared in Pyrex beakers fitted with removable stainless steel baffles constructed from sheet metal and wire. Overhead mixers (CAT R17, Staufen, Germany) fitted with 4-blade propeller type impellers were used to agitate the bentonite slurries. Propellers are used when strong vertical currents are desired, for example, when heavy solid particles are to be kept in suspension (McCabe et al. 2001). Both the impellers and the removable baffles were purpose-built at the School of Chemical Engineering workshop at the University of Adelaide. The dimensions of the apparatus are given in Figure 7.1.

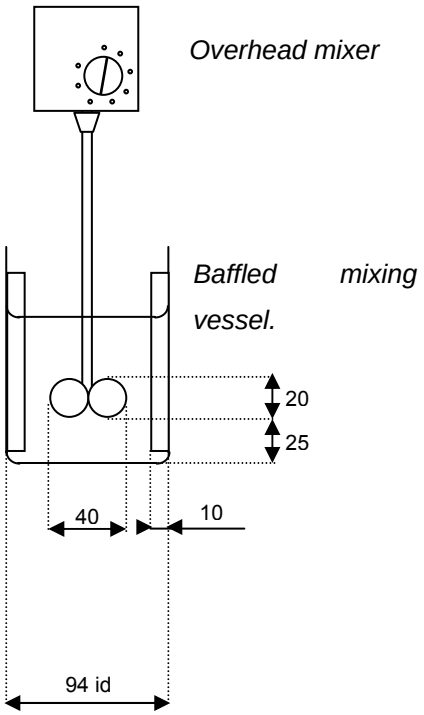


Figure 7.1: Mixer arrangement (All dimensions are given in mm). Note that there are actually 4 evenly spaced baffles. The two that run into the page are omitted from the diagram for clarity.

7.2.3 Fining Trial

A fining trial was conducted on the wine. The purpose of this was to assess the dose required for stability and to establish what doses would be suitable for subsequent experiments to compare the bentonite prepared in different manners.

The preparation protocol for the fining trial was to prepare 500 mL of 50 g/L bentonite in RO water. 25 g of bentonite was added in 3 batches (10g, 10g and 5g) at 10 minute intervals to 491 ml of RO water. Before each addition, and 10 minutes after the final addition, the baffles were rotated 1/8th of a turn so that any clumps of bentonite that had become wedged between the wall and the baffles were dislodged. The overhead mixer was run at 1000 rpm and the slurry was mixed for 5 hours (starting from when the first batch of bentonite was added to the water) before addition to the wine. 5 hours may be shorter than sometimes practiced in industry; however, the level of agitation achieved in such a small laboratory mixing vessel is likely to be much more vigorous than bentonite prepared on an industrial scale.

100 mL batches of wine had been placed in 100 mL stoppered glass measuring cylinders. Before addition of bentonite, these were inverted once to ensure homogeneity. Bentonite aliquots (0 – 2 mL) were then added with an autopipette. Cylinders were inverted twice and left to settle for 2 days.

After 2 days, lees volumes were recorded and 50 mL of supernatant withdrawn using a 10mL autopipette and collected in 50 mL capped polypropylene centrifuge tubes. Turbidity was measured using a turbidimeter (Hach 2100N, Loveland, CO, USA). Samples were refrigerated until heat test analysis could be undertaken.

7.2.4 Variations in bentonite preparation

A variety of different bentonite preparation conditions were trialled. A measure of lees volume and a measure of the protein removal effectiveness of the bentonite were required. Based on the results of the fining trial, doses of 0.5 g/L and 1 g/L were used for each comparison. 1 g/L was chosen because this was the approximate dose required for stability. A dose of 0.5 g/L was used to enable a more accurate comparison of each bentonite preparation's heat-stabilising effect. At the higher dose of 1 g/L the small quantity of residual protein makes it impractical to distinguish between subtle differences in protein removal effectiveness.

The experimental scheme was to manipulate one variable and to keep all other variables at the standard preparation conditions utilised in the fining trial. A control at the standard preparation condition was always prepared and all results have been normalised against this control. Experiments were conducted in duplicate. This duplicate is a repeat of the

experiment on a separate day with freshly prepared bentonite slurries. The sub-sections below describe the variables manipulated.

7.2.5 pH

Apart from the bentonite prepared without pH adjustment (pH 10), pH 3 and pH 7 slurries were prepared by addition of 3M L(+)-Tartaric acid 1 hour after beginning slurry preparation.

7.2.6 Fluid

Bentonite was prepared in RO and mains water and also in the wine itself. In a subsequent experiment, bentonite was also added directly (dry) to the wine without any pre-swelling.

7.2.7 Concentration

Bentonite was prepared at 30 g/L, 50 g/L and 70 g/L.

7.2.8 Time

Bentonite was prepared 1 hour, 5 hours and 24 hours before addition to the wine.

7.2.9 Temperature

Bentonite was prepared (and maintained) at 40˚C and 60˚C on hot plates in addition to the control at ambient conditions (~20˚C). For bentonite prepared at 40˚C and 60˚C, considerable evaporation was evident over the 5 hour preparation period. Therefore, the bentonite slurry volume was measured before addition, and the aliquot added was modified accordingly to account for any evaporation.

7.2.10 Speed

Bentonite was prepared with the agitator rotating at 600, 1000 or 2000 rpm.

7.2.11 Mixing

In addition to the control, which was mixed for 5 hours at 1000 rpm (“medium”), one lot of bentonite slurry was agitated at 2000 rpm for 1 hour and then at 600 rpm for 4 hours (“high-low”) and another was agitated for 2000 rpm for 1 hour and then stopped for 4 hours and then mixed for 5 minutes at 1000 rpm before use (“high-stop”).

7.2.12 Re-suspension

For this experiment, the same batch of bentonite slurry was used and addition was in the standard manner. The control was allowed to settle as normal for 2 days ("no resuspension"). For one other set of cylinders, the bentonite was gently resuspended by inverting the cylinders twice after 1 day before allowing it to settle for a further day ("gentle"). Another set of cylinders were also agitated after 1 day but instead of gently inverting them, they were vigorously shaken for 30 seconds before being left to settle for 1 further day ("violent"). Preliminary experiments had demonstrated that settling was complete in several hours.

7.2.13 Heat test and measurement of heat-unstable protein concentration

The heat test was similar to that suggested by Pocock and Rankine (1973) as this test is widely adopted in the Australian wine industry. Samples were all tested in duplicate. Each sample was filtered using low protein binding syringe filters (Acrodisc 25mm with 0.45µm Supor membrane; Pall, East Hills, NY, USA) into two 20 mL screw-capped glass tubes. Sample turbidities were measured. Samples were then placed in a water bath at 80˚C for 6 hours. They were cooled in iced water for 15 minutes and were then left at ambient conditions for a further hour before the turbidity was measured once again. Samples from a given trial were always tested in the same batch to mitigate the effects of any external influences.

While filtering the samples for heat test analysis, samples were also collected in glass hplc vials. Samples from the preliminary fining trial and other samples of particular interest (selected on the basis of the heat test results) were analysed directly for heat-unstable protein concentration using RP-HPLC as described by Muhlack et al. (2006). These were performed in duplicate and concentrations are expressed in terms of a protein standard, cytochrome c.

7.3 Results & discussion

7.3.1 Fining trial at standard preparation conditions

Figure 7.2 displays the results from the fining trial. The heat test turbidity at a given dose and the volume of wine occluded at that dose are reported. Approximately 1 g/L was required for stability. The volume of wine occluded at this dose was 9%.

The volumes of occluded wine given in Figure 7.2 are likely to exaggerate the volume of wine that would be occluded in industrial practice. Using small laboratory measuring cylinders, wall effects may be significant, whereas they are unlikely to be in a large tank. In addition, compressive forces on the lees in production-scale tanks will be larger because of the height of wine (and the higher sections of lees) pushing down on the lees.

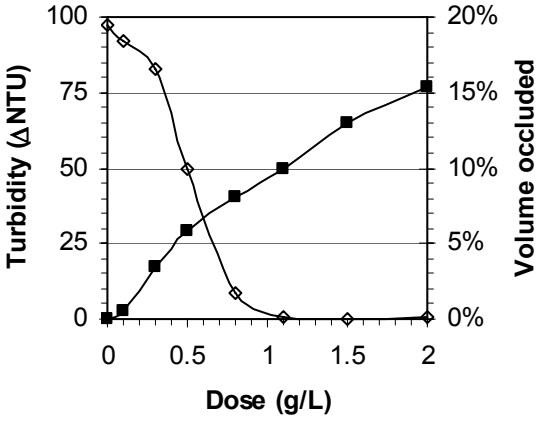


Figure 7.2: Fining trial results at standard preparation conditions.
(◇Heat test turbidity, ■ Volume occluded)

The turbidity of the supernatant (the turbidity measured after the supernatant has been collected off the bentonite lees) decreased dramatically with bentonite addition. The turbidity of the untreated wine was 22 NTU but this was reduced to approximately 3 NTU with the addition of 1 g/L of bentonite. However, this is not necessarily a true representation as some suspended bentonite flocs that did not settle under the influence of gravity were visible in the supernatant, but were not collected during the pipetting of the supernatant into sample tubes, as they were larger than the pipette tip orifice.

7.3.2 Different preparation conditions

Heat test results for each experiment were used to estimate the potential haze prevented, using the heat test haze produced by the untreated wine as a basis. Table 7.2 presents normalised experimental results for the different bentonite preparations relative to the

control preparation. The extra haze prevented at 0.5 g/L compared with the control is shown, along with the extra lees formed at 1 g/L.

The pH modified slurries were greyer in colour than the unadjusted slurry, and appeared to be slightly less viscous. They were less effective at removing protein on a mass basis and formed more lees than the unadjusted slurry, which had a pH of 10.05.

The preparation fluid had a noticeable effect on both protein removal and lees volume. Preparation in mains water reduced lees volume by approximately 20% at 1 g/L, but the haze removed at 0.5 g/L was approximately 30% less than the control. This reduction in protein removal corresponds with the observations of Zoecklein et al. (1999) and Rankine (2004). The effect of preparation in wine was even more marked. Lees volume was reduced by approximately 75% at 1 g/L, but protein adsorption was reduced to such an extent as to render the wine-swelled bentonite ineffective. Swelling of bentonite in wine is cautioned against by Rankine (2004) but it is practised in some circumstances, for example, if excessive water has been added to the wine to be treated. The Australian Standards (FSANZ, 2005) only allow 3 % v/v of water in the final product through the addition of additives and processing aids. These results suggest that bentonite should not be prepared in this manner. Experiments were also conducted involving direct addition of dry bentonite to wine. These were not successful. Lees volumes at 1g/L were less than 1%, but there was no measurable removal of protein at this dose. A granular bentonite was used for this experiment; the result may have been different if a powder bentonite was used with more agitation as Marchal et al. (2002 and 2003) did find some success with the direct addition of dry bentonite to wine.

Slurry concentration also had some effect on bentonite effectiveness. Bentonite prepared at 30 g/L performed similarly to that prepared at 50 g/L while bentonite prepared at 70 g/L was less effective at removing protein but also formed less lees.

Increased preparation time resulted in greater protein adsorption but a corresponding increase in bentonite lees. Temperature and speed had a similar effect. This is not surprising as these parameters are all expected to cause greater separation of the unit layers of the bentonite, resulting in an increased surface area for protein adsorption.

From information obtained on this scale, it is difficult to conduct a detailed cost-benefit analysis of bentonite prepared in different manners. However, estimates suggest that techniques that produced a smaller lees volume per gram of bentonite, would likely result in a similar lees volume for the heat stable wine, since a greater mass of bentonite would be required. On the basis of this, it seems sensible to prepare the bentonite in as pure water as possible with vigorous mixing. The bentonite dose required for stability is therefore minimised.

The experimental results reiterate that fining trials should mimic bentonite preparation techniques practiced in the cellar as closely as possible, otherwise large inconsistencies

may result. For example, the same type of water should be used. RO water should not be used in the laboratory if mains water will be used in the plant.

Resuspension of bentonite lees showed the most interesting results. This experiment was conducted to test the hypothesis that the resuspended bentonite may possibly resettle with an altered packing and form a smaller volume of lees. The key result from this experiment was that resuspension resulted in approximately 20% more (potential) haze removal compared with samples that were not resuspended. Lees volume was similar to the control, however supernatant turbidity was slightly higher. To corroborate this extra haze prevention result, samples from both replicates were analysed directly for heat-unstable protein concentration by RP-HPLC. These analyses showed a similar trend to the results derived from the heat test. Heat-unstable protein concentration was reduced by approximately 13%. To achieve this extra protein removal without the additional mixing, approximately 0.08 g/L extra bentonite would have been required.

Bentonite addition is a mixing problem and there are possibilities for reducing overall lees by contacting the bentonite more effectively with the wine. After addition of the bentonite slurry to the measuring cylinder, followed by inversion of the cylinders twicee, the bentonite appeared to be well dispersed and the mixture homogeneous. Despite protein removal being shown in a number of studies to be rapid (McLaren et al. 1958, Armstrong and Chesters 1964, Lee 1986, Blade and Boulton 1988), there was potential for further protein removal when the lees were resuspended 1 day later. A possible explanation for this increased protein adsorption after lees resuspension is the fact that bentonite quickly flocculates on addition to wine. On lees resuspension, the flocs may have been broken up, exposing further sites for protein adsorption.

Table 7.2: Summary of effectiveness of bentonite preparation method[a]

Condition	Haze prevented[b]	Lees
	(0.5 g/L)	*(1g/L)*
pH		
3	0.92	1.21
7	0.92	1.04
10.05*	1.00	1.00
Fluid		
RO water*	1.00	1.00
Mains water	0.72	0.79
Wine	0.13	0.27
Concentration		
30 g/L	1.03	1.01
50 g/L*	1.00	1.00
70 g/L	0.75	0.90
Time		
1 hr	0.85	0.86
5 hr*	1.00	1.00
24 hr	1.20	1.17
Temp.		
20 ˚C*	1.00	1.00
40 ˚C	1.16	1.15
60 ˚C	1.18	1.17
Speed		
600 rpm	0.93	0.90
1000 rpm*	1.00	1.00
2000 rpm	1.01	1.07
Mixing		
Medium*	1.00	1.00
High – low	0.93	0.94
High – stop	0.96	0.93
Resuspension		
No resuspension*	1.00 (1.00)	1.00
Gentle	1.21 (1.14)	0.99
Violent	1.20 (1.12)	0.96

[a]Results have been normalised against the standard preparation condition (denoted *)

[b]Where heat-unstable protein concentration was measured, protein removal was also normalised against the standard preparation condition and is shown in brackets.

7.4 Concluding remarks

In general, bentonite preparation techniques that resulted in decreased lees volumes were accompanied by decreased protein removal effectiveness. It is estimated that with the higher doses of bentonite required for stability, approximately the same volume of lees would be produced. Based on this, it seems most sensible to prepare bentonite in pure water with vigorous mixing.

The further protein removal observed after resuspension of bentonite lees highlights the importance of good contacting between the bentonite and wine. This improved protein adsorption is postulated to be due to the breakage of bentonite flocs exposing (previously fouled) sites for protein removal. Improved mixing that results in breakage of bentonite flocs may decrease the bentonite dose required for heat stability. A lower dose could produce smaller lees, and hence wine and value losses may be minimised.

8 In-line dosing of bentonite: Contact-time requirement?

8.1 Introduction

One example of a more efficient method for bentonite fining may be in-line dosing. This concept involves adding bentonite to wine in a pipe as it is pumped from one tank to another (or elsewhere). With sufficient contact time the bentonite may adsorb and remove the haze-forming proteins from the wine and render it heat stable. The bentonite would be separated from the wine by centrifugation or filtration.

The contact time to stabilise a wine by in-line dosing should only be a matter of minutes, as protein adsorption by bentonite is believed to occur relatively rapidly. For example, Blade and Boulton (1988) observed that maximum adsorption of bovine serum albumin (BSA) on bentonite occurred within 30 s. Whilst similar behaviour has not been directly demonstrated for protein in wine, Lee (1986) found that a Gewürztraminer wine became stable within one minute of bentonite addition. This might minimise any potential for adverse quality outcomes. Another obvious advantage would be a substantial decrease in processing time. Furthermore, use of centrifugation or filtration should compact the bentonite lees during separation from the wine, therefore reducing the volume of lees, and thus, the volume of occluded wine. This would maximise product recovery and minimise the value losses arising from quality downgrading of occluded wine recovered from the lees.

In-line dosing methods for bentonite fining are already used by some wineries and anecdotal reports suggest that commercial applications are growing. Despite this, however, there appears to be little published information and only a few explicit references to it can be found. Rector (1983) described in-line dosing of bentonite in a review of theories and uses of fining agents. Commercial use of the method in California during the 1980s was noted by Boulton (1986). Blade and Boulton (1988) refer to industry practices for bentonite fining as including the addition of the bentonite in-line with a retention time of 2 min before clarification by centrifugation. Zoecklin (1988) also suggested that protein removal from wine could be achieved by an in-line dosing approach. Unfortunately no specific examples of industrial application or substantial details of process configurations or performance were given in each of these instances. In particular it was not described what minimum contact time was necessary; if bentonite dose levels similar to batch processing were required; what pipe work arrangement and flow conditions were used; which methods were most efficient for separating the bentonite from wine or juice; whether treatment effects on wine quality were different (compared with batch fining); and what production efficiency gains or cost savings (if any) were achieved. Such information would clearly be invaluable for those contemplating in-line dosing, and also for wineries that are already exploiting the method for bentonite fining.

However, there was initial skepticism from industry that in-line dosing was a viable process; “otherwise wouldn’t it already be widely employed by now?” In particular it was doubted that protein adsorption could occur rapidly enough to avoid substantial pipe lengths or installation of sizable contact tanks. Furthermore, could additional compaction be achieved by centrifugation? Concerns were also raised that there might be an adverse sensory difference between batch-fined and in-line dosed wine.

This and the following sections (9 and 10) of this report document and presents the results of various investigations to fill some of these gaps in knowledge and to provide information that could permit commercial evaluation of in-line dosing as an alternative processing strategy for bentonite fining.

This first section presents the results of preliminary mathematical modeling of the in-line dosing process that were designed to overcome initial doubts regarding contact-time requirements. The favorable outcome of this preliminary mathematical modeling helped to persuade the industry sponsor, HWC, to commission a field test of in-line dosing at a commercial winery under production-scale conditions.

8.2 Contact time validation: Mathematical modelling

8.2.1 Model equations

The mathematical model describes protein adsorption by bentonite following bentonite injection into a contact pipe carrying the wine. The model equations make the following assumptions.

- Flow in the pipe is turbulent and hence bentonite particles are immediately and evenly dispersed in the wine.
- The frame of reference is moving with particles as they flow with wine along the pipe.
- Axial dispersion effects may be neglected.
- Plug flow: radial variations in particle transit time and concentration may be ignored.
- Adsorption occurs by external-film mass transfer of protein from bulk-phase to particle surface then adsorption on the particle surface.
- Adsorption may be described by a multi-component Langmuir equilibrium adsorption isotherm.

The dimensionless model equation describing the protein concentration in the bulk-phase is:

$$\frac{dc_i}{d\tau} = -\xi_i (c_i - c_{pi}) \quad \text{for } i = 1, 2, ..., N_S \qquad (8.1)$$

where $\tau = \frac{t}{(L/v)}$; $c_i = \frac{C_i}{C_{i0}}$; $c_{pi} = \frac{C_{pi}}{C_{i0}}$; $\xi_i = k_i a \frac{L}{v}$; t = time; N_S = number of species; C = bulk-phase concentration; C_p = liquid-phase concentration at particle surface; C_{i0} =

concentration value used to non-dimensionlise concentration of species i; L = pipe length; v = pipe velocity; k = external film mass-transfer coefficient; and a = particle surface area per unit volume.

The dimensionless model equation describing the protein concentration in the liquid-phase adjacent the particle surface is

$$\frac{dc_{pi}}{d\tau} = \xi_i \left(c_i - c_{pi}\right) - \frac{dc^s_{pi}}{d\tau} \qquad (8.2)$$

where $c^s_{pi} = c_{bi} \frac{C^s_{pi}}{C_{i0}}$; and C^s_{pi} = particle phase concentration.

The multi-component equilibrium Langmuir adsorption is:

$$C^s_{pi} = \frac{C^\infty_{pi} K_i C_{pi}}{1 + \sum_{j=1}^{N_S} K_j C_{pj}} \qquad (8.3)$$

where C^∞_p = maximum adsorption capacity; and K = Langmuir equilibrium constant.

For numerical convenience, the multi-component equilibrium Langmuir adsorption may be described by its non-equilibrium version to provide the final model equation for the particle-phase protein concentration:

$$\frac{dc^s_{pi}}{d\tau} = Da_{ai}\, c_{pi}\, c^\infty_{pi} \left(1 - \sum_{j=1}^{N_S} \frac{c^s_{pj}}{c^\infty_{pj}}\right) - Da_{di}\, c^s_{pi} \qquad (8.4)$$

where $Da_{ai} = \frac{k_{ai} C_{0i} L}{v}$; $Da_{di} = \frac{k_{di} L}{v}$; and k_{ai} & k_{di} = rate constants for adsorption and desorption processes, respectively, which are related to the equilibrium isotherm parameter by $K_i = \frac{k_{ai}}{k_{di}}$. Equation 8.3 simulates equilibrium conditions when Da_{ai} and Da_{di} are set sufficiently high (>1000) (Colby *et al.* 1996).

8.2.2 Numerical solution

Equations 8.1, 8.2, and 8.4 constitute a system of ordinary differential equations. These were solved to predict the bulk-phase concentration of protein as a function of contact time in the pipe using the “stiffr” differential equation solver of Mathcad 2000 Professional (Mathsoft, Inc.).

8.2.3 Model parameters

The key model parameters necessary to allow successful simulation of the process include mass-transfer coefficients and equilibrium isotherms for the residual grape

proteins in the wine; the adsorption surface area per unit volume; the initial protein concentrations; the bentonite dose level; and the diameter and flowrate of wine in the pipe.

The protein content of wine can be principally described by two major protein fractions (Waters *et al.* 2005): a 24 kDa fraction containing thaumatin-like protein; and a 32 kDa fraction consisting of chitanases. The adsorption equilibrium of these protein fractions with bentonite in real wines is not known. However, this project (refer Section 6) has measured values of adsorption for a purified thaumatin-like protein on a commercial sodium (Na) bentonite, Volclay WG, in model wine solutions. Values obtained for Langmuir adsorption isotherm parameters were approximately 0.45 mg/mg and 0.015 L/mg for c_p^{∞} and K, respectively. Consequently it was assumed that protein content in wine could be described by a single lumped fraction possessing these values of Langmuir adsorption isotherm parameters.

The internal diameter pipe and wine flowrate were 4" and 20000 L/hr, respectively. The viscosity was assumed to be 2 cP at a temperature of 10 °C. The Reynolds number (Re) for flow under these conditions was 33000. With turbulent flow in the pipe, the mass transfer coefficient was conservatively estimated assuming a Sherwood number (Sh) of 2. The protein diffusion coefficient (required for the Sh number) was estimated from a semi-empirical equation presented by Polson (1950). The molecular weight was assumed to be 32 kDa. Particle size measurements of several commercial bentonites by laser scattering suggested a mean-volume particle diameter of approximately 10 μm.

Protein concentrations in wine generally appear to vary between 30 to 250 mg/L depending on grape variety, viticultural practices and harvesting and processing conditions (Muhlack 2006). A value of 150 mg/L was used. V

Values given for available surface area of bentonites appear to range from 400 up to 800 ft^2/g (Muhlack 2006). A value of 500 ft^2/g was adopted. The bentonite dose was set to 0.5 g/L.

8.3 Results & discussion

The results of the simulation are shown in Figure 8.1. It suggests that protein adsorption will be rapid and complete within one to three minutes of bentonite addition.

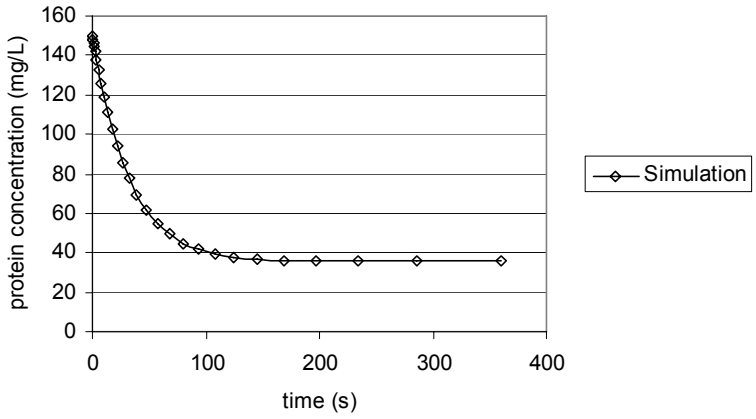


Figure 8.1: Protein concentration vs. contact time for initial mathematical modelling of in-line dosing process

8.4 Concluding remarks

The very simple model and simulation of in-line dosing with bentonite produced results that corroborate previous laboratory observations of rapid protein adsorption. This provides confidence that an industrial in-line dosing paradigm for bentonite fining would only require relatively short contact times to achieve the desired treatment effect.

