PROTEIN STABILISATION OF NEW ZEALAND SAUVIGNON BLANC

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Protein Stabilisation of New Zealand Sauvignon Blanc

by

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Identifying alternatives to bentonite fining and/or reducing bentonite use to achieve protein stability has long been a goal of the wine industry. Unstable proteins in white and rosé wines have been demonstrated to be pathogenesis-related proteins which originate from the grape, survive vinification and remain soluble in finished wine. This thesis contributes information about protein in and stabilisation of New Zealand wines. The aims of this study were first to develop methods for protein quantification and characterisaton, and then further to investigate viticultural and oenological influences on wine protein stabilisation. This study was carried out on Sauvignon blanc from the Marlborough region in consecutive vintages, 2007 and 2008. Three methods, Coomassie Brilliant Blue (CBB) assay, lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS-PAGE) and sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE), were utilised to investigate effects of vintage, vineyard site, pruning, pH adjustment, timing of bentonite addition, stabilisation test parameters and type of adsorbent.

Wine protein recovery by acetone precipitation was more effective than that by ultrafiltration (10 kDa molecular weight cut-off); the latter gave a 21% loss of total protein by

SDS-CGE. Proteins with molecular weight between 19 and 33 kDa were the predominant fractions determined by both LDS-PAGE (70%) and SDS-CGE (98%), but the 64 kDa fraction observed by LDS-PAGE (21%) was not detectable in SDS-CGE. However, the SDS-CGE provided better resolution than LDS-PAGE with 8 fractions (9.6, 19, 21 22, 24.6, 26.5, 27.6 and 32 kDa) detected. For a range of wines (n = 18) surveyed results indicated that the CBB assay resulted in wine protein concentrations (average 113.3 mg L^{-1}; CV = 16%) about 42% higher compared to LDS-PAGE (r^2 = 0.80; CV = 8%) and about 4.2 times lower compared to SDS-CGE (r^2 = 0.62; CV = 16%). The rapid and simple CBB assay coupled with the finding that a narrow range of protein content (10 to 25 mg L^{-1}) remained in most stabilised wines (n = 102) could be a good method to predict bentonite requirement.

Among 5 vineyard sites in Marlborough, one consistently showed the lowest protein concentration in juice and wine, lowest haze formation and lowest bentonite requirement regardless of pruning treatment and vintage, whereas other vineyards varied when pruning treatments and/or vintages were compared. Two juice protein peaks at 22 and 28 kDa in SDS-CGE appeared to be related to two main wine protein fractions at 22 and 26 kDa, respectively. The 28 kDa fraction was reduced and became heterogeneous after fermentation, while the 22 kDa fraction remained unaffected. Bentonite requirement determined by a standard hot/cold test (80°C for 6 hours followed by 4°C overnight) was correlated with total and individual protein concentrations and haze level; there was a good correlation of bentonite requirement with the 26 and 32 kDa fractions (r^2 = 0.78; $p < 0.001$), but less good with protein haze, total protein concentration and the predominant 22 kDa fraction ($r^2 < 0.50$; $p < 0.05$).

The presence or absence of bentonite during fermentation seemingly did not affect fermentation kinetics to below 0 °Brix regardless of pH, although lower juice pH (2.80 and

3.00) tended to result in sluggish fermentation. The presence of bentonite in the ferment improved the rate of completion of fermentation for the slower fermentation (pH 2.80). Adjusting the pH of juice and/or wine modified the bentonite requirement by two mechanisms: reduced wine pH improves protein adsorption efficiency by bentonite fining, and decreased juice pH results in a lower wine protein content. Bentonite addition during fermentation was the most efficient in terms of protein removal but fining after fermentation resulted in the lowest overall dosage. The molecular weight profile of proteins from wines produced at microvinification and commercial scales were identical, although different fermentation scales slightly affected wine protein content. Protein content and molecular weight profiles in stabilised wines were affected by the original juice pH with more complex patterns from high pH juice.

Heat treatment at 90 °C for 1 hour produced the most protein haze and was the most sensitive in haze reduction in response to incremental bentonite fining compared to 80 °C for 2, 6 and 15 hours treatments. There seemed to be a coincident point at approximately 3 nephelometric turbidity units which gave the same predicted bentonite requirement for all heat tests. Na bentonite was the most efficient adsorbent in protein removal, followed by NaCa bentonite, polymeric resin and cation-exchange resin. Protein adsorption isotherms from the Langmuir model indicated that the adsorption capacity for Na bentonite was about 2 and 3 times higher compared to that for NaCa bentonite and polymeric resin, respectively. Na bentonite showed slightly higher binding affinity (Langmuir constant) than NaCa bentonite, but much greater when compared to polymeric resin. The polymeric resin was favorable for use in a regenerable continuous process but suffered from recovery inefficiency. Some tendency for selective removal by bentonite was noted between wine protein fractions based on molecular weight examined by SDS-CGE. However, this was not affected by wine pH (2.80 and 3.85) or bentonite type (Na or NaCa). Based on pH effects and lees volume

considerations, NaCa bentonite is suitable for low pH wines and high bentonite requirement, whereas Na bentonite is suitable for high pH wines or low bentonite requirement.

Keywords: Adsorption isotherm, bentonite, capillary gel electrophoresis, Coomassie blue, electrophoresis, fermentation, fining, haze, heat test, juice protein, lees, Marlborough Sauvignon blanc, pH, protein assay, protein stability, resin, New Zealand, stabilisation, turbidity, unstable protein, vine size, vineyard site, wine protein.

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Contribution of Authors

Dr. Roland Harrison and Dr. Jim D. Morton supervised the entire project. Dr. Mike C.T. Trought helped with sampling of grape, juice and wine for the regional trial and access to materials and facilities at the Marlborough Wine Research Centre. Mr. Andy Frost provided winemaking consultation and access to materials and facilities at the Brancott Winery, Pernod Ricard (NZ) Ltd.

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Chapter 1

General Introduction

1.1 Research background

Excess protein remaining soluble in white and rosé wines is one of the major causes of a wine spoilage known as haze (Ferreira et al. 2002, Waters et al. 2005). Protein is only present in wines in small amounts but may denature during storage causing aggregates that settle in bottled wines (Pocock and Waters 2006). Brightness and clarity of wines are the most apparent and vital characteristics to consumers and are thus of major concern to oenologists. Any unattractive haze or amorphous sediment forming in wines can ruin the quality and the value of these products, causing a severe economic problem for producers. Therefore, protein depletion in white and rosé wines is a crucial issue in winemaking as well as the process of protein haze formation.

Unstable wine proteins have been found mainly to be derived from the grape and most are pathogenesis-related (Waters et al. 1996, Dambrouck et al. 2003). Natural grape proteins have been thought as a prerequisite, together with other unknown compounds/factors in wine, to form turbidity and precipitate (Pocock et al. 2007, Batista et al. 2009). To control this problem, removal of troublesome proteins has been commonly achieved by batch-wise bentonite fining during winemaking (Waters et al. 2005). Unfortunately, this treatment not only causes a loss in volume of up to 10% but also downgrades wine quality, resulting in a loss of economic value (Høj et al. 2000). The adverse effects are due to the removal of wine aroma by bentonite fining (Miller et al. 1985) and the need for recovery treatments of wine from bentonite lees. Reducing bentonite use and/or identifying alternatives to batch-wise bentonite fining such as proteolytic degradation (Pocock et al. 2003), in-line dosing (Muhlack et al. 2006), use of a regenerable adsorbent in a continuous process (Salazar et al.

2006) and release of haze protective material from yeast cell wall (Gonzalez-Ramos et al. 2009) would be desirable to the wine industry.

To date, Sauvignon blanc remains the most important variety to the New Zealand wine industry accounting for 47% of the total producing vineyard area (31,057 hectares) and 62% of the total crush (285,000 tonnes) in 2009 (New Zealand Winegrowers Annual Report 2009). In 2008, Sauvignon blanc contributed 76% of New Zealand wine exports by volume and 84% of the total Sauvignon blanc producing vineyard area is located in the Marlborough region (New Zealand Winegrowers Statistical Annual 2008). This variety has a relative high level of grape-derived protein and requires higher bentonite addition rates to achieve protein stability compared to Chardonnay, White Riesling, Sultana and Sylvaner (Hsu and Heatherbell 1987b, Duncan 1992, Pocock and Waters 2006). Thaumatin-like protein (~22 kDa) has been found to be the most heat unstable protein compared to other protein fractions (Waters et al. 1991, 1992, Pocock et al. 2007) and also thaumatin-like protein was noted to be the major protein of natural precipitate in a Sauvignon blanc wine (Esteruelas et al. 2009b). Consequently, the high proportion of thaumatin-like protein in Sauvignon blanc wine (70%) reported by Peng et al. (1997) may be responsible for the high instability of this variety. Understanding the protein composition of Marlborough Sauvignon blanc as well as identifying viticultural and oenological practices that affect heat stability would contribute to knowledge to prevent or reduce bentonite use, and hence improve wine quality.

1.2 Thesis structure

This thesis comprises eight chapters including this general introduction. Chapter 2 reviews the literature related to wine protein characteristics and stabilisation, together with other factors affecting protein instability. Chapter 3 outlines research objectives, analytical methods and trials in this study. Chapters 4, 5, 6 and 7 are experiment results prepared in a style for journal

submission. Chapter 8 is a general discussion and conclusion which draws all the results together.

Chapter 2

Literature Review

2.1 Introduction

Proteins responsible for wine haze have been found to be pathogenesis-related (PR) proteins (Waters et al. 1996). These proteins are the major proteins found in wine and generally possess molecular weight (MW) higher than 10 kDa and an isoelectric point (pI) below 6 (Hsu and Heatherbell 1987b). PR proteins are a prerequisite, together with other unknown wine components/factors, for the formation of hazes in wines (Pocock et al. 2007, Batista et al. 2009). This literature review contains a wide range of information concerning white wine protein occurrence, characterisation and stabilisation as well as factors in wines and vineyards affecting protein content and instability.

2.2 Proteins responsible for wine instability

Protein induced turbidity has been found to be the major haze in beverages (Siebert 2006). The process of protein haze formation includes protein denaturation and aggregation, followed by flocculation with other wine components into a suspended haze and finally precipitation in bottled wines (Waters et al. 2005). Protein denaturation and haze formation can occur in wines after a period of time during which they might be transported and/or stored under poor conditions or through an accelerated protein-denaturing “hot/cold test” process; this hot/cold test has been commonly used to predict wine haze potential (Pocock and Waters 2006).

In early studies, heat-formed sediments were found to contain a similar composition of hydrolyzed amino acids to naturally formed haze and two main electrophoretic fractions showed different sensitivity to heat (Koch and Sajak 1959). Similarly, wine proteins were

further fractionated and analyzed leading to a conclusion that fractions of low pI and low MW were heat-labile proteins (Moretti and Berg 1965). By using ultrafiltration to separate proteins and 2-dimensional electrophoresis, it was postulated that proteins of MW from 12 to 30 kDa and pI from 4.1 to 5.8 were responsible for protein instability and haze formation in white wines (Hsu and Heatherbell 1987b).

In addition, wine proteins were fractioned and separated using a combination of salting out with ammonium sulphate and ultrafiltration by Waters et al. (1991, 1992). Protein fractions were confirmed electrophoretically and added back to protein-free wines. It was found that proteins of 24, 28 and 32 kDa were the most responsible for heat instability in wines. Subsequently, these protein fractions were identified as PR proteins using N-terminal sequencing by the same authors (Waters et al. 1996). The 24 kDa fraction showed homology to plant thaumatin-like proteins and the 28 kDa fraction showed homology to plant chitinases. Peptides of the digested 32 kDa fraction also had sequence homology to plant chitinases. The 32 kDa fraction found in natural wine haze has recently been identified as β-(1-3)-glucanase (also one of the plant PR protein family) (Esteruelas et al. 2009b).

Wine proteins of lower MW (20 to 30 kDa) and low pI (< 6) belonging to plant PR protein family have now been demonstrated to be the major proteins, causing heat instability in wine worldwide (Ferreira et al. 2002, Waters et al. 2005).

2.3 Proteins from grape to wine

Typically, protein content of unfined wines are in the range 15 to 230 mg L^{-1} (Ferreira et al. 2002) and may be up to 300 mg L^{-1} (Waters et al. 2005). Juice and wine protein concentrations of up to around 700 mg L^{-1} have also been reported (Santoro 1995, Vincenzi et al. 2005a). Protein content is significantly influenced by grape variety (Duncan 1992, Dorrestein et al. 1995, Fukui and Yokotsuka 2003). Also, depending on protein recovery and

quantification methods, total protein concentration in the same wine may differ (Vincenzi et al. 2005a). It was found that only 25% of initial juice proteins remained in Champagne wine (Luguera et al. 1998) and around 60% of initial juice PR proteins remained in Sauvignon blanc wine (Pocock and Waters 1998).

In addition to grape berries, a variety of sources including grapevine tissues, bacteria, fungi and autolyzed yeast also contribute soluble proteins to wines (Kwon 2004). Yeast autolysis during fermentation has been reported to release peptides to wine occasionally resulting in higher protein concentration in wine compared to the corresponding juice (Bayly and Berg 1967). However, it was considered that approximately 90% of wine proteins were derived from the proteins present in juice (Fukui and Yokotsuka 2003).

A progressive reduction in protein content and fraction number is normally observed during the winemaking process form grape to wine. Hsu and Heatherbell (1987a) reported that up to forty-one protein fractions were detected in grape extracts with MW from 11.2 to 190 kDa, but only around 25 protein fractions were detected in juice and wine with most in the 11.2-65 kDa range. Pueyo et al. (1993) also indicated that a general reduction in protein content after fermentation coincided with the disappearance or diminished intensity of some electrophoretic bands and there appeared to be a modification of protein pI during vinification with pI values from 3.6 to 4.5 being more prevalent in musts and 4.6 to 5.0 in wines.

Protein modification during fermentation was also observed using different analytical methods. Polyacrylamide gel isoelectric focusing patterns of proteins of must and resultant wine showed only very slight differences for the acidic bands with pI from 3.6 to 4.8, but showed the decrease or disappearance of bands with pI from 5.0 to 9.0 (Santoro 1995). Varied immunological patterns between juice and resultant wine proteins also indicated the modification of protein during vinification (Ferreira et al. 2000). A more detailed

examination indicated that there were a large number of wine proteins exhibiting similar MW but different charge and this might be the result of limited proteolysis during fermentation (Monteiro et al. 2001). Limited proteolysis of PR proteins (chitinases) has been implied to occur during grape processing to wine (Waters et al. 1998).

2.4 Origin of wine proteins

Protein in wine is generally believed to be largely from grape berries. Ferreira et al. (2000) analysed other possible sources of wine protein including grape skin, pulp, seed, stem and leaf and yeast using a modified Lowry assay. As expected, yeast cells were the richest in protein at 780 μg g^{-1} fresh weight. The grape seed was particularly rich in protein at 290 μg g^{-1} fresh weight followed by the leaf, pulp and the stem at 192, 95.6 and 71 μg g^{-1} fresh weight, respectively. The grape skin had a relatively low protein content at 1.6 μg g^{-1} fresh weight. However, when modern immunological techniques (wine proteins specific) were applied to these sources, wine proteins were found to originate entirely from the pulp (Ferreira et al. 2000). Similar immunodectection (protein specific from various sources) was also used by Dambrouck et al (2003). These authors pointed out that most wine proteins originated from grapes, and yeast also contributed high MW mannoproteins to wine during alcoholic fermentation.

Other findings supporting the idea that wine proteins mainly originate from berry pulp have been reported. Tattersall et al. (1997) used highly specific antibodies to search for *Vitis vinifera* thaumatin-like (VVTL) proteins in grapevine tissues. Equal amount of protein extracts from berry skins, berry pulp, seeds, roots, flowers, tendrils and young and mature leaves were electrophoresed and subjected to Western blotting analysis. Only the berry pulp and berry skin extracts contained detectable amounts of VVTL protein. The level of VVTL protein in the pulp extracts was many times higher than the levels found in the berry skin,

and VVTL protein in berry skin extracts might be due to the small amounts of pulp adhering to the berry skin during preparation (Tattersall et al. 1997). However, Pocock et al. (1998) found similar amounts of the main PR proteins (VVTL1 and chitinases) per berry in both skin and pulp homogenates by using HPLC in conjunction with previous identification.

Other sources contributing proteins to wine have also been identified. Kwon (2004) utilised nano-high performance liquid chromatography mass spectrometry to profile soluble proteins in a commercial Sauvignon blanc wine. Twelve identified proteins were from yeast with MW in the range of 21.8 and 121 kDa, particularly in the range of 34.1 to 63.5 kDa. Five identified proteins were from grape with MW in the range of 14.6 and 71.5 kDa. Two identified proteins from bacteria and one identified protein from fungi were in the MW range 41.2 and 77.1 kDa.

2.5 Protein accumulation during berry development

It has been pointed out that total soluble protein concentrations in juice and resultant wine increased as grapes matured, and that the proportion of wine protein MW fractions by SDS-PAGE was also affected by the grape maturity (Murphey et al. 1989). It was further determined using a combination of various separation techniques that total protein concentration (PR proteins) was increased but its diversity was decreased from the early stages of berry development until maturity (Monteiro et al. 2007).

Tattersall et al. (1997) recorded two main protein fractions in SDS-PAGE patterns with apparent MW at 24 and 32 kDa (thaumatin-like protein and chitinase, respectively) from 13 weeks postflowering for cv Muscat of Alexandria grape. Robinson et al. (1997) detected that chitinase activity started from the onset of ripening for cv Shiraz berries. Salzman et al. (1998) identified a thaumatin-like protein (27 kDa), two chitinases (29 and 32 kDa) and a lipid-transfer protein (9 kDa) starting to appear in SDS-PAGE patterns from the onset of

ripening for cv Concord berries. Pocock et al. (2000) also observed that the accumulation of thaumatin-like protein and chitinases started at berry softening for Muscat of Alexandria, Sultana, Shiraz cultivars, Sauvignon blanc and Pinot noir cultivars.

Appearance at the onset of ripening and accumulation throughout the berry development for grape PR proteins has led to speculation that PR proteins are developmentally-regulated proteins directly or indirectly produced at the onset of sugar accumulation, possibly due to the presence of regulatory genes (Robinson et al. 1997, Tattersall et al. 1997). Although, thaumatin-like protein and chitinase were reported to be fruit-specific PR proteins (only found in berry, especially in the pulp) (Robinson et al. 1997, Tattersall et al. 1997), induction in grapevine leaves and berries under pathological and chemical treatments were identified (Jacobs et al. 1999, Jayasankar et al. 2003, Monteiro et al. 2003b). Therefore, major proteins (PR proteins) of grapevine, which can cause haze in wine, are not only present in sound berries but can be induced by biotic or abiotic stresses during vegetative growth.

2.6 Characteristics of wine proteins

Protein MW and pI are the two protein characteristics commonly studied. Hsu and Heatherbell (1987b) reported that the major wine proteins had MW between 11.2 and 65 kDa and pI between 4.1 and 5.8. These ranges for the dominant wine proteins are generally confirmed worldwide (Waters et al. 1991, Pueyo et al. 1993, Santoro 1995, Ferreira et al. 2000, Yokotsuka et al. 2007). Based on fractionation of wine protein and back addition to protein-free wine, these two protein characteristics have been shown to significantly influence heat-induced haze formation behaviour. The 24 kDa fraction (thaumatin-like protein) gave rise to twice as much haze as the 32 kDa fraction (chitinase), whereas the higher MW fraction (63 kDa with polysaccharide) was heat stable (Waters et al. 1991, 1992, Pocock et al. 2007).

Three protein pI ranges, high (≥ 7.0), medium (5.94 to 4.65) and low (< 4.65) developed compact sediment, flocculation and then precipitation in large aggregates, and suspended haze, respectively (Dawes et al. 1994). An interaction effect between protein pI and wine pH on haze formation was also observed with lower wine pH resulting in smaller particle size, and more haze formed when wine pH approached protein pI through the mechanism of isoelectric precipitation (Batista et al. 2009). In addition, wine proteins with pI lower than wine pH have also been reported in some instances (Murphey et al. 1989, Marchal et al. 1996) and MW of the identical proteins can differ slightly depending on analytical methods. For instance, the MW of thaumatin-like proteins and chitinases determined in SDS-PAGE were several kDa higher compared to those determined by mass spectrometry (Pocock et al. 2000).

It has been suggested that glycoproteins (12-30 kDa) in wine were the ones responsible for wine protein instability owing to their necessary removal for heat stability (Hsu and Heatherbell 1987b). Glycoproteins and mannoproteins with grape and yeast origins, respectively, have been observed in many wine protein studies (Yokotsuka et al. 1991, Waters et al. 1994b). The degree of protein glycosylation showed a wide range for red wine protein fractions (0.3-16.1% w/w) (Yokotsuka and Singleton 1997) and for white wine protein fractions (up to 5.2% w/w) (Waters et al. 1995b). Glycosylation of proteins may depend on grape variety (Nakopoulou et al. 2006). It has been shown that a 24/25 kDa protein in a Champenois Chardonnay wine was a N-glycosylated protein and underwent no modification during fermentation (Marchal et al. 1996), whereas degradation or modification of the sugar moieties of the glycoproteins (12-30 kDa) was found to occur during vinification for a hybrid grape variety (Muscat Bailey A) (Yokotsuka and Singleton 1997). The hydrolysis of the sugar chains of grape derived glycoproteins by glycosidase treatment was found to increase turbidity with seed phenols in a model wine (Nishihata et al. 2004). On the other hand, yeast-derived mannoproteins (420 and 31.8 kDa) could contribute a stabilisation effect on

wine proteins, reducing haze formation (Waters et al. 1994b, Moine-Ledoux and Dubourdieu 1999). Yeast derived mannoproteins (10 to 30 kDa) possessing both composition of the hydrophobic and hydrophilic protein domains and mannose moity also improved the foaming properties in sparkling wines (Núñez et al. 2006).

Grape-derived major proteins (24 and 32 kDa) have been suspected to be inherently resistant to proteolysis with no degradation even after 2 weeks incubation with a peptidase preparation (Vinozym P) at 15 °C (Waters et al. 1992). The resistance of grape juice protein to proteolysis was also shown for a selection of commercial proteases (Duncan 1992). This proteolytic resistance of PR proteins under winemaking conditions was further demonstrated to be an intrinsic characteristic and not due to either phenolic association or glycosylation (Waters et al. 1995b), which is not surprising since the major wine proteins are PR proteins and possess anti-fungal properties (Waters et al. 1996, Monteiro et al. 2003b). It has been stated that vinification can be seen as a purification process for grape PR proteins (Ferreira et al. 2004). In addition, the hydrolytic stability of wine PR proteins has also been hypothesized owing to their conformations in wines (Heatherbell et al. 1984). This is supported by the finding that denaturation of grape-derived proteins with alcohol precipitation or thermal treatments did markedly degrade proteins enzymatically (Conterno and Delfini 1994, Pocock et al. 2003). However, little is known about wine protein conformations. On the other hand, limited protease activity during fermentation has been proposed (Lamikanra and Inyang 1988) and demonstrated (Waters et al. 1998, Dizy and Bisson 1999). Major wine proteins and corresponding hydrolysed products were also observed in a stabilised wine (Okuda et al. 2006). However, proteolytic degradation of protein during fermentation was not able to affect the heat-induced haze forming potential of the final wines (Dizy and Bisson 2000).

Two basic thaumatins were found to be much sweeter than sucrose on a molar basis (Van der

Wel and Loeve 1972). Peng et al. (1997) evaluated the sensory properties of thaumatin-like proteins from Sauvignon blanc as they are the predominant proteins (70%) and are homologous to thaumatin. Thus, their removal during winemaking for stability may have an impact on wine quality. Aqueous solutions containing protein addition rates up to 2200 mg L^{-1} of isolated 82% thaumatin-like proteins (much higher than might be found in a wine) were not significantly determined as sweet compared to water by a panel of 26 participants. Peng et al. (1997) explained the lack of the putative sweet taste resulted from the amino acid sequence and to lower levels of basic residues (lysine) of wine thaumatin-like proteins compared to authentic thaumatin. Wine proteins reported in the literature are generally composed of similar amino acids with high proportions in asparagine, glycine, alanine, serine, threonine and glutamine but low levels of lysine (Yokotsuka et al. 1991, Waters et al. 1995b). Therefore, protein in wine might not have a direct effect on wine sweetness and taste. However, when wine proteins were mixed with phenols in wine-like solutions (10% ethanol and pH 3.0), the presence of proteins at low concentration, but not at higher concentration, decreased the taste intensity of phenols (Fukui et al. 2002). It was hypothesized that protein might contribute to taste intensity at higher concentrations in presence of phenols (Fukui et al. 2002).

2.7 Factors in wine affecting wine protein instability

Protein-polyphenol interaction is the most commonly implicated and studied mechanism for wine instability and protein haze formation (Somers and Ziemelis 1973, Yokotsuka et al. 1991, Dawes et al. 1994). Waters et al. (1995a) reported that both heat-induced and natural haze contained procyanidins with a content ranging from 0.02 to 4.9% (w/w), and procyanidins were only weakly associated (i.e. not covalent bonding) with heat-induced haze and soluble wine proteins. The presence of procyanidins was necessary for wine proteins to form turbidity as wine proteins alone (isolated and back added to a model wine) did not cause turbidity (Waters et al. 1995a). Analysis of a natural precipitate from a Sauvignon blanc wine also

revealed that proteins (mainly thaumatin-like proteins) and phenolics only contributed 10% and 7% of the dry weight of precipitate, respectively, with the remainder being polysaccharides (4%) and unknown components (Esteruelas et al. 2009b).

In model system studies, the amount of haze formed depended both on the concentrations of protein and polyphenol and on their ratio, and a conceptual model for the interaction between haze-active polyphenol and haze-active protein was proposed (Siebert et al. 1996a). Conceptually, "haze-active" polyphenols are thought of as having at least two sites (phenol groups) that can bind to proteins, and "haze-active" proteins have a finite number of sites which polyphenols can bind to. Thus, the largest network, corresponding to the largest particle size and the greatest light scattering, would occur when the number of polyphenol binding sites matches the number of protein binding sites, whereas either protein-rich or polyphenol-rich solutions resulted in smaller particles and less light scatting (Siebert et al. 1996a). For haze-active polyphenol, increased polymerization of wine tannin and interaction with isolated must proteins resulted in increased turbidity (Yokotsuka et al. 1983). For haze-active proteins, it is likely that proline is a necessary component of the polyphenol binding site and other amino acids in the peptide may exert steric influence on binding site (Siebert et al. 1996a). Evidence was given in a study of Muscat Gordo Blanco wine protein in which the 24 kDa protein with 7% proline caused 50% more heat-induced haze than 32 kDa protein with only 1% proline (Waters et al. 1992, Waters et al. 1995b). Wine proteins are generally composed of a relative low percentage of proline (< 7%) (Yokotsuka et al. 1991) compared to beer proteins (> 10%), for example, (Iimure et al. 2009). To accelerate wine protein haze formation, thermal shocks are often utilised as heating could expose more protein active sites for haze-active polyphenol binding (Siebert et al. 1996a) and cooling could reduce the solubility of protein-polyphenol complexes (Yokotsuka and Singleton 1995).

In addition, pH and ethanol content are two other factors often implicated in protein haze formation. Moretti and Berg (1965) noted that wines with lower pH (≤ 3.0) were protein stable with little turbidity after a heat test and ascribed this to the isoelectric precipitation of proteins. Murphey et al. (1989) also found that juice with lower pH tended to end with lower wine protein concentration and lower bentonite requirement to heat stability. Turbidity of a protein-polyphenol complex generally increased with increasing pH from 2.5 to 3.7 in a model wine study (10% v/v ethanol) (Yokotsuka and Singleton 1995). Maximum light scattering was detected at pH 4 (2.5 to 4.5) for a model solution regardless of ethanol content (0-12% v/v) (Siebert et al. 1996b). Isolated Arinto wine proteins dissolved in water and model wines with various pH values showed a peak of instability centred at around pH 4.0 which coincided with the isoelectric point of most Arinto wine proteins (Batista et al. 2009). This demonstrated the presence of an isoelectric precipitation mechanism in wine protein haze formation. Furthermore, high ethanol addition (50% v/v) does cause protein instability as it was used to induce wine haze (Esteruelas et al. 2009a). In a model solution study, turbidity gradually increased (100 to 120 NTU) in response to increases in ethanol content (6 to 12% v/v) (Siebert et al. 1996b). In real wine, however, this ethanol effect may not influence wine protein instability. It was found that a wine (12.1% v/v ethanol) with slight alcohol additions (up to 2% v/v) did not interfere with the haze formation profile to heat tests (Mesquita et al. 2001). This was also supported in a wine survey study in which the ethanol concentration showed no significant effect on turbidity development in wines (Sarmento et al. 2000a).

Pocock et al. (2007) pointed out that sulphate was one previously unknown essential factor promoting haze formation in the absence of phenolic compounds in a model wine. It was deemed to be the acceleration of protein denaturation and/or competition between sulphate anions and proteins at wine alcohol concentrations for water of solvation that caused a loss of water from the protein surface, resulting in the protein aggregation (Pocock et al. 2007). Besse

et al. (2000) observed that protein haze formation in heat-induced wines coincided with an apparent decrease in copper concentration, implying that copper was involved in the protein haze complex. However, copper ions in model wines with isolated wine proteins did not cause heat-induced haze (Pocock et al. 2007). Copper found in wine protein precipitate by heat treatment may be copper involved in the oxidation and polymerization of wine phenolics (Clark et al. 2003).

Mesquita et al. (2001) investigated the effects of wine matrix (protein-free wine) on haze formation profiles to increasing temperatures (30, 40, 50, 60, 70 and 80 °C) among various wines by back addition of isolated proteins. Interestingly, when wine "A" was depleted of its own proteins by sufficient bentonite addition and proteins from wine "B" or of non-wine origin (BSA) were added, its heat-induced turbidity profiles behaved like wine "A". It was concluded that the presence of proteins is a prerequisite for turbidity formation, but it is the wine matrix, not the protein themselves, that determines the pattern of turbidity formation to heat tests (Mesquita et al. 2001). Some compounds in the wine matrix, including anions such as acetate, chloride, citrate, phosphate and tartrate and cations Fe^{2+}/Fe^{3+}, have been excluded as causes of heat-induced protein haze in model wines (Pocock et al. 2007). Thus, other compounds or interactions may be responsible for the wine matrix effect.

2.8 Vineyard factors affecting wine protein instability

By using a principal component analysis and clustering techniques, Sarmento et al. (2001) pointed out that the most important factor affecting wine protein profile was the grape cultivar, and that growing region was also somewhat influential, whereas vinification practice (commercial or laboratory scale) on the same varietal wines did not show a major effect. Wine protein fractions have been shown to respond differently to bentonite fining and in heat turbidity (Hsu and Heatherbell 1987b, Waters et al. 1991, 1992). Thus, varietal effects on heat

stability would be expected.

Hsu and Heatherbell (1987b) observed varied wine protein concentrations and bentonite requirements to heat stability between Gewürztraminer, White Riesling, Sauvignon blanc and Sylvaner cultivars with Gewürztraminer wine showing the highest protein concentration and bentonite requirement. In a varietal survey, Duncan (1992) also found that the average protein contents (mg L^{-1}) and bentonite requirements (g L^{-1}) for heat stability for Chardonnay, Sauvignon blanc, Gewürztraminer, Muscat Gordo Blanco and Riesling juices were 68/0.68, 103/1.15, 136/1.5, 117/1.68 and 76/0.57, respectively. Although Gewürztraminer had the highest average level of juice protein, Muscat Gordo Blanco juice required the largest average amount of bentonite for heat stability (Duncan 1992). Pueyo et al. (1993) noted differences among varieties for juice and wine proteins in native electrophoresis, SDS electrophoresis and isoelectric focusing patterns. Pocock et al. (2000) further identified the differences in PR protein concentrations (mg L^{-1}) among varieties for Muscat of Alexandria, Sauvignon blanc, Sultana, Pinot noir and Shiraz juices with thaumatin-like proteins/chitinases at 119/118, 119/76, 23/44, 35/21 and 18/9. Furthermore, differences in protein profiles between varieties examined by trap mass spectrometry or high-performance capillary electrophoresis have been proposed for varietal differentiation of juice and wine (Hayasaka et al. 2001, Chabreyrie et al. 2008).

Proteins originating from grapevine tissues, either during normal development or induced by abiotic stimulation, have been identified and show a diverse range with PR proteins being predominant (Sarry et al. 2004, Deytieux et al. 2007, Martinez-Esteso et al. 2009). Therefore, stresses from the pathogenic conditions and/or mechanical wounding would possibly enhance wine protein instability as observed in wines produced from a field study with pathogenic infection (Girbau et al. 2004). It was also noted that the infection of

Botrytis cinerea reduced proteins (20-30 and 62/64 kDa) in must and wine proteolytically (Marchal et al. 1998, Marchal et al. 2006), whereas plant nonspecific lipid-transfer proteins (10 kDa) were induced (Girault et al. 2008). The chitinases seem more susceptible to proteolytic degradation than the thaumatin-like proteins (Manteau et al. 2003, Pocock et al. 2003). Differences in induction of PR proteins may depend on the grapevine tissue (leaf or berry) and/or cultivar and/or type of infection pathogen (Bezier et al. 2002, Robert et al. 2002, Girbau et al. 2004).