9 In-line dosing of bentonite: field testing

9.1 Introduction

The section of the report presents results obtained from field testing of in-line dosing of bentonite for commercial application at the Stanley Winery in Buronga, Australia. The field tests were performed under production conditions using a purpose-designed in-line dosing system with centrifugation employed for bentonite separation and product clarification. The primary goals of the field tests were to (i) identify the contact period necessary for the stabilisation effect to be achieved and (ii) establish whether there was any significant difference in the treatment effectiveness between in-line dosing and conventional batch fining. The testing was conducted on a Gordo juice and Sultana wine using two commercial bentonites: Vitiben and SIHA-Active-Bentonite G. The heat stabilisation treatment effect was measured by heat-test (80 ºC for 6 hr) and HPLC analysis of "heat-unstable" protein content. Sensory effects were evaluated by duo-trio difference tests.

9.2 Materials & methods

9.2.1 Wine, juice and test site

The Stanley Winery, located at Buronga in the Sunraysia district of Victoria, has an annual processing capacity of 80,000 tonnes and a packaging capacity of approximately 40 million litres. The Sultana wine and Gordo juice originated from vineyards in the Sunraysia district and were processed at the winery during the 2004 Vintage. Single batches of wine and juice from individual tanks were used for the field tests. The tanks containing the wine and juice were continuously agitated and maintained at constant temperatures of 11 and 1 °C, respectively. This ensured that all testing was performed at a consistent wine or juice quality. Table 9.1 shows the values of alcohol, sugar, pH, acidity and SO_2 from analyses by the winery's laboratory and the heat unstable protein concentration and heat test turbidity of the wine and juice from samples taken from the tanks at the commencement of field testing.

Table 9.1: Physical and chemical properties of Gordo juice and Sultana wine[1].

Property	Sultana wine	Gordo juice
Alcohol, %	10.4	nd[2]
Residual sugar, g/L	1.9	31.4
pH	3.6	3.2
Volatile acidity, g/L	0.14	0.34
Total acid (pH 8.2), g/L	4	7
Free SO_2, mg/L	36	3
Total SO_2, mg/L	183	116
Heat unstable protein concentration, mg cyt. *c*/L	100	266
Heat test turbidity, NTU	4.1	100

[1]All analyses except heat unstable protein concentration were performed by the winery laboratory using standard methods.

[2]nd: Not determined.

9.2.2 Bentonites

Vitiben (BPM Minerals, Denver, USA) is a Wyoming bentonite of sodium (Na^+)-type. SIHA-Active-Bentonite G (E. Begerow GmbH & Co., Langenlonsheim, Germany) is a mixture of natural sodium and calcium (Na^+/Ca^{2+}) bentonite. Both Vitiben and SIHA-Active-Bentonite G are commercial products presently used by the wine industry for bentonite fining of wine. Information about physical and chemical properties of each product is available from their suppliers.

9.2.3 In-line dosing field tests

The in-line dosing field tests were undertaken during a five day time period between 20 and 24 September, 2004. Table 9.2 summarises the test conditions that were achieved, in chronological order. It was not feasible to randomise the order in which wine or juice and bentonite type were tested. Consequently, tests were performed sequentially with one wine or juice-bentonite combination at a time; however, the order of bentonite dose levels for each wine or juice-bentonite combination was randomised. Additionally the order of sample collection from sampling points was randomised.

Table 9.2: Test conditions achieved during field test, in chronological order.

Day	Wine or Juice	Bentonite	Dose, g/L
1	Gordo	Vitiben	0.51
			0.24
			0.97
2	Gordo	SIHA-Active-Bentonite G	1.49
			0.42
3	Sultana	Vitiben	0.05
			0.54
			0.31
			0.13
4	Sultana	SIHA-Active-Bentonite G	0.18
			0.87
			0.34
			0.59

9.2.4 In-line dosing system

The in-line dosing system was purpose designed for the field tests and almost entirely assembled from existing equipment and materials at the test site (Figures 9.1 and 9.2).

Untreated wine was pumped from a storage tank to an injection point, where bentonite slurry was continuously injected into the wine or juice. A static mixer was used to disperse the slurry and ensure even contact between bentonite and wine. A contact period of up to 5 min in pipe work was allowed before centrifugation with a Westfalia SC 150-06-777 clarifier (Westfalia Separator AG, Oelde, Germany) to separate the bentonite from the juice or wine.

All rigid pipe work in the in-line dosing system was 316 stainless steel. Various types of sanitary food-grade flexible hose available at the winery were employed to provide connections between rigid pipe work. The diameter of the pipe work or hose was either 3" (76.2 mm) or 4" (101.6 mm) for sections of pipe or hose conveying wine or juice, and 1" (25.4 mm) for the bentonite slurry.

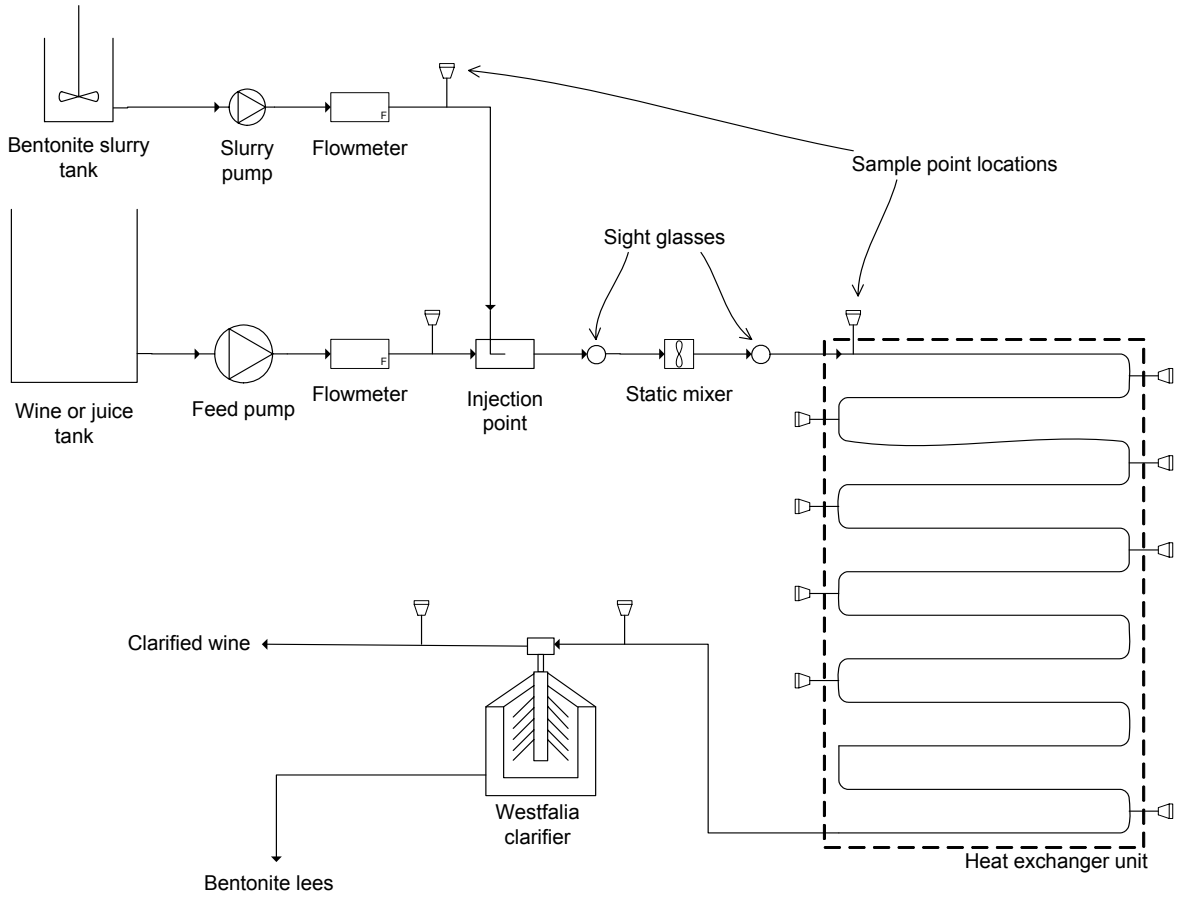


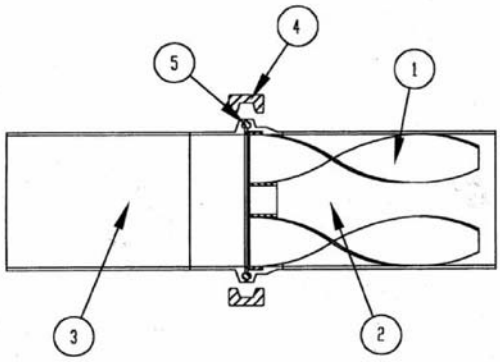
Figure 9.1: Process flowsheet illustrating the in-line dosing arrangement for field tests at the Stanley Winery.

An existing trolley-mounted pump unit (Mono Pumps (Aust) Pty Ltd, Mordiallac, Australia) used at the winery for transfer operations was employed as the feed pump for wine or juice. The pump unit consisted of a Packo FP2 50-200 single stage centrifugal pump head driven by an ABB three-phase, 2-pole, 18.5 kW AC motor. The power supply frequency to the pump motor was modulated using a Danfoss VLT 5000 Series variable frequency drive, which allowed control of motor speed, and thus pump flowrate.

The injection point was manufactured at the winery's workshop by winery technical staff. It was constructed from a segment of 3" pipe into which a ¾" (19.1 mm) centre-aligned hooked pipe was welded for injection of the bentonite slurry (Figure 9.2a). This configuration is only one of a number of feasible engineering designs for the injection point, which would enable bentonite slurry of differing concentrations and viscosities to be effectively introduced at the required flowrates into the pipe conveying the wine or juice.



(a)



Item No.	Description
1	Twister
2	Twister retaining tube
3	Clamp ferrule tube
4	Tri-clover clamp s/s heavy duty 3"
5	Tri-clover seal standard EPDM 3"

(b)

Figure 9.2: (a) Picture of injection point and (b) Drawing illustrating the internal arrangement of in-line static mixer.

The static mixer was of proprietary design and supplied by WE Ware & Co. (Adelaide, Australia). It consisted of a 3" pipe segment containing a central open ring with 3 twisted ribbon elements spaced equally around the ring's annulus (Figure 9.2b).

An existing shell-and-tube heat exchanger unit (manufactured by Industrial Refrigeration Services Pty Ltd, Port Adelaide, South Australia) was used to provide the contact pipe work. The single tube section of the heat exchanger was divided into 12 straight segments connected by bends. The diameter of the tube was 4" (101.6 mm). The purpose of the heat exchanger was solely to provide contact time between bentonite and wine or juice following the injection point and prior to the clarifier; there was no heating or cooling.

The Westfalia SC 150-06-777 clarifier is a self-cleaning disc-stack centrifuge that is widely employed in the Australian wine industry for juice or wine clarification. The bowl has a diameter of 782 mm, is hermitically sealed and operates at a speed of approximately 4800 rpm during operation producing a centrifugal 'G' force of 10070×*g* at the bowl periphery. The total bowl volume is 62 L with a sediment holding space of 25 L. At Stanley Winery this clarifier is usually operated at flowrates between 8000 and 30000 L/hr depending on

solids content of the wine or juice and the clarification objective. Partial-ejection of solids is normally performed during operation. When a partial injection occurs, solids in the bowl are ejected from the sediment holding space to a storage chamber. The solids are subsequently flushed from this storage chamber with water and discharged from the clarifier. Ejection events are either time-dependent (operator set) or automatically controlled based on transmittance of clarified product.

For the purposes of the field tests the flowrate selected for operation of the inline dosing system and to the clarifier was 12000 L/hr. This value was selected in consultation with technical personnel at the winery, who, based on their operating experience, believed that this would be sufficient for achieving effective separation of bentonite from the wine or juice. The residence time of wine or juice in the bowl of the clarifier at this operating flowrate was approximately 18 s. The operation of the centrifuge was set to intermittent partial discharge with discharge events activated by timer every 10 min.

The volumetric flowrates of bentonite slurry and wine or juice (prior to bentonite injection) were monitored by ABB MagMaster 1" and 3" electromagnetic flowmeters (ABB Australia Pty Limited, Sydney, Australia), respectively. Rigid pipe work equivalent to at least 10 pipe diameters in length were fixed to the inlet and outlet flanges of each flowmeter to ensure that the flow was axisymmetric and free of swirl. The flowmeters had previously been calibrated with water by the manufacturer. These calibrations would be unaffected by the differing fluid viscosity or density of the wine, juice or bentonite slurry. The accuracy of flow measurement by the electromagnetic flowmeters according based on the manufacturer's specifications was approximately ± 0.5% for the wine and ± 1-2% for the bentonite slurry based on the pipe velocities at the flowrates that occurred during the field tests.

Sample cocks (¼", 6.4 mm) (WE Ware & Co., Adelaide, Australia) were installed to allow collection of samples from various pipe work locations (refer Figure 9.1 and Table 9.3): bentonite slurry dosing pipe work pre-injection point; raw wine or juice pre-injection point; at inlet and selected hairpin bends of contact pipe work; and at the inlet and outlet of centrifuge.

Table 9.3: Locations and contact times (where applicable) for sampling points.

No.	Location[1]	Contact time (s)
-1	Bentonite slurry, pre-injection point	-
0	Untreated wine, pre-injection point (Raw wine or juice)	0
	Heat exchanger	
2	- Inlet	10
3	- Bend 1	33
4	- Bend 2	55
5	- Bend 3	78
6	- Bend 4	100
7	- Bend 5	123
8	- Bend 6	146
9	- Bend 8	191
10	- Bend 11	260
	Centrifuge	
11	- Inlet	360
12	- Outlet	-

[1]Refer to Figure 9.1.

9.2.5 Bentonite preparation and dosing system

Bentonite slurries were prepared in two existing tanks already used for this purpose at the winery. The tanks were both cylindrical and flat-bottomed with an approximate diameter of 1.5 m and height of 2.3 m. The tanks were baffled and fitted with identical top-mounted mixing units (manufacturer unknown), each with a 1 kW motor and a single impeller consisting of two right-angle blades located at the base of the shaft. The impeller position was approximately two thirds of the depth of the tank. An existing positive displacement pump (manufacturer unknown) was also used for 'pumping-around' slurry in the tank during preparation.

The bentonite slurry (Vitiben or SIHA Active-Bentonite-G) was prepared by hydration in potable tap water as 4 % w/v slurry. The preparation tanks were filled with water and the bentonite was slowly added manually to each tank until the required concentration was achieved. It took approximately 30 min for bentonite addition to be completed. During bentonite addition and thereafter until testing was complete, the tanks were continuously agitated by the mixing units to disperse and keep the bentonite slurry suspended. As well, 'pumping-around' of the bentonite slurry was performed during the first 2 hr after bentonite addition to assist with de-agglomeration and dispersing of bentonite particles.

The testing period on each day was between 10 and 14 hr. Weiss et al. (2001) observed that there was no difference in performance of bentonite fining between slurries hydrated

for one day and longer. Therefore the bentonite slurry was always prepared the previous day in the morning. This ensured approximately 24 hr of hydration prior to first use on the day of testing.

The bentonite slurry was pumped from the preparation tanks to the injection point using a Mono Pump PLF50211J8/C helical screw positive displacement pump with direct current motor (Mono Pumps (Aust) Pty Ltd, Mordiallac, Australia). The pump's motor speed, and thus slurry dosing rate, was controlled with a MotorMaster 20000 series electrical speed controller (Minarik, Glendale, USA).

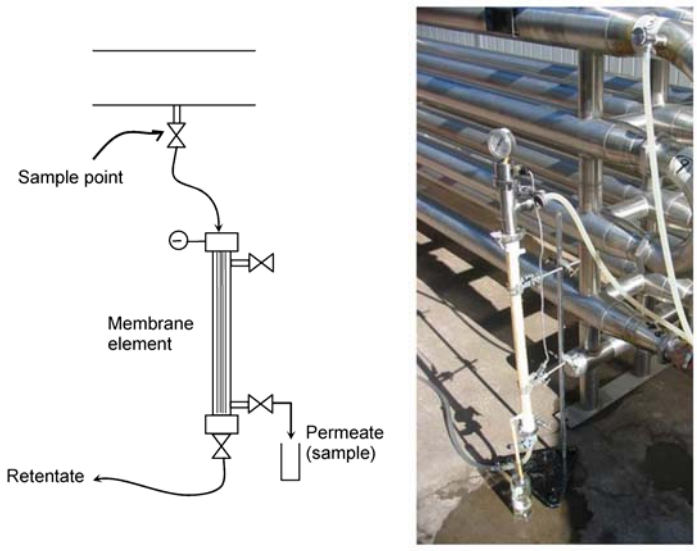
9.2.6 Sampling system

A cross-flow microfiltration sampling system was employed to separate bentonite from wine or juice at sampling points following the injection point and prior to the clarifier. Two identical sampling systems for the field tests were constructed. The arrangement of these sampling systems is shown schematically in Figure 9.3a, and a picture of one of the sampling systems as set up at the winery during the field tests is shown in Figure 9.3b. During field testing the sampling systems were connected to sample cocks at sampling points of the in-line dosing system. The sample cocks were opened to allow bentonite-laden wine or juice to flow through the lumen of the microfiltration membrane element. The membrane elements were allowed to operate under cross-flow conditions to prevent bentonite particles from collecting on and fouling the membrane surface. Part of the wine or juice in the lumen passed across the membrane into the shell of the membrane element and was discharged from an outlet port as permeate or filtrate and collected for analysis. The flow rate of filtrate was controlled by adjusting the transmembrane pressure (between lumen and shell).

The microfiltration sampling systems were connected to sample cocks with ½" (12.7 mm) silicone (platinum cured) Masterflex® tubing (Cole-Palmer Instruments, Vernon Hills, USA) flexible tubing. At the microfiltration sampling system, this tubing was attached to a short section of ½" 316 stainless steel tube pipe welded to a 316 stainless steel tri-clover flange. This flange was attached to a 1.5" (38.1 mm) tri-clover flanged 316 stainless steel tee piece with a standard bourdon-type pressure gauge (Ametek USG, Feasterville, USA). The tee piece was mounted on the inlet fascia of the membrane element. An unlabelled ½" threaded plastic ball valve (manufacturer unknown) fitted to a 1.5" plastic tri-clover type flange was mounted on the outlet fascia of the membrane element. The ball valve was used to throttle the flow through the membrane element, and thus, permit regulation of the transmembrane pressure.

The membrane fibres in the microfiltration elements were polysulfone with a nominal pore rating of 0.1 μm, lumen of 3 mm internal diameter and nominal flow path of 60 cm. There

were 33 fibres in each element providing a total membrane area of 0.17 m^2 with lumen and shell volumes of 145 and 120 mL, respectively. The nominal pore rating of 0.1 μm for the membrane was selected after particle size analysis by laser scattering with a Malvern System 2600 Particle Sizer (Malvern Instruments Ltd, Malvern, England) of aqueous suspensions for each bentonite. The suspensions were prepared by hydration in Milli-Q water for 24 hr. The mean particle volume diameters recorded were 6.9 and 10.1 μm for Vitiben and SIHA Active-Bentonite-G, respectively. In each case, no particles less than 0.8 μm were observed. Therefore the 0.1 μm-rated membrane was able to effectively separate the bentonite from the juice or wine.



(a) (b)

Figure 9.3: (a) Basic schematic of microfiltration sampling system; and (b) Picture of sampling system in operation during field tests.

9.2.7 Sampling system: field test sampling protocol

The microfiltration sampling systems were connected to sample cocks, which were opened. The ball valve at the outlet of the membrane element was adjusted to increase trans-membrane pressure to approximately 15 to 20 psi (100 to 140 kPa) and achieve the desired flow of filtrate. The sampling protocol adopted for collecting filtrate samples for analysis was as follows. The shell side was operated in an empty or drained mode, so that filtrate emerging onto the lumen external surface would flow by gravity down the lumen to the base of the membrane shell where it would accumulate and discharge from the permeate port. A 375 mL volume of filtrate was allowed to discharge from the permeate port before a sample was collected. This was designed to wash away any residual filtrate present on the lumen and in the shell housing from the preceding test condition. The sampling system was allowed to operate for a further 20 min before samples of filtrate were collected.

For analysis of treated wine or juice by heat test, samples were collected in a 375 mL (flint claret) bottle. A small amount of dry ice was added to each bottle prior to sealing with a natural cork. Samples for HPLC analysis of heat test turbidity were collected in 10 mL capped polypropylene tubes; no dry ice was added.

Samples of raw and clarified wine or juice were also collected for sensory analysis. These samples were not micro-filtered. The samples were collected in 750 mL (heavy sparkling style) bottles; 6 × 750 mL bottles were obtained for each sample. Dry ice was added and the bottles crown sealed.

All samples collected during the field tests were stored at 4 °C for later analysis.

9.2.8 Sampling system: potential for protein binding on membranes

Prior to field tests, a laboratory experiment was performed to investigate potential for protein binding by membrane elements of the microfiltration sampling systems. Model wine solution (150 L) was prepared in a 200 L 316 grade stainless steel tank. The model wine solution was Milli-Q water with 1 g/L potassium bitartrate, pH adjusted to 3.2 using L-tartaric acid. BSA (96 % pure, Cohn V fraction; Sigma-Aldrich, Missouri, USA) was added to achieve a concentration of approximately 80 mg/L. A Masterflex® peristaltic pump with Model 7529-20 pump head (Cole-Palmer Instruments, Vernon Hills, USA) was used to pump the model wine solution from the tank through the membrane element. All pipe work connections were with silicone (platinum cured) Masterflex® tubing (Cole-Palmer Instruments, Vernon Hills, USA) flexible tubing.

The sampling system was flushed with Milli-Q water and then drained prior to commencing the experiment. The model wine solution was pumped through the sampling system. The flowrate and transmembrane pressure were 5 L/min and 10 psi (65 kPa), respectively. This was the maximum flowrate that could be achieved with the peristaltic pump unit. The field test sampling protocol was followed. After 20 min, samples of the model wine solution from the tank and of the filtrate from the sampling system were collected. The protein concentration of the samples was compared by HPLC using the same technique employed for analysis of heat unstable protein.

9.2.9 Sampling system verification during field tests: steady state operation

There was no opportunity to validate the sampling protocol for the microfiltration sampling systems prior to the field tests. Therefore, samples from the microfiltration sampling system were collected every minute from a sampling point during a transition in operating condition for one of the field test scenarios. The samples were analysed by HPLC for heat unstable protein to observe whether stable or steady state conditions were achieved by the field test sampling protocol. The flowrate of retentate and permeate were also

measured using a cylinder and stopwatch, to evaluate the hydraulic conditions present in the microfiltration element.

9.2.10 Quantification of bentonite carryover during field tests

During the field tests there was carryover of bentonite observed during the centrifugal clarification step. This was not expected to occur, especially as the operating flowrate through the centrifuge had been reduced as a precautionary measure. Several samples were taken during fining of Sultana wine with SIHA-Active-Bentonite G to evaluate the extent of carryover. The samples were taken 5 min after a discharge event. The solids content was quantified by spin testing to measure the volume of sediment present in samples. The spin tests were conducted in 10 mL tapered tubes using a laboratory centrifuge available at the site, which was supplied with the Westfalia SC150 system when originally installed. The spin tests were performed at 3000 rpm (approximately 1400×*g*) for 5 min. The volume of solids in the clarified wine or juice was compared with that in bentonite-dosed samples taken before the centrifuge to estimate the proportion of bentonite carry over.

9.2.11 Batch-wise bentonite fining trials

The batch fining trials were undertaken separately by winery personnel after the in-line dosing field tests were completed. These trials were performed in cylindrical 4 kL tanks normally used at the winery for yeast preparation. The bentonite slurries were prepared identically to that for in-line dosing field tests using the same equipment. Appropriate bentonite doses were determined by bench-top batch fining tests in 100 mL graduated measuring cylinders at the winery's laboratory. The required volume of bentonite slurry was added to tanks by pumping it through the racking valve at the bottom of the tank. The tanks were subsequently pumped around for several minutes to ensure even dispersion and contact of the bentonite with the wine or juice. Settling was allowed to occur for six days. Clarified wine was collected from the top of the tank and samples were taken for heat testing to evaluate heat stability. The samples were collected and handled in an identical manner to the samples taken for in-line dosing tests. Wine or juice was judged to be heat stable if the difference in turbidity after heat test was less than 2 NTU, the winery's criterion for heat stability. If the wine or juice was not heat stable, batch fining was repeated until this was achieved. Samples were then collected for sensory comparison with the samples from in-line dosing field tests.

9.2.12 Heat tests

All heat testing was performed in the winery's analytical laboratory with a procedure similar to that of Pocock and Rankine (1973). Turbidity measurements were performed with a 2100N Hach turbidimeter (Hach Company, Colorado, USA) and recorded as nephelometric turbidity units (NTU). The sample was filtered using a disposable 0.45 μm cellulose nitrate membrane filter and dispensed into 2 × 20 mL (original and duplicate) capped glass tubes. Initial turbidity in each tube was measured. The tubes were placed in an oven at 80 ˚C for 6½ hr, after which they were immediately cooled to room temperature by immersion in an ice bath. The final turbidity values in each tube were measured and the difference between final and initial values recorded as the heat test turbidity value.

9.2.13 HPLC analysis for heat-unstable protein and BSA

The HPLC method was similar to a reversed phase technique that has been developed by Peng et al (1997) to identify and quantify the principal heat-unstable proteins in juice or wine. These are believed to primarily consist of chitanases and thaumatin-like proteins (Waters et al. 2005).