Proteins of 27, 32, 34 and 38 kDa were induced and synthesized in Chardonnay leaves elicited with salicylic acid or strains of *Botrytis cinerea*, leading to the speculation that in grapevines, like in herbaceous species, glucanases and chitinases are involved in defence mechanisms (Renault et al. 1996). Various basic and acidic chitinases were identified in different grapevine tissues (winter-resting stem internodes, roots, berries and leaves) (Derckel et al. 1996). Wounding, chemical treatment and/or pathogenic infection were found to provoke β-1,3-glucanase and chitinases in leaves and berries within a few days (Derckel et al. 1998, Kraeva et al. 2000, Renault et al. 2000). From a survey of 21 different grapevine (*Vitis ssp.*) genotypes, there was a good correlation between resistance rating to powdery mildew and activity of chitinase and β-1,3-glucanase in spring but not in summer (Giannakis et al. 1998). Chitinase and β-1,3-glucanase were demonstrated to have antifungal activity in inhibiting germ tube growth and showed a synergistic effect when both enzymes were present (Giannakis et al. 1998).

Grapevine thaumatin-like protein was demonstrated to possess antifungal activity by Jayasankar et al. (2003) in anthracnose-resistant grapevine (*Vitis vinifera* L. cv. Chardonnay) and by Monteiro et al. (2003b) in grapevines of *Vitis vinifera* L. cvs. Moscatel, D. Maria, and F. Pires using immunological methods and N-terminal amino acid sequencing coupled

with antifungal bioassays. Jayasankar et al. (2003) identified two secreted proteins (21.6 and 22 kDa), differentially expressed by *in vitro*-selected embrogenic cultures and regenerated plants. The 21.6 kDa protein showed high degree of homology to *V. vinifera* thaumatin-like protein 2 (VVTL2 = grapevine osmotin; Acc no. CAA71883). The 22 kDa protein showed inhibition of fungal growth *in vivo* and high degree of homology to *V. vinifera* thaumatin-like protein 1 (VVTL1; AAB61590). In addition, Monteiro et al. (2003b) also characterised osmotin and thaumatin-like proteins having antifungal properties. Both proteins can block the growth of *Phomopsis viticola* and *Botrytis cinerea* mycelia and exhibit a remarkable inhibitory action on spore germination and germ tube growth of *U. necator*, *Phomopsis viticola* and *Botrytis cinerea*. There was also a synergistic effect on antifungal activity when both osmotin and thaumatin-like proteins were present simultaneously (Monteiro et al. 2003b).

It had been suspected that damage to berries caused by mechanical harvesting might induce PR proteins as a result of a grape defence mechanism before subsequent pressing (Pocock et al. 1998). However, it was found that little protein was induced in response to mechanical harvesting, whereas juice from mechanical harvesting coupled with long transport time resulted in increased PR protein content in juice and resultant wine, thus increasing heat instability (Pocock et al. 1998, Pocock and Waters 1998). This was ascribed to the prolonged contact time of grape skins and solids of broken berries with juice during transport, resulting in longer extraction time (Pocock et al. 1998).

Pocock et al. (2000) found that grapes from irrigated vines tended to contain either the same or more proteins on a per berry basis than those from drought-stressed non-irrigated vines, whereas juice from the irrigated vines had lower protein concentration than that from drought-stressed non-irrigated vines on a per volume basis. It was concluded that drought

stress either had little effect on PR protein accumulation or inhibited production in Shiraz berries (Pocock et al. 2000). Furthermore, wine protein concentration was found to increase linearly with increased nitrogen fertilization rates in the vineyard, and the bentonite requirement for heat stability paralleled wine protein concentration (Spayd et al. 1994). In addition, UV-C irradiation has also been reported to induce defence responses resulting in accumulation of both chitinase and β-1,3-glucanase activity in grapevine leaves of *V. rupestris* and *V. vinifera* (Bonomelli et al. 2004). It has been emphasised that the environmental and/or pathogenetic conditions during grapevine growth determined the protein content and profile as grape proteins from the same variety and vineyard but different vintages showed significant differences (Monteiro et al. 2003a).

2.9 Protein stabilisation treatments in wines

Bentonite fining remains the most common and effective practice for protein stabilisation in the wine industry (Ferreira et al. 2002, Waters et al. 2005). Bentonite fining tended to remove proteins with higher pI (5.8- 8.0) and intermediate MW (32-45 kDa) preferentially, shown in 2-dimentional gel electrophoresis (Hsu and Heatherbell 1987b), whereas the amount of protein depletion correlated approximately linearly with the level of bentonite addition in chromatofocusing profiles implying no selectivity based on protein pI (Dawes et al. 1994). Contrasting results might be due to different analytical methods. It was found that a relatively large amount of bentonite was required to remove a small portion of proteins (1-2 mg L^{-1}) which were most resistant to bentonite fining before achieving heat stability (Hsu and Heatherbell 1987b). At lower bentonite addition rates (0.1 g L^{-1}), other wine species were removed first rather than proteins (Duncan 1992).

Bentonite-protein interaction was found to be rapid and complete within 30 seconds after addition in a model wine solution (Blade and Boulton 1988) and within 120 seconds for a

in-line dosing system (Muhlack et al. 2006). Adsorption was independent of temperature but varied slightly with protein concentration, ethanol concentration and pH, and was strongly affected by bentonite type (Blade and Boulton 1988). Achaerandio et al. (2001) proposed that ethanol molecules could disperse bentonite layers and improve the protein adsorption capacity. Wine pH and protein pI were believed to govern the effectiveness of protein removal by bentonite fining through a cation-exchange mechanism (Ferreira et al. 2002). In a model study, however, lower pH wine resulted in lower protein adsorption capacity by bentonite fining due, it was speculated, to increased competition of hydrogen ions with proteins (Blade and Boulton 1988). Additionally, on a weight basis, sodium bentonite was more effective than calcium bentonite for protein removal, but a much greater volume of lees were formed (Leske et al. 1995). Higher adsorption capacity but more lees production for sodium bentonite compared to calcium bentonite was due to the high swelling ability of sodium bentonite (Blade and Boulton 1988, Leske et al. 1995).

Bentonite addition can occur before, during or after fermentation. Miller et al. (1985) reported that addition to the ferment was the most efficient to achieve heat stability with a bentonite requirement of 0.8 g L^{-1} for juice, 0.6 g L^{-1} for fermenting juice and 1.2 g L^{-1} for wine. Similar results were also reported by Ewart et al. (1980) in a commercial-scale trial. On the other hand, Somers and Ziemelis (1973) reported that bentonite addition after fermentation was the most efficient to remove proteins. Muhlack et al. (2006) utilised a continuous in-line dosing process followed by centrifugation as an alternative to batch fining. Although there was a 30% bentonite carry-over effect, which means a portion of bentonite still present in centrifuged wine, higher quality wine could be recovered from 96.9% of initial wine volume for batch fining to 99.8% of initial wine volume for in-line dosing without detectable sensory modification (Muhlack et al. 2006).

Bentonite dosage required to achieve wine protein stability is normally determined in the winery laboratory by conducting bentonite fining trials coupled with a procedure to force protein haze formation and examination. The heat test is the most common accelerated procedure to mimic protein haze formation in real storage conditions. Pocock and Rankine (1973) compared a combination of various temperatures and durations of heating and concluded that the 80 °C for 6 hours procedure produced the most haze. Recently, based on storage trials, Pocock and Waters (2006) suggested the less severe 80 °C for 2 hours procedure to predict bentonite requirement for wines stored in the short to medium term. However, there are many other procedures to test wine protein stability. Sarmento et al. (2000a) indicated that the heat test was a good indicator of total protein content and less affected by other wine components compared to ethanol and tannin precipitation tests. Esteruelas et al. (2009a) further indicated that the high temperature test (90 °C for 1 hour) precipitated haze with a similar composition to natural haze when compared to low temperature, bentotest, tannin, ammonium sulphate, trichloroacetic acid, ethanol and prostab tests. Behaviour of wine protein precipitation by heating correlated well with protein removal by bentonite fining, and a higher temperature (> 60 °C for 30 minutes) was required to precipitate thaumatin-like proteins and chitinases which are the dominant and most unstable proteins in wine (Sauvage et al. 2010).

Flores et al. (1990) investigated the effect of ultrafiltration on Riesling and Gewurztraminer wine composition and stability. Wines treated by ultrafiltration were significantly reduced in colour (420 nm), total phenol, protein and haze. Although wine protein stability was achieved with a MW cut-off of 10 kDa, trace instability could remain and some bentonite addition was required (Flores et al. 1990). This observation is in accord with Hsu et al. (1987) who noted that up to 99% of the protein in wine was removed with ultrafiltration of 10 or 30 kDa MW cut-off, eliminating or reducing the bentonite requirement by up to 95%.

However, ultrafiltration treatment was also reported to change the wine aroma profile, affecting wine flavour, and particular care was needed to avoid wine/air contact and microbiological contamination of the filtration equipment (Miller et al. 1985).

A protective material which reduces wine protein haze to heat treatment was found in wines, and the mechanism of haze protection was due to decreased particle size of the haze rather than preventing protein precipitation (Waters et al. 1993). Haze protective material was shown to reduce wine haze formation to heat treatment and in a storage trial (Brown et al. 2007). It was proposed that haze protective material was able to compete with wine proteins for the unknown wine component(s) required to form haze (Dupin et al. 2000b), and the glycan structure was found to be crucial component for the haze protective activity (Schmidt et al. 2009). Two haze protective materials were identified as a mannoprotein (420 kDa) released during yeast fermentation and a wine arabinogalactan-protein (210 kDa) (Waters et al. 1994b, a). A N-glycosylated mannoprotein (31.8 kDa) corresponding to a invertase fragment released from *Saccharomyces cerevisiae* cell wall and responsible for improving the protein stability of white wines aged on their lees was also identified (Moine-Ledoux and Dubourdieu 1999). Haze protective materials remained soluble and active in wine after heating and could be obtained by isolation from the fermentation procedure or by extraction of yeast cell walls through enzymatic digestion and/or chemical reagents (Waters et al. 1993, Moine-Ledoux and Dubourdieu 1999, Dupin et al. 2000a, Lomolino and Curioni 2007).

Proteins as the sole nitrogen source for yeast cell growth has been observed (Conterno and Delfini 1994). Despite PR proteins being intrinsically resistant to proteolytic degradation by enzymatic treatment or during wine making processes (Waters et al. 1992, 1995b), a limited proteolysis of chitinases may occur by acid catalysis (Waters et al. 1998). Gradual disappearance of chitinase and thaumatin-like protein was coincident with the occurrence of

acid protease activity during Champagne winemaking and low MW fragments were linked to chitinase, indicating proteolysis of the chitinase (Manteau et al. 2003). High temperature treatment (90 ^{o}C for 1 minute) resulted in reduced content of chitinases but had little effect on thaumatin-like proteins, whereas a combined heat and protease treatment showed a significant degradation of both chitinases and thaumatin-like proteins (Pocock et al. 2003). At lower temperature (45 ^{o}C), protease treatments required up to more than 7 hours for proteolytic degradation of PR proteins (Pocock et al. 2003). With wine and juice protected from air, 90 °C for several-minutes was found to have little effect on aroma, whereas treatments at 45 °C for three weeks did change their sensory characteristics (Francis et al. 1994).

Despite bentonite being the most effective material for protein removal, regenerable adsorbents have been proposed and evaluated to substitute for batch-wise bentonite fining for wine protein stability (Sarmento et al. 2000b). Due to the strong interaction between tannin and protein, tannic acid derivatives were investigated and immobilised as a protein adsorption material in column treatment (Weetall et al. 1984, Powers et al. 1988). Tannic acid derivatives showed the ability to remove wine proteins but suffered from a reduction in protein binding capacity after a small number of regeneration cycles (Powers et al. 1988). Three adsorbent resins were studied using packed columns by Gump and Huang (1999). Juice and wine proteins were successively removed by all resins along with phenolics, heavy metals, flavours and a reduction in wine colour (420 nm). However, the regeneration ability of resins was not assessed. Pachova et al. (2002) compared various forms of zirconium and alumina oxides on adsorption of three standard proteins in a model wine and concluded that various protein characteristics (pI, MW and dimensions) affected protein adsorption behaviour.

The zirconium oxide in power form was further investigated and showed the ability to selectively remove unstable wine proteins (20-30 kDa) in a percolated bed. Improved

adsorption capacity after heat regeneration was due to increased pore diameter and mesopore area (Pashova et al. 2004b, 2004a). Little change of treated wine was observed in physicochemical properties and phenolic composition except for acidity and wine colour (420 nm) (Pashova et al. 2004b, Salazar et al. 2006, 2007). Specific removal of wine protein was also observed using a chitin polymer in batch and continuous treatments (Vincenzi et al. 2005b). On the basis of same total wine protein content, the specific removal of chitinase resulted in greater haze reduction compared to bentonite fining, and this was due to the specific chitin-binding domain of chitinase (Vincenzi et al. 2005b). In general, materials used to remove wine proteins, including bentonite, also remove polyphenols and polysaccharides but often at different rates (Vincenzi et al. 2005b, Salazar et al. 2006, Bruijn et al. 2009).

An interesting finding in a study to collect proteins responsible for foam formation and stabilisation in Champagne wine led to the possibility of being further used in wine protein stabilisation. Brissonnet and Maujean (1991, 1993) fractionated and analysed the foam induced by blowing gas into the base wine. It appeared that proteins were eluted preferentially in the first fractions of foam, and protein concentration consistently reduced from the first fraction to the last one (Brissonnet and Maujean 1991), implying that proteins could be concentrated and removed through this method. Protein analysis of the foam wine (from the collapsed foam) and remaining wine showed no differences in protein MW and pI, but hydrophobic proteins contributed more to the foam wine (Brissonnet and Maujean 1993). This technique is now regarded as flotation used in clarifying grape must before fermentation as an alternative to gravity settling or centrifugation (Ferrarini et al. 1995, Marchal et al. 2003). However, few studies have been published with regard to grape protein removal and stability. Flotation treatment may be further studied and developed for wine protein removal.

Although a variety of techniques have been developed to stabilise white wine, bentonite fining predominates with resulting effects for wine sensory characteristics and thus the main purpose of this thesis will be to understand and optimise the use of bentonite for Sauvignon blanc.

Chapter 3

Objectives and Methodology

3.1 Research objectives

1. To develop analytical methods/protocols for quantifying and characterising juice and wine proteins.
2. To survey the variation of protein content and composition of Sauvignon blanc within Marlborough region which might correlate with heat instability.
3. To investigate effects of pH adjustment within commercial oenological parameters on protein concentration, composition and heat stability.
4. To compare the ability of different hot/cold tests to predict bentonite requirement.
5. To compare bentonite type and other adsorbents in terms of protein adsorption capacity.

3.2 Protein analyses

Three analytical methods were developed to quantify and/or characterise juice/wine proteins. They were a modified Coomassie Brilliant Blue (CBB) assay, lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS-PAGE) and sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE). By using these three analytical methods, it was possible to quantify effects of viticultural, oenological and stabilisation treatments on protein concentration and molecular weight profile. These protein data can further correlate with wine protein stability to heat test (haze formation and bentonite requirement) which will then build up relationships between treatments, protein data and heat stability, identifying optimal practices to reduce wine protein instability and bentonite use.

3.2.1 Coomassie Brilliant Blue assay

The CBB assay optimised in this study is based on the Bradford protein assay (Bradford

1976) with suggested modification of sample alkalisation to improve sensitivity and uniformity of protein-dye interaction (Stoscheck 1990, Boyes et al. 1997). This assay can quantify polypeptides with molecular weight greater than 3 kDa (Sedmak and Grossberg 1977) which is suitable for studying heat unstable proteins that have molecular weight greater than 10 kDa in juice and wine (Hsu and Heatherbell 1987a, b).

3.2.2 Lithium dodecyl sulphate polyacrylamide gel electrophoresis

LDS-PAGE is the conventional method to determine wine protein molecular weight (Hsu and Heatherbell 1987a). Commercial gels and protocol and a protein mixture with bovine serum albumin (BSA) were utilised. Wine protein concentrates were heat-denatured in the presence of lithium dodecyl sulphate. Gels were stained with Coomassie blue and imaged. Each protein was identified by comparison with protein standards and quantified by integrating peak area with calibration to known quantity BSA peak in the same electropherogram, similar to others reported (Sauvage et al. 2010).