A Brownlee Aquapore C4 column (Perkin Elmer, Massachusetts, USA), 100 × 2.1 mm, 7 μm, 300 Å was used. The column was equilibrated in solvent A [10 % v/v acetonitrile (ACN), 0.1 % v/v trifluoroacetic acid (TFA)] and held at 40˚C. The initial conditions were maintained for 4 min after injection of the 100 μL sample. From 4 to 6 min the solvent B [90 % v/v ACN, 0.1 % v/v TFA] increased to 28 %, then to 48 % at 11 min, 55 % at 14 min, 58 % at 15 min and a maximum of 60 % B at 18 min before re-equilibrating in solvent A for 2 min prior to injection of the next sample. Flow rate was 0.8 mL/min and peaks were detected at 220 nm. An Agilent 1100 series system was used with auto-sampler and diode-array detector.

The heat unstable protein fractions were identified from their retention time (Peng et al. 1997, Pocock et al. 1998, 2000, 2003, Waters et al. 1998). Protein concentration was quantified by comparison with measured peak areas of a protein standard: horse heart cytochrome *c* (cyt. *c*).

9.2.14 Sensory analysis

The sensory analysis took place at AWRI seven months after the field tests. It was only conducted on the Sultana wine. The sensory comparisons were performed as balanced reference duo-trio tests following the methods described by Australian Standard (AS) 2542.2.4 - 1988: Sensory analysis of foods – Specific methods – Duo-trio test. Panellists were presented with a reference sample and two test samples of which one ~~is~~ was the

same as the reference. Each test sample was presented as the reference an equal number of times. The panellists were required to indicate which test sample matched the reference. The results were analysed using a binomial test.

Prior to testing, 400 mL lots of each sample from 2 × 750 mL bottles were clarified with a Beckman laboratory centrifuge at 4 °C at 12000 × *g* (max.) for 20 min. Clarified samples were combined in a 1.5 L bottle and stored overnight before analysis.

The sensory panel consisted of fifteen experienced, screened and qualified assessors. The samples were presented to the assessors in 30 mL aliquots in a 3-digit coded, covered, ISO standard wine glass at approximately 22 °C, in isolated booths under sodium lighting. The presentation order was randomised and replicated. Data analysis was performed as specified in AS 2542.2.4. As a replicated test with fifteen assessors was carried out, the method outlined in Stone & Sidel (1993) was also used to establish whether the response between first and second replicate was significantly different.

9.2.15 Curve fitting and regression analysis

Plots of various experimental data, e.g. heat unstable protein and heat test turbidity, collected during the field trials were prepared to investigate system behaviour, e.g. in-line dosing response *vs.* contact period. This system behaviour was often non-linear. Curves of different functionality (as described later in Results) were therefore fitted to the experimental data to aid in interpretation and better illustrate or verify behavioural trends.

This was performed by ordinary least squares (OLS) non-linear regression (Bates and Watts, 1988). Best fit regression parameters for the function describing the curve were determined using the Microsoft Excel Solver tool (Frontline Systems, Inc. Nevada, USA) to minimise the sum of the squares of residuals or errors, i.e. SSE, between predicted curve values and experimental response data. The Microsoft Excel Solver tool uses the Generalized Reduced Gradient (GRG2) nonlinear optimization code (developed by Leon Lasdon, University of Texas at Austin, and Allan Waren, Cleveland State University).

The curve fits were also used to identify outliers in experimental data. Studentised residuals for individual data points were estimated (Comuzzi et al. 2003). Generally, a data point was considered to be an outlier if the studentised residual exceeded a threshold value of 2. Where appropriate, outliers were removed from experimental data and curve fitting repeated with the amended data set.

9.3 Results

9.3.1 Sampling system: Potential for membrane protein binding

The BSA concentration in the model wine solution and permeate as determined during the filter validation test was 82 and 83 mg cyt. *c*/L, respectively, averaged from two separate HPLC analyses. This indicates an absence of protein binding by the membrane which otherwise could have affected results during field tests.

9.3.2 Field test sampling protocol validation

The field test sampling protocol validation was performed at a contact time of 260 s following a transition in bentonite dose level from 0.5 to 0.3 g/L for in-line dosing of Sultana wine with Vitiben. The transmembrane pressure of the microfiltration element was 8 psi (55 kPa), and the retentate and permeate flowrates were 21 L/min and 84 mL/min, respectively. Under these conditions, the cross flow velocity of wine in the lumen was 1.5 m/s, and the flux rate – the filtrate flowrate per unit membrane area – was *ca.* 30 L/m^2hr.

Figure 9.4 shows the heat unstable protein concentration of permeate samples taken every minute by the sampling system. A sigmoidal-type function was fitted to experimental data:

$$f_1(t) = a_1 + \frac{a_2}{1+e^{a_3 t - a_4}}$$

where $f_1(t)$ is the predicted heat unstable protein concentration, t is time, and a_1 to a_4 are regression parameters. The resulting curve fit is overlayed on experimental data in Figure 9.4. The F statistic for the regression was highly significant (*P* value < 10^{-8}) and the R^2 statistic was 0.93.

Extrapolation of the curve fit beyond the terminal data point at 25 min suggests that, even though filtrate composition had not stabilised, i.e. reached its final steady state value, the response in heat unstable protein concentration was nearly 90 % complete by 20 min (the sampling period employed during the field tests). For this particular instance the error in heat unstable protein concentration from sampling at 20 min (instead of later, i.e. 30 min, when the response had stabilised) was approximately 1.5 mg/L. If this had been repeated at other sampling points and transitions in operating conditions it would have contributed to an error in the recorded values of heat unstable concentration and heat test turbidity. However, when compared with the change in heat unstable protein concentration from the initial value of 100 mg/L in the untreated Sultana wine, this error can be considered comparatively small. Furthermore the randomisation of dose levels during the field tests would have distributed this error relatively evenly between positive and negative values.

Therefore the experimental data collected during the field test results provide an accurate and valid indication of the fining response which occurred.

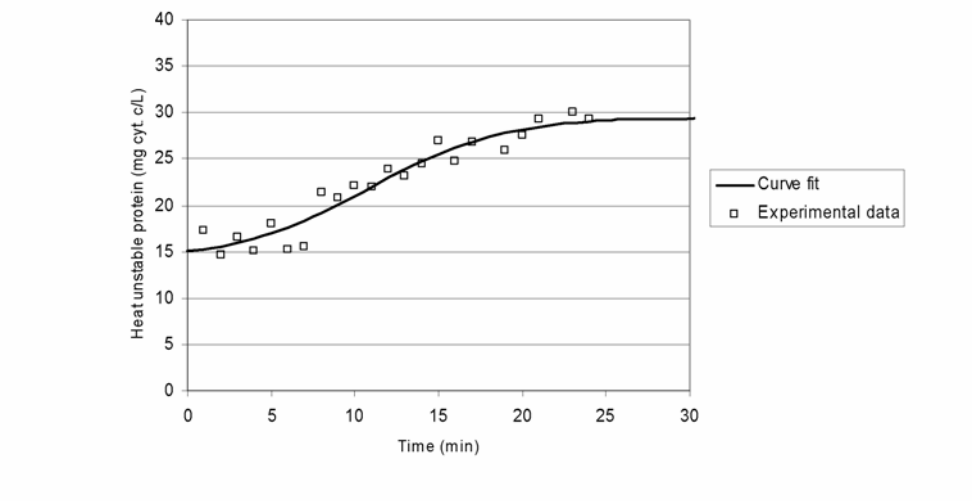


Figure 9.4: Heat unstable protein concentration (mg cyt. *c*/L) in permeate sample versus sample time (min) at a contact time of 260 s following a dose transition from 0.5 to 0.3 g/L of Vitiben during in-line dosing field tests of Sultana wine.

9.3.3 In-line dosing field tests

Figures 9.5a and b show the effect of contact time and bentonite dose on heat test turbidity and heat unstable protein, respectively, for the Gordo juice fined with Vitiben. To better illustrate the fining behaviour presented in Figures 9.5a and b, an exponential decay-type function was fitted to the experimental data for each bentonite dose:

$$f_2(t) = b_1 - b_2 e^{-b_3 t}$$

where $f_2(t)$ is the predicted heat unstable protein concentration or heat test turbidity, and b_1 to b_3 are regression parameters.

The fining responses observed in Figures 9.5a and b for both heat test turbidity and heat unstable protein are virtually identical. Furthermore, most of the fining effect occurred within at least a minute and was practically complete after 2 minutes contact time. This was observed at all testing conditions irrespective of wine or juice, bentonite, or dose.

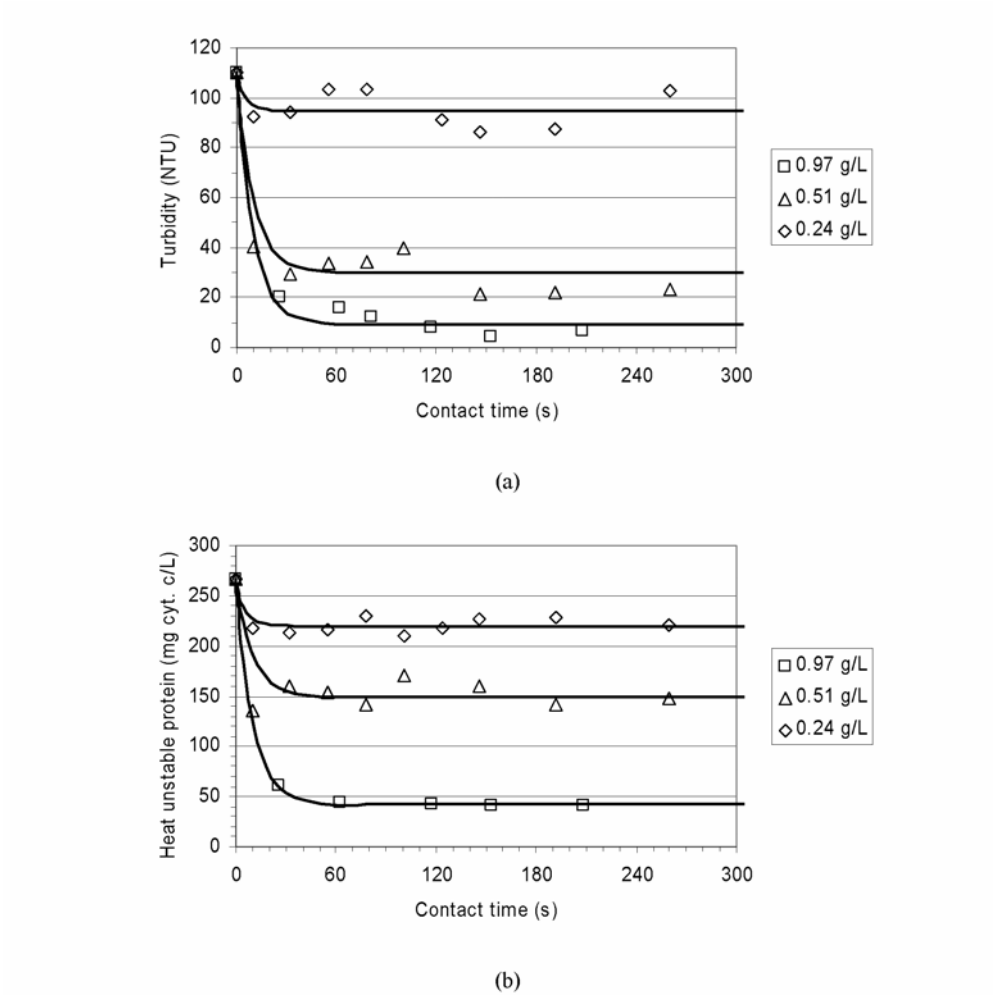


Figure 9.5: Effect of contact time (s) and bentonite dose (refer legend) on (a) heat test turbidity (NTU) and (b) heat unstable protein (mg cyt. *c*/L) for Gordo juice fined with Vitiben during in-line dosing field tests.

Figures 9.6a and b summarise relationships for heat test turbidity and heat unstable protein versus bentonite dose level after in-line dosing at a 2 min contact time under all testing conditions. The 2 minute data were obtained by interpolation between experimental data from curve fits. Note that straight lines have been drawn to connect data points in Figures 9.6a and b to better illustrate the relationship between turbidity or protein concentration and bentonite dose.

Interestingly, the performance of Vitiben and SIHA-Active-Bentonite G appear almost indistinguishable for fining of the Sultana wine.

Based on the data in Figure 9.6a, the bentonite dose required to achieve the heat test criterion of the Stanley Winery (<2 NTU) by in-line dosing of the Sultana wine would be in the order of 0.2 to 0.3 g/L for either Vitiben or SIHA-Active-Bentonite G.

With the Gordo juice, the winery's heat test criterion was not achieved at any of the bentonite dose levels used in the field tests.

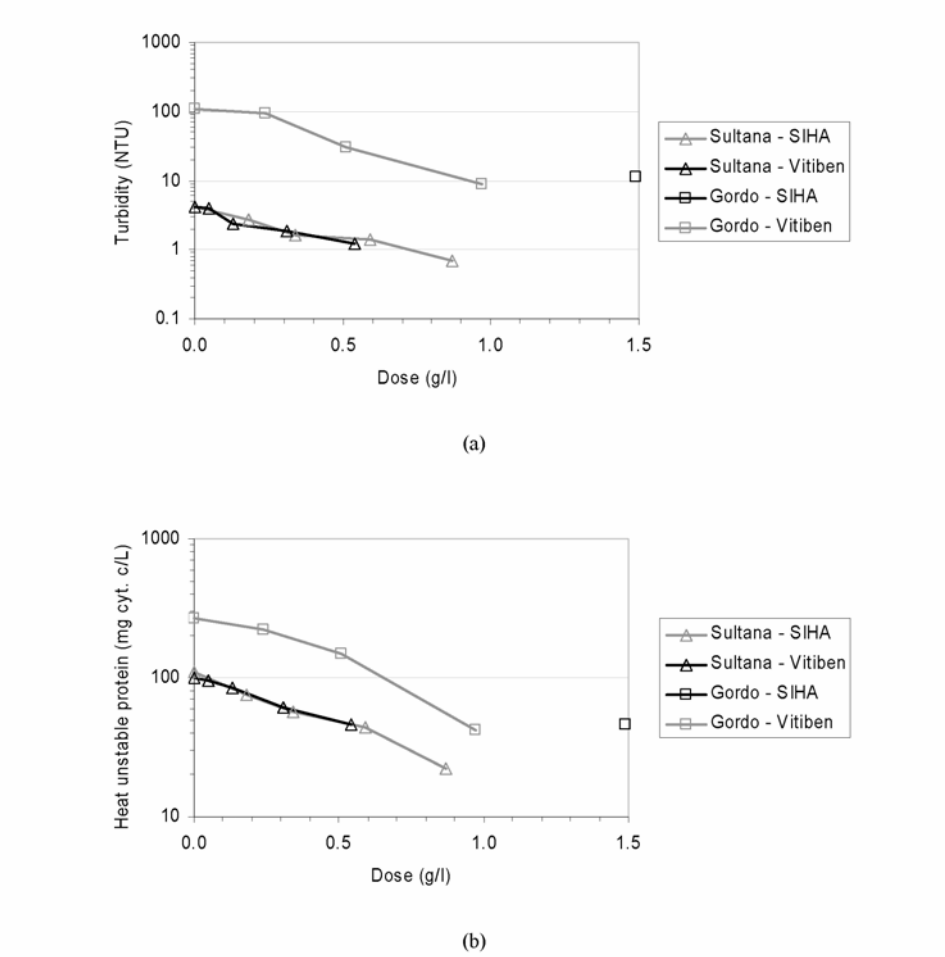


Figure 9.6: (a) Heat test turbidity (NTU) and (b) heat unstable protein (mg cyt. *c*/L) versus bentonite dose (g/L) for in-line dosing of Gordo juice or Sultana wine with Vitiben or SIHA-Active-Bentonite G at 2 min contact time. Note: log scale on vertical axis in both plots.

9.3.4 Batch fining performance

The bentonite doses for batch fining were determined by the winery's laboratory. The recommended dose level for fining of the Sultana wine with Vitiben to attain the winery's protein stability criterion of < 2 NTU was 0.19 g/L. This is at the lower limit of the 0.2 to 0.3 g/L range suggested from in-line dosing performance data. For batch fining of the wine with SIHA-Active-Bentonite G, a dose of 0.4 g/L was recommended. This appears to exceed that which was required by in-line dosing.

9.3.5 Bentonite carryover during in-line dosing

An analysis of spin testing on spot samples that were taken during fining of Sultana wine with SIHA-Active-Bentonite G is summarised in Table 9.4. These data show the solids content of the wine before and after the centrifuge. Prior to the field tests, the wine had

already been clarified by centrifugation. We therefore assumed that the solids present in the wine were predominantly bentonite lees.

The data in Table 9.4 shows that the carryover of the SIHA-Active-Bentonite G was between 15 and 30 %. We expect that Vitiben, a sodium bentonite, which swells more and is less dense, may have produced even greater carryover. Observations on site during field tests confirmed that difference.

Table 9.4: Results of spin tests during in-line dosing of Sultana wine with SIHA-Active-Bentonite G and estimated % carryover of bentonite from centrifuge.

Bentonite Dose, g/L	Pre-centrifuge, % solids v/v	Post-centrifuge, % solids v/v	Carryover, % bentonite addition
0.18	0.100	0.015	15
0.34	0.260	0.065	25
0.59	0.375	0.120	32
0.87	0.530	0.135	26

9.3.6 Sensory analysis

Sensory analysis was only performed with the Sultana wine. There was no significant difference between the two sessions used to conduct the duo-trio difference tests. The final results of comparisons obtained by the tests are included in Table 9.5.

No significant difference ($P<0.05$) was detected between the untreated and in-line dosed wine with Vitiben at 0.54 g/L. There was also no significant difference between the untreated and batch-fined wine with Vitiben at 0.19 g/L, and between the in-line dosed (0.54 g/L) and batch-fined wine (0.19 g/L) with Vitiben.

However a significant difference was detected between in-line dosing with Vitiben (0.54 g/L) and in-line dosing with SIHA-Active-Bentonite Active (0.87 g/L). Conversely, there was no significant difference when comparing the wine after it was batch fined with each of the bentonites (Vitiben at 0.19 g/L and SIHA-Active-Bentonite Active at 0.4 g/L).

Table 9.5: Sensory comparisons performed by balanced reference duo-trio difference tests. Number of responses by panellists correctly identifying which test sample matched the reference is given as well as the calculated *P* value.

Test	Sample A		Sample B	Correct responses[1]	*P*-value[2]
1	Untreated wine	*vs.*	Batch-fined, 0.19 g/L Vitiben	18	0.18
2	In-line dosed, 0.54 g/L Vitiben	*vs.*	Untreated wine	19	0.10
3	In-line dosed, 0.54 g/LVitiben	*vs.*	Batch fined, 0.19 g/L Vitiben	13	0.82
4	In-line dosed, 0.54 g/LVitiben	*vs.*	In-line dosed, 0.87 g/L SIHA-Active-Bentonite G	21	0.02*
5	Batch fined, 0.19 g/L Vitiben	*vs.*	Batch fined, 0.4 g/L SIHA-Active-Bentonite G	17	0.29

[1]Total number of responses = 30.

[2]Significant *P*-values (α <0.05) indicated by *.

9.4 Discussion

9.4.1 Site observations: in-line mixing and hydraulic conditions

The optimal hydraulic conditions for in-line dosing would be to achieve good mixing with dispersion and intimate contact of bentonite particles with wine or juice, as well as an even period of contact time between each particle and the wine or juice before they are separated. The hydraulic environment that favours this is highly turbulent flow at the injection point and in the contact pipe work, which will produce cross mixing and approximate plug flow conditions, i.e. even flow across the pipe. The Reynolds (Re) number is an engineering parameter used to predict whether fluid flow in pipes is laminar or turbulent. It is described by the following function.

$$Re = \frac{\rho v D}{\mu}$$

where ρ is the fluid density, v is the mean fluid velocity in the pipe, D is the pipe diameter and μ is the fluid viscosity.

When the Re number exceeds 2100, flow in a pipe will begin to change from laminar to turbulent flow. At Re numbers above 10000, turbulent flow is said to be fully developed where cross mixing and plug flow effects are maximised.

At the flowrate employed during the field tests it is estimated that the Re number was at least greater than 11000 for the juice and much higher for the wine. Therefore it exceeded the threshold value of 10000 for fully developed turbulent flow. Hence, there should have been adequate mixing to ensure that bentonite remained in suspension and fully dispersed and did not settle out following injection. There also should have been ample turbulent effect to ensure that the bentonite was rapidly mixed and dispersed in the wine or juice at injection. In this respect the static mixer may not have been needed. Sight glasses were located on the pipe work during the field tests following the injection point and static mixer. Observations of the bentonite and wine or juice suspension during the field tests indicated that the dispersion of the bentonite was already relatively homogenous following the injection point (and prior to the static mixer).

9.4.2 Microfiltration sampling system: membrane fouling?

Any bentonite fouling on membrane surfaces of the microfiltration sampling systems may have produced a filter effect leading to additional protein adsorption from the filtrate, thus potentially causing erroneous readings of heat test turbidity and heat unstable protein concentration. To consider whether bentonite fouling may have occurred, the value of flux rate for the microfiltration sampling systems during the field tests (*ca.* 30 L/m^{2}hr) may be compared with the critical flux condition, i.e. the value of flux rate when cake deposition or

fouling during cross-flow microfiltration is believed to commence. When the flux rate exceeds this critical value the rate of transport of particles to the membrane surface caused by filtration is greater than particle transport away from the membrane arising from convection, back-diffusive and/or axial mass transfer mechanisms (Kim and Park 2002).

Kim and Park (2002) have previously measured the critical flux value for microfiltration of a bentonite suspension in a hollow-fibre microfiltration membrane with identical pore size rating. In their experiment, the bentonite particle size was approximately 6.3 μm, the cross-flow velocity was 1.65 m/s, and the transmembrane pressure was 5 psi (30 kPa). They recorded a critical flux value greater than 90 L/m^{2}hr. This is larger than the flux rate measured for the microfiltration sampling systems during the field tests. All other sampling during the field tests was performed under similar operating conditions. Furthermore, visual inspections were periodically made in between test conditions of the lumen in the microfiltration elements. No deposition of bentonite on membrane surfaces was observed. Therefore, bentonite fouling of microfiltration elements did not occur.

9.4.3 Protein adsorption kinetics & implications for fining efficiency

The short contact time required by in-line dosing during the field tests to saturate the fining effect validates earlier studies which have identified that protein adsorption (Blade & Boulton 1988) and heat stabilisation of wine (Lee 1986) by bentonite is rapid.

The HPLC technique employed in this study measures the principal heat unstable proteins in a wine or juice. However there may be other proteins present that are not detected by this method. Whilst these other proteins do not substantially affect wine protein stability, they may be adsorbed by bentonite, and thus, increase the final dose that must be added.

Furthermore there is evidence that different protein fractions require distinct bentonite concentrations for removal (Ferreira et al. 2002). Differences between protein classes in their adsorption equilibria and kinetics could be significant in determining how quickly the various protein fractions are adsorbed from wine. If the haze-forming proteins adsorb faster than heat-stable proteins, this could potentially decrease the bentonite dose required by in-line dosing to stabilise the wine.

There are also other reasons why one might expect better fining performance from in-line dosing. Hydraulic mixing during in-line dosing should present a superior environment to initially disperse the bentonite and ensure even contact with the wine.

Zocklein (1988) has suggested that prolonged contact between bentonite and wine may cause adsorbed protein to ‘slough off’ the particles. For batch fining, this might necessitate a higher dose to compensate.

Practically speaking though, any potential benefit of more efficient fining by in-line dosing may be moot. The reason for this is simple: dose levels at wineries are most often

established by a laboratory fining trial. These are performed under ideal conditions, which provide good contact between bentonite and wine because of their small scale and better mixing conditions. Therefore, laboratory fining trials might already recommend close to the minimum dose possible with in-line dosing. If anything, batch fining should require a higher dose than the laboratory determined value to achieve an equivalent performance.

9.4.4 Fining of Gordo juice

Most heat unstable or haze-forming proteins in wine originate from the grape berry (Waters et al. 1996, 2005, Tattersall et al. 2001, Ferreira et al. 2002). Therefore, fining of a juice should reduce the concentrations of heat unstable protein present after fermentation.

Bentonite fining of juice may be used to avoid post fermentation fining, and thus, additional handling of a wine (Ribéreau-Gayon et al. 2000). Alternatively, it may be employed to reduce dose levels required after fermentation for fining of a wine. This might lessen value losses by reducing lees volume. (However this would only prove advantageous if savings achieved outweighed any added expense and product loss that might be involved with fining of the juice.) Another advantage of juice fining is that it might minimise adverse sensory effects where this is of concern (Miller et al. 1985). It is difficult to find actual comparisons of juice versus wine fining. The information that exists (e.g. Miller et al. 1985, Zoecklein 1988, Ribéreau-Gayon et al. 2000) suggests that only a similar or slightly higher dose is needed or used for fining of juice. However it was not always indicated in these studies whether a subsequent fining of the wine was necessary to achieve heat stability.

For in-line dosing, the fining of the Gordo juice was unable to achieve the winery's heat stability criterion of < 2 NTU. Therefore, fining following fermentation of the juice may have been required in this instance.