3.2.3 Sodium dodecyl sulphate capillary gel electrophoresis

SDS-CGE separates wine proteins based on molecular weight with similar mechanism to LDS-PAGE but in a highly automoted operation (Rodríguez-Delgado et al. 2002). A commercial kit (Beckman Coulter; Fullerton, CA) was used with modification to adapt to the capillary electrophoresis machine (Agilent; Waldbronn, Germany), similar to others reported (Lee 2000). A front marker (benzoic acid) and standard protein (BSA) with various concentrations were required for correction of migration time and for calibration of wine protein concentration, respectively, as reported by others (Lee et al. 2002).

3.3 Protein recovery

Two methods of organic solvent precipitation (acetone and ethanol) were compared with ultrafiltration (10 kDa molecular weight cut-off) for establishing a more economical and

simple protein recovery method for use in electrophoresis. Proteins recovered from juice and wine by acetone or ethanol addition for various analytical methods have been studied and reported in the literature (Yokotsuka et al. 1991, Vincenzi et al. 2005a). With standardised protein recovery methods, analysis using LDS-PAGE and SDS-CGE for a large number of samples becomes possible.

3.4 Hot/cold test and bentonite requirement

A hot/cold test (80°C for 6 hours followed by 4°C overnight) commonly used in the wine industry and literature (Pocock and Waters 2006) was adopted as a standard protein stability test with comparison to other existing hot tests. Bentonite dose rate required to stabilise a wine was defined as the quantity to achieve light scattering characterised by an increase of 2 nephelometric turbidity units (NTU) during the hot/cold test. Bentonite requirement (g L^{-1}) was determined by linear interpolation between the two closest bentonite fining rates and two decimal places were presented.

3.5 Viticultural treatments

Sauvignon blanc from 5 vineyards with 2- and 4-cane pruning regimes within the defined wine region of Marlborough, New Zealand, were studied over two consecutive vintages (2007 and 2008). Five vineyards, representing vineyard areas of Marlborough Sauvignon blanc, have been managed in an identical manner from 2004 by staff from the Marlborough Wine Research Centre. Grapes from a trunk diameter trial within one of the studied vineyards in 2007 (Trought et al. 2008) was also included. The variability within the defined wine region and within the same vineyard regarding protein content, molecular weight composition and heat stability was investigated.

3.6 Winemaking treatments

Variations in pH do have significant effects on wine protein stability but few investigations

under winemaking conditions have been carried out (Murphey et al. 1989, Batista et al. 2009). Both Sauvignon blanc juice and wine pH adjustments were proposed within the range encountered in commercial winemaking to attempt to determine pH effects for each. Three timings of bentonite addition were also superimposed on the juice pH adjustment trial with the aim of establishing optimal practice in protein stabilisation as there are contrasting results in the literature (Somers and Ziemelis 1973, Ewart et al. 1980).

3.7 Stabilisation treatments

Effects of bentonite type on protein adsorption capacity and lees volume formation are major (Leske et al. 1995) but there are no clear guidelines for optimal practice. Requirements for two commercial bentonites (Na and NaCa forms) were compared for wines with pH from 2.80 to 3.85 and on bentonite lees formation at incremental addition to a pH 3.34 wine. Optimal selection of bentonite type according to wine pH and addition rate could be derived. In addition, two bentonites and two resins were screened by studying protein adsorption isotherms with the aim of developing a resin-packed column for a continuous protein removal process (Sarmento et al. 2000b).

Chapter 4

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Comparative Studies of Three Protein Assays on Marlborough Sauvignon Blanc Wine

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Short title: **Comparative Studies of Wine Protein Assays**

Category: protein structure and analysis; electrophoretic techniques

Abstract

Three protein assays were investigated and compared as well as protein recovery methods for use in wine protein analyses. The Coomassie Brilliant Blue (CBB) assay provided a rapid method for quantification. Lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE) gave both quantitative and qualitative results on a molecular weight (MW) basis. When using an identical calibration standard protein, the CBB assay resulted in 40% higher concentration of wine proteins (108.5 mg L^{-1}) compared to LDS-PAGE (77.2 mg L^{-1}), and about 5 times lower than SDS-CGE (532.1 mg L^{-1}). Protein recovery by acetone precipitation was more effective than that by ultrafiltration (10 kDa MW cut-off); the latter gave a 21% loss of total protein by SDS-CGE. Proteins with MW of 19-33 kDa were the predominant fractions in both LDS-PAGE (70%) and SDS-CGE (98%), but the 64 kDa fraction present in LDS-PAGE (21%) was not detectable in SDS-CGE. The SDS-CGE provided better resolution than LDS-PAGE with 8 fractions (9.6, 19, 21 22, 24.6, 26.5, 27.6 and 32 kDa). The CBB assay detected low protein levels with a narrow range (10 to 25 mg L^{-1}) remaining in most protein stabilized wines.

Keywords: Wine; Protein assay; Stability; Coomassie; Electrophoresis; SDS-CGE

Introduction

Proteins in wine are mainly derived from the grape, survive during the winemaking process and remain soluble in the final wine [1,2]. Most wine proteins are of low isoelectric point (pI, 4.1-5.8) and low molecular weight (MW, 20-30 kDa) [3,4]. Unless soluble proteins are removed during winemaking, protein haze development can occur in bottled white/rose wines during storage. For protein removal, fining with a certain quantity of bentonite is conventionally applied in the wine industry [5,6]. This requires a trial to determine the appropriate bentonite fining rate. Unfortunately, the cumbersome hot/cold test remains the most common and effective procedure [7,8]. Improved methods to predict bentonite requirement for wine protein stability control as well as to quantify and characterize wine proteins are desirable [9].

Quoted wine protein concentrations commonly range from tens up to 300 mg L^{-1} [5], depending on the grape variety [10], growing conditions [11], winemaking [12] and analytical method [13]. An ideal method for quantifying juice/wine protein would be rapid, insensitive to other juice/wine constituents and easy to perform. The Bradford assay using Coomassie dye is the most common assay for proteins due to its simplicity, reproducibility and rapidity [13], requiring only one dye reagent. This assay can determine a variety of proteins and polypeptides with MW greater than 3 kDa [14]. Three forms (cationic, neutral and anionic species) of Coomassie dye exist in equilibrium with λ_{max} at 470, 650 and 595 nm, respectively, and the development of the color relies on the anionic species binding to proteins, result in absorbance increase at 595 nm [15]. Disadvantages of this assay have been the large variation of color yields to different proteins [16] and slight nonlinearity [17]. Some other drawbacks reported in the literature relating to grape juices and wines are underestimation of protein concentration [18], longer incubation time [19] and pretreatment for protein recovery [20].

In addition to quantifying juice/wine proteins, their intrinsic characteristics are of interest with regard to MW, pI, amino acid composition and sequence, hydrophobicity and

glycosylation [3,21,22]. Protein MW is conventionally analyzed using slab gel based sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) but this requires time-consuming and labor-intensive staining and destaining processes and only provides semi-quantitative results. Capillary electrophoresis has the advantages of a smaller required quantity of analyte, high speed and full automation capability for wine protein analysis [23]. Protein separation by sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE) has been demonstrated to take place through the same mechanism as in SDS-PAGE based on MW [24-26]. The protein sieving medium in SDS-CGE is a polymer solution rather than a physical gel in SDS-PAGE so that automation could be achieved by simply replacing the polymer solution. However, few studies using SDS-CGE have been reported for wine proteins analysis [25], or for their quantification. Challenges still encountered in optimizing the SDS-CGE analysis include sample preparation, peak resolution and area quantification [25,26]. Ease of sample preparation, short analysis time, good sample peak resolution and reliability from run to run are desirable for this analytical method.

In this study, the Coomassie Brilliant Blue (CBB) assay, lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and SDS-CGE were optimized and evaluated for their ability to analyze Sauvignon blanc wine proteins. Bovine serum albumin (BSA) was chosen as a calibration standard for the comparison of the three assays for protein quantification. Protein MW profile and relative concentration by LDS-PAGE and SDS-CGE was also compared, together with sample preparation.

Furthermore, the performance of the CBB assay to determine protein concentrations in juice samples was also investigated in order that the relationship between juice protein and wine protein concentration could be investigated. Juice samples were also analyzed by SDS-CGE since this method is inherently more amenable to large sample runs and traces are easier to integrate using existing software solutions compared to LDS-PAGE.

Additionally, in attempting to explore the possibility of using the fast and easy CBB assay

optimized in this study as an alternative to the time-consuming hot/cold test, protein concentration in wines before and after bentonite fining to achieve protein stability was investigated.

Materials and methods

Chemicals and reagents

Coomassie Brilliant Blue dye concentrate, semi-micro cuvettes (polystyrene, 1 cm path and 1.5 mL) and a primary protein MW standard (10-250 kDa, Precision Plus Protein All Blue Standards) were purchased from Bio-Rad (Hercules, CA). Bovine serum albumin (Cohn V fraction, ≥ 96%, lyophilized powder), 2-mercaptoethanol (14.3 M) and benzoic acid were from Sigma (St. Louis, MO). Acetone, methanol, acetic acid and sodium hydroxide were from MERCK (Darmstadt, Germany). Commercial polyacrylamide gel (NuPAGE® 4-12% Bis-Tris Gel, 1.0 mm X 10 well), lithium dodecyl sulfate (LDS, 4X, 10 mL) and NuPAGE® MES SDS running buffer (20X, 500 mL) were purchased from Invitrogen (Carlsbad, CA). Staining reagent (GelCode Blue Stain Reagent, Coomassie G-250) was purchased from Pierce (Rockford, IL). A sodium bentonite (Volclay KWK, American Colloid Company) and a sodium-calcium bentonite (NaCalit PORE-TEC, Erbslöh Geisenheim AG) were used to remove wine protein.

A SDS-MW Analysis Kit from Beckman Coulter (Fullerton, CA) was used. This kit included a mixture of protein sizing standards (10, 20, 35, 50, 100, 150 and 225 kDa), sample buffer (1% SDS in 100 mM Tris-HCl, pH 9.0), gel buffer, hydrochloric acid (HCl, 0.1 M) and sodium hydroxide (NaOH, 0.1 M). A rinse solution composed of 2-fold diluted sample buffer was prepared to rinse the end of the capillary. Fused-silica capillaries with 50 μm internal diameter (Biotaq Inc., Gaithersburg, MD) were trimmed to 31 cm (effective length from cathodic end to the detector 22.5 cm) for use.

Juice and wine samples

Sauvignon blanc juice (21.4 °Brix and pH 3.24) and corresponding wine (alcohol 13.6% v/v

and pH 3.34) from the 2008 vintage (provided by Pernod Ricard (NZ) Ltd.) were used as standard samples. Wines (n = 102) collected from 2007 and 2008 vintages either from commercial or laboratory scale winemaking were obtained for protein determination by the CBB assay before and after achieving protein stability by bentonite fining. Some of the wines (n = 18) were also used for comparison between three analytical methods. Soluble solids content, pH and alcohol content were measured by an infrared technique using WineScan™ FT120 (Foss, Denmark).

Protein recovery

Frozen samples were first thawed and filtered through 0.45 μm cellulose nitrate filter (47 mm; Sartorius, Geottingen, Germany) or centrifuged at 10,000 g for 10 min. Proteins recovered from wine by ultrafiltration (Microcon Centrifugal Filter Device YM-10) were washed 2 times with 100 μL deionized water before additional ultrafiltration to desired volume (~30 μL). Wine protein was also recovered by acetone precipitation. One volume of wine was mixed with four volume of ice-cold acetone and then left at -20°C for 1 hour before recovery by centrifugation (10,000 g for 5 min).

Bentonite fining and protein stability test

Bentonite fining trials were conducted by adding amounts of 5% (w/v) bentonite slurry to 50 mL wines, mixing for 1 min and then left settling for 35 min before subjecting to centrifugation using a Heraeus Megafuge 1.0 at 3,220 g for 10 min (DJB Labcare Ltd, Buckinghamshire, England) and filtration (Sartorius). A hot/cold test (80°C for 6 h followed by 4°C overnight) was used as a protein stability test. Wines with differences in turbidity higher than 2 nephelometric turbidity units (NTU; Model 2100P; Hach, Loveland, CO) before and after the hot/cold test were considered as protein unstable. Protein remaining in stabilized wine (NTU = 2) was determined by linear interpolation between the two closest bentonite fining rates.

CBB assay

Wine (200 μL) and BSA standards (200 μL, 0-200 mg L^{-1}) were mixed with 100 μL 1 M NaOH, followed by 5 min incubation and centrifugation (10,000 g for 5 min). Alkalized samples (60 μL) were then mixed with 1200 μL 5-fold diluted dye reagent (5DDR) in 1.5 mL cuvettes. Absorbance readings were made against deionized water at 595 nm after 10 min incubation. Triplicate analyses were conducted for each sample except for protein stabilized wines for which duplicate analyses were performed. Protein concentrations were determined with respect to the BSA standards and expressed as mg L^{-1} BSA equivalents. Experiments were conducted to examine the effect of sample/5DDR volume ratio, base addition and protein-dye incubation time on the estimation of wine protein concentration.

The same series of experiments was carried out using a juice sample for which a preliminary test indicated that juice dilution was necessary to prevent protein underestimation. Consequently, juice samples of 75 or 100 μL were diluted to 200 μL.

LDS-PAGE procedure

Protein concentrates (30 μL) from 250 μL juice/wine were mixed with 10 μL sample buffer (a mixture of 152 μL LDS and 8 μL 2-mercaptoethanol) and then denatured in boiling water (5 min). Denatured protein samples (25 μL) were loaded onto the gel. BSA (7 μg) was also loaded onto both sides of the gel for protein MW referencing and quantification. Electrophoresis was run in 20-fold diluted MES SDS running buffer with constant voltage mode (200 V) at room temperature for 55 min. The gel was stained in the staining reagent for at least 1 hour to overnight after protein fixation in a fixing solution (50% methanol and 7% acetic acid, v/v) for 15 min, followed by destaining in deionized water for 1-2 hours. Both gel staining and destaining were conducted on the Rocker Platform (BellcoGlass, NJ, USA).

Destained gels were analyzed using Gel Doc EQ™ (Bio-Rad Laboratories, USA) gel imaging hardware and Quantity One® (Bio-Rad Laboratories, USA) imaging and analysis software. Protein MW and quantity for bands in each sample lane were determined with

respect to the electrophoretic distance of MW references and electrophoretic intensity (optical trace area) of BSA band (60 kDa), respectively. Total protein concentration was the sum of the main protein bands found and expressed as mg L^{-1} BSA equivalents.

SDS-CGE procedure

The Agilent Capillary Electrophoresis System (Waldbronn, Germany) equipped with external pressure (nitrogen gas) was utilized in CE+p mode and controlled by the ChemStation system software (Rev. A.10.02). Signals were detected at 214 nm and temperature was regulated to 25°C. Injection was carried out in hydrodynamic mode by applying a pressure of 50 mbar for 2 min. A constant voltage of -20 kV (reversed voltage polarity) was applied and the observed electric current was approximately 41 μA.

At the beginning of each day, capillaries were flushed with 0.1 M NaOH at 3 bar for 30 min. Prior to electrophoresis, the following solutions were flushed from the inlet vial to the waste vial by applying 940 mbar: 0.1 M NaOH (5 min), 0.1 M HCl (5 min) and gel buffer (10 min), followed by dipping the capillary inlet end in the rinse solution (~5 sec). After sample injection, electrophoresis was run with capillary ends positioned at vials also containing the gel buffer. After electrophoresis, capillaries were flushed with deionized water at 3 bar for 3 min. All materials were refreshed after every six runs. At the end of each day, capillaries were thoroughly flushed at 3 bar with 0.1 M NaOH (10 min) and deionized water (10 min) before drying with air (10 min).

All protein migration times (MT) and peak areas (PA) were corrected with the internal reference (benzoic acid) and expressed as relative migration time (RMT) and relative peak area (RPA). Total protein concentration was the sum of detectable protein peaks and expressed as mg L^{-1} BSA equivalents.

Protein concentrates from 300 μL wine were made up to 54 μL with sample buffer, and then mixed with 3 μL benzoic acid (1 mg mL^{-1}) and 3 μL 2-mercaptoethanol. For comparison, protein concentrates from 300 μL of juice were also included in this experiment. A

calibration curve for protein quantification was derived from a series of BSA dilutions to final concentrations of 2, 1, 0.5, 0.25, 0.125 and 0.0625 g L^{-1}. Final samples and BSA standards contained 5% v/v 2-mercaptoethanol and 0.005% w/v benzoic acid. The prepared MW reference was a mixture of 10 µL sizing standards, 53 µL sample buffer, 2 µL benzoic acid and 5 µL 2-mercaptoethanol. All protein samples were denatured in a 95°C water bath for 10 min before analysis.

Statistics

One-way analysis of variance with Fisher 95% simultaneous confidence intervals was performed using MINITAB 15 statistical software in order to estimate the lowest detectable protein concentration using the CBB assay and protein dilution in LDS-PAGE.

Results

CBB assay optimization

The CBB assay was optimized in terms of sample/5DDR volume ratio, base addition and protein-dye incubation time. Three base additions (100 µL of 0.5, 1.0 and 1.5 M NaOH) and three 5DDR volumes (1200, 1800 and 2400 µL) were compared using BSA standards with absorbance readings at 595 nm after 30 minutes incubation. Irrespective of the base addition, greatest sensitivity (slope) was obtained with the 1200 µL 5DDR. All assays resulted in good linearity ($r^2 > 0.996$). Conditions of 1.0 M NaOH and 1200 µL 5DDR were further used to study the effect of incubation time up to 60 min in 10 min intervals. The slope gradually decreased with increasing incubation time from 0.0093 A_{595} μg^{-1} BSA at 0 min to 0.0076 A_{595} μg^{-1} BSA at 60 min. The highest slope and r^2 value were obtained with 10 min incubation. In addition, a tendency for flocculation and coagulation of the protein-dye complex was observed for BSA standards of higher than 100 mg L^{-1} after 30 min incubation. Optimized conditions were 1.0 M NaOH, 1200 µL 5DDR and 10 min protein-dye incubation time.

Linearity, reproducibility and sensitivity limit of CBB assay

The high linearity and reproducibility of this CBB assay were demonstrated from the results

of day-to-day BSA calibration curves (n = 33) over a period of three years (Table 1). The linear range for BSA content extended from 0 to 60 μg with a mean r^2 value of 0.995 and a coefficient of variation (CV) of 0.2%. The mean response (slope) was 0.0101 A_{595} μg^{-1} BSA with a CV of 4.9%.

Using this optimized CBB assay, total protein concentration of the Sauvignon blanc standards were measured at 110.2 ± 6.7 mg L^{-1} (mean ± SD) for juice and 108.5 ± 2.8 mg L^{-1} for the corresponding unfined wine. The protein concentration of collected wines (n = 102) ranged between 60 and 150 mg L^{-1}. When wines achieved protein stability, the remaining protein content ranged between 5 and 35 mg L^{-1} with 90% of the wines in the range of 10 and 25 mg L^{-1}.

Protein recovery

Wine protein recovered by 4-volume acetone precipitation resulted in higher electrophoretic intensity of the 21 kDa fraction on LDS-PAGE (2358 ± 23) (average of triplicate preparations ± SD) than that recovered by ultrafiltration (2194 ± 46). There was also a marked depletion in electrophoretic intensity of the 33 kDa band from the ultrafiltration treatment. The ultrafiltration treatment recovered 21% less wine protein than 4-volume acetone precipitation as measured by SDS-CGE (Table 2). Therefore, 4-volume acetone precipitation was used as the standard method for wine protein recovery in LDS-PAGE and SDS-CGE. For juice protein recovery, only the ultrafiltration treatment was applicable since acetone precipitation resulted in protein concentrate with high liquid volume, presumably as a result of the high soluble solids content.

Variability of LDS-PAGE

BSA loadings from 1.5 to 15 μg were shown to correlate linearly with the optical trace area from 706 to 1865 (r^2 = 0.956) and are suitable for wine protein MW referencing, containing a major band at 60 kDa. Other minor impurities at 8.8, 11.8, 22.5 30, 39 and 150 kDa as determined by the primary protein MW standard were also detected. A dilution series of

wine protein concentrate was used to test the sensitivity and variability of LDS-PAGE in duplicate gels. Samples of 30 to 2 μL (in 4 μL decrements) of 30 μL protein concentrate from 250 μL wine were made up to 40 μL. Good linearity ($r^2 > 0.937$) and significant differences ($p < 0.001$) between wine dilutions and protein contents for each protein fraction were evident. Nevertheless, increasing dilution also increased the derived wine protein concentrations using BSA as a standard from 77.2 to 305.1 mg L^{-1} (Table 3) and little improvement of peak resolution was observed (Fig. 1). The major wine protein fractions were 10, 21, 33 and 64 kDa with the 21 kDa being the predominant fraction (> 58% of the total). Overall, significant gel to gel variation in the electrophoretic intensity of the BSA reference (7 μg loadings) was observed (CV of 26.3% for 15 gels with two values from each).

Accuracy and reproducibility of SDS-CGE

Plotting the log MW of the protein standards (10-225 kDa) (y axis) versus 1/RMT (x axis) gave a linear regression by which protein separation based on MW was corroborated (Fig. 2). However, an improved regression value was obtained using a quadratic function ($r^2 = 0.999$) or by using only standards of low to medium MW (10-100 kDa) ($r^2 = 0.996$) in the range of juice and wine proteins. Using the regression line derived from 10 to 100 kDa standards, the determined MW of 10, 20, 35, 50 and 100 kDa sizing standards were 9.5 ± 0.1, 20.7 ± 0.3, 36.2 ± 0.6, 52.8 ± 0.8 and 93.1 ± 1.7 kDa (average of 5 runs ± SD), respectively. Results of migration times (MT) and peak areas (PA) and relative to the internal reference (Table 4) were used to examine reproducibility. Good reproducibility (CV < 1.6%) was obtained for MT and RMT, whereas both PA and RPA showed greater variation (CV < 16.4%). Nevertheless, reliable results were obtained for juice and wine proteins (Table 2) with CV values below 4.5%.

Protein analyses by SDS-CGE

The BSA calibration curve for protein quantification showed high linearity ($r^2 > 0.999$) over

the range of 0.0625 to 2 g L^{-1} (Fig. 3). The derived BSA MW (66 kDa was the supplier's indication, Sigma) shifted gradually from 65.8 kDa at the highest concentration (2 g L^{-1}) to 68.5 kDa at the lowest concentration (0.0625 g L^{-1}). Sauvignon blanc protein peaks found were classified into six MW fractions (Table 2). Although total protein concentration in juice and the resultant wine were similar, fermentation changed the composition of proteins. The 26 kDa fraction (53%) was the predominant protein in juice followed by the 22 kDa fraction (38%), whereas it reversed in the resultant wine with the 22 kDa fraction (66%) being predominant followed by the 26 kDa fraction (25%). These two fractions, however, contributed more than 90% of total proteins regardless of protein concentration in juice or corresponding wine and recovery methods (Table 2). Moreover, wine proteins recovered by acetone precipitation have better resolution than ultrafiltration with distinguishable peaks at 9.6, 19, 21 22, 24.6, 26.5, 27.6 and 32 kDa (Fig. 4). Protein peaks at 21 and 22 kDa corresponded to the 22 kDa fraction, and protein peaks at 24.6, 26.5 and 27.6 kDa corresponded to the 26 kDa fraction.

Comparison of three analytical methods

Protein concentrations of 18 wines examined by the CBB assay, LDS-PAGE and SDS-CGE averaged 113.3, 79.5 and 483.6 mg L^{-1}, respectively. The CBB assay resulted in wine protein concentration about 42% higher compared to LDS-PAGE (CV = 8%) and about 4.2 times lower compared to SDS-CGE (CV = 16%). Quantification from three analytical methods were positively correlated between each other. The CBB assay showed higher linear correlation to the LDS-PAGE (r^2 = 0.80) compared to SDS-CGE (r^2 = 0.62).

Discussion

Base treatment to reduce variation in color yield among various proteins and enhance sensitivity for the Coomassie dye-based assay has been previously reported [27,28]. Following base addition, juice and wine samples were further diluted 3- and 1.5-fold, respectively. Thus, our optimized CBB assay had the additional advantage of diluting soluble

solids and ethanol content as well as other potential interfering compounds, such as phenols and polysaccharides [20,28,29], while retaining a good response for the protein-dye reaction. Glucose, fructose and ethanol are known not to interfere with protein determination under standard conditions [30]. It has been claimed that about half of proteins in wine are bound to phenolics [31] and this interaction results in protein underestimation [18,19]. In addition, a pectin concentration of less than 5 g L^{-1} has been shown to have little effect on the Coomassie dye assay [29]. Consequently, base addition to juice and wine samples potentially improved the sensitivity and accuracy of CBB assay by two factors: increasing sample pH may weaken hydrogen bonds, releasing proteins from interfering compounds and exposing more protein active sites for dye binding [28]; diluting samples may reduce concentrations of interference compounds to below their effective thresholds, favoring Coomassie dye-protein complex formation [29]. The survey of wines showed that a low concentration of protein in a narrow range remained in stabilized wines and is supported by other studies [32-34]. The possibility therefore exists that results from the CBB assay in this narrow protein range (10-25 mg L^{-1}) could be used as an indicator of protein stability and hence provide an alternative to the hot/cold test to predict bentonite requirement as others have proposed [9,34].

Our SDS-CGE procedures and conditions, especially the short capillary effective length (22.5 cm) and small ID (50 μm), contrasts with other studies [26,35] and showed good separation and reproducibility for the sizing standards and long term stability for sample analysis. For qualitative and quantitative analysis, the use of hydrodynamic injection, together with an internal reference is essential to minimize run-to-run variation [25,36]. As a consequence, high reliability for the MW calibration curve (Fig. 2) was achieved similar to other studies [25,26] and good quantitative reliability for juice/wine protein analysis (Table 2) as reported by others [36]. The higher variation of RPA for MW sizing standards (Table 4) might be the result of their low concentrations and unstable base line, resulting in integration bias.

Furthermore, results that acetone precipitation was more effective than ultrafiltration for wine

protein recovery in both LDS-PAGE and SDS-CGE is in accordance with the findings on the recovery of BSA from protein-free wine by SDS-PAGE [37]. The loss of wine protein (21%) into filtrate after ultrafiltration in our SDS-CGE analysis (Table 2) is in agreement with the observations using the Bradford assay in which filtrates could account for 28-61% of the initial wine values [20]. However, this apparent higher protein loss percentage could also be attributed to the fact that the Bradford assay quantifies polypeptides and proteins with MW higher than 3 kDa, or to interference compounds [14,20]. Protein recovery of retentate and filtrate after ultrafiltration were additive to that by acetone precipitation alone in our SDS-CGE results (Table 2). In addition, protein recovery from juice by acetone precipitation is not suitable owing to the large quantity of sugar that was also precipitated [38].

The protein concentrations of the standard wine determined by the CBB assay and LDS-PAGE (108.5 and 77.2 mg L^{-1}, respectively) were in the range commonly reported (15-300 mg L^{-1}) [5,6], whereas SDS-CGE analysis gave a much higher protein level (532.1 mg L^{-1}). Protein concentrations up to and over 700 mg L^{-1} have been reported [37,39,40]. The trend of differences in protein concentration between the three analytical methods is similar for all studied wine samples (n = 18). Similar values and good correlation between the CBB assay and LDS-PAGE are expected due to the same quantification mechanism. SDS-CGE quantification giving results 4.2 times higher than the CBB assay is similar to the findings that protein quantification by amino acid summation was 4.3 times and 2-5 times higher compared to that by modified Coomassie assays for juice protein and wine protein fractions, respectively [18,41]. This implies that values of wine protein concentration determined by the SDS-CGE might be similar to that quantified according to amino acid summation of hydrolysed protein.

Thus, the differences between our results can probably be assigned to the methods utilized. Quantification in the CBB assay and LDS-PAGE were based on the same mechanism; i.e. enhancing the coloration through forming of a Coomassie dye-protein complex [15], whereas

absorption of peptide bond at 214 nm is detected in SDS-CGE. The wine protein MW profile in LDS-PAGE was similar to that in SDS-CGE, except the 64 kDa fraction which was not detectable in SDS-CGE (Fig. 4). Similarly, other workers found that the relative percentage of 65 kDa fraction in SDS-PAGE was much higher than that in chromatography-based separation with 210 nm absorption, resulting in overestimation of the 65 kDa fraction in SDS-PAGE [42]. Also, differences in the percentage of protein in MW fractions between LDS-PAGE and SDS-CGE might be due to their different analytical mechanisms. Variable amino acid composition and glycosylation between protein fractions has been reported [3,18,22]. Arginine and lysine are the main amino acids responsible for the color reaction in Coomassie blue bye-based assay [15], whereas the ultraviolet absorption (210-220 nm) chiefly resulted from protein peptide bonds was less sensitive to changes of protein amino acid composition [43]. Consequently, different results between different analytical methods in protein MW composition and quantification were expected.

Conclusions

Our optimized CBB assay is a simple, fast and reliable protein quantification method which is suitable for industry application as well as for scientific study regarding juice and wine proteins. It is possible to utilize the CBB assay after bentonite fining trials to examine protein residues in fined juice and wine as an alternative to the hot/cold test in determining required bentonite dosage. Acetone precipitation is an easy, more efficient and possibly more economical protein recovery method than ultrafiltration for wine protein analysis as demonstrated in our LDS-PAGE and SDS-CGE results. Protein MW profiles of Sauvignon blanc juice and resultant wine were similar to each other and to other *Vitis vinifera* varieties but different in relative percentage. By using an identical calibration standard (BSA), analysis by SDS-CGE resulted in much higher protein concentrations than the CBB assay and LDS-PAGE. In addition, the highly automated SDS-CGE method provided more resolved peaks and quantitative convenience over conventional LDS-PAGE method.

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Table 1

Summary of calibration curve parameters with BSA contents ranged from 0 to 60 μg (0-200 mg L^{-1}) by CBB assay from day-to-day analyses (n = 33).

	Slope	Intercept	r^2	A_{595} max	A_{595} min
Average	0.0101	0.5805	0.9951	1.172	0.565
SD	0.0005	0.0158	0.0023	0.035	0.016
CV	4.9%	2.7%	0.2%	3.0%	2.8%
Range	0.0093-0.0117	0.5501-0.6163	0.9874-0.9984	1.129-1.297	0.533-0.602

BSA: bovine serum albumin.

CBB: Coomassie Brilliant Blue.

SD: standard deviation.

CV: coefficient of variation.

Table 2

Protein concentration and composition of Sauvignon blanc juice and resultant wine by SDS-CGE analysis.

Treatments		Protein MW fractions (%)						Total protein
		<19 kDa	19 kDa	22 kDa	26 kDa	32 kDa	>32 kDa	(mean ± SD)
Juice	Retentate[a]	2.3	0.0	38.4	52.8	4.9	1.6	546.1 ± 24.4[d]
Wine	Acetone[b]	2.1	5.0	65.6	24.6	2.7	0.0	532.1 ± 9.3[d]
	Retentate[a]	3.5	0.0	67.5	23.1	5.0	1.0	421.8 ± 10.5[e]
	Filtrate[c]	0.0	16.4	34.5	42.5	0.0	6.7	88.8 ± 22.3[e]

[a] Protein recovered by ultrafiltration (10 kDa MW cut-off filter).

[b] Protein recovered by 4-volume acetone precipitation.

[c] Protein recovered from the filtrate of ultrafiltration by acetone precipitation.

[d] Average from two sample injection time at 80 and 120 sec.

[e] Average of duplicate preparation.

SDS-CGE: sodium dodecyl sulfate capillary gel electrophoresis.

MW: molecular weight.

Table 3

Effect of dilution factor on total wine protein concentration determined and relative percentage of MW fractions by LDS-PAGE.

Concentrate[a]	Protein MW fractions (%)				Wine protein[b]
(μL)	10 kDa	21 kDa	33 kDa	64 kDa	(mg L^{-1})
30	13.7	58.0	7.8	20.5	77.2 ± 0.3
26	14.0	60.8	7.5	17.7	80.7 ± 0.6
22	13.6	63.4	7.1	15.9	87.0 ± 0.5
18	12.5	65.7	6.6	15.2	98.9 ± 1.2
14	12.2	67.2	5.4	15.2	112.0 ± 2.0
10	11.4	69.2	4.8	14.6	138.4 ± 0.9
6	11.9	72.3	3.8	12.0	188.6 ± 11.9
2	11.8	75.5	3.4	9.3	305.1 ± 19.5

[a] Samples of 30 to 2 μL (in 4 μL decrement) of 30 μL protein concentrate from 250 μL wine were made up to 40 μL finals with 25 μL loadings in duplicate.

[b] Quantification was by reference to BSA standard.

LDS-PAGE: lithium dodecyl sulfate polyacrylamide gel electrophoresis.

MW: molecular weight.

Table 4

Relative migration time (RMT) and relative peak area (RPA) reproducibility of the protein sizing standards utilised in SDS-CGE analysis.