9.4.5 Compaction of lees by centrifugation

It was not possible to accurately quantify and compare the volumes of lees that were produced as a result of centrifugation during in-line dosing field tests or gravity settling in the batch fining trials performed at the Stanley Winery. The bentonite lees discharged from the centrifuge were diluted by water used to flush them from the clarifier storage chamber. For batch fining trials, there was no racking arm in the tanks that could be used to ascertain the location of the interface between clarified wine or juice and lees.

The results of spin tests may be used as a guide to consider the additional lees compaction achieved by centrifugation. However these results will overestimate the volume of lees produced by centrifugation due to wall friction support of the pellet present

in the 10 mL tapered test tubes. Furthermore the compressive force applied to lees in the bowl of an industrial centrifuge (e.g. estimated at up to 1500 psi (100 atm) in a Westfalia SC 150-06-777 clarifier) is significantly higher than that experienced during a spin test (*c* 20 psi), and thus, a lower level of compaction would occur.

SIHA-Active-Bentonite G produced a pre-centrifuge lees volume of 0.53 % v/v during the spin test of Sultana wine containing a dose level of 0.87 g/L (Table 9.4). Morant (1990) measured the volume of lees produced by gravity settling for eight days of SIHA-Active-Bentonite G after fining of a 1990 Padthaway Riesling. The dose level was 1 g/L. A lees volume of 3 % was observed. Assuming that this result can be extrapolated directly to this present study, the equivalent lees volume at a dose of 0.87 g/L would be approximately 2.6 %. Therefore, centrifugal compaction during the spin test may have reduced the lees volume by five-fold. Taking into account the higher compressive force present in industrial centrifuges and presence of wall friction effects in spin-test test tubes, centrifugal compaction can evidently reduce the volume of lees to a fraction of that produced by batch fining. Furthermore, sodium bentonites which swell more may experience even greater centrifugal compaction than a sodium-calcium bentonite.

9.4.6 Bentonite carryover during in-line dosing

A proposed advantage of in-line dosing is that efficient separation of bentonite and wine or juice will minimise the volume of lees. A carryover of bentonite during this clarification step would still produce lees in the receiving tank, albeit a lower volume, and create value losses.

The estimations in Figure 9.7 give a quantitative perspective to this problem. These notional calculations estimate product recovery under three fining scenarios for a 275 kL volume (a common tank size in some large wineries) of wine:

(a) batch fining followed by tank settling and racking, which generates a 5 % v/v of tank lees;

(b) in-line dosing with a 30 % bentonite carry-over followed by tank settling and racking, which produces approximately 1.5 % v/v of tank lees;

(c) in-line dosing with zero bentonite carry over and no tank lees.

The calculations assume 5 % w/v bentonite slurry; a bentonite dose of 1 g/L; recovery of wine from tank lees by RDVF; a five-fold compaction of lees volume by centrifugation; and that no further wine is recoverable from compacted lees produced by centrifugal separation. They also take into account the increase in initial wine volume arising from addition of the bentonite slurry.

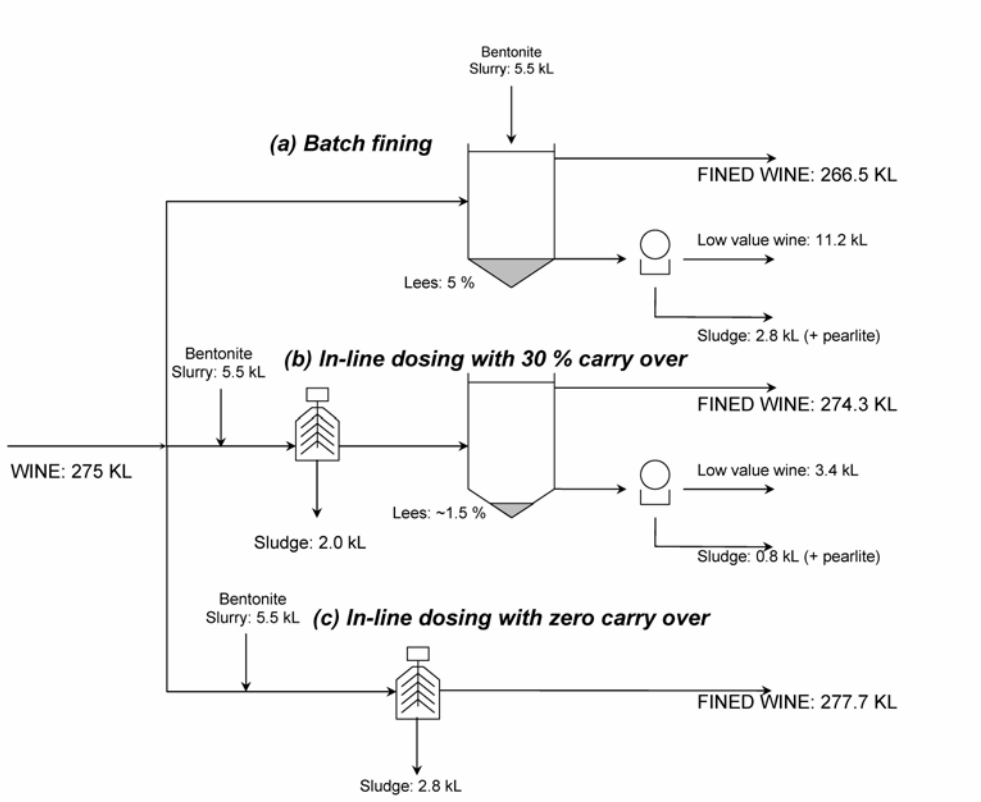


Figure 9.7: Estimations to quantify wine recovery under three bentonite fining scenarios: (a) batch fining with 5 % tank lees; (b) in-line dosing with 30 % carryover and approximately 1.5 % tank lees; (c) in-line dosing with zero carry over.

The results indicate that

(a) for batch fining, the wine recovered (based on original volume) would be 96.9 % of higher quality wine with 4.1 % as lesser quality wine (recovered from tank lees by RDV filtration);

(b) with in-line dosing and a 30 % carryover, recovery of higher quality wine would be 99.8 % with a 1.2 % recovery of lesser quality wine; and

(c) for in-line dosing with zero carry-over, a 101 % recovery of higher quality wine with no lesser quality wine would be achieved.

In all scenarios the total recovery of (higher and lower) quality wine exceeds the original wine volume because of the increase in wine volume arising from water added with the bentonite. Based on these calculations we suggest that significant savings in value losses could be achieved by implementation of in-line dosing and these will be maximised if carryover during centrifugal clarification is minimised or eliminated. There are a number of process strategies that could be explored to achieve this, including the following.

1. A lower operating flowrate. However this would increase processing time, although a solution to this problem could be to utilise two or more centrifuges in parallel.

2. Multiple clarification steps. For example a second centrifuge in series or an additional pass performed subsequently.
3. A centrifuge with higher clarification efficiency.
4. A bentonite that is more efficiently clarified. However if this bentonite has lower fining performance and requires higher doses it might still produce the same quantity of carryover.
5. Counter-fining with another fining agent during in-line dosing of bentonite that will improve clarification efficiency. Counter-fining is a common strategy already exploited in the wine industry to enhance settling and compaction of lees in tanks (Zoecklein 1988, Boulton et al. 1998).

Each of these options may involve a cost penalty that would have to be balanced against the savings achieved. In addition to value losses, the savings would include the expenses avoided with a further processing step to recover wine occluded in bentonite lees during batch fining.

9.4.7 Sensory effects

In the wine industry there appear to be two schools of thought: those who do not believe that bentonite fining has an effect and those who consider that it does. Published literature (although not always with experimental data) can be found to support both view points (Miller et al. 1985, Rankine 1989, Leske et al. 1995, Martinez-Rodriguez and Polo 2003, Pocock et al. 2003). The truth is probably somewhere in between. Taking into account all of the other factors that may have contributed to differences in wine quality of the samples collected for sensory analysis, the evidence in this study tends to lean towards a conclusion that bentonite fining did not produce a detectable effect on the Sultana wine. Furthermore, even if fining did affect wine quality, in-line dosing and batch fining had very similar effects.

9.5 Concluding remarks

The fining effect for in-line dosing of the Sultana wine and Gordo juice was achieved within 2 min of contact time after addition of bentonite. Whilst in-line dosing has potential to improve efficiency of bentonite use compared with batch fining, this may be difficult to realise in practice because dose levels will usually be decided by laboratory fining trials regardless of which fining method is used.

In this study, in-line dosing did not produce a significant effect on sensory quality of the Sultana wine as assessed by duo-trio difference testing comparing both untreated (or raw) and batch fined wine. However, further sensory evaluation would allow a more conclusive result to be obtained.

A significant challenge confronting commercial application of in-line dosing is bentonite carryover if the bentonite and wine or juice is not efficiently separated. This carryover will form lees in the receiving tank, which will either need to be resuspended and clarified again by filtration or centrifugation, or allowed to gravity settle so wine can be recovered by racking. If the carryover problem can be resolved, in-line dosing could eliminate significant value losses currently experienced by wineries when recovering wine occluded in bentonite lees by batch fining.

10 In-line dosing: Simulation of multi-component adsorption

10.1 Introduction

From protein quantification by HPLC analysis of field test samples, it was possible to deduce values of protein adsorption that provide equilibrium values for the two principal heat unstable protein fractions (thaumatin-like protein and chitanases) present in wine at the different dose levels for the commercial bentonites that were employed. These equilibrium values occurred when the fining response had stabilized. This allowed the multi-component Langmuir adsorption isotherm parameters C_p^{∞} and K for the two protein fractions to be determined. With this information, it was possible to extend the process simulation model used to initially predict contact time for protein adsorption during in-line dosing (presented in Section 8), to consider multi-component adsorption. Whilst this exercise in itself may not of immediate practical value, it demonstrates how mathematical tools widely used in other industries can be extrapolated to wine production processes. With further development and validation, such tools could be used to help optimize wine production operations and improve production efficiencies in the Australian wine industry.

10.2 Method

The equilibrium values of protein adsorption during field tests were employed to obtain the multi-component Langmuir adsorption isotherm parameters C_p^{∞} and K (refer Sections 5, 6 and 8 for various presentations and descriptions of the multi-component Langmuir adsorption) for the two protein fractions. This was performed by non-linear least squares regression of the equilibrium data on Microsoft Excel. One outlier was arbitrarily excluded in order to attain rational values.

The adsorption isotherm parameters were used to simulate the fining response of the two protein fractions under the same equations, assumptions parameter values for the previously presented mathematical model for in-line dosing (Section 8) except a value of 6.9 μm was employed for the volume mean particle diameter of Vitiben based on laser scattering particle size measurements (Muhlack 2006).

10.3 Results & discussion

10.3.1 Langmuir adsorption parameters

Table 10.1 presents the final values obtained for the two haze forming proteins and summarizes the regression statistics.

Table 10.1: Isotherm parameters and regression statistics from non-linear regression of multi-component Langmuir adsorption isotherm to equilibrium data for in-line fining of juice and wine with Vitiben.

Protein fraction (or i)	Thaumatin-like protein (VVLT1)	Chitanase
C_p^{∞} (mg/mg)	0.191	0.159
K (L/mg)	0.059	0.048
Regression statistics		
R^2	0.64	
F_0	6.00	
p	0.013	

10.3.2 Multi-component process simulation

Figure 10.2 shows the results for one of the process simulations for in-line dosing of wine with Vitiben at 0.54 g/L under field test conditions. Good agreement between model predictions and experimental data is observed. Similar concurrence between model predictions and experimental data was obtained at other field testing conditions.

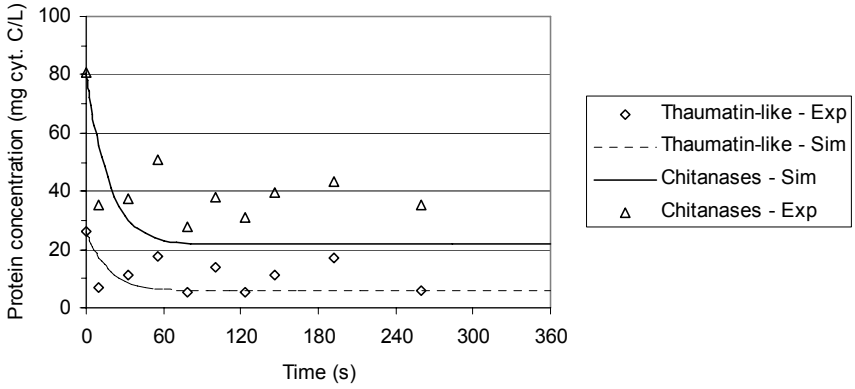


Figure 10.2: Process simulation of multi-component protein adsorption by thaumatin-like proteins and chitanases during in-line dosing of 0.54 g/L Vitiben in wine.

10.4 Concluding remarks

A mathematical model has been developed to simulate the in-line dosing process. This may be a valuable tool for future optimization studies.

11 Improved quantification of wine proteins by HPLC

11.1 Introduction

Wine proteins range in size from 10 to 65 kDa, with most possessing a low isoelectric point (pI, 4.1 to 5.8 (Hsu and Heatherbell 1987)) and low molecular weight (MW, 20 to 30 kDa (Hsu and Heatherbell 1987, Waters et al. 1991, Waters et al. 1992)). The majority of the wine proteins responsible for haze are grape pathogenesis related (PR) proteins, thaumatin-like proteins and chitinases, with MW between 20 and 30 kDa (Waters et al. 1996). The concentration of protein in untreated white wines differs by variety and ranges up to 300 mg/L (Waters et al. 2005).

Quantitative methods based on reversed phase (RP) and size exclusion chromatography (SEC) high performance liquid chromatographic (HPLC) separation of the proteins prior to detection have been found to be useful in the measurement of wine proteins (Tyson et al. 1981, Dubourdieu et al. 1986, Interesse et al. 1987, Modra and Williams 1988, Polo et al. 1989, González-Lara and González 1991, Ledoux et al. 1992, Santoro 1985, Peng et al. 1997, Waters et al. 1998, Pocock et al. 1998, Pocock and Waters 1998, Pocock et al. 2000, Pocock et al. 2003, Muhlack et al. 2006, Nordestgaard et al. 2007). RP-HPLC utilises a hydrophobic stationary phase. As the hydrophobicity of an initially polar mobile phase is increased, more hydrophobic compounds elute.

Size exclusion chromatography, often referred to as gel filtration or gel permeation, separates compounds based on molecular size. Following sample injection, molecules diffuse in and out of solvent-filled pores of particles of the stationary phase. Molecules larger than the pores are totally excluded and are first to elute at the void volume. Increasingly smaller molecules elute next. Molecules that are below a certain size and totally penetrate the pores elute together unresolved at the permeation volume. Separation is primarily by molecular size and not purely by molecular weight. Adsorption of proteins onto the stationary phase is discouraged by the use of buffers and salts in the mobile phase.

Somers and Ziemelis (1973) used this form of chromatography to separate total wine proteins from other wine components, by using a stationary phase with a fractionation range of 1 to 5 kDa, and loaded untreated wine samples directly onto the column. Each analysis took at least 100 minutes to complete. Tyson et al. (1981) used a stationary phase that had an exclusion limit of 80 kDa in an HPLC system. One peak contained the total soluble protein. The time for analysis exceeded 10 minutes.

Dubourdieu et al. (1986), Interesse et al. (1987) and Modra and Williams (1988) also used this technique and injected untreated must and wine samples directly onto HPLC columns with exclusion limits of 70 to 500 kDa. These methods utilised relatively long columns (600 mm) and resolved proteins into three or four fractions and required more than 60 minutes

to complete. Polo et al. (1989) used a more conventional length HPLC column (250 mm) with a fractionation range of 4 to 400 kDa and dialysed must samples before injection. This method resulted in a number of incompletely resolved peaks and was complete within 18 minutes.

As part of work involved with the GWRDC project, a size exclusion HPLC method to quantify proteins in wine was developed and compared with the more widely used reversed-phase method. The impact of various method parameters such as flow rate, injection volume, salt concentration, pH and the use of a denaturant were investigated.

11.2 Experimental

11.2.1 Wines

Eight commercial dry white wines, which had been clarified but not fined with bentonite, were supplied in volumes ranging from 80 L to 240 L by two companies. These wines were delivered to the Hickinbotham Roseworthy Wine Science Laboratory of the University of Adelaide at Urrbrae, South Australia over a four month period. The wines were transported in either 20 L polyethylene containers or, in one case in a 200 L polyethylene drum, and on arrival were stored in a temperature controlled room maintained at 0 ˚C. Samples were taken for analysis. Details of the wines are presented in Table 11.1. If necessary, sulphur dioxide was added to bring the free SO_2 level to that suitable for the pH of the wine.

Table 11.1: Analysis of wines before bottling.

Variety	Source	pH	Alcohol (%v/v)
Sauvignon Blanc	Riverland, SA	3.34	10.5
Semillon A	Adelaide Hills, SA	3.22	11.0
Semillon B	Hunter Valley, NSW	3.29	9.9
Chardonnay (unwooded)[a]	Langhorne Creek, SA	3.42	11.9
Chardonnay (wooded)[a]	Langhorne Creek, SA	3.36	11.9
Sultana	Mildura, VIC	3.43	12.1
Riesling	Eden Valley, SA	2.97	12.1
Gordo	Mildura, VIC	3.47	12.5

[a]Both wines made from the same lot of juice; unwooded held in stainless steel tank, wooded held in stainless steel tank containing planks

11.2.2 HPLC

SEC was conducted with a BioSep SEC-S2000 (300 × 7.8 mm, 5 µm particle size, 145 Å pore size, Phenomenex, Lane Cove, NSW, Australia) column with a Phenomenex SecurityGuard guard cartridge (4 × 3 mm) which contained the same phase as the column. The manufacturer claims this column has an exclusion range of 300 to 1 kDa for native proteins. All SEC chromatography was conducted at 25 ˚C.

Molecular weight calibration was performed using a protein mixture (Phenomenex) containing Bovine Thyroglobulin (670 kDa), IgG (150 kDa), Ovalbumin (44 kDa), Myoglobin (17 kDa), Uridine (244.2 Da). These were dissolved in 0.10 M sodium dihydrogen phosphate (NaH_2PO_4) adjusted to pH 6.8, and stored at -20 ˚C until required.

Mobile phases trialled included 0.05 M and 0.10 M NaH_2PO_4 either at natural pH or adjusted to pH 3.2 by the addition of 5 M hydrochloric acid (HCl) or to pH 6.8 by the addition of 5 M sodium hydroxide (NaOH). Addition of 5 g/L of sodium dodecyl sulphate (SDS) was also investigated. Flow rates of 0.2 to 1 mL/min and injection volumes of 10 to 100 µL were trialled.

Wine protein concentration was also determined using the RP-HPLC method described by Muhlack et al. (2006). A Brownlee Aquapore C4 column (100 × 2.1 mm, 7 µm particle size, 300 Å pore size, PerkinElmer, Wellesley, MA, USA) was used. The column was equilibrated in Solvent A [10% v/v acetonitrile (ACN), 0.1% v/v trifluoroacetic acid (TFA)] and held at 40 ˚C. The initial conditions were maintained for 4 minutes after injection of the 100 µL sample. From 4 to 6 minutes the solvent B [90% v/v ACN, 0.1% v/v TFA] increased to 28% and then to 48% at 11 minutes, 55% at 14 minutes, 58% at 15 minutes and a maximum of 60% B at 18 minutes before re-equilibration in solvent A for 2 minutes prior to injection of the next sample. Flow rate was 0.8 mL/min. Fractions were identified by comparison with previous RP-HPLC of wine and fractionated wine proteins (Peng et al. 1997, Waters et al. 1998, Pocock et al. 1998, Pocock et al. 2000, Pocock et al. 2003).

An Agilent 1100 series HPLC system was used with auto-sampler and diode-array detector (Agilent, Santa Clara, CA, USA). All samples were centrifuged (9300g, 5 minutes, room temperature) to remove particulate matter prior to injection. Each sample was tested in duplicate.

Protein concentration was quantified by comparison with the peak area of a protein standard: cytochrome c from bovine heart (Sigma, St. Louis, MO, USA) dissolved in Milli Q water at concentrations of 12.5 to 400 mg/L.

11.2.3 Heat test

Heating at 80 ˚C for 6 hours, as suggested by Pocock and Rankine (1973), was used to determine the stability of wines against protein haze. Samples were filtered using low

protein binding syringe filters (Acrodisc 25 mm with 0.2 µm Supor membrane, Pall, East Hills, NY, USA). Sample turbidity was measured using a turbidimeter (2100N; Hach, Loveland, CO, USA) before samples were placed into 20 mL screw capped glass tubes in an 80 ˚C water bath. After 6 hours, the sample tubes were removed and cooled in iced water for 15 minutes and then allowed to stand for an hour before sample turbidity was once again measured. Untreated wine samples were tested in duplicate. The reported heat test result is the difference in turbidity between the heated and unheated samples.

11.2.4 Bentonite fining trial

40 g/L Vitiben bentonite (BPM Minerals, Houston, TX, USA) slurry was prepared 15 hours prior to use. Fining was conducted in 50 mL polypropylene tubes by the addition of aliquots of slurry (0 to 2 mL) to 40 mL batches of Sauvignon Blanc wine. All tubes were vortex mixed for approximately 30 seconds, after which they were placed sideways on a shaker table (approximate dimensions and orbiting speed: 50×50 cm, 100 rpm) for a further hour, followed by further vortex mixing. Samples were centrifuged (2000g, 30 minutes, room temperature) to remove bentonite and adsorbed proteins. Heat test and HPLC analysis followed.

11.2.5 Heating experiment

40 mL aliquots of Gordo wine were heated in closed polypropylene tubes in a 55˚C water bath for 24 hours. Samples were collected at intervals and measured for protein concentration by HPLC and heat stability by the heat test.

11.2.6 PVPP fining

40 mL aliquots of the Sauvignon Blanc and unwooded Chardonnay wines were treated at 5 g/L with Polyvinylpolypyrrolidone (PVPP) (Sigma) by the addition of 2.9 mL of a 70 g/L PVPP slurry (prepared in Milli Q water the previous day). Samples, along with untreated controls, were vortex mixed, agitated on an oscillating table (approximately dimensions and orbiting speed: 50×50 cm, 100 rpm) for 2 hours. Samples were centrifuged (875g, 15 minutes, room temperature) before supernatant was drawn for SEC-HPLC analysis. The remaining supernatant was collected and dosed a second time at 5 g/L with PVPP and mixed and centrifuged as before. Samples were again taken for SEC-HPLC analysis.

11.2.7 Ultrafiltration

Ultrafiltration was performed using 5 and 10 kDa nominal molecular weight limit Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA, USA). Samples of Sauvignon Blanc and unwooded Chardonnay wine were initially filtered through low protein binding

syringe microfilters to remove particulates (Acrodisc 25 mm with 0.2 µm Supor membrane, Pall). Centrifugation using the ultrafilters was conducted according to the manufacturer's instructions commencing with 3.5 mL then centrifuging until approximately 3.2 mL of permeate had collected. Both permeate and retentate were brought to the original volume, 3.5 mL, by the addition of model wine. The model wine was 12% ethanol, 2 g/L potassium bitartrate adjusted to pH 3.4 by the addition of L-(+)-Tartaric acid. Samples were taken for SEC-HPLC analysis.

11.3 Results & discussion

11.3.1 SEC-HPLC method development

The ability of the column to separate standard proteins according to molecular size is illustrated in Figure 11.1. Bovine Thyroglobulin (MW = 670 kDa) and Uridine (MW=244.2 Da) were used to determine the void and permeation volumes, respectively, of the column. The 3 proteins within the exclusion range specified by the manufacturer were used for molecular weight calibration.

The Sauvignon Blanc wine was used for development of a method to separate and quantify proteins in wine. Wines made from Sauvignon Blanc grapes typically have a high protein concentration and was therefore a suitable wine on which to focus initially.

There was little difference in separation for flow rates between 0.2 and 1 mL/min (Figure 11.2a). Injection volumes of 10 µL, 20 µL and 50 µL gave similar resolution (Figure 11.2b). A mobile phase salt concentration of 0.05 M NaH_2PO_4 resulted in a slightly sharper peak 'B' (thought to contain most of the protein – see Figure 11.3 for designation of peaks) than with 0.10 M solution (Figure 2c). For both salt concentrations, analysis was conducted without pH adjustment of the mobile phase (pH~4.5) and with pH adjustment to 3.2 or 6.8 with 5M HCl or 5M NaOH respectively. Peak 'B' was sharpest at pH 6.8 and became wider at pH 4.5 and at pH 3.2, there appeared to be 2 poorly resolved peaks (Figure 11.2d).

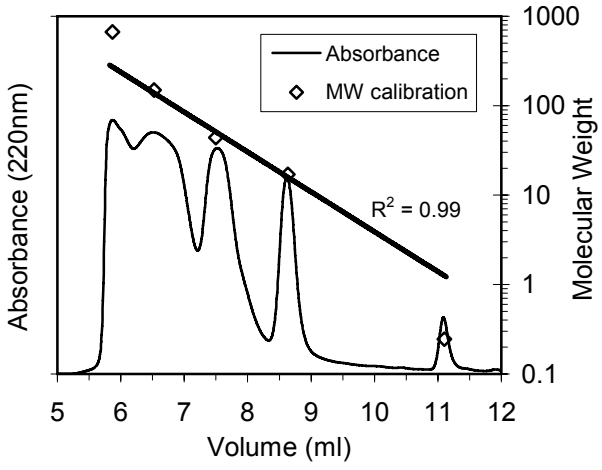


Figure 11.1: SEC-HPLC molecular weight calibration using a protein mixture. Eluent – 0.05 M NaH_2PO_4, pH 6.8; Injection volume – 20 µL; Flow rate – 1.0 mL/min.