Peak number[a]	MW (kDa)	RMT (n = 5)		RPA (n = 5)	
		Time(min)	CV (%)	Area (mAU)	CV (%)
0	Reference[b]	1.000	—	1.000	—
1	10	1.722	0.3	1.767	5.3
2	20	2.028	0.3	1.937	10.2
3	35	2.328	0.4	1.119	9.9
4	50	2.584	0.5	2.360	10.1
5	100	3.099	0.6	1.137	11.7
6	150	3.420	0.8	0.463	15.4
7	225	3.757[c]	1.1	0.641[c]	10.3

[a] Peak numbers correspond to electrophoretic peaks in Fig. 2.

[b] Benzoic acid with MT = 5.172 ± 0.026 and PA = 33.3 ± 1.9 (average of 5 runs ± SD).

[c] n = 4.

SDS-CGE: sodium dodecyl sulfate capillary gel electrophoresis.

CV: coefficient of variation.

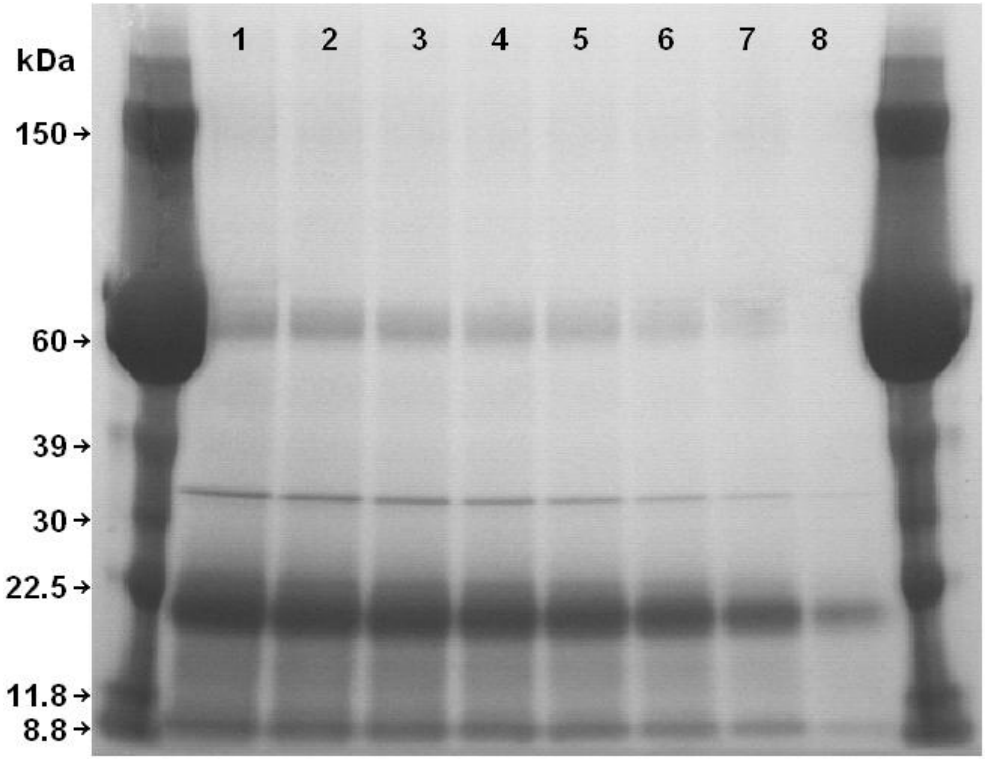

Fig. 1. Electropherogram of Sauvignon blanc wine protein (in a series dilution) by LDS-PAGE analysis. Lane 1 to 8 indicated 25 μL loadings of 40 μL finals consisted of 30 to 2 μL (in 4 μL decrement) of 30 μL protein concentrate from 250 μL wines, respectively. Lanes on both sides of the gel were 10.5 μg loadings of BSA used as molecular weight referencing (arrows in the left) and quantification.

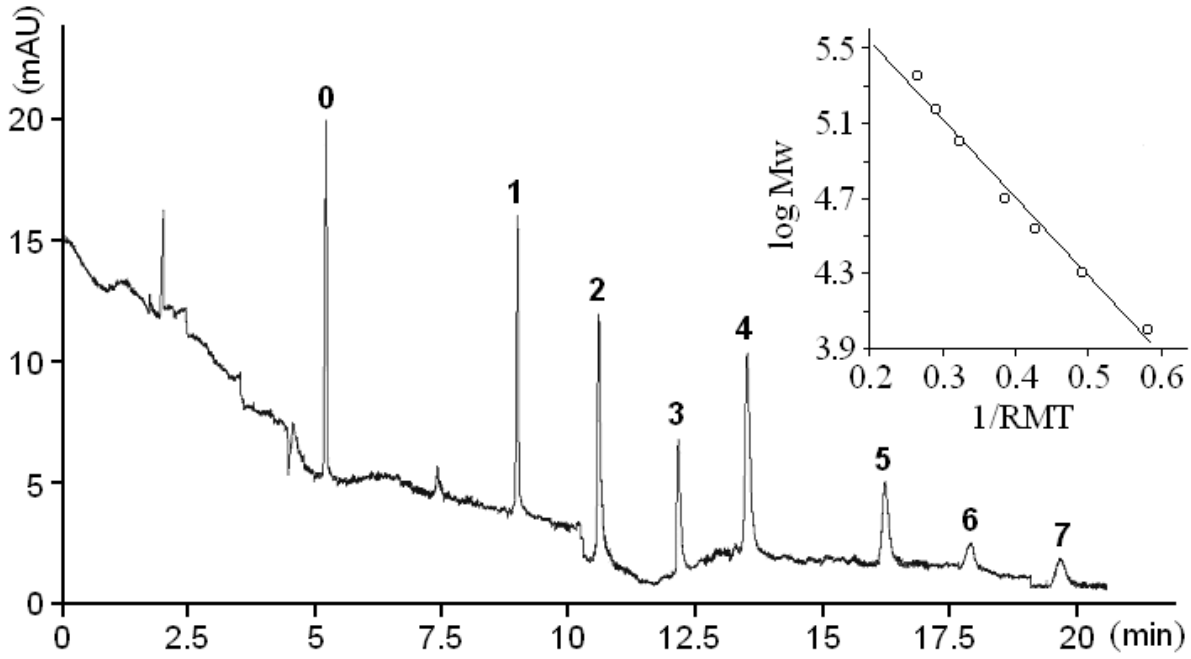

Fig. 2. SDS-CGE separation of protein sizing standards (peak 1-7) and the internal reference (peak 0, benzoic acid). Peak numbers from 1 to 7 represent 10 to 225 kDa standards as detailed in Table 3. Plot of molecular weight logarithm of sizing standards versus their 1/RMT is present on the right top of the electropherogram.

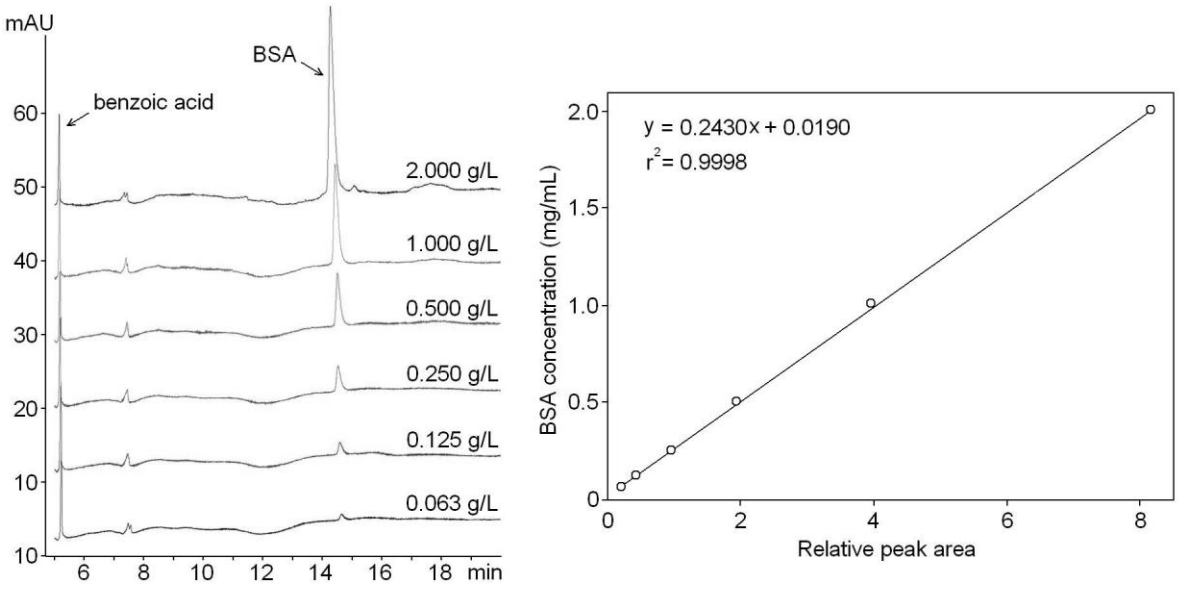

Fig. 3. Electropherograms of 6 BSA concentrations from 2 to 0.063 g L^{-1} (left) by SDS-CGE. Number over the curve represents the BSA concentration of that curve in g L^{-1}. A protein calibration curve (right) derived from 6 BSA concentrations (left).

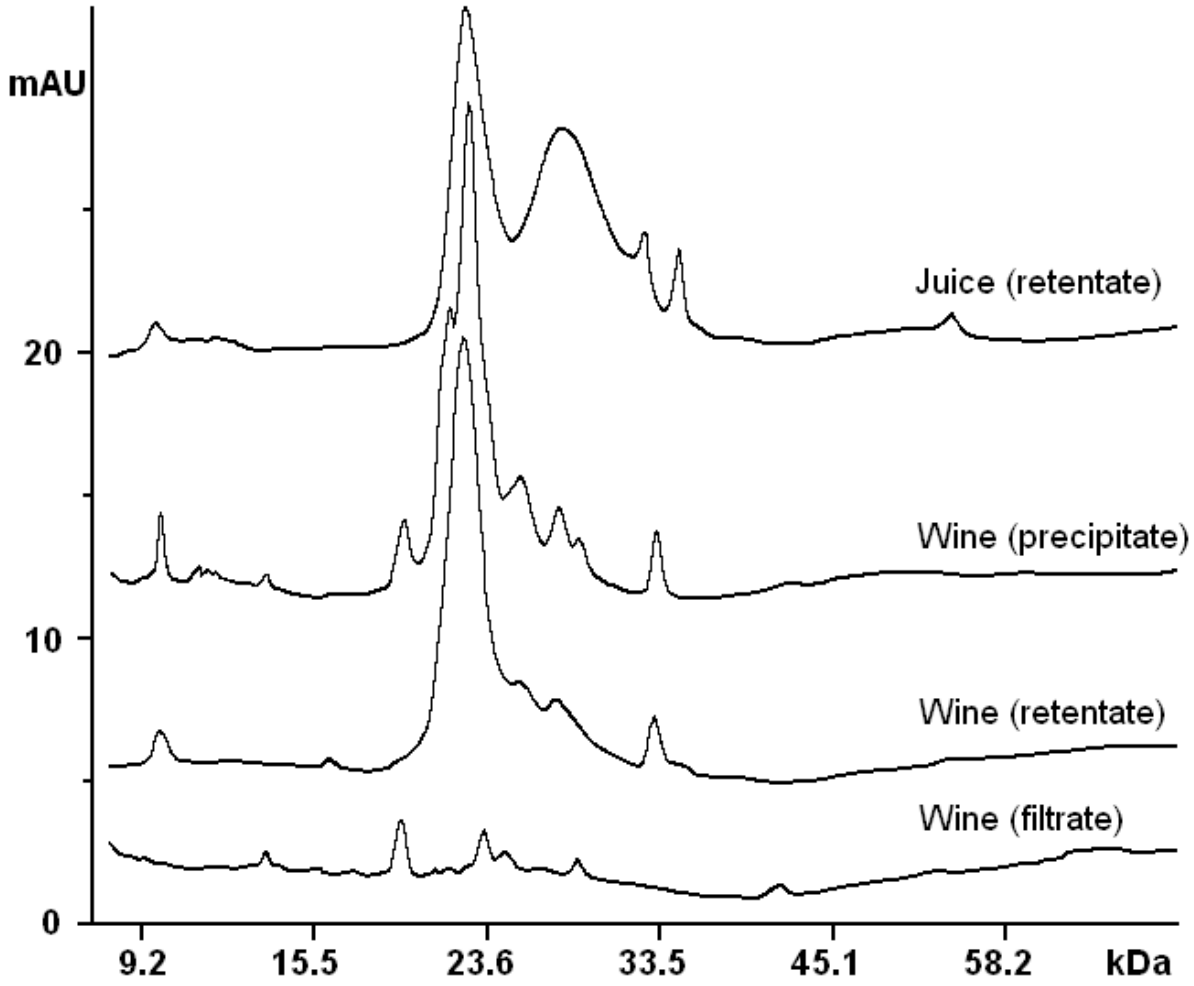

Fig. 4. Electropherograms of Sauvignon blanc juice and resultant wine by SDS-CGE analysis with two protein recovery methods: acetone precipitation and ultrafiltration (10 kDa MW cut-off) as described in materials and methods. The wine filtrate from ultrafiltration was further subjected to acetone precipitation.

Chapter 5

(*A preparation for submission for* Australian Journal of Grape and Wine Research)

Protein Quantification of Marlborough Sauvignon Blanc and Correlation with Bentonite Requirement

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Short running title: **Protein Stability of Marlborough Sauvignon Blanc**

Abstract

Protein instability has long been a technical issue in white wine production. Proteins in Sauvignon blanc juices and wines from five selected Marlborough, New Zealand vineyards with two pruning regimes (2- and 4-cane) were studied over two consecutive vintages. Proteins were quantified and characterised by Coomassie Brilliant Blue (CBB) assay, lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS-PAGE) and sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE). Bentonite fining coupled with a hot/cold test (80°C for 6 hours followed by 4°C overnight) was used to determine the bentonite requirement for wine protein stability. One vineyard consistently showed the lowest protein concentration in juice and wine, haze formation and bentonite requirement regardless of pruning treatments and vintages, whereas others varied when pruning treatments and/or vineyard sites and/or vintages were compared. Two prevalent juice protein peaks at 22 and 28 kDa in SDS-CGE corresponded to two main wine protein peaks at 22 and 26 kDa, respectively. The 26 kDa fraction was reduced and became heterogeneous after fermentation, while the 22 kDa fraction remained unaffected. There was a good correlation between bentonite requirement with the 26 kDa fraction ($r^2 = 0.78$), but not with protein haze, total protein concentration and the predominant 22 kDa fraction ($r^2 < 0.50$).

Keywords: *Bentonite; Coomassie; Electrophoresis; Protein Stability; SDS-CGE*

Introduction

Residual proteins are considered the most important nitrogenous compounds in white wine since they are involved in the formation of commercially unacceptable hazes or deposits during storage of bottle wines (Ferreira et al. 2002, Waters et al. 2005). Depending on storage conditions, proteins in wine will denature with time followed by flocculation and coagulation and finally precipitate in the wine bottle (Pocock and Waters 2006). To minimise the protein haze potential in bottled wine, batch-wise fining of bentonite has been commonly performed in winery, resulting in quantitative and qualitative loss in wine as bentonite lees (Høj et al. 2000).

Protein concentrations in un-fined white wine vary and typically range up to 300 mg L^{-1} (Ferreira et al. 2002, Waters et al. 2005). Proteins remaining in white wine after fermentation are mainly those proteins stable at acidic wine conditions and highly resistant to proteolysis. They have been demonstrated to be members of plant pathogenesis-related (PR) proteins, originating from grapes (Waters et al. 1996, Dambrouck et al. 2003, Sauvage et al. 2010). In early studies, proteins with molecular weight (MW) at 20-30 kDa were shown to be the major proteins regardless of variety and vintage (Hsu and Heatherbell 1987a, Murphey et al. 1989, Waters et al. 1992, Pueyo et al. 1993). Ubiquitous grape PR proteins with MW between 20 and 30 kDa from various cultivars were confirmed as thaumatin-like proteins and chitinases, possessing similarity in MW but differences in relative quantity (Pocock et al. 2000). In addition, slight variations in electropherograms between varieties were noticeable (Pueyo et al. 1993, Ferreira et al. 2000). These minor variations have lead to the possibility of utilising PR protein profiling for varietal differentiation (Hayasaka et al. 2001, Chabreyrie et al. 2008). However, such application has been shown to be challenging as environmental conditions and vine growth also determine the protein profile (Sarmento et al. 2001, Monteiro et al. 2003).