5g/L of SDS was added to the mobile phase. Its addition resulted in a decrease in retention time of the likely proteinaceous peaks, 'A' and 'B', and did not appear to enhance separation (Figure 11.2e).

Based on the findings of the initial trials, the proposed method uses a 0.05 M NaH_2PO_4 solution adjusted to a pH of 6.8 (with 5M NaOH) for the mobile phase, a flow rate of 1

mL/min and an injection volume of 20 µL.

Figure 11.3 shows a typical excerpt from the chromatograms for 2 of the 8 wines. The key peaks are labelled along with an approximate molecular weight based on the molecular weight calibration (Figure 11.1). Figure 11.4 shows chromatograms for the same 2 wines using the reversed phase method.

In the size exclusion method, it was evident that some wine compounds were eluting after the permeation volume. Additional sample preparation, other mobile phase conditions, or running each sample for longer may be ways to overcome this problem. The last strategy would probably be the most practical in terms of speed, but would only be a possibility if it could be demonstrated that the compounds eluting after the permeation volume were not protein.

To probe the identity of the compounds, several investigations were undertaken. Firstly, a trial was conducted on the Sauvignon Blanc wine and the unwooded Chardonnay wine. These wines had shown higher peaks well after the permeation volume. Each wine was treated with PVPP and also by ultrafiltration to remove smaller non-proteinaceous substances. PVPP is a synthetic high molecular weight fining agent with an affinity for low molecular weight phenolic compounds in wine (Zoecklein et al. 1999).

Excessive PVPP fining marginally reduced the peaks appearing after the permeation volume for both wines. The first fining reduced the total peak area after the permeation volume by 20% for the Sauvignon Blanc and 26% for the Chardonnay. The second fining reduced the total peak area after the permeation volume to 34% and 33% of the original peak area for the Sauvignon Blanc and Chardonnay, respectively. These results do not include the effect of dilution of the wine by the PVPP slurry. The dilution was 7% after the first fining and 15% after the second fining.

The effect of ultrafiltration was more dramatic. The retentate for both wines, through both size filters, exhibited a significantly reduced post permeation volume peak area (94 to 97%). The centrifugal ultrafilters used must be left with some liquid in the retentate chamber. This is a likely reason for any peaks after the permeation volume for the retentate. Figure 11.5 shows the Chardonnay before ultrafiltration through a 10 kDa nominal MW cut-off filter as well as the retentate and permeate after model wine addition. Clearly, the permeate contained the compounds that are interacting with the stationary phase and eluting after the permeation volume. The identity of these compounds was not explored further as the focus of this work was the wine proteins. In this and other trials, processing of 27 mL of mobile phase was found to be sufficient for all compounds to elute and therefore 27 minutes was adopted as the time between sample injections.

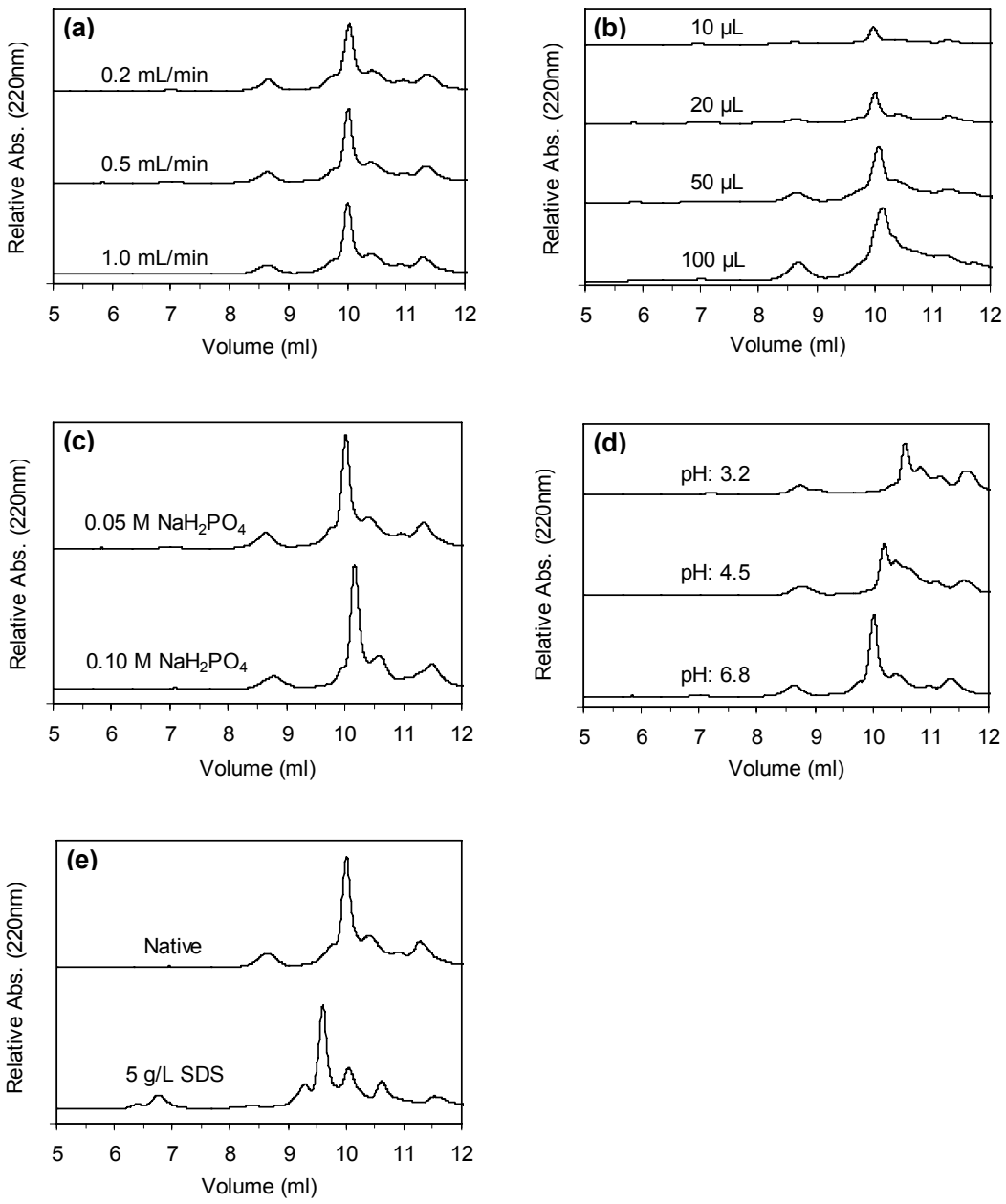


Figure 11.2: Effect of chromatographic parameters on SEC-HPLC separation after injection of Sauvignon Blanc wine: (a) *Flow rate* - 0.05 M NaH_2PO_4, pH 6.8, 20 µL injection; (b) *Injection volume* - 0.05 M NaH_2PO_4, pH 6.8, 1.0 mL/min; (c) *Salt concentration* - pH 6.8, 20 µL injection, 0.5 mL/min; (d) *pH* - 0.05 M NaH_2PO_4, 20 µL injection, 0.5 mL/min; (e) *Denaturant addition* - 0.05 M NaH_2PO_4, pH 6.8, 20 µL injection, 1 mL/min.

For samples of Sauvignon Blanc and unwooded Chardonnay processed using both size exclusion and reversed phase chromatography, spectra between 220 and 700 nm for all peak maxima, including those eluting after the exclusion limit, were analysed to further identify them. Altman (2003) suggests some common features of protein spectra: (1) Little-to-no absorbance between 310-340nm, (2) A shoulder at approximately 290nm, (3) A peak at approximately 280nm, (4) A trough at approximately 245-250nm, (5) A rise between 245-240nm, (6) for a pure protein the ratio of absorbance at 260nm to

absorbance at 280nm should be 0.5 – 0.7. Using these heuristics, all the peaks labelled in Figures 11.3 and 11.4 are proteinaceous whilst the unlabelled peaks are not. It is of importance to note that no proteins appear to elute after 11 minutes in the size exclusion method (after the permeation volume of the column).

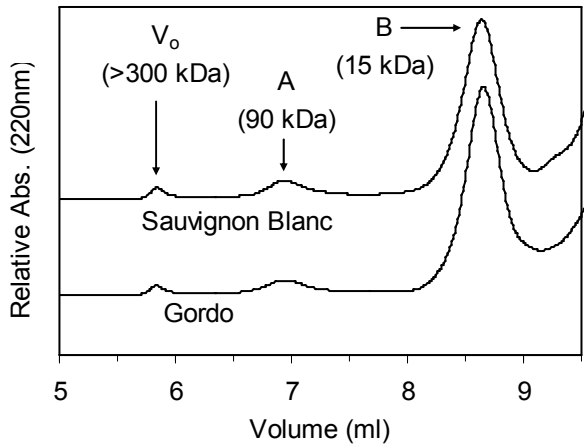


Figure 11.3: SEC-HPLC of the Sauvignon Blanc and Gordo wine. Eluent – 0.05 M NaH_2PO_4, pH 6.8; Injection volume – 20 µL; Flow rate – 1.0 mL/min.

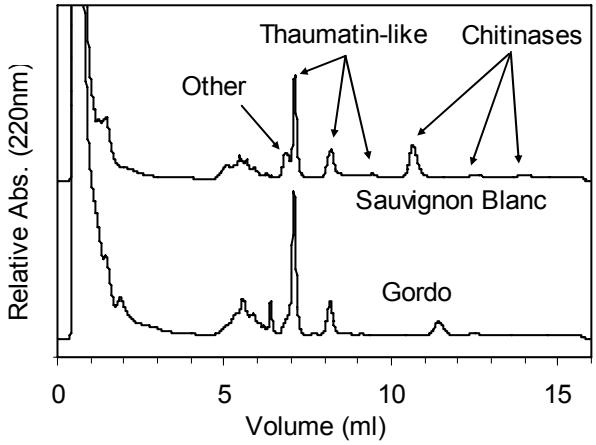


Figure 11.4: RP-HPLC of the Sauvignon Blanc and Gordo wine. Buffer A: 10% acetonitrile, 0.1% trifluoroacetic acid in water; Buffer B: 90% acetonitrile, 0.1% trifluoroacetic acid in water.

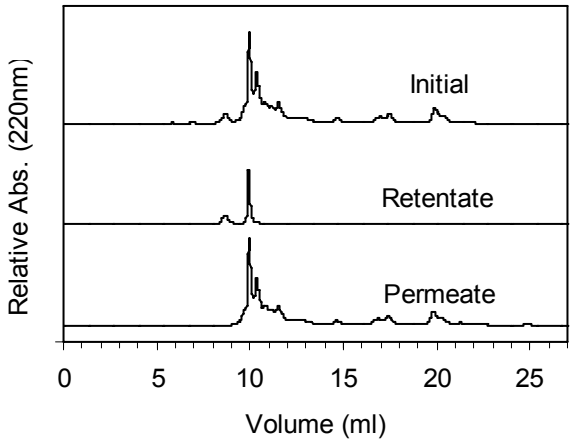


Figure 11.5: SEC-HPLC of the unwooded Chardonnay processed using a nominal 10 kDa ultrafilter. Eluent – 0.05 M NaH_2PO_4, pH 6.8; Injection volume – 20 µL; Flow rate – 1.0 mL/min.

Table 11.2 provides a summary of the protein concentrations of all 8 wines. Peak 'B' correlates well with the sum of the thaumatin-like and chitinase proteins determined by the reversed phase method. From the molecular weight calibration regression (Figure 11.1), peak 'B' has an approximate MW of 15 kDa, while the MW of the thaumatin-like and chitinase proteins of wine, determined by electrospray mass spectrometry, are 22 kDa and 25 kDa respectively (Hayasaka et al. 2001). From the results with 8 different wines, it seems likely that peak 'B' corresponds to the sum of thaumatin-like and chitinase proteins. For the unwooded and wooded Chardonnay, Sultana and Riesling, the agreement between the estimated concentrations is not as close. In Figure 11.4, it is evident that the 'other' peak is not perfectly separated from the major thaumatin-like protein fraction in the reversed phase method. This overlap may be one reason for the difference between the two chromatographic methods, and it is noticeable that the wines with poorer agreement between the estimated concentrations tend to have a relatively high concentration of the 'other' fraction.

Peak 'A' is likely to be grape invertase. Grape invertase, a relatively heavily glycosylated protein (Dambrouck et al. 2005), is not detected by the reversed phase method, likely because it does not bind to this resin under the conditions of chromatography. All peak assignments for the SEC method should be further confirmed by mass spectrometry of collected fractions.

In general, the elution of proteins from the size exclusion method showed a similar wine protein pattern to that observed by other authors (Tyson et al. 1981, Dubourdieu et al. 1986, Interesse et al. 1987, Modra and Williams 1988, Polo et al. 1989). Using columns that were twice as long and more expensive, Dubourdieu et al. (1986), Interesse et al. (1987) and Modra and Williams (1988) achieved some separation of peaks likely to

correspond to thaumatin-like and chitinase proteins; however, the peaks were still poorly resolved.

Table 11.2: Protein composition of untreated wines.

Wine	Heat Test[a] (ΔNTU)	Size exclusion HPLC (mg/L[b])			Reversed phase HPLC (mg/L[b])			
		V_o[c]	A[c]	B[c]	T[d]	C[e]	T+C[f]	Other
Sauvignon Blanc	59.5	5.3	23.8	218.7	156.1	67.6	223.7	41.5
Semillon A	38.5	5.3	21.9	175.0	123.7	55.8	179.5	43.9
Semillon B	32.2	5.4	20.8	92.5	83.0	11.5	94.5	51.7
Chardonnay (unwooded)	64.3	4.5	15.3	133.1	169.5	13.1	182.6	73.0
Chardonnay (wooded)	50.1	5.7	17.4	125.0	139.1	6.4	145.5	71.8
Sultana	6.4	6.1	18.9	46.6	24.8	5.5	30.2	25.5
Riesling	54.0	3.8	8.7	136.5	146.7	4.9	151.6	55.6
Gordo	170.2	4.0	14.9	243.7	196.2	41.0	237.3	24.7

[a] ΔNTU < 2.0 is considered as heat stable

[b] Expressed in mg/L of the surrogate protein, cytochrome C

[c] Approximate molecular weight (kDa) determined from calibration (Figure 11.1):

V_o: >300 kDa (void fraction for the column)

A: 90 kDa

B: 15 kDa

[d] Thaumatin-like proteins

[e] Chitinase proteins

[f] Thaumatin-like + chitinase proteins

11.3.2 Application of the method to a bentonite fining trial

The protein composition, assessed by size exclusion and reversed phase HPLC, of a bentonite fining trial is summarised in Table 11.3.

By reversed phase chromatograhy, the fraction of thaumatin-like proteins removed is similar to that of the chitinases at equivalent bentonite doses. Higher doses of bentonite were required to remove the 'other' fraction and some of this fraction remains in the heat stabilised wine.

Grape invertase required higher bentonite doses for removal than the thaumatin-like and chitinase proteins, but was completely removed at a dose of 1.5 g/L bentonite. This result differs from Dambrouck et al. (2005) who found a good correlation between the decrease in the grape invertase content and the total protein content of wines, following bentonite treatments. The fraction with molecular weight > 300 kDa fraction, corresponding to the

void fraction on the size exclusion column, was not completely removed at a 2 g/L addition of bentonite but the initial concentration was extremely low.

Table 11.3: Fraction of each protein removed from the Sauvignon Blanc wine after fining with bentonite.

Dose (g/L)	Heat Test [a] (ΔNTU)	Fraction removed					
		Size exclusion HPLC			Reversed phase HPLC		
		>300 kDa	Grape Invertase	Thaumatin-like + Chitinase	Thaumatin-like	Chitinases	Other
0	65.7	0%	0%	0%	0%	0%	0%
0.1	69.8	7%	0%	19%	12%	6%	14%
0.2	53.6	3%	23%	30%	28%	28%	21%
0.4	28.6	6%	31%	63%	69%	62%	31%
0.7	1.8	15%	44%	94%	96%	99%	49%
1.1	2.7	22%	76%	100%	97%	97%	73%
1.5	0.5	37%	100%	100%	98%	99%	87%
2	0.6	37%	100%	100%	100%	99%	94%

[a] ΔNTU < 2.0 is considered as heat stable

11.3.3 Application of the method to a heating trial

Table 11.4 summarises the results of changes in protein composition after heating a Gordo wine at 55 ˚C. The chitinase fraction was quickly removed from solution after exposure to heat (within 15 minutes). The thaumatin-like fraction was also heat labile. As expected from the results of Waters et al. (1991), grape invertase was relatively thermostable. Subsequent experiments showed that exposure of the Gordo wine to a standard 6 hours at 80 ˚C heat test resulted in complete removal of the thaumatin-like proteins, the chitinase proteins as well as the grape invertase.

Table 11.4: Fraction of each protein removed from the Gordo wine after heating at 55˚C.

Time (hrs)	Heat Test [a] (ΔNTU)	Fraction removed					
		Size exclusion HPLC			Reversed phase HPLC		
		> 300 kDa	Grape Invertase	Thaumatin-like + Chitinase	Thaumatin-like	Chitinase	Other
0	168.7	0%	0%	0%	0%	0%	0%
0.25	125.6	7%	-4%	25%	16%	99%	17%
1	60.4	11%	12%	57%	56%	98%	72%
6	2.2	17%	18%	100%	95%	100%	77%
24	3.7	23%	19%	100%	99%	98%	85%

[a] ΔNTU < 2.0 is considered as heat stable

11.4 Concluding remarks

A size exclusion HPLC method was optimised to quantify wine protein in less than 30 minutes. The method can be used without any sample preparation other than centrifugation or microfiltration to remove particulate matter. This method separated a peak likely to be grape invertase from a peak likely to contain the heat-unstable thaumatin-like and chitinase proteins. A reversed phase method was superior at separating the similarly sized thaumatin-like and chitinase proteins. In tandem, these two methods were used to analyse a bentonite fining trial of one wine and exposure of another wine to heat. Thaumatin-like and chitinase proteins were removed at lower doses for a Sauvignon Blanc wine than grape invertase. During heating of a Gordo wine at 55 ˚C, chitinases were removed from solution relatively quickly followed by the thaumatin-like proteins. The fraction likely to be grape invertase was the most thermostable, but it also was removed after exposure to the standard wine industry heat test of 6 hours at 80˚C.

12 Bentonite fining: Can we improve efficiency and decrease value losses?

12.1 Introduction

In this section of the report, comments about more effective use of bentonite for fining is considered outside of those areas already considered. In particular, a quantitative approach is taken to evaluating research that is being performed and/or identifying knowledge gaps and opportunities that exist. A number of questions are posed and evaluated to determine whether answers already exist. This type of analysis is valuable for guiding future research and development to maximise commercial outcomes for the industry.

12.2 Heat stability testing & Dose Optimisation

The heat stability test most commonly employed by industry is to observe whether a wine sample produces excessive (e.g. > 2 NTU) turbidity following heating at 80 °C for 6 hrs. However, recent research at the Australian Wine Research Institute (Pocock & Waters 2006) has discovered that this test does not always accurately predict haze formation during long-term storage trials of white wines. In fact, it may over estimate the bentonite dose required for stability under some storage conditions. Table 12.1 summarises the data obtained by the AWRI study. Note the results obtained for the Riesling. It was still stable during long-term storage at a dose of 0.4 g/L, whereas heat testing at 80 °C for 6 hrs indicated that a dose level double this value, 0.8 g/L, was required. Even a less severe version of the heat test of 80 °C for 2 hrs indicated a much higher value of bentonite dose than was necessary. A similar pattern can be seen for the other wines tested although it is difficult to accurately quantify exactly what lower values of bentonite dose satisfactorily achieved stability during long-term storage *versus* heat test because of the sparsity of the dose levels included in the study.

Nevertheless, if this is the case, this presents a significant opportunity to decrease bentonite dose and reduce volume of wine occluded in bentonite lees. Obviously, these results require additional validation. In particular, what is the effect of a longer storage period on haze formation? Furthermore, how does one correctly judge how much bentonite dose could be reduced from the value obtained from using a heat test at 80 °C for 6 hrs (or 2 hr)? The current heat test procedure may need to be modified or an alternative test that more accurately predicts whether haze formation will occur could be required.

Table 12.1: Summary of data from recent AWRI research (Pocock & Waters 2006) of how well stability tests predict haze formation during storage.

Wine	Bentonite dose that achieved stability (g/L)				Dose levels employed for each wine (g/L)
	Storage trial, 20 °C for 12 months*	*Storage trial*, 35 °C for 1 months*	*Heat test, 80 °C, 2 hr*	*Heat test, 80 °C, 6 hr*	
Unwooded Chardonnay	0	0.8	0.8	0.8	0, 0.8 & 1.2
Wooded Chardonnay	0	0.7	0.7	0.8	0, 0.7, 0.8 & 0.9
Gordo	1.2	1.2	1.2	1.4	0, 1.2 & 1.4
Reisling	0.4	0.4	0.7	0.8	0, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 & 1.1
Sauvignon Blanc	1.1	1.1	1.2	1.4	0, 1.1, 1.2, 1.4 & 2.2
Semillon (1)	0	0.8	0.8	0.8	0, 0.8 & 1.8
Semillon (2)	0.9	0.9	1.1	1.1	0, 0.9, 1.1 & 1.2
Sultana	0	0	0.4	0.5	0, 0.4, 0.5 & 2.3

* By visual assessment of clarity following storage period.

12.3 Bentonite selection: Na versus Ca

Sodium (Na) bentonite finds widest application in the United States, whereas calcium (Ca) bentonite is widely accepted in Europe (Zoecklin 1988). There doesn't seem to be any data as to popularity of either form in Australia but it seems that industry usage is predominated by Na bentonites or Na/Ca blends. It is well established that Na bentonites swell more Ca bentonites, and thus produce a greater volume of lees for an equivalent dose, but are more efficient at adsorbing proteins, and hence, require a lower dose (than Ca bentonites) to achieve heat stability (Zoecklin 1988). However, there is little quantitative data that shows what these relationships actually are. An exception is a study from more than a decade ago by AWRI (Morant 1990). Figure 12.1 summarises the results obtained by this study. Analysis of these results (not shown) suggests that Ca bentonites, whilst requiring (on average) a dose three times higher than Na bentonites to achieve heat stability, will produce one quarter of the lees. Furthermore, Na/Ca bentonites required a dose 50 % greater than Na bentonites but generated one half the volume of lees.

The data presented indicates that using Na/Ca or Ca bentonites could significantly reduce values losses arising from bentonite fining. For example, estimates from the above data suggest that a Na bentonite dose of 0.4 g/L produces 6 % v/v lees whereas a Ca bentonite, by reducing lees volume, could regain up to 5 % of the original volume of the wine that would ordinarily be occluded in Na bentonite lees.

However, if this is the case why are Na/Ca or Ca bentonites not widely used? There are a number of possible reasons. First, higher doses of Ca bentonite require greater volume of slurry or more concentrated slurry. Calculations assuming a 2 % constraint on water addition to the wine suggest that for a Na bentonite dose of 1 g/L, 5 % w/v slurry would be required. At the higher doses of Na/Ca and Ca bentonite necessary to achieve the same treatment effect, the slurry concentrations are 7.5 and 15 % w/v, respectively. It may not be possible or practical to achieve these higher slurry concentrations and thus Na bentonites are favoured. However, Ca bentonites swell less and higher slurry concentrations may be possible when compared with Na bentonites. Investigations of the rheology of bentonite slurries would provide valuable data to answer this question.

Second, Ca bentonites release Ca during bentonite fining and may contribute to tartrate instability. However, this might only be problematic if bentonite fining was performed subsequent to tartrate stabilisation.

Third, Ca bentonites may contribute to heavy metal pick up (Boulton *et al.* 1995), and thus, their use may be undesirable at elevated bentonite dose rates. However, this was not observed by Morant (1990) when she measured trace metal concentrations for Arsenic and Copper in wine following fining. In fact, no difference in heavy metal content was observed between the Na, Na/Ca and Ca bentonites.

Nevertheless, the question remains – at low bentonite dose rates (e.g. < 0.5 g Na bentonite/L), is a Ca bentonite preferable if it significantly reduces the volume of lees?