Thaumatin-like proteins and chitinases are the two major proteins in Sauvignon blanc grape juices and wines with the thaumatin-like proteins contributing 70% of total protein in wine (Peng et al. 1997, Pocock et al. 1998, Pocock and Waters 1998). Proteins of MW at 20-30 kDa have been reported as responsible for heat- and chemical-induced and natural haze in wine (Hsu and Heatherbell 1987b, Esteruelas et al. 2009a) and it has also been shown that model and protein-free wines with thaumatin-like proteins cause more haze than with chitinases (Waters et al. 1992, Pocock et al. 2007). However, Sarmento et al. (2000) suggested that the total protein concentration is a good indicator for wine protein stability as all protein fractions showed similar tendency to precipitation during wine storage. In addition, although doubling the PR protein concentration in a wine resulted in twice the bentonite requirement for

stabilisation (Pocock and Waters 1998), the order of total protein concentration for different white cultivars was slightly different to the order of bentonite requirements both for juices (Duncan 1992) and wines (Hsu and Heatherbell 1987b). The relationships between wine protein stability, bentonite requirement and protein concentration as well as individual proteins remain unclear.

In this study, Sauvignon blanc from 5 vineyards within the Marlborough region were utilised over two consecutive vintages to investigate protein concentration, MW profile and bentonite requirement. In addition two pruning treatments were studied. There were two main aims: 1) to describe the variability of wine proteins from a single variety over two vintages and relate this variability to site and pruning regimes and 2) to determine whether a correlation exists between total protein and individual protein fractions and bentonite dosage required for heat stability. In addition, various vine sizes, even within a vineyard, were noticeable at many vineyards in the Marlborough region (Trought et al. 2008). A vine size trial within one of the studied vineyards in 2007 was also included in this study to examine the variability within the same vineyard with regard to protein content, MW composition and heat stability.

Materials and methods

Juice and wine samples

Samples were obtained in 2007 and 2008 from a pre-existing trial, consisting of five representative vineyard areas (Table 1) of Marlborough Sauvignon blanc (clone MS UCD1, grafted onto SO4 rootstock and planted in 1994) which had been managed in an identical manner from 2004 by staff from the Marlborough Wine Research Centre. For each area, two pruning treatments (2-cane and 4-cane) were imposed in a randomised block design with 4 replicates. Trial plots consisted of four vines planted in bays between intermediate wooden posts. At one of the representative areas (SQ) and for the 4-cane treatment, plots were selected with vines that had a medium trunk circumference (c. 187 mm) based on measurements of 8 complete vineyard rows (Trought et al. 2008). Plots representing extra small (c. 165 mm) and extra large (c. 220 mm) trunk circumferences were also included in 2007.

In 2007, grape samples (10 bunches per plot) from replicated blocks of each treatment were hand-harvested at a target maturity of 21.5 ºBrix and processed using standard protocols to obtain juice samples. In 2008, grapes were also hand-harvested and replicate blocks pooled before triplicate sub-samples were obtained. In both years, bulk samples from each treatment were pooled and sub-sampled for wine-making in triplicate, small lots (< 30 L) again according to a standard research protocol. Samples could not be obtained from the 4-cane treatment at one site (VI) in 2008 because of disease.

Protein recovery and analysis

Methods as reported previously were utilised (Hung et al. 2010). Briefly, total protein concentration was determined using reaction with Coomassie Brilliant Blue (CBB) dye following alkalization of the sample. Protein concentrates for LDS-PAGE and SDS-CGE procedures were prepared following recovery by ultrafiltration (10 kDa MW cut-off, Millipore) for juice samples and by precipitation with ice-cold acetone and centrifugation for wine samples. LDS-PAGE was carried out using commercial polyacrylamide gels (NuPAGE® 4-12% Bis-Tris Gel, 1.0 mm X 10 well) and SDS-CGE utilised a capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) with external pressure in CE+p mode and controlled by ChemStation system (Rev. A.10.02). Gels were run at constant voltage for 55 min at room temperature and destained gels were analyzed using Gel Doc EQ™ (Bio-Rad Laboratories, USA) gel imaging hardware and Quantity One® (Bio-Rad Laboratories, USA) imaging and analysis software. For capillary electrophoresis, samples were injected by applying pressure (50 mbar) for 2 minutes and constant voltage was applied for electrophoresis with detection at 214 nm. Triplicate analyses were averaged for each sample in CBB assay. Means of one electrophoresis on two of triplicate wines in LDS-PAGE were reported, whereas duplicate electrophoresis was performed on one of triplicate wines in SDS-CGE.

Bentonite fining and protein stability test

Procedures for bentonite fining and protein stability test were utilised as previously reported (Hung et al. 2010). Bentonite fined wines with differences in turbidity higher than 2 nephelometric turbidity units (NTU; by Model 2100P; Hach, Loveland, CO) before and after the hot/cold test (80°C for 6 hours followed by 4°C overnight) were considered as protein unstable. The dose rate required to stabilise a wine was defined as the quantity to achieve light scattering characterised by an increase of 2 NTU during the hot/cold test. Bentonite requirement was determined by linear interpolation between the two closest bentonite fining rates.

Other analyses

Soluble solids contest, pH, titratable acidity and alcohol content were measured by Fourier Transform Infrared Spectoscopy (FTIR) interferometer (WineScan™ FT120, Foss, Denmark).

Statistics

The results of determinations of juice samples obtained in 2007 (except those involving different trunk circumferences) were analyzed with a linear mixed model in which site and pruning interactions were designated fixed effects and site and block interactions were

designated random effects using Genstat 11. For trunk circumference data, one-way analysis of variance (ANOVA) was performed using Minitab 15. Data for juice from 2008 and wine in both 2007 and 2008 were analyzed by two-way ANOVA with site and pruning treatment as factors (Minitab 15). In addition, pair-wise t-tests were applied to test for statistical differences of applicable variables between years (Minitab 15). Linear regression analysis was performed to correlate bentonite requirement with variables (Minitab 15).

Results

Juice and wine samples

Grape harvest date and resultant wine composition for all samples are summarised in Table 2. Generally, grape from 2-cane vines were harvested several days earlier than 4-cane vines. The average alcohol content and pH of all wines were 13.0 (%, v/v) and 3.10 with coefficients of variation (CVs) at 3.7% and 1.9%, respectively. In addition, the average CV for ethanol and pH for triplicate wines from the same juice lot was 0.5% and 0.2%, respectively. The effect of vinification on wine protein concentration by CBB assay, haze formation and bentonite requirement for each treatment also showed good replication with average CVs of 2.8%, 7.4% and 3.0%, respectively. As a consequence, reliable replication of vinification was implied.

Juice and wine protein analyses by CBB assay

Total protein concentrations in juices and resultant wines from the vineyard trial over two vintages are presented in Table 3. Generally, total protein content decreased after fermentation. Protein concentration in wine was from 74.2 to 97.1 % of that in juice except for samples BO4 and VI4 in 2007 vintage and BO4 and SE4 in 2008 vintage which was slightly higher than 100 %.

In 2007, both juice and wine from the BO site had the lowest protein concentrations and those from the OY site had the highest protein concentrations regardless of the pruning regime. Similar protein contents between juices from 2-cane and 4-cane vines at the same site were noted, but wines from 4-cane vines had significantly higher protein content than those from 2-cane vines. In 2008, juice from 2-cane vines had significantly higher protein contents than 4-cane vines, but similar protein contents between wines from the same site were observed irrespective of the pruning regime. Generally, among two consecutive vintages, the BO site produced consistent low protein concentrations in juices and resultant wines, whereas the OY site showed the greatest variation between vintages (CV > 20%). Significant differences in juice and wine protein concentration among sites but not between vintages ($p > 0.09$) were evident. No clear effect of pruning regime (2-cane and 4-cane) on juice and resultant wine protein concentrations could be identified.

Wine protein haze and bentonite requirement

Protein haze levels of un-fined wines and bentonite dosages required for protein stabilisation are also presented in Table 3. Wines from the BO site showed the lowest protein haze formation and bentonite requirement irrespective of pruning treatments and vintages. Wines from the VI site had the highest protein haze levels for two vintages, while wines from the SE site in 2007 and the SQ site in 2008 required the highest bentonite dosages. Additionally, wines from 2-cane and 4-cane vines at the same sites in 2007 showed only small differences in wine protein haze formation and bentonite requirement, whereas higher protein haze and bentonite dosage were noticed for wines from 2-cane vines compared to 4-cane vines in 2008. In general, significant differences in wine protein haze formation between sites and between vintages (p = 0.010) were evident, whereas bentonite requirement differed significantly only between sites but not between vintages (p = 0.753). The influence of pruning regime on haze formation and bentonite requirement varied in magnitude between vintages.

Wine protein analysis by LDS-PAGE

Wine protein concentration and MW composition examined by LDS-PAGE are detailed in Table 4. Total protein content estimated using the intensity of protein bands ranged between 60.1 and 96.6 mg L^{-1}. Wines made from the BO site had the lowest protein concentrations regardless of pruning regime and vintage. The highest wine protein concentrations were from OY, SE and VI4 treatments in 2007 and SQ and VI2 treatments in 2008. Small differences in protein concentration between wines from the same site were observed (CV < 5%) except for wines from the VI site in 2007 and OY site in 2008 (CV > 10%). Significant differences in wine protein concentration between sites were determined, whereas only small differences were noted between the two pruning regimes and between vintages (p = 0.019).

The electrophoretic patterns of wine proteins for all samples were similar with differences in relative intensity (Table 4). Commonly, five bands were observed with MW at 10, 21, 23, 33 and 64 kDa. A major peak at 21 kDa, in all cases, was accompanied with a distinct shoulder at 23 kDa in densitometric scans of LDS-PAGE. These two fractions (21 and 23 kDa) contributed 50-70% (average 62%) of total wine protein concentrations. Proteins of 10, 33 and 64 kDa fractions averaged at approximately 10, 8 and 20% of total protein contents, respectively. Furthermore, a similar percentage of 21 kDa fraction between wines from the same site over two vintages was observed except for wines from the BO site and for wines from the OY site in 2008. Significantly lower percentages of 23 kDa fraction in wines from 4-cane vines than 2-cane vines were determined only at the VI site in 2007 and BO and OY sites in 2008. In general, small differences in MW composition between wines from the same

site and vintages ($p \leq 0.046$, except the 21 kDa fraction with $p = 0.129$) were observed, whereas significant differences between wines among sites were determined.

Juice and wine protein analyses by SDS-CGE

Total protein contents in wines from 2007 and in juices and wines from 2008 are presented in Table 5 and 6, respectively. A general decrease in total protein content after fermentation was noticed. Protein concentration in wine was between 54.7 and 88.7% of that in juice except for sample SQ4 which was slightly higher than 100%. Juices and wines from the BO site contained, again, the lowest protein concentration irrespective of pruning regimes and vintages. Wines from the same sites showed small differences in protein contents (CV < 9%) except for that from the SQ site in 2008 (CV = 17%). The highest wine protein concentrations were from the OY and SE sites in 2007 and SQ and VI sites in 2008. Generally, significant differences in wine protein contents between sites and between pruning regimes were evident, while differences were significant only between sites for juice protein. No clear effect on wine protein content among vintages was observed ($p = 0.514$).

The protein MW profiles between wines in 2007 (Table 5 and Figure 1) and between juices and resultant wines in 2008 (Table 6 and Figure 2) showed a high degree of similarity with small differences in relative percentages regardless of sites, pruning regimes, and vintages. Small differences in percentage for each protein fraction among juice samples were observed ($p \geq 0.005$) with the 22 and 28 kDa fractions being predominant, contributing more than 88% of the total proteins. Other common peaks were at 7.4, 9.6 and 32 kDa. A faint peak at 37 kDa and a small peak at 54 kDa in some cases were also noticed. Approximately equal percentage of the 22 and 28 kDa fractions in juice proteins was observed from the BO and OY sites, whereas 10% higher for 28 kDa fraction than 22 kDa fraction was noticed in that from the SE, SQ and VI sites. Moreover, the wine protein MW profile was similar to juice proteins with peaks at 9.6, 19, 22, 26 and 32 kDa for all wines regardless of treatments and vintages. The 26 kDa fraction in wine, representing heterogeneous peaks between 24 and 28 kDa, corresponds to the 28 kDa fraction in juice (Figure 2). Unlike in juice, the 22 kDa fraction was the predominant protein in wine with more than half (56% on average) the total protein concentration. In combination the 22 and 26 kDa fractions accounted for over 90% of total wine protein concentration. In general, the site and pruning regimes significantly influenced the main wine proteins (22 and 26 kDa), whereas only the site showed significant effect on main juice proteins (22 and 28 kDa). Significant differences in MW composition of wine proteins among vintages were obvious ($p < 0.05$) except for the 19 kDa fraction ($p = 0.514$).

In addition, there were distinguishable differences in the 26 kDa fraction for wines between

treatments. In 2007, the profile of the 26 kDa fraction was identical between wines from the same site (2-cane and 4-cane vines) but varied between wines from different sites (Figure 1). In 2008, marked decrease in the 26 kDa fraction in wines from the 4-cane vine compared to 2-cane vines was observed at the BO and OY sites but not others (Figure 2). Wines from the SQ site also showed marked degradation of the 26 kDa fraction in 2007 compared 2008 (Figures 1 and 2).

Effects of vine size

Smaller trunk circumference vines (SQ4S) resulted in wines with higher alcohol content than those from the larger trunk circumference (SQ4L) (Table 2) as a result of higher grape maturity from the SQ4S (20.3 °Brix) compared to SQ4L (18.2 °Brix) harvested on the same day. No significant effect of trunk circumference on juice and resultant wine protein concentrations was found by using the CBB assay for both ($p > 0.210$) and LDS-PAGE for wine ($p = 0.084$), whereas significant differences in wine protein haze formation ($p = 0.001$) and bentonite requirement ($p < 0.001$) were evident. Wines from the SQ4S showed higher protein haze formation (71 NTU) but required less bentonite to achieve stability (0.67 g L^{-1}) compared to the SQ4L (50 NTU and 0.78 g L^{-1}). The electrophoretic pattern of 2007 wine proteins from vines at the SQ site with different trunk circumference is illustrated in Figure 3. Similar protein MW but significant differences in relative percentages were observed ($p \leq 0.05$, except for the 10 kDa fraction), especially for the 23 kDa fraction ($p = 0.001$).

Correlations between bentonite requirement and protein contents

The relationships of bentonite requirements with wine protein haze levels (hot/cold test) and total juice/wine protein concentrations (CBB assay, LDS-PAGE and SDS-CEG) all showed significant effects ($p < 0.03$) with positive correlations. However, their linear correlation coefficients were quite low ($r^2 < 0.50$). Among them, juice protein concentration gave the best linear correlation ($p < 0.001$; $r^2 = 0.50$) and protein haze gave the poorest linear correlation ($p = 0.022$; $r^2 = 0.25$) with bentonite requirement. Furthermore, individual proteins separated by LDS-PAGE and SDS-CEG were investigated. Three protein MW fractions at 23 kDa in LDS-PAGE and 26 and 32 kDa in SDS-CEG showed a significant effect on bentonite requirement ($p \leq 0.004$). However, the 26 and 32 kDa fractions showed the best linear correlations ($r^2 = 0.78$) with bentonite requirement.

Differences in bentonite requirement for wines appeared to correspond to changes in the 23 kDa band in LDS-PAGE and 26 kDa peak in SDS-CGE. For instance, the significant decreases in bentonite requirement for wines from 4-cane vines compared to 2-cane vines at the BO and OY sites in 2008 coincided with apparent decreases in the 23 kDa fraction in

LDS-PAGE (Figure 4) and the 26 kDa fraction in SDS-CGE (Figure 2). The marked increase in 26 kDa peaks in SDS-CGE from the SQ site in 2008 compared to 2007 also reflected on increase in bentonite requirement. Furthermore, the significantly higher intensity of the 23 kDa band in LDS-PAGE for wines from the SQ4L in the vine size trial coincided with higher bentonite rates required.

Discussion

Protein concentration in Sauvignon blanc wines from the Marlborough region determined by CBB assay and LDS-PAGE are consistent with other reported results (Ferreira et al. 2002, Waters et al. 2005). The considerably larger values for wine protein content estimated by SDS-CGE have also been reported in the literature by different methods (Dorrestein et al. 1995, Santoro 1995, Vincenzi et al. 2005a). Discrepancies between these results are attributed to various mechanisms as discussed in a previous study (Hung et al. 2010). Reduction in protein concentration after fermentation for many samples regardless of analytical methods (CBB assay and SDS-CGE) is generally accepted and ascribed to proteolysis, yeast cell adsorption and alcohol precipitation (Luguera et al. 1997, Canals et al. 1998, Manteau et al. 2003). Nevertheless, some wines with more protein than the corresponding juices were noticed. Release of yeast proteins and partial degradation of grape proteins to wine during fermentation have been corroborated (Waters et al. 1998, Dambrouck et al. 2003, Okuda et al. 2006) and may contribute to the total protein concentration. Additionally, lower protein recovery rate by ultrafiltration used for juice proteins compared to the acetone precipitation method as used for wine proteins for SDS-CGE analysis as ascribed previously (Hung et al. 2010) may also be important.