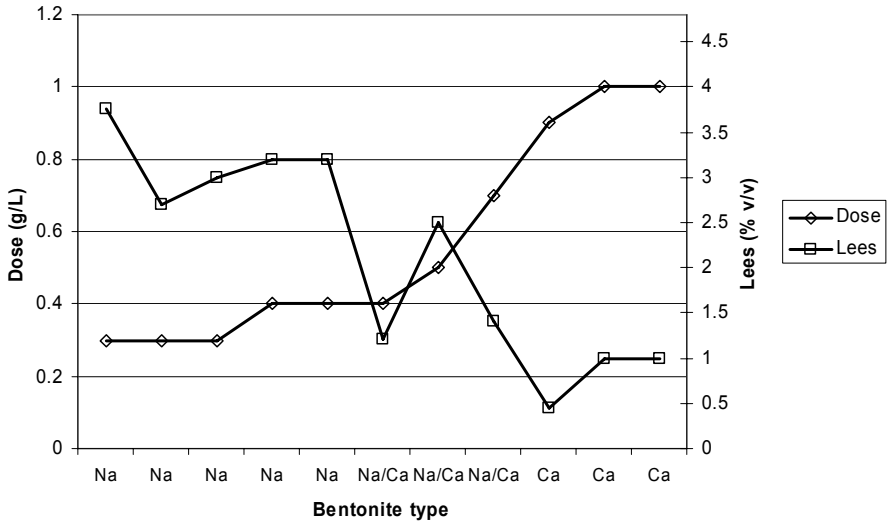


Figure 12.1: Values of dose to achieve stability and volume of lees produced at that dose for different Na, Na/Ca and Ca bentonites, from data obtained by Morant (1990).

12.4 Lees Compaction

Bentonite swells considerably upon hydration and by the time it gravity settles as lees in wine it occupies approximately 150 times its original dry volume. Therefore, considerable scope exists for compression and compaction to reduce the volume of lees, and thus, value losses.

Figure 12.2 illustrates the effect that lees compaction might have on the volume of wine recovered after Na bentonite fining at a dose level of 0.4 g/L producing 6 % v/v lees. Note that in the figure, the % wine recovery is based on the original wine volume. Furthermore, the maximum volume of wine recoverable is greater than 100 % because it includes the water added to the wine with the bentonite slurry.

If lees were compacted by further 20 % (to 4.8 % v/v) this could produce to an increased recovery in racked (i.e. high value) wine of 1.2 % (of the original wine volume). This would be equivalent to a 0.6 % increase in total wine product value when recovered wine from RDV is accounted for. If the lees could be compacted five-fold (i.e. 80 % compaction), the additional racked wine recovered could be approximately 5 %. In this scenario, all occluded wine potentially recoverable by RDV would probably have been extracted from the lees. Thus, there would be no need for further reprocessing.

The question is how much compaction is required and how would this additional compaction be achieved? For gravity settled lees, it might be conceivable to achieve up to 20 % additional compaction (an uneducated guess). In these situations, unless the tank could be pressurised (to achieve greater compressive force on the lees), the only

practical way this could be achieved is through attaining a higher packing density in the lees. How bentonites flocculate, settle and pack during fining of wines is very poorly understood (if at all). It could be that how a bentonite is prepared, added and mixed with the wine strongly influences the volume of lees. The answer to this question could be readily obtained by laboratory experimentation.

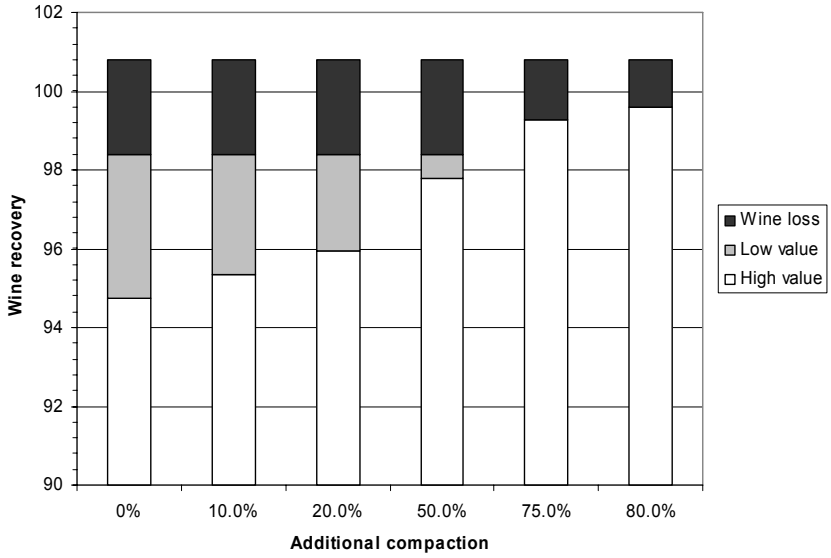


Figure 12.2: Effect of additional compaction on wine recovery from a Na bentonite at 0.4 g/L producing 6 % v/v lees.

Another strategy to compact the lees is already practised by winemakers: bentonite fining during cold stabilisation. The precipitated tartrate crystals are believed to assist with bentonite lees compaction (Zoecklin et al. 1988). However, while there are several references that suggest this happens, no quantitative data is available to support this assertion. Another advantage of bentonite fining at the same time as cold stabilisation is a reduction in number of processing steps, thereby minimising product loss. Low *et al.* (2006) has recently investigated the costs of cold stabilisation *versus* alternative technologies for tartrate stabilisation, including electrodialysis and nanofiltration. These alternative technologies all require separate bentonite fining. After accounting for this, combined cold stabilisation and bentonite fining remained the most cost-effective process for tartrate stabilisation.

Attaining compaction exceeding 20 % would likely involve additional compaction of the lees above that achieved by gravity, for example, by applying centrifugation. As already mentioned industrial centrifuges can substantially increase the compressive pressure acting on the lees. The additional compaction could be achieved during bentonite separation from the wine, as in the case of the in-line dosing field tests that were performed by the authors, or by separate processing of gravity settled lees following

racking of the wine. In the latter case, tubular cross-flow microfiltration has been tested at a winery but results do not yet appear publicly available.

12.5 Fining juice

Bentonite fining of juice is commonly cited as a method for avoiding post fermentation fining. The advantages of fining juice are said to be as follows. It avoids additional handling of a wine (Ribéreau-Gayon *et al.* 2000). Of course, this is only applicable if stability is actually achieved. Where stability is not achieved juice fining will reduce dose levels required after fermentation for fining of a wine, and may lessen value losses by reducing lees volume. However this would only prove advantageous if savings achieved outweighed any added expense and product loss that might be involved in fining of the juice. As well, juice fining minimises adverse sensory effects where this is of concern (Miller et al. 1985). This benefit occurs as juice components are adsorbed onto bentonite not fermentation or barrel aging constituents (Zoecklin, 1988). However, what if these juice components are important flavour and aroma pre-cursors?

It is very difficult to find actual comparisons of juice *versus* wine fining. The information that exists (e.g. Miller et al. 1985, Zoecklein 1988, Ribéreau-Gayon et al. 2000) suggests a similar (or slightly higher dose) is required for fining of the juice. Unfortunately, it was seldom indicated in these studies if a subsequent fining of the wine was necessary to achieve heat stability.

What is known is that juice will contain more protein than the wine. As an example, data obtained by Murphey *et al.* (1989) suggests that protein levels may decrease by between 10 and 40 % during vinification. The proteins that remain in the wine are predominantly grape-derived, pathenogenesis-related, are highly resistant to low pH and enzymatic and non-enzymatic hydrolysis, and principally comprise thaumatin-like proteins and chitanases (Waters *et al.* 2005).

A higher level of protein in the juice would be expected to require a greater bentonite dose rate. This should produce more lees but it is not known whether bentonite swelling and packing density of lees is greater or smaller in juice. Clearly, a larger volume of juice (compared to wine if fining was performed post-fermentation) occluded in the lees is likely. Whilst is often said that juice does not possess the same monetary value as wine when the potential benefits of juice fining are mentioned (e.g. Zoecklin 1988), surely there is an opportunity cost associated with juice occluded in the lees that would arise from its fermentation into wine.

To consider this further, fining-dose response data for a wine from a recent laboratory investigation was analysed. The following assumptions were made. Prior to fermentation the juice contained 25 % more protein. The bentonite dose required was proportional to

the protein concentration, which may not be correct as distinct protein fractions have been observed to require differing bentonite dose levels for removal (Ferreira et al. 2002). The volume of lees was also proportional to the dose, which is again probably not true as evidence exists (e.g. compare data of Morant 1990 *versus* Leske et al. 1995 for same bentonites at differing dose levels) to suggest that while higher doses produce larger volumes of lees, these compact more. The lees produced by fermentation would be approximately 3 % v/v. Both juice and wine would be fined to achieve heat stability.

Under these conditions, juice fining was estimated to produce 10.9 % v/v lees whereas fining of the wine following fermentation would result in 8 % v/v lees. For the wine, up to 60 % of the wine occluded in the lees can be recovered by RDV filtration, albeit diminished in quality and value as a result. The question is whether juice would be recovered from juice fining lees? If not, a significant absolute loss of potential wine occurs. This proposition would clearly not make sense and it was assumed that the juice lees would be reprocessed by RDV filtration. The recovered juice would be returned for fermentation.

Question: is there a possible adverse quality impact on wine involved with RDV re-processing of juice lees? Furthermore, juice re-processing of lees must be performed immediately, and thus could be problematic at a commercial winery in the throes of vintage.

Further analysis of the value gain in juice fining *versus* wine fining if juice in lees was recovered by RDV filtration suggest that juice fining may only increase total wine value by approximately 0.5 % (v/v). This assumes that wine value was independent of when fining was performed (i.e. before or after fermentation), which may not be the case.

Another issue to be considered with juice fining is how to ascertain whether the resulting wine will be heat stable. The incidence and intensity of protein haze may be modulated by pH, ethanol, metal ions, polysaccharides and phenolic compounds (Waters *et al.* 2005). Even though a juice may be heat stable by heat test, it is possible that a resulting wine could be heat unstable, requiring an additional fining. If additional fining is required, the benefits of juice fining may become marginal unless there is another driving force for performing it, e.g. superior sensory outcome. A reliable way to ensure juice fining achieved heat stability in the wine may be to directly quantify protein content.

An additional problem with juice fining is evidence suggesting it can reduce fermentation rate (Weiss & Bisson 2002) and may cause stuck fermentations and hydrogen sulphide generation (Zoecklin 1988). However, it has also been observed (Weiss & Bisson 2002) that no adverse effect occurs if bentonite remains present during the fermentation. Adding bentonite during fermentation is also practised by winemakers. The bentonite is believed to assist in compaction of the fermentation lees (Zoecklin 1988). However, there appears to be no quantitative data available to support this claim.

Clearly, juice fining, and even bentonite addition during fermentation, requires further investigation and analysis. In particular, experiments that provide side-by-side performance comparisons of juice fining *versus* bentonite addition during fermentation *versus* wine fining would be advantageous to answer some of these questions.

12.6 Concluding remarks

This section of the report has attempted to construct a quantitative basis around some of the current gaps in knowledge that exist regarding traditional and new methods of bentonite fining. It is evident that significantly more information is required to ensure that process selection decisions can be made with the confidence to maximise quality and financial outcomes. Areas which deserve more attention include; how bentonite preparation and addition affect compaction of gravity settled bentonite lees; the compaction of lees and performance of bentonite fining if applied during cold stabilisation; processing methods for compacting bentonite lees during in-line dosing or separately which do not adversely impact on wine quality; and the technical performance and economic value of juice fining *versus* bentonite addition during fermentation *versus* wine fining.

13 Combined protein/enzyme treatment

13.1 Introduction

Previous studies have shown that acid proteases have high activity for hydrolysing grape proteins remaining in white wine (Cordonnier and Dugal, 1968; Feuillat *et al*, 1980; Heatherbell *et al*, 1987; Pocock *et al*, 2003, Tucker and Woods, 1995). These acid proteases, also known as aspartic proteases or aspartyl proteases are a widely distributed subfamily of proteolytic enzymes, which belong to the endopeptidase family and have been found in vertebrates, plants, viruses and retroviruses.

The concept of using acid proteases for the removal of wine proteins is not in itself unique. From 1980 till present a number of proteases have been investigated with varying levels of success (Cordonnier and Dugal, 1968; Feuillat *et al*, 1980; Heatherbell *et al*, 1987; Tucker and Woods, 1995). Conditions in these studies were not optimised for high protease activities. These non-optimal conditions include the low pH and cellaring temperature of wine and also inhibition caused by sulphur dioxide, alcohol and tannins in wine.

Consequently, recent studies have investigated both the sensory impact and process improvements gained by applying enzymes at elevated temperatures (Pocock *et al*, 2003; Heatherbell *et al*, 1987). However, these studies unfortunately exceeded temperature optima and still added the acid protease in a low activity temperature range. Due to the parabolic nature of the optimum temperature curve, this produced similar results in terms of enzyme kinetics to having the enzyme applied at or near room temperature.

The work presented in this section of the report was undertaken to screen seven different commercially available acid proteases by applying combined heat and enzyme treatments to a real wine under closer to optimal conditions. In this way enzymes with the strongest activity against wine proteins were chosen for empirical optimisation and kinetic studies.

As mentioned in the Methodology section to this report, this work was commenced by the second PhD student. This student decided to leave after twelve months into the project. Hence, this work could not be extended further as part of the GWRDC project. The information presented in this section is taken from the sole conference paper that was prepared by the PhD student.

13.2 Materials & methods

13.2.1 Gordo Wine

White wine prepared from Gordo grapes was obtained from SouthCorp (Lindemans, Karadoc, Vic) and was not fined with bentonite. Gordo wine was chosen for its high heat unstable protein content. Six 20L containers of clarified wine were held in cold storage at 0ºC for four months. 750ml aliquots were filtered bright (0.45µm) through filter pads and

bottled. Bottles were held in bottle storage at 18ºC until use. Analytical data for the wine determined by The Australian Wine Research Institute was pH=3.47, and alcohol content 12.5 %v/v. Protein content was analysed before usage on the 27/09/04 with 184mg.L thaumatins and 71mg/L chitinases.

13.2.2 Proteolytic Enzymes

Seven enzymes were chosen for screening. Enzymes were chosen based on two key criteria; high activity at low pH and high activity at temperatures below 60ºC in order to minimise any sensory impacts at elevated temperatures. The enzymes chosen were Papain 6000L (Enzyme Solutions, Austrlaia), Crystalzyme Vino Pro (Enzyme Solutions, Austrlaia), GC 106 (Enzyme Solutions, Austrlaia), Optidex L300 (Enzyme Solutions, Austrlaia), Acid protease II (Amano Enzymes Inc.), Acid protease A (Amano Enzymes Inc.), and Trenolin Mash (Erbsloh, Germany). Solutions were stored at 4ºC until use.

13.2.3 Heat/Enzyme Treatment of Wine

Glass tubes (volume 20ml, diameter 1cm) were filled with 18ml of Gordo wine, closed with a screw cap and treated with 5 different treatments and 7 different proteases. Each test was performed with one repeat and all results reported within this work are the average of the two trials.

The five different treatments were chosen to investigate the viability of the process with the effectiveness of pre heat treatment and the importance of precise protease addition temperature being the two key investigations. These treatments have been numbered 1-5 (Note: this numbering system will be used throughout this section).

1. Heat to 70ºC (hold 4 minutes); cool to 45ºC and add enzyme (hold 30 minutes).
2. Heat to 70ºC (hold 4 minutes); cool to 55ºC and add enzyme (hold 30 minutes).
3. Stabilize sample at 20ºC and add enzyme (hold 30 minutes).
4. Stabilize sample at 45ºC and add enzyme (hold 30 minutes).
5. Stabilize sample at 55ºC and add enzyme (hold 30 minutes).

Due to the use of laboratory heating baths in this experiment a considerable amount of time was required to heat the sample to the required initial temperature. For example heating to 70ºC required 190 seconds in a 70ºC water bath. Quoted times above are indicative of the actual time at the stated temperature. Figure 13.1 shows the complete temperature profiles of the wine samples when subjected to the five different processing regimes.

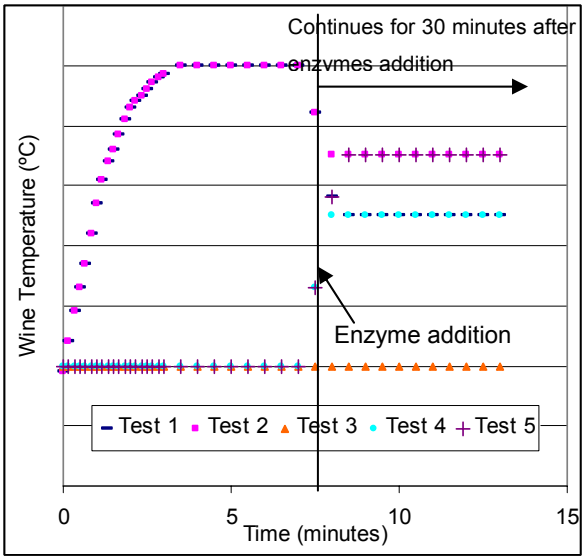


Figure 13.1: Temperature profiles for the 5 different testing methods.

As mentioned above the protease addition step required addition at two temperatures which when combined covered the range of optimal temperature cited by the manufacturers for the individual proteases. Proteases were also added at concentrations recommended by the manufacturer, as detailed in Table 13.1.

Table 13.1: Enzyme Characteristics

Enzyme	Concentration (mL/L)	Optimum Temp (ºC)	Optimum pH
Papain 6000L	1.05	47	5.0-7.0
Crystalzyme Vino Pro	1.05	45	2.0-4.0
GC 106	1.05	47	2.0-4.0
Optidex L300	1.05	54	2.0-4.0
Acid Protease II	2.63	45	3.0-5.0
Acid Protease A	2.63	54	3.0-6.0
Trenolin Mash	1.05	45	2.0-4.0

13.2.4 Haze forming protein content by heat test

The heat test method was used to determined protein stability (Pocock AND Rankine,1973; Iland et al, 2000). Wine samples filtered through a 0.45µm syringe filter before being incubated at 80ºC for 6 hours and then cooled rapidly in an ice-bath before storage at 4ºC overnight. As well as visual examination for hazes the turbidity of the solution was measured before and after heating using a Hach turbidimeter. A wine was considered heat unstable if the difference in turbidity exceeded 2NTU.

13.2.5 Protein Analysis by SDS Gel Electrophoresis

The supernatant obtained after centrifugation of 500µL of the treated wine was reduced to an aliquot of 150µL and evaporated at 50ºC in the presence of nitrogen to concentrate the sample. This was diluted using 25µL of gel loading buffer solution (50mM Tris-Cl (pH6.8), 100mM dithiothreitol, 2% (w/v) SDS (electrophoresis grade), 0.1% bromophenol blue, 10% (w/v) glycerol). Samples were then immersed in boiling water for four minutes to denature the proteins present followed by cooling to room temperature before loading into the gel.

SDS-agarose gels were purchased from Gradipore (4-20% iGels NG21-420). The electrophoresis buffer consisted of 25mM Tris base, 250mM glycine (electrophoresis grade) (pH 8.3) and 0.1% SDS. The samples were then loaded onto the gel and run with a constant voltage of 100V for approximately 2 hours. Finally the gels were stained with Stain- Coomassie Brilliant Blue R-250 and de-stained with methanol: water: glacial acetic acid (5:4:1). Gel images were recorded using a HP Deskscan and quantitative data was derived using Quantity One (BioRad, USA).

13.3 Results

13.3.1 Heat Stability

The results of the heat stability test on the prepared samples are shown in Figure 13.2. The heat test was able to show that the addition of protease in all cases had an affect on the stability of white wine. The standard shown in each case is the average value for eight samples. The average percentage standard deviation from the mean value for the difference in turbidity was 14% however this value ranged from 1% to 25% depending on the sample and in particular on the protease present.

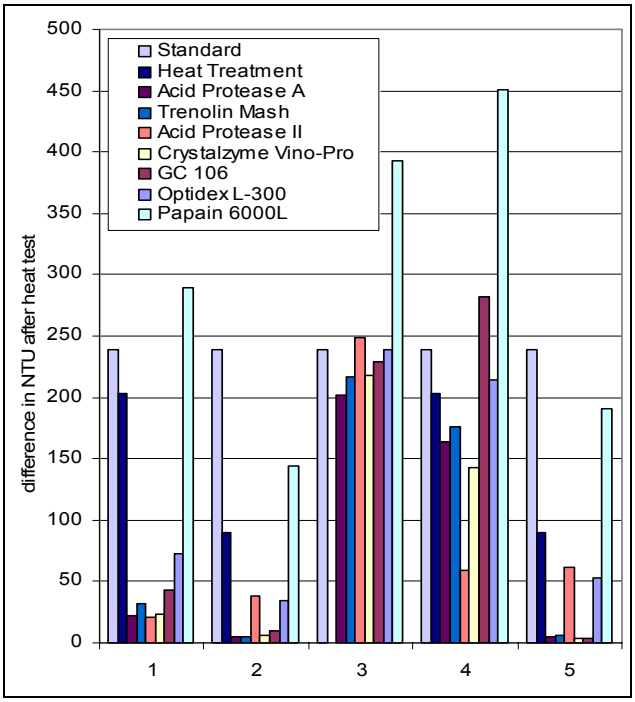


Figure 13.2: Results of heat stability testing.

Key to treatments:

1. **Heat to 70ºC (hold 4 minutes); cool to 45ºC and add enzyme (hold 30 minutes).**
2. **Heat to 70ºC (hold 4 minutes); cool to 55ºC and add enzyme (hold 30 minutes).**
3. **Stabilize sample at 20ºC and add enzyme (hold 30 minutes).**
4. **Stabilize sample at 45ºC and add enzyme (hold 30 minutes).**
5. **Stabilize sample at 55ºC and add enzyme (hold 30 minutes).**

13.3.2 SDS Gel Electrophoresis

Examples of key results from the gel electrophoresis of the treated wine samples are shown in Figure 13.3.

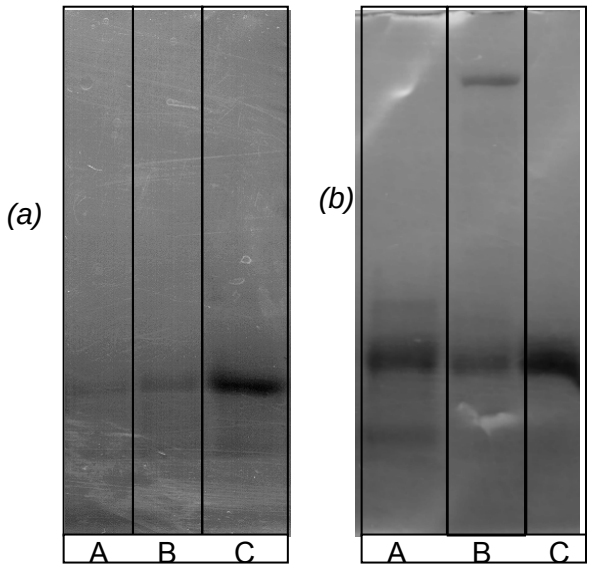


Figure 13.3: Key SDS-PAGE gel electrophoresis samples.

(a) A= Trenolin Mash (treatment 5), B= Acid Protease 2 (treatment 2), C= Standard Wine.
(b) A= GC106 (treatment 3), B= Optidex L-300 (treatment 1), C= Standard Wine.

All samples were visually scored as a first attempt to validate the heat test results. Subsequent to this a gel analysis computer program Quantity One (Bio-rad) was used to perform quantitative gel analysis. Key results for the most promising enzyme/treatment combinations are shown below in Table 13.2. Standard deviations for these results were all under 20%. Figure 13.3 shows a number of important characteristics of both the wine and the treatment enzymes. Lane C of both gel (a) and (b) are untreated samples of Gordo wine. The strong band evident is VVTL1, with the other thaumatin like and low molecular weight proteins clearly visible. Chitinases A,B,C, and D are not as clearly visible however were quantifiable using the Quantity One software. The Gordo wine was chosen for these characteristics because anecdotal evidence has shown that VVTL1 is the most difficult wine protein to remove. Lanes A and B of gel (a) show examples of treatments which markedly reduced the haze forming content of the wine. Lanes A and B of gel (b) show examples of treatments where minimal VVTL1 degradation resulted from the treatment. In addition to this these lanes also show strong protease bands at both high and low molecular weight.

The results for three of the proteases tested indicated high activity against the wine proteins. Table 13.2 shows the relative concentration of protein remaining in the wine after treatment as compared to the standard wine for these three proteases.

Table 13.2: Quantitative results from gel analysis using Quantity One.

Treatment Number	Enzyme	Relative Concentration
2	Acid Protease A	3.76
5	Acid Protease A	3.50
3	Acid Protease A	9.53
1	Acid Protease II	5.20
4	Acid Protease II	6.86
3	Acid Protease II	11.18
2	Trenolin Mash	4.24
5	Trenolin Mash	4.64
3	Trenolin Mash	7.80
Standard	N/A	11.17

13.3.3 Protein Quantification Method Comparison.

In order to aid in the validation of the methods used a comparison between the protein measured using a heat test and protein concentration using SDS-PAGE was performed. Figure 13.4 shows this comparison in graphical format.

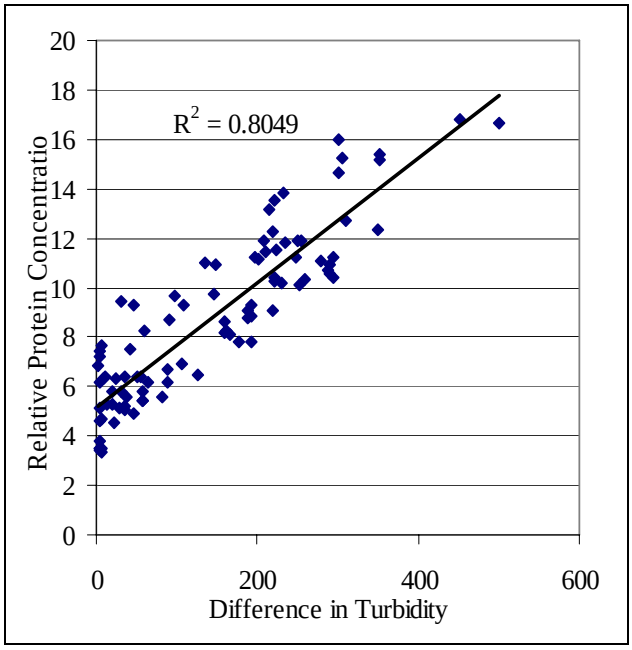


Figure 13.4: Comparison of protein quantification methods. Relative Protein Concentration is derived from SDS-PAGE and Difference in Turbidity from a heat test.

13.4 Discussion

13.4.1 Protein Quantitation Method Comparison and Validation

The literature gives little insight into the exact mechanism of haze formation in white wine. It is unclear as to whether total protein content or concentration of particular protein is the key driver of the degree of haze formation. Given this uncertainty as to the exact mechanism it is important to validate that the methods being used for determining the effect of different proteases and treatment give comparable results.

Figure 13.4 shows that the total protein content compares well with the heat test which is the industry standard for determining wine stability. However there was some variation from a perfect linear relationship through the origin of the graph. There are a number of reasons for this discrepancy.

- Not all wine proteins form haze. These proteins however do contribute to the total protein concentration determined by SDS-PAGE.
- The protease concentrations used in this experiment is significant when compared to total wine protein. The fact that some proteases are stable above the heat test temperature and some are not will affect the results. This was confirmed when neglecting the protease bands in the total protein content by electrophoresis yield a linear relationship with R^2=0.91.
- Methods compared more accurately when only considering samples from one gel. Comparison between gels requires very accurate background readings which is difficult on gels with many low concentration protein fractions.

13.4.2 Comparison of Treatment Methods

The two methods of protein quantification provided analogous results. Therefore looking at both Figure 13.2 and the complete results from the electrophoresis gels (not presented here) the different treatments can be compared.

Heat treatment alone reduced the haze production by an average of 60% for treatment 2 and 50% for treatment 1. This agrees with a previous study which found that protein content continued to decrease as temperature increased (Pocock, 2003).

As inferred by the activity curves of the proteases, the degradation of protein for all samples undergoing treatment at 20ºC (treatment 3) was negligible.

For most samples where protease was added at elevated temperatures (treatments 1, 2, 4 and 5) the degradation of protein was greater than that without protease addition. The treatments that were an exception to this were; all treatments using Papain 6000L as the protease, and treatment 4 with GC106 and Optidex L-300 as the proteases. In all cases pre-heat treatment at 70ºC provided some advantage in terms of protein denaturation

over simple heating to protease optimum temperature. However it is important to note that the best treatment is dependent on the protease activity curve.

13.4.3 Comparison of Proteases

Papain 6000L was completely ineffective and induced more haze in the wine. All other proteases reduced wine protein content however not all proteases behaved the same way:

- Optidex L-300 was observed to occlude almost 70% of the treated wine sample during filtering. This indicates that the lowering of haze formation is due to coalescence not proteolysis.
- GC 106 and Crystalzyme Vino Pro showed reduction in wine protein but not at significant levels. And also GC106 occluded around 35% of the wine again indicated a large degree of coalescence instead of proteolysis.
- Acid protease A, Acid Protease II and Trenolin Mash showed the most promising results, with very low coalescence.

13.5 Concluding remarks

This study has confirmed that the approach of combining heat treatment with acid proteases is valid for the removal of haze forming proteins in white wine.

Five different processing regimes were investigated. The regime with no heating (3) was considerably less successful that the other four regimes, failing in most cases to provide any reduction in wine protein levels. Processing regimes with preheat treatment of 70ºC for four minutes showed slightly better protein reduction than treatments without this step.

Seven different enzymes were used in this study, Papain 6000L, Crystalzyme Vino Pro, GC 106, Optidex L300, Acid Protease II, Acid Protease A and Trenolin Mash. The latter three enzymes produced wines which had heat test turbidity difference as low as 2% of that in the standard wine.

Commercial application of this process will require further investigation into the optimum processing conditions, and accurate scale up including kinetic modelling.

14 SAM coated nanoparticles

14.1 Introduction

During the GWRDC project, another alternative to bentonite for heat stabilisation was identified. This new technology involved use of self assembled monolayer (SAM) coated sand particles. A SAM consists of an ordered layer of adjacent multiple linear organic molecules (usually several to ten alkyl groups) with coupling agent at one end (which is attached to the implant surface) and a functionalized end group at the other. The coupling agent binds to the external surface of sand particles. The functionalized end group is the functional component of the SAM, which could be used to effect adsorption, and thus, removal of protein from wine.

The use of SAM-coated sand particles was proposed by Professor Peter Majewski of the Ian Wark Research Institute, University of South Australia. Professor Majewski supplied 30 g of SAM particles with a sulphonate ($-SO_3H$) functional group and a particle size of approximately 100 microns for evaluation. This evaluation involved a fining trial where it was tested for its ability to remove heat unstable proteins from white wine. For comparison purposes, the same white wine was also to be fined with Vitiben bentonite, a sodium bentonite used extensively in the wine industry for removing heat unstable proteins.

This evaluation was essentially a proof-of-concept to determine if the SAM-coated sand particles supplied by Professor Majewski were able to successfully heat stabilise a wine. An informal sensory analysis was also performed to establish whether the SAM-coated sand particles might produce an adverse sensory effect on wine.

The wine used for the fining trial was a heat unstable Sauvignon Blanc obtained from AWRI.

14.2 Materials & methods

Very briefly, a 40 g/L bentonite slurry was prepared 24 hours prior to use and a 40 g/L nanoparticle slurry was prepared approximately an hour before use.

Fining was conducted in 50 ml polypropylene tubes by the addition of aliquots of slurry to 40ml batches of wine. 7 different doses of nanoparticles and 7 different doses of bentonite were used as well as 2 untreated wine controls.

For informal sensory analysis, a further 8 tubes of wine dosed at 1.1 g bentonite/L, 8 tubes dosed at 3 g nanoparticles/L, and 8 tubes of untreated wine were prepared. All tubes were vortex mixed for approximately 30 seconds after which they were placed sideways on a shaker table (orbiting at ~ 100 rpm) for a further hour, before tubes were

once again vortex mixed. Samples were centrifuged at 3000 rpm (~ 2000 ×g) for 30 min to remove the majority of solids. The supernatant of samples to be used for sensory analysis was removed and collected in 3 × 250 ml glass bottles. The supernatants from the remaining tubes were filtered through 0.2 µm low protein binding syringe filters in preparation for heat tests to assess heat stability.

Sensory samples were taken to AWRI for and informal tasting by experienced tasters.

Heat stability tests were conducted the following day. The turbidity of samples was measured. Samples were then heated at 80 ˚C for 6 hours in a water bath, cooled for 15 minutes in an ice bath, and left to stabilise to room temperature for a further hour, after which, sample turbidity was again measured.

14.3 Results & Discussion

14.3.1 Heat stability

The results of the heat stability tests are shown below in Figure 14.1. The Vitiben bentonite was able to successfully heat stabilise the wine with a dose < 1 g/L. The SAM coated nanoparticles did not appear to have any significant effect on the heat stability of the wine. It was concluded that the most likely reason for this was that the preparation of the SAM coating on this batch of sand particles had failed.

Also noted was the much larger diameter of the sand particles. As a consequence, it would have taken a dose several hundred times of that for bentonite to provide the same reactive surface area for protein adsorption. For any evaluation to be realistic much smaller sand particles, approximately the same size and bentonite (i.e. 6 to 10 microns) should be provided.

The mechanism of protein adsorption for the SAM-coated sand particles will essentially be cation-exchange, based on dissociation of the sulphonate functional group. Whether this explicit use of an ion-exchange mechanism in this manner for heat stabilisation would be acceptable to the Australian wine industry is not clear.

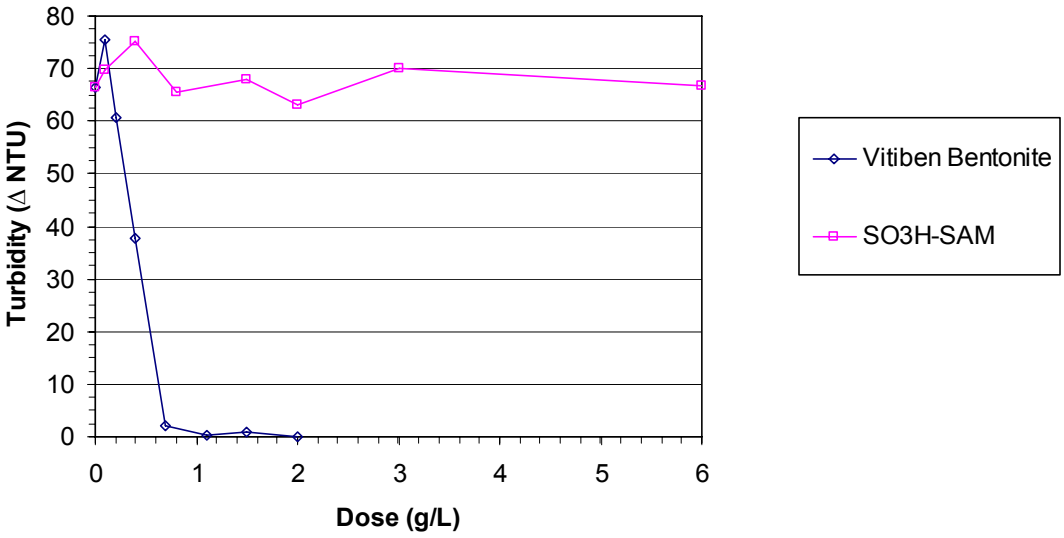


Figure 14.1: Heat Stability tests from Sauvignon Blanc fining trial with Vitiben bentonite and SAM coated nanoparticles.

14.3.2 Informal sensory analysis

Informal sensory analysis suggested that the untreated, bentonite fined and nanoparticle fined samples were similar and had no obvious gross defects. Both tasters seemed to find it difficult to smell/taste any difference between the bentonite and nanoparticle fined wine but thought the untreated wine was slightly more aromatic. One taster suggested that the nanoparticle fined wine was slightly more acidic on the palate. This may have been because the sulphonate functional group is hydrogen exchanged.

14.4 Concluding remarks

In this study, SAM coated sand particle proved ineffective at achieving heat stabilisation of a wine. However, it was unlikely to succeed given the large particle diameter and apparent difficulty in attaching the SAM layer. These problems could be overcome. The results have been reported back to Professor Majewski and it is understood he is revising his approach to produce another batch of SAM coated sand particle for further testing. This alternative technology has promise but there would be many barriers to overcome before acceptance by the Australian wine industry. This includes explicit use of an ion-exchange mechanism for protein adsorption and whether the SAM-coated nanoparticles would be acceptable as a food-grade reagent suitable for use with wine.

15 Outcome/Conclusion

The project has undertaken a diverse range of investigations of how current methods of bentonite fining can be improved and of alternative technologies for protein haze prevention in white wines. The outcomes from the project are summarised below.

- The project produced new data and knowledge regarding the properties and performance of different commercial bentonites (Sections 5 and 11). This included adsorption capacities, settling rates and volume of lees included. This information will allow industry to make better decisions when selecting bentonites to improve fining efficiency and reduce wine losses.
- The project has provided new data and knowledge that will allow industry to identify the optimal conditions for bentonite slurry preparation (Section 7). It was found that the most practical approach to bentonite slurry preparation is to use pure water and vigorous mixing for at least a day. Furthermore, bentonite performance may be improved by re-suspending bentonite lees in wine after they have settled. This could reduce the bentonite dose necessary to achieve stability, and thus, decrease wine losses.
- The project has provided a better understanding of what factors affect protein adsorption during bentonite fining (Sections 5 and 6). The most important factors appear to be slurry age and wine pH and potassium concentration. The effect of potassium suggests that bentonite fining may be more effective before tartrate stabilisation of the white wine when potassium concentration is highest.
- The project developed a new method for purification of gram quantities of heat unstable proteins from juice for R&D applications (Section 6). This new method will be valuable for future research investigating improvements in protein haze prevention methods.
- The project has identified that modification of commercial bentonites by heat treatment has the potential to improve the performance of bentonite fining by reducing the volume of lees and achieving more rapid settling (Section 5). In particular, it was found that some heat treated bentonites reduce the volume of lees three-fold but a higher dose was required.
- The project has demonstrated that in-line dosing of bentonite combined with centrifugal clarification is a more efficient method for bentonite fining, which can; reduce contact-time requirements to < 3 min; decrease wine losses by up to 80 %; and potentially avoid subsequent lees processing by rotary drum vacuum filtration (Section 9).

- The project has demonstrated that mathematical modelling tools used widely by other industries can also be applied for process simulation, analysis and optimisation of wine production operations (Sections 5, 6, 8 and 10). These tools were invaluable for experimental design and validating and understanding experimental observations. In particular, mathematical modelling of in-line dosing of bentonite helped to persuade industry to perform field testing
- The project has produced a new rapid HPLC method for quantifying protein content in wine and identifying those proteins responsible for haze formation (Section 11).
- The project has verified that combined/heat enzyme treatment has potential as an alternative technology to bentonite fining (Section 12).

Whilst the work in this project has been extensive there are many areas that deserve further attention. Many of these can be achieved by implementation of the findings contained in this report. Others involve acting on and/or extending some observations made regarding existing industry practices. Those that particularly stand out are as follows.

- The wine industry should develop more comprehensive methods for selecting and evaluating bentonite performance. Current decision making often appears to be based on bentonite price not on wine loss, which is the key cost driver in bentonite fining.
- It is recommended that wine industry associations and companies collaborate and work together with bentonite suppliers and/or research agencies to develop improved forms of bentonite with performance characteristics that optimise bentonite fining.
- The industry should work with bentonite suppliers and/or research agencies to develop best practice methods for preparation of bentonite slurry that ensure it can achieve its optimal performance when used.
- In-line dosing of bentonite with centrifugal clarification should be considered in place of batch-wise addition where practical. To optimise performance of this process, the industry should further work with suppliers of centrifugation equipment and/or research agencies to minimise observed problems with bentonite carry-over. Furthermore, the argument for application of in-line dosing would be strongly aided by availability of several industrial case studies that include calculation of wine and cost savings.
- Further research should be invested in investigating non-bentonite haze prevention alternatives. This includes enzyme addition with or without heat treatment.
- This project found during field testing on in-line dosing that bentonite did not cause any adverse sensory impacts on wine. If this is the case, the current trend towards

juice fining may not be advantageous when the opportunity cost of juice (which could be converted into wine) occluded in lees is considered.

- A more convenient method for measuring the heat stability of juice and wine would be invaluable for the industry. Direct quantification of heat unstable protein content would be ideal for this purpose but current methods for this purpose involve time-consuming and expensive HPLC, which is not available at most wineries. This area clearly deserves further attention.

Appendix 1: Communication

This project was undertaken in close association with the industry partner for the project, Hardy Wine Company. There was regular reporting and updates to the industry partner and they will receive a copy of this report. The AWRI also collaborated in the project and results are available to them to disseminate to industry through their industry outreach/extension activities.

Various results of the project have also been published in refereed journals, presented at conferences, and in Dr Muhlack's PhD thesis. The citations for these publications are given below.

The GWRDC project team contributed to a review article "Preventing protein haze in bottled white wine" prepared by Dr Elizabeth Waters for publication in the Australian Journal of Grape and Wine Research. This citation is included in the list below.

Also included in the citations below are relevant papers/presentations that have arisen from the contribution of the Chief Investigator to the AWRI project investigating use of HPF in wine.

The project has been profiled in Wine Industry Journal and in the GWRDC publication R&D@Work. Citations for these articles are listed below under "other publications".

This project has produced large volume of information and data which is still being analyzed by the Chief Investigator, which will lead to additional publications/presentations in the near future (i.e. next twelve months).

The Chief Investigator would welcome any additional opportunities/suggestions to further communicate the project outcomes.

Conference presentations/papers (refereed)

1. Colby CB, O'Neill BK, Waters EJ and Nordestgaard S (2006). In-line dosing of bentonite: A new processing strategy for the wine industry. Chemeca 2006 - 34th Australasian Chemical Engineering Conference, Auckland, New Zealand, 17 - 20 September 2006
2. Macintyre OJ, Colby CB, Chambers PJ, O'Neill BK, Pretorius IS and WatersEJ (2006). Optimisation of production of a wine haze protective factor. Chemeca 2006 - 34th Australasian Chemical Engineering Conference, Auckland, New Zealand, 17 - 20 September 2006.
3. Nordestgaard SJ, Colby CB, Chuan YP, O'Neill BK and Waters EJ (2006). Bentonite Lab Fining Trials with White Wine. Chemeca 2006 - 34th Australasian Chemical Engineering Conference, Auckland, New Zealand, 17 - 20 September 2006.

4. Colby CB, Nordestgaard S, Waters E and O'Neill BK (2006). Bentonite fining: Can we improve performance and efficiency and decrease value losses? Winery Engineering Australia Conference 2006 - Engineering for the Competitive Edge, Adelaide, Australia, 21 - 22 June 2006.
5. Lloyd FK, Colby CB, O'Neill BK, Waters E (2005), Combined Heat/Proteolytic Enzyme Treatment for the Removal of Protein Haze in Wine. Chemeca 2005 - 33rd Australasian Chemical Engineering Conference, Brisbane, Australia, 25 - 28 September 2005.
6. Muhlack RA, Waters EJ, O'Neill BK, Lim A and Colby CB (2004). New insights into the adsorption of haze-forming proteins by bentonite during winemaking. Chemeca 2004: 32nd Australasian Chemical Engineering Conference, Sydney, Australia: 26 - 29 September 2004.

Journal publications (refereed)

1. Waters, E.J., Alexander, G., Muhlack, R., Colby C., O'Neill, C., Høj, P.B. and Jones, P. (2005), *Preventing protein haze in bottled white wine*, Aust. J. Grape Wine R., 11, 215-225.
2. Muhlack, R., Nordestgaard, S., Waters, E., O'Neill, B.K. Colby, C.B. (2006) In-line dosing for bentonite fining of wine or juice: contact time, clarification, product recovery & sensory effects. Australian Journal of Grape and Wine Research, 12(3), 221-234
3. Nordestgaard S, Colby C, Chaun YP, O'Neill B, Waters E, Deans L and Policki P (2007) In-Line Dosing of White Wine for Bentonite Fining with Centrifugal Clarification. American Journal of Enology and Viticulture 58(2)
4. Muhlack RA, Waters EJ, Lim A, O'Neill BK and Colby CB (2007) An alternative method for purification of major thaumitin-like grape protein (VVTL1) responsible for haze formation in white wine. Asia-Pacific Journal of Chemical Engineering, Published Online: 15 Apr 2007

Other publications

1. Methods to improve the bentonite fining process become clearer; Wine Industry Journal, 22(2), p 24.
2. Engineering fine tunes bentonite; R&D@Work: GWRDC, January 2006

PhD theses

1. Muhlack, R. (2006) *Development of alternative technologies for bentonite treatment of heat-unstable white wine*. PhD thesis, University of Adelaide, Australia.

Appendix 2: Intellectual Property

The intellectual property associated with this project includes the data obtained from laboratory experiments and field testing and desk-top and feasibility studies. Much of this data has already been published (including in this report), and therefore, is in the public domain, except for Dr Muhlack's PhD thesis which is still embargoed by the University. The embargo on Dr Muhlack's thesis will soon lapse and there is no intention to extend it. There is presently no intention to, nor any perceived merit, in restricting disclosure of other unpublished data unless at instigation of the Grape & Wine Research & Development Corporation, Australian Wine Research Institute or Hardy Wine Company.

Appendix 3: References

Achaerandio, I., Pachova, V., Güell, C., López, F. (2001) Protein Adsorption by Bentonite in a White Wine Model Solution: Effect of Protein Molecular Weight and Ethanol Concentration. American Journal of Enology and Viticulture, 52(2), 122-126

Altin, O., Özbelge, Ö., Dogu, T. (1999) Effect of pH in an Aqueous Medium on the Surface Area, Pore Size Distribution, Density and Porosity of Montmorillonite. Journal of Colloid and Interface Science, 217, 19-27

Altman, J. (2003), *Protocol for using the Cary Spectrophotometer at the Altman laboratory at the Emory Vaccine Centre*, http://www.microbiology.emory.edu/altman/f_protocols/f_instruments/spectrophotometer

Armstrong, D. E., Chesters, G. (1964) Properties of Protein-Bentonite Complexes as Influenced by Equilibration Conditions. Soil Science, 98, 39-52

Asenjo, J. A., Chaudhuri, J. B. (1996) "Innovative separation methods in bioprocessing" In Separation processes in the food and biotechnology industries: Principals and applications. A. S. Grandison & M. J. Lewis (eds), Woodhead Publishing Ltd, Cambridge, pp 179-206

Bates, D. M., Watts, D. G. (1988) Non linear Regression Analysis and Its Applications. John Wiley and Sons, Inc., New York

Bailey, J. E., Ollis, D. F. (1986) Biochemical Engineering Fundamentals. McGraw-Hill, Inc., New York

Bayly, F. C., Berg, H. W. (1967) Grape and Wine Proteins of White Wine Varietals. American Journal of Enology and Viticulture, 18, 18-32

Blade, W.H., Boulton, R. (1988) Adsorption of Protein by Bentonite in a Model Wine Solution. American Journal of Enology and Viticulture, 39(3), 193-199

Bojemueller, E., Nennemann, A., Lagaly, G. (2001) Enhanced pesticide adsorption by thermally modified bentonites. Applied Clay Science, 18, 277-284

Bosma, J.C., Wesselingh, J.A. (1998) pH Dependence of Ion-Exchange Equilibrium of Proteins. American Institute of Chemical Engineers Journal, 44(11), 2399-2409

Boulton, R. (1986), *Experience in the application of centrifuges to wine production in California*, In: Innovations in Enology – New factors in wine clarification, Proc. of an International Symposium, 28-29 May 1986, Stuttgart, Fed. Rep. of Germany, pp. 46-52.

Boulton, R., Singleton, V.L., Bisson L.F. and Kunkee, R.E. (1996) Principles and Practices of Winemaking. Aspen Publishers, Inc.: Gaithersburg, Maryland.

Bradley, W.F., Grim, R.E. (1948) Colloid Properties of Layer Silicates. Journal of Physical and Colloid Chemistry, 52, 1404-1413

Cabaroglu, T., Razungles, A., Baumes, R., Gunata, Z. (2003) Effect of fining treatments on the aromatic potential of white wines from Muscat Ottonel and Gewürztraminer cultivars. Sciences des Aliments, 23, 411-423

Calvet, R., Prost, R. (1970) Cation migration into empty octahedral sites and surface properties of clays. Clays and Clay Minerals, 19, 175-186

CAMO A/S (1994) The Unscrambler v 5.5 User's Guide: Software for multivariate data analysis applying PCA, PCR and PLS, including experimental design. CAMO A/S Trondheim, Norway

CAMO A/S (2005) The Unscrambler v 9.2 software online help: "MLR vs. PCR vs. PLS". CAMO A/S Trondheim, Norway

Chorom, M., Rengasamy, P., Murray, R. S. (1994) Clay Dispersion as Influenced by pH and Net Particle Charge of Sodic Soils. Australian Journal of Soil Research, 32, 1243-1252

Chorom, M., Rengasamy, P. (1995) Dispersion and zeta potential of pure clays as related to net particle charge under varying pH, electrolyte concentration and cation type. European Journal of Soil Science, 46, 657-665

Chorom, M., Rengasamy, P. (1996) Effect of heating on swelling and dispersion of different forms of a smectite. Clays and Clay Minerals, 44(6), 783-790

Churchman, G. J. (1999) Procedures for the efficient recycling of bentonite used for the removal of proteins from wine. Final Report to Grape and Wine Research and Development Corporation, Project CSS 98/1

Colby, C.B., O'Neill, B.K. and Middelberg, A.P.J. (1996), *Simulation of compression effects during scale-up of a commercial ion-exchange process*, Biotechnology Progress, 12, 662-681.

Commonwealth of Australia, Department of Foreign Affairs and Trade (1994) "Agreement between Australia and the European Community on Trade in Wine, and Protocol", Australian Treaty Series 1994 No 6, Entry into force: 1 March 1994

Comuzzi, C, Polese, P., Melchior, A., Portanova, R., Tolazzi, M. (2003) SOLVERSTAT: A new utility for multipurpose analysis. An application to the investigation of deoxygenated Co(II) complex formation in dimethylsulfoxide solution. Talanta 59, 67-80.

Constantinides, A., Mostoufi, N. (1999) Numerical Methods for Chemical Engineers with MATLAB Applications. Prentice Hall, Inc., Upper Saddle River, New Jersey

Cordonnier, R., Dugal, A., (1968). *"Les activities proteolytiques du raisin".* Ann. Technol. Agric., 17, 189-206.

Coulson, J. M., Richardson, J. F. (1979), Chemical Engineering: Volume 3. Chemical Reactor design, Biochemical Reaction Engineering including Computational techniques and Control. 2 ed. Pergamon Press Ltd., Oxford, pp 1-106

Cusack, M., Peirpoint, W. S. (1988) Similarities Between Sweet Protein Thaumatin and a Pathogenesis-Related Protein From Tobacco. Phytochemistry, 27(12), 3817-3821

Dambrouck, T., Marchal, R., Cilindre, C., Parmentier, M., Jeandet, P. (2005), *Determination of the Grape Invertase Content (Using PTA-ELISA) following various Fining Treatments versus Changes in the Total Protein Content of Wine. Relationships with wine foamability*, Journal of Agriculture and Food Chemistry. 53, 8782-8789

Dawes, H., Boyes, S., Keene, J., Heatherbell, D. (1994) Protein Instability of Wines: Influence of Protein Isoelectric Point. American Journal of Enology and Viticulture, 45(3), 319-326

Deshpande, S. S. (1995) "Affinity Chromotography" In: Bioseparation Processes in Foods. R. K. Singh and S. S. H. Rizvi (eds), Marcel Dekker, Inc., New York, pp 297-332

Dillon, W. R., Goldstein, M. (1984) Multivariate Analysis: Methods and Applications. John Wiley and Sons, Inc. New York

Dubourdieu, D., Llauberes, R. and Ollivier, C. (1986), *Estimation rapide des constituants macomoleculaires des mouts et des vins par chromatographie liquide haute pression (CLHP) de tamisage moleculaire*, Connaiss. Vigne Vin 20, 119-123.

Duncan, B. (1992) Varietal Differences in White Grape Protein: Implications for Bentonite Fining. Australian and NZ Wine Industry Journal, Aug, pp 189-193

Dunn, M. J. (1989) "Electrophoretic Analysis Methods" In: Protein purification methods: a practical approach. E. L. V. Harris, S. Angal (eds.), IRL Press at Oxford University Press, New York

Ensminger, L.E., Gieseking, J.E. (1939) The Adsorption of Proteins by Montmorillonite Clays. Soil Science, 48, 467-473

Fargues, C., Bailly, M., Grevillot, G. (1998) Adsorption of BSA and Hemoglobin on Hydroxyapatite Suort: Equilibria and Multicomponent Dynamic Adsorption. Adsorption, 4, 5-16

Ferreira, R. B., Piçarra-Pereira, M. H., Monteiro, S., Loureiro, V. B. and Teixeira, A. R. (2002) The wine proteins. Trends in Food Science & Technology, 12, 230-239.

Feuillat, M., Brillant, G., Rochard, J., (1980). *"Mise en evidence d'une production de proteases exocellulaires par les levures au cours de la fermentation alcoolique du mount raisin."* Connaiss Vinge Vin, 14, 37-52.

Fils-Lycaon B. R., Wiersma P. A., Eastwell K. C., Sautiere P. (1996) A Cherry Protein and Its Gene, Abundantly Expressed in Ripening Fruit, Have Been Identified as Thaumatin-Like. Plant Physiology, 111, 269-273

Finlayson, B. A. (1980) Nonlinear Analysis in Chemical Engineering. McGraw-Hill, Inc., New York

Food Standards Australia New Zealand (2005) Standard 4.5.1 - Wine production requirements.

Foust, A. S., Wenzel, L. A., Clump, C. W. Maus, L. Andersen, L. B. (1980) Principals of Unit Operations 2nd Ed, John Wiley & Sons, Inc., New York, pp 629-631

Francis, I. L., Sefton, M. A., Williams, P. J. (1994) The sensory effects of pre- or post-fermentation thermal processing on Chardonnay and Semillon wines. American Journal of Enology and Viticulture, 45, 243-251

Geankoplis, C. J. (1978) Transport Processes and Unit Operations, Allyn and Bacon, Inc., Boston, pp 309

Gieseking, J. E. (1939) The Mechanism of Cation Exchange in the Montmorillonite-Beidellite-Nontronite Type of Clay Minerals. Soil Science, 47, 1-14

Gishen, M., Coulter, A. (2003) Personal communication regarding the pH, ethanol, sugar, phenolic, and metal ion composition of juice and wine, Australian Wine Research Institute

González, R. and González, L.M. (1991), *Analysis of the soluble proteins in grape musts by reversed-phase HPLC*, Chromatographia, 32, 463-465

Gourgeon, R. D., Reinholdt, M., Delmotte, L., Miehe-Brendle, J., Chézeau, J. M., Le Dred, R., Marchal, R. and Jeandet, P. (2002) Direct observation of polylysine side-chain interaction with smectites interlayer surfaces through ^{1}H-^{27}Al heteronuclear correlation NMR spectroscopy. Langmuir, the American Chemical Society Journal of Surfaces and Colloids, 18, 3396-3398

Gourgeon, R. D., Soulard, M., Reinholdt, M., Miehe-Brendle, J., Chézeau, J. M., Le Dred, R., Marchal, R. and Jeandet, P. (2003) Polypeptide adsorption onto a synthetic montmorillonite: A combined solid-state NMR, X-ray diffraction, thermal analysis and N_2 adsorption study. European Journal of Inorganic Chemistry, 2003, 1366-1372

Graham, E. E., Pucciani, A., Pinto, N. G. (1987) A Comparative Study of Models to Predict Protein Adsorption. Biotechnology Progress, 3(3), 141-145

Grandison, A. S. (1996) “Ion-exchange and electrodialysis” In: Separation processes in the food and biotechnology industries: Principals and applications. A. S. Grandison & M. J. Lewis (eds),Woodhead Publishing Ltd, Cambridge, 155-178

Grim, R. E. (1968) Clay Mineralogy. 2 ed. McGraw Hill Book Company: New York

GWRDC (July 2002), Benefit Cost Analysis Guidelines for Researchers

Hayasaka Y., Adams K. S., Pocock K. F., Baldock G. A., Waters E. J., Høj P. B. (2001) Use of Electrospray Mass Spectrometry for Mass Determination of Grape (*Vitis Vinifera*) Juice Pathogenesis-Related Proteins: A Potential Tool for Varietal Differentiation. Journal of Agriculture and Food Chemistry, 49, 1830-1839.

Heatherbell, D., Ngaba, P., Fombin, J., Watson, B., Garcia, Z., Flores, J., Hsu, J. (1984) "Recent developments in the application of ultrafiltration and protease enzymes to grape juice and wine processing." In: Proceedings of the International Symposium on Cool Climate Viticulture and Enology. D. A. Heatherbell, P. B. Lombard, F. W. Bodyfelt, S. F. Price, eds., Oregon State University, Corvallis, OR

Hendricks, S. B., Jefferson, M. E. (1938) Structure of Kaolin and Talc-Pyrophyllite Hydrates and Their Bearing on Water Sorption of Clays. American Mineralogist, 23, 863-875

Høj, P.B., Tattersall, D.B., Adams, K., Pocock, K.F., Hayasaka, Y., van Heeswijck, R. and Waters, E.J.(2000) *The 'Haze Proteins' of wine – A summary of properties, factors affecting their accumulation in grapes, and the amount of bentonite required for their removal from wine*. Proc. of the ASEV 50th Anniversary Meeting held at Seattle, Washington, USA, pp. 144-154.

Hsu, J. C., Heatherbell, D. A. (1987a) Isolation and Characterisation of Soluble Proteins in Grapes, Grape Juice, and Wine. American Journal of Enology and Viticulture, 38(1), 6-10

Hsu, J., Heatherbell, D. A. (1987b) Heat-Unstable Proteins in Wine. I. Characterization and Removal by Bentonite Fining and Heat Treatment. American Journal of Enology and Viticulture, 38(1), 11-16

Hsu, J., Heatherbell, D. A. (1987c) Heat-Unstable Proteins in Wine. II. Characterization and Removal by Ultrafiltration. American Journal of Enology and Viticulture, 38(1), 17-22

Interesse, F.S., Alloggio, V., Lamparelli, F. and D'Avella, G. (1987), *Proteins in musts estimated by size-exclusion HPLC*, Food Chemistry, 23, 65-78.

Joshi, R. C., Achari, G., Horsfield, D., Nagaraj, T. S. (1994) Effect of Heat Treatment on Strength of Clays. Journal of Geotechnical Engineering, 120, 1080-1088

Kim, S. and Park, H. (2002) Applicability assessment of subcritical flux operation in crossflow microfiltration with a concentration polarization model. Journal of Environmental Engineering 128, 335-340.

Kline, P., (1994) An Easy Guide to Factor Analysis. Routledge, London

Koch, J., Sajak, E. (1959) A Review and Some Studies On Grape Protein, American Journal of Enology and Viticulture, 10, 114-123

Ledoux, V., Dulau, L. and Dubourdieu, D. (1992), *Interprétation de l'amélioration de la stabilité protéique des vins au cours de l'élevage sur lies*, J. Int. Sci. Vigne Vin 26, 239-251.

Lee, T.H. (1986) Protein instability: Nature, characterisation and removal by bentonite. In: Physical Stability of Wine – Proceedings of a seminar held on 10 October 1985 at

Reynella, South Australia. pp. 23-39. Australian Society of Viticulture and Oenolgy, Glen Osmond, SA.

Leske, P.A., Bruer, N.G.C., Capdeboscq, V. An evaluation of some characteristics of commercial bentonites. Wine Industry Journal 10(1), 73-77.

Linthorst, H. J. M. (1991) Pathogenesis-Related Proteins of Plants. Critical Reviews in Plant Science, 10(2), 123-150

Low, L., Colby, C.B., O'Neill, B.K., Ford C., Godden J., Gishen, M. (2006) Review and economic evaluation of new technologies for tartrate stabilisation of wines. Journal of Food Engineering: Submitted 28 April 2006.

Marchal, R., Weingartner, S., Voisin, C., Jeandet, Ph. and Chatelain, F. (2002) Use of mathematical laws for optimising the doses of swelled and dry bentonite during the fining of white wines. Part I: Clarification and colloidal stability. Journal International des Sciences de la Vigne et du Vin 36, 169-176.

Marchal, R., Weingartner, S., Voisin, C., Jeandet, Ph. and Chatelain, F. (2003) Use of mathematical laws for optimising the doses of swollen and dry bentonite during the fining of white wines. Part II: Influence on the volume of lies and economics. Journal International des Sciences de la Vigne et du Vin 37, 123-130.

Martinez-Rodriguez, A.J. and Polo, M.C. (2003) Effect of addition of bentonite to the tirage solution on the nitrogen composition and sensory quality of sparkling wines. Food Chemistry 81, 383-388.

McCabe, W.L., Smith, J.C. and Harriott, P. (2001) Unit operations of chemical engineering. Sixth addition. McGraw-Hill, Singapore.

McLaren, A.D., Peterson, G.H. and Barshad, I. (1958) The adsorption and reactions of enzymes and proteins on clay minerals: IV. Kaolinite and Montmorillonite. Proceedings of the soil society of America 22, 239-244.

Miller, G.C., Amon, J.M., Gibson, R.L. and Simpson, R.F. (1985) Loss of aroma attributable to protein stabilization with bentonite or ultrafiltration. The Australian Grapegrower & Winemaker, April 1985, 46-50

Modra, E.J. and Williams, P.J. (1988), *Are proteases active in wines and juices?*, Australian Grapegrower Winemaker 292, 42-46

Monteiro, S., Piçarra-Periera, M. A., Mesquita, P. R., Loureiro, V. B., Teixeira, A., Ferreira, R. B. (2001) The Wide Diversity of Structurally Similar Wine Proteins, Journal of Agriculture and Food Chemistry, 49, 3999-4010

Montgomery, D. C. (2001) Design and Analysis of Experiments. John Wiley and Sons, Inc. New York

Morant, I. (1990) Evaluation of bentonites available on the Australian market. AWRI Technical Review 67, 16-33.

Moretti, R. H., Berg, H. W. (1965) Variability Among Wines To Protein Clouding. American Journal of Enology and Viticulture, 16, 69-78

Muhlack, R., Nordestgaard, S., Waters, E., O'Neill, B.K. Colby, C.B. (2006) In-line dosing for bentonite fining of wine or juice: contact time, clarification, product recovery & sensory effects. Australian Journal of Grape and Wine Research, 12(3), 221-234

Muhlack, R. (2006) *Development of alternative technologies for bentonite treatment of heat-unstable white wine*. PhD thesis, University of Adelaide, Australia.

Murphey, J.M., Spayd, S.E. and Powers, J.R. Effect of grape maturation on soluble protein characteristics of Gewürztraminer and white Riesling juice and wine. American Journal of Enology and Viticulture 40(3), 199-207.

Ngaba, P. R., Heatherbell, D. A. (1981) "Protein instability in wine. I. Characterisation of unstable proteins. II. The application of protease enzymes as bentonite substitutes in protein stabilization wine." In: Proceedings of the 32nd Annual Meeting of the American Society of Enologists, San Diego, CA.

Norde, W., Lyklema, J. (1978) The Adsorption of Human Plasma Albumin and Bovine Pancreas Ribonuclease at Negatively Charged Polystyrene Surfaces I: Adsorption Isotherms, Effects of Charge, Ionic Strength and Temperature. Journal of Colloid and Interface Science, 66(2), 257-265

Norde, W. (1986) Adsorption of Proteins from Solution at Liquid-Solid Interface. Advances in Colloid and Interface Science, 25, 267-340

Nordestgaard N., Colby, C.B., Chuan, Y.P., O'Neill, B.K., Waters E.J., Deans L., Policki, P (2007). In-line Dosing of White Wine with Bentonite with Centrifugal Clarification. American Journal of Enology & Viticulture 58(2).

Pashley, R. M. (1981) DLVO and Hydration Forces between Mica Surfaces in Li^+, Na^+, K^+ and Cs^+ Electrolyte Solutions: A Correlation of Double-Layer and Hydration Forces with Surface Cation Exchange Properties. Journal of Colloid and Interface Science, 83(2), 531-546

Pashova, V., Güell, C. and López, F. (2004) White wine continuous protein stabilization by packed column. Journal of Agricultural and Food Chemistry, 52, 1558-1563.

Peng, Z., Pocock, K. F., Waters, E. J., Francis, I. L. and Williams, P. J. (1997) Taste properties of grape (*Vitis vinifera*) pathogenesis-related proteins isolated from wine. Journal of Agricultural Food Chemistry, 45, 4639-4643

Perry, R. H., Green D. W. editors, (1997) Perry's Chemical Engineers' Handbook. 7th Ed, McGraw-Hill, Inc., New York, pp 5-70, 16-16

Peynaud, E. (1984) Knowing and making wine. Wiley, New York.

Pocock, K. and Rankine, B. (1973) Heat test for detecting protein instability in wine. Australian Wine Brewing and Spirit Review 91, 42-43.

Pocock, K.F., Hayasaka, Y., Peng, Z., Williams, P.J. and Waters, E.J. (1998) The effect of mechanical harvesting and long-distance transportation on the concentration of haze-forming proteins in grape juice. Australian Journal of Grape and Wine Research 4, 23-29.

Pocock, K.F., Hayasaka, Y., McCarthy, M.G., Waters, E.J., (2000). "*Thaumatin-like Proteins and Chitinases, the Haze-Forming Proteins of*

Wine, Accumulate during Ripening of Grape (Vitis vinifera) Berries and Drought Stress Does Not Affect the Final Levels per Berry at Maturity" J. Agric Food Chem. 48, 1637-1643.

Pocock, K.F., Hoj, P.B., Adams, K.S., Kwiatkowski, M.J., Waters, E.J., (2003). *"Combine dheat and proteolytic enzyme treatment of white wines reduces haze forming protein content without detrimental effect".* Australian Journal of Grape and Wine Research, 9(1), 53-63.

Pocock, K.F., Waters, E.J. (2006) Protein haze in bottled white wines: how well do stability tests and bentonite fining trials predict haze formation during storage and transport. Australian Journal of Grape & Wine Research. 12(3), 212-220.

Pollnitz, A.P., Capone, D.L., Caldersmith, M.C. and Sefton, M.A. (2003) The effect of various wine bottle closures and fining agents on flavour and aroma compounds in wine. In: Grapegrowing at the Edge; Managing the Wine Business; Impacts on Wine Flavour. Proceedings of a seminar held on 10-11 July 2003 at Tanunda, South Australia. (Australian Society of Viticulture and Oenology: Adelaide, South Australia) pp. 59-63

Polo, M.C., Cáceres, I., Palop, Ll., Dizy, M., Pueyo, E., Martín-Alvarez, P.J. (1989), *Study of the proteic fraction of grape musts by high performance liquid chromatography and electrophoretical techniques. Variety Differentiation*, in Charalambous, G. (Ed), *Flavours and Off-Flavors, Proceedings of the 6th International Flavor Conference*, Rethymon, Crete, Greece, 5-7 July 1989, pp. 87-101

Polson, A. (1950), *Some Aspects of Diffusion in Solution and a Definition of a Colloidal Particle*, J. Phys. Colloid Chem., 54, 649.

Powers, J. R., Nagel, C. W., Weller, K. (1988) Protein Removal from a Wine by Immobilised Grape Proanthocyanidins. American Journal of Enology and Viticulture, 39(2), 117-120

Rankine, B. (2004) Making good wine: A manual of winemaking practice for Australia and New Zealand. Macmillan, Sydney.

Rector, B. (1983) Theories and uses of fining agents. Practical Winery, January-February 1983, 43-48.

Ribéreau-Gayon, Dubourdieu, D. Donèche, B. and Lonuaud, A. (2000) Juice treatments and the advisability of bentonite treatments. In: Handbook of Enology, Vol. 1 – The

microbiology of wine and vinification. John Wiley & Sons Ltd: New York, USA; pp 390-391.

Richardson, J. F., Zaki, W. N. (1954) Sedimentation and Fluidisation: Part I. Transactions of the Institution of Chemical Engineers, 32, 35-53

Rodriguez, J.L.P., Weiss, A., Lagaly, G. (1977) A Natural Clay Organic Complex from Andalusian Black Earth. Clay and Clay Minerals, 25, 243-251

Rosenbrock, H. H. (1963) Some general implicit processes for the numerical solution of differential equations. The Computer Journal, 5(4), 329-330

Santoro, M. (1995) Fractionation and Characterisation of Must and Wine Proteins. American Journal of Enology and Viticulture, 46(2), 250-254

Sarmento, M. R, Oliveira, J. C, Boulton, R. B. (2000) Selection of low swelling materials for protein adsorption from white wines, International journal of Food Science and Technology, 35, 41-47

Saywell, L.G. (1934) Clarification of wine. Industrial and Engineering Chemistry 26, 981-982.

Skidmore, G. L., Morstmann, B. J., Chase, H. A. (1990a) Modelling Single-Component Protein Adsorption to the Cation Exchanger S Sepharose FF. Journal of Chromatography, 498, 113-128

Skidmore, G. L., Chase, H. A. (1990b) Two-Component Protein Adsorption to the Cation Exchanger S Sepharose FF. Journal of Chromatography, 505, 329-347

Stockley, C. (2002) Personal communication regarding the export of Australian wines treated by ion exchange. The Australian Wine Research Institute

Somers, T.C. and Ziemelis, G. (1973), *Direct determination of wine proteins*, American Journal of Enology and Viticulture, 24, 47-50.

Stone, H. and Sidel, J.L. (1993) Sensory Evaluation Practices. Second Ed. Academic Press Inc.: Orlando, Florida.

Suen, S. (1996) A Comparison of Isotherm and Kinetic Models for Binary-Solute Adsorption to Affinity Membranes. Journal of Chemical Technology and Biotechnology, 65, 249-257

Tattersall, D. B. (1999) Identification and Characterisation of v*itis vinifera* Pathogenesis-Related Proteins That Accumulate During Berry Ripening. PhD Thesis, University of Adelaide

Tatttersall, D.B., Pocock, K.F., Hayasaka, Y., Adams, K., van Heeswijck, R., Waters, E.J. and Høj, P.B. (2001) Pathogenesis related proteins in grapes during berry growth and their involvement in white wine instability. Current knowledge and future perspectives in relation to winemaking practices. In: Molecular Biology and Biotechnology of the Grapevine. Ed. K.A. Roubelakis-Angelakis (Kluwer Academic Publishers: Dordrecht, Netherlands), pp. 183-201.

Tucker, G.A., Woods, L.F.J, Ed. 1995. *"Enzymes in Food Processing"*. Glasgow, Blackie Academic & Professional.

Tyson, P.J., Luis, E.S., Day, W.R., Walker, B. and Lee, T.H. (1981), *Estimation of soluble protein in must and wine by high-performance liquid chromatography*, American Journal of Enology and Viticulture, 32, 241-243

van Loon, L. C. (1985) Pathogenesis-related proteins. Plant Molecular Biology, 4, 111-116

van Olphen H. (1963) An Introduction to Clay Colloid Chemistry, John Wiley & Sons

Vidal S., Williams P., Doco T., Moutounet M., Pellerin P. (2003) The polysaccharides of red wine: total fractionation and characterization. Carbohydrate Polymers, 54(4), 439-447

Volclay Pty Ltd (1997), Specification Data and Material Safety Data Sheet for Volclay WG KWK

Volk, W (1969) Applied Statistics for Engineers. 2nd ed., McGraw-Hill, Inc, New York

Walas, S. M. (1997) "Chemical Reactors", in R. H. Perry, D. W. Green (eds.), Perry's Chemical Engineers' Handbook. 7 ed., Mcgraw-Hill, New York, Section 23

Waters E. J., Wallace W., Williams P. J. (1991) Heat Haze Characteristics of Fractionated Wine Proteins. American Journal of Enology and Viticulture, 42(2), 123-127

Waters, E. J. (1991) Heat Unstable Wine Proteins and their Interactions with Wine Polysaccharides. PhD Thesis, University of Adelaide

Waters, E. J., Wallace, W., Williams, P. J. (1992) The identification of heat-unstable wine proteins and their resistance to peptidases. Journal of Agriculture and Food Chemistry, 40, 1514-1519

Waters, E. J., Wallace, W., Tate, M. E., Williams, P. J (1993) Isolation and Partial Characterisation of a Neutral Haze Protective Factor from Wine. Journal of Agriculture and Food Chemistry., 41, 724-730

Waters, E., J., Pellerin, P., Brillouet, J-M. (1994) A *Saccharomyces* mannoprotein that protects wine from protein haze. Carbohydrate Polymers, 23, 185-191

Waters, E.J., Shirley, N.J. and Williams, P.J. (1996) Nuisance proteins of wine are grape pathogenesis-related proteins. Journal of Agricultural and Food Chemistry 44, 3-5.

Waters, E.J. Hayasaka, Y., Tattersall, D.B., Adams, K.S. and Williams, P.J. (1998) Sequence analysis of grape (*Vitis Vinifera*) berry chitanases that cause haze formation in wines. Journal of Agricultural and Food Chemistry 46, 4950-4957.

Waters, E.J., Alexander, G., Muhlack, R., Colby C., O'Neill, C., Høj, P.B. and Jones, P. (2005), *Preventing protein haze in bottled white wine*, Aust. J. Grape Wine R., 11, 215-225.

Weetall, H. H., Zelko, J. T., Bailey, L. F. (1984) A New Method for the Stabilisation of White Wine. American Journal of Enology and Viticulture, 35(4), 212-215

Weiss, K.C., Bisson, L. (2002) Effect of bentonite treatment of grape juice on yeast fermentation. American Journal of Enology and Viticulture 53(1), 28-36.

Weiss, K. C., Lange, L. W. and Bisson, L. F. (2001) Small-scale fining trials: Effect of method of addition on efficiency of bentonite fining. American Journal of Enology and Viticulture 52, 275-279.

Whitley, R.D., R. Wachter, F. Liu., Wang, N.H.L. (1989) Ion-Exchange Equilibria of Lysozyme, Myoglobin and Bovine Serum Albumin. Journal of Chromatography 465, 137-156

Zoecklin, B. (1988) Bentonite fining of juice and wine. Virginia Cooperative Extension Service (United States of America): Publication 463-014.

Zoecklein, B. K., Fugelsang, B. Gump, and Nury, F. (1999) Wine Analysis and Production. Kluwer Academic/Plenum Publishers, New York.

Appendix 4: Staff

The principal staff involved in the project was as per original GWRDC Application – refer Table below. However, this list does not include the technical personnel and winemakers at Berri Estates Winery that contributed to the project during field testing. Associate Professor Brian O'Neill was the Project Supervisor. Dr Chris Colby was the Chief Investigator.

Name	Title	Position	Qualification	Agency
1. Brian O'Neill	Associate Professor	Deputy Head of School	BE (Chem) Hons PhD, *UQld*	School of Chemical Engineering, University of Adelaide
2. Chris Colby	Dr.	Senior Lecturer	BE (Chem) Hons PhD, *UAdel*	School of Chemical Engineering, University of Adelaide
3. Peter Høj	Professor	Director	MSc PhD, *Copenhagen*	Australian Wine Research Institute
4. Elizabeth Waters	Dr.	Principle Research Biochemist	BSc PhD, *UAdel*	Australian Wine Research Institute
5. Audrey Lim	Ms.	Group Oenologist	BSc Hons, *UMelb*, GradDipOenol, *UAdel*	BRL Hardy Ltd
6. Dr Richard Muhlack	Mr.	Postgraduate Student	BE (Chem) Hons, *UAdel*	School of Chemical Engineering, University of Adelaide
7. Ms Felicity Lloyd		Postgraduate Student		School of Chemical Engineering, University of Adelaide
8. Mr Simon Nordestgaard		Research Engineer		School of Chemical Engineering, University of Adelaide

Appendix 5: Budget reconciliation

Refer following page(s) for completed copy(ies) of Form B or equivalent.