Identification of two major fractions between 20 and 30 kDa in electropherograms for juice and wine proteins are in great agreement with other studies using various analytical methods on Sauvignon blanc proteins (Peng et al. 1997, Kwon 2004, Esteruelas et al. 2009a) and on protein comparison among varieties (Hsu and Heatherbell 1987a, Weiss et al. 1998, Ferreira et al. 2000, Hayasaka et al. 2001). Studies suggest that these proteins are ubiquitous PR proteins found in grape and wine, presumably thaumatin-like proteins and chitinases (Peng et al. 1997, Pocock et al. 2000). Peaks at 22 and 26 kDa in SDS-CGE corresponds to bands at 21 and 23 kDa in LDS-PAGE, respectively, as both mechanisms of protein separations were identical and based on MW size (Rodríguez-Delgado et al. 2002). Proteins with similar MW to the 33 kDa band in LDS-PAGE and 32 kDa peak in SDS-CGE, which have rarely been reported and characterised in wine protein studies (Hsu and Heatherbell 1987a, Hayasaka et al. 2001, Kwon 2004), have recently been identified as β-(1-3)-glucanase and involved in

Sauvignon blanc wine protein haze formation (Esteruelas et al. 2009a, bb). The absence of this fraction in many studies may be a consequence of it being variety specific and/or its low concentration and vulnerable characteristics to bentonite and thermal treatments (Hsu and Heatherbell 1987b, Sauvage et al. 2010). The loss of this fraction after ultrafiltration (10 kDa MW cut-off) was observed in our previous studies (Hung et al. 2010). Furthermore, the 64 kDa fraction identified by LDS-PAGE (about 20 %) but not detected by SDS-CGE was elucidated in the study by Marangon et al (2009) who found that Coomassie staining has higher affinity to the invertase (65 kDa) than ultraviolet absorption (210 nm) resulting in its overestimation. Fractions at around 10 kDa in LDS-PAGE and SDS-CGE and 64 kDa in LDS-PAGE were with similar MW to proteins confirmed as lipid transfer protein and vacuolar invertase, respectively, and are relatively stable to heat and bentonite fining (Okuda et al. 2006, Sauvage et al. 2010).

The high similarity of Sauvignon blanc proteins in MW profiles (SDS-CGE) between juices and between wines but slightly different between juice and resultant wine is in agreement with a varietal study by Pueyo et al. (1993). The reduction of 28 kDa peak in juice after fermentation may be a result of proteolysis as reported by Waters et al. (1998) and Manteau et al. (2003) where low MW fragments were detected and linked to chitinases. The peak which appeared at 19 kDa in SDS-CGE for wines but not for juices also implied protein hydrolysis during vinification as observed by Okuda et al. (2006). Consequently, the reduction and heterogeneity of the fraction at 26 kDa in wine were observed. Various isoforms of chitinases in different varieties have also been observed (Pocock et al. 2000). Chitinases have been shown to be involved in berry development and the defense system against abiotic and biotic stresses (Robinson et al. 1997, Derckel et al. 1998). Noticeable variations of the 26 kDa fraction in electropherograms between studied wines, even from the same site and vintage, may reflect the environmental conditions as environmental factors were crucial in determining the protein profile reported by others (Sarmento et al. 2001, Monteiro et al. 2003). However, specific growing conditions affecting proteins in grape are still not fully elucidated, although during the harvest season (March and April in Marlborough), there was more precipitation in 2008 (164 mm) compared to 2007 (61 mm) (http://www.wineresearch.org.nz/weatherdata.htm retrieved on 3rd March, 2010). As the 4-cane vines are generally harvested several days after 2-cane vines, the effect of pruning regime on protein concentration/composition, haze formation and bentonite requirement was more significant in 2008 than 2007. However, as the wine matrix, apart from proteins, also affects the haze formation behavior (Mesquita et al. 2001), effects of other wine composition changes can not be ruled out.

The range in bentonite rates required to stabilise wines is comparable to other published data (Pocock and Waters 2006). As can be expected, the total juice/wine protein concentration and haze level significantly correlated with bentonite requirement. However, the 26 and 32 kDa fractions by SDS-CGE showed the greatest significance ($p < 0.001$) and linear correlations ($r^2 = 0.78$) with bentonite requirement. The 32 kDa fraction is unlikely to be the factor determining the bentonite requirement as it was low in concentration and removed at the early stage of bentonite fining before stability was achieved (Hsu and Heatherbell 1987b, Sauvage et al. 2010). The 26 kDa fraction, presumably the chitinase and one of the most heat unstable proteins reported (Waters et al. 1991, 1992, Waters et al. 1996), may play an important role in bentonite rate determination. Although, it has been shown that the thaumatin-like proteins alone produce half more haze than the chitinases alone in model and protein-free wines (Waters et al. 1992, Pocock et al. 2007), the specific removal of chitinases by chitin polymers resulted in a more marked decrease in protein haze formation than the non-specific removal of wine proteins by bentonite (Vincenzi et al. 2005b). Sauvage et al. (2010) also noted that progressive removal of chitinases in wine until completion was achieved at 0.6 g L^{-1} bentonite (80% reduction in total protein content) close to stability, whereas 30% of thaumatin-like proteins still remained in stabilised wine even at 1.5 g L^{-1} bentonite addition. In addition, various PR proteins remaining in stabilised wine by bentonite fining were detected and characterised except for the chitinases (Okuda et al. 2006). These recent findings explain results from the present work that, in a real wine system, a good correlation between 26 kDa protein content and bentonite requirement was obtained. The poor correlations of bentonite requirement with total wine protein concentration and haze level may be the result of complexity of haze formation, involving non-proteinaceous components and chemicals in wine (Waters et al. 1995, Pocock et al. 2007, Batista et al. 2009, Esteruelas et al. 2009a).

Conclusions

The protein concentration in juice and wine as determined by the CBB assay and LDS-PAGE were in the typical ranges, whereas quantification by SDS-CGE resulted in much higher protein concentration. A general reduction in protein content after fermentation was found with some exceptions. Wines from the BO site were consistently low in juice and wine protein concentration, wine protein haze and bentonite requirement regardless of pruning treatments and vintages, while others varied. The protein MW profile of Sauvignon blanc from Marlborough region, New Zealand is similar to that found in other *Vitis vinifera* varieties worldwide. Similarity of protein MW profiles for wines from five vineyards over two consecutive vintages was noticed with minor variations in the 26 kDa fraction. Neither

the protein haze nor the total protein concentration as quantified by CBB assay, LDS-PAGE and SDS-CGE correlated well with bentonite dosage required for protein stabilisation. Good correlation of bentonite requirement with the 26 kDa fraction was observed, but not with the predominant 22 kDa fraction. This implies that the depletion of 26 kDa fraction in wine is a good indicator for protein stability by bentonite fining.

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Table 1. Details of Sauvignon blanc sample sources used in this study, consisting of five subregional vineyards within Marlborough region, New Zealand.

Notation	Vineyard	Subregion	Contributor
BO	Brancott Estate, Booker Vineyard	Brancott Valley	Pernod Ricard NZ Ltd
OY	Oyster Bay Vineyard	Upper Wairau Plains	Delegat's Wine Estate Ltd
SE	Awatere Estate, Seaview Vineyard	Awatere Valley	Pernod Ricard NZ Ltd
SQ	Stoneleigh Vineyard, Squire Estate	Central Rapaura	Pernod Ricard NZ Ltd
VI	Villa Maria Winery Block	Fairhall	Villa Maria Estate Ltd

Table 2. Analyses of Sauvignon blanc wines from 5 vineyards within Marlborough region, New Zealand in two vintages.

Treatment[a]	2007 vintage				2008 vintage			
	Harvest Date	Alcohol (% v/v)	pH	TA (g/L)	Harvest Date	Alcohol (% v/v)	pH	TA (g/L)
BO2	27 Mar	13.47	3.11	8.1	20 Mar	12.90	3.01	9.0
BO4	08 Apr	12.93	3.16	7.2	04 Apr	13.40	3.04	7.8
OY2	10 Apr	13.63	3.22	7.9	19 Mar	12.53	3.00	9.7
OY4	10 Apr	13.47	3.16	8.1	04 Apr	13.10	3.06	7.9
SE2	26 Mar	13.40	3.07	9.1	29 Mar	13.30	3.10	8.5
SE4	30 Mar	12.80	3.15	8.2	09 Apr	13.17	3.07	8.1
SQ2	26 Mar	13.07	3.07	9.0	22 Mar	12.40	3.07	9.7
SQ4	28 Mar	12.87	3.09	8.5	31 Mar	12.30	3.14	8.2
VI2	03 Apr	13.03	3.13	7.6	03 Apr	13.10	3.12	7.4
VI4	19 Apr	12.77	3.18	7.1		n.d.		
SQ4S	28 Mar	13.17	3.15	7.8		n.d.		
SQ4L	28 Mar	11.53	3.01	9.7		n.d.		

[a] Notation indicates vineyard (see Table 1), 2-cane or 4-cane and where applicable, extra small (S) or extra large (L) trunck circumference.

TA: titratable acidity expressed as g/L tartaric acid.

n.d. = not determined.

Table 3. Sauvignon blanc protein concentration and heat stability from 5 vineyards with two pruning regimes in two vintages[a].

Treatments		2007 vintage				2008 vintage			
		Protein (mg/L)[b]		Haze[c]	Bentonite[d]	Protein (mg/L)[b]		Haze[c]	Bentonite[d]
Site	Cane	Juice	Wine	(NTU)	req. (g/L)	Juice	Wine	(NTU)	req. (g/L)
BO	2	113.9_{ab}	96.5_{a}	56.4_{a}	0.56_{a}	106.1_{bc}	78.7_{a}	38.5_{a}	0.59_{b}
	4	108.8_{a}	111.5_{bc}	68.4_{ab}	0.55_{a}	76.7_{a}	81.6_{ab}	35.9_{a}	0.51_{a}
OY	2	161.5_{c}	136.4_{de}	90.6_{de}	0.80_{cd}	120.1_{de}	95.4_{c}	61.0_{b}	0.71_{c}
	4	163.5_{c}	142.7_{e}	87.6_{cde}	0.83_{cd}	99.1_{b}	92.2_{bc}	31.7_{a}	0.59_{b}
SE	2	126.7_{abc}	119.7_{c}	71.4_{abc}	0.88_{d}	129.0_{ef}	112.7_{d}	72.5_{b}	0.86_{d}
	4	149.9_{bc}	132.2_{d}	83.0_{bcde}	0.87_{d}	112.1_{cd}	114.6_{d}	41.2_{a}	0.68_{c}
SQ	2	135.5_{abc}	105.3_{b}	76.6_{bcd}	0.82_{cd}	142.1_{g}	113.7_{d}	65.7_{b}	1.06_{e}
	4	142.5_{abc}	114.5_{c}	78.6_{bcd}	0.71_{b}	138.0_{fg}	134.0_{e}	67.1_{b}	1.08_{e}
VI	2	120.3_{ab}	115.5_{c}	86.5_{cde}	0.75_{bc}	142.1_{g}	129.5_{e}	88.6_{c}	0.90_{d}
	4	117.7_{ab}	136.9_{de}	98.7_{e}	0.76_{bc}	n.d.			
Significance	Site	***	***	***	***	***	***	***	***
	Cane	n.s.	***	**	n.s.	***	**	***	***
	Site×Cane	n.s.	**	n.s.	**	**	***	***	***

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). Two-way analysis excluded VI for 2008. n.s. = not significant (> 0.05); ** = significant at ≤ 0.01 level; *** = significant at ≤ 0.001 level.

[b] Analysis by a modified Coomassie Brilliant Blue (CBB) assay and expressed in mg/L BSA equivalents.

[c] Means of nephelometric haze measures on un-fined triplicate wines.

[d] Means of bentonite dosages required for stabilising triplicate wines to a hot/cold test.

n.d. = not determined.

Table 4. LDS-PAGE analysis of wines from 5 vineyards with two pruning regimes in two vintages[a].

Treatments		2007 vintage[c]						2008 vintage[c]					
		Protein	Composition (%) of protein fractions (kDa)					Protein	Composition (%) of protein fractions				
Site	Cane	(mg/L)	10	21	23	33	64	(mg/L)	10	21	23	33	64
BO	2	75.2_{a}	11.5_{bc}	37.5_{a}	23.3_{bcd}	8.2_{bcd}	19.4_{c}	63.8_{a}	8.2_{ab}	35.5_{bc}	20.4_{cd}	9.2_{bcd}	26.7_{cd}
	4	75.7_{a}	11.6_{bc}	42.8_{b}	23.3_{bc}	6.1_{ab}	16.2_{ab}	60.1_{a}	9.2_{b}	40.7_{d}	15.9_{b}	10.5_{cd}	23.7_{bc}
OY	2	96.6_{c}	11.0_{abc}	36.9_{a}	19.3_{a}	9.7_{d}	23.1_{d}	75.9_{c}	7.4_{ab}	28.0_{a}	21.1_{cde}	9.0_{bc}	34.5_{e}
	4	91.6_{bc}	12.2_{c}	38.3_{a}	20.9_{ab}	8.5_{d}	20.0_{c}	66.1_{ab}	7.1_{a}	41.4_{d}	12.6_{a}	9.9_{bcd}	29.0_{d}
SE	2	92.0_{bc}	9.8_{ab}	35.4_{a}	27.8_{ef}	7.5_{bcd}	19.5_{c}	72.9_{bc}	9.4_{b}	32.5_{b}	22.4_{cde}	8.6_{b}	27.1_{cd}
	4	94.3_{bc}	10.0_{ab}	36.5_{a}	26.1_{de}	8.4_{cd}	18.9_{bc}	71.9_{bc}	8.3_{ab}	35.0_{bc}	20.2_{c}	9.6_{bcd}	26.8_{cd}
SQ	2	78.9_{a}	11.2_{abc}	47.5_{c}	23.1_{bc}	4.5_{a}	13.7_{a}	79.0_{cd}	9.0_{ab}	34.9_{bc}	25.0_{f}	10.5_{cd}	20.7_{b}
	4	84.5_{ab}	9.1_{a}	49.9_{c}	21.0_{ab}	6.0_{ab}	14.0_{a}	83.9_{de}	8.3_{ab}	37.1_{c}	23.1_{def}	10.9_{d}	20.5_{b}
VI	2	80.6_{a}	10.6_{abc}	38.2_{a}	29.6_{f}	6.3_{abc}	15.3_{a}	89.8_{e}	12.5_{c}	44.5_{e}	23.8_{ef}	5.6_{a}	13.6_{a}
	4	93.6_{bc}	9.7_{ab}	38.3_{a}	25.8_{cde}	7.6_{bcd}	18.5_{bc}	n.d.					
Significance	Site	***	**	***	***	***	***	***	**	***	***	**	***
	Cane	*	n.s.	***	**	n.s.	n.s.	*	n.s.	***	***	**	**
	Site×Cane	**	*	**	**	**	***	***	n.s.	***	***	n.s.	*

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). Two-way analysis excluded VI for 2008. n.s. = not significant (> 0.05); * = significant at ≤ 0.05 level; ** = significant at ≤ 0.01 level; *** = significant at ≤ 0.001 level.

[b] Means of one electrophoresis on two of triplicate wines by lithium dodecyl sulphate polyacrylamide gel electrophoresis (LDS-PAGE) and proteins were expressed in mg/L BSA equivalents for total protein content and in percentage among protein fractions.

n.d. = not determined.

Table 5. SDS-CGE analysis on wines from 5 vineyards in 2007.

Treatments		Wine[a]					
		Protein	Composition (%) of protein fractions (kDa)				
Site	Cane	(mg/L)	<19	19	22	26	32
BO	4	400.1	2.8	1.9	61.8	32.4	1.0
OY	4	825.0	3.3	2.1	64.2	28.5	1.9
SE	2	576.9	3.2	2.7	55.0	35.7	3.5
	4	543.6	3.3	2.4	56.7	34.7	2.9
SQ	2	458.2	2.3	3.2	57.9	33.7	2.9
	4	425.5	2.5	4.2	59.6	32.6	1.1
VI	2	493.9	3.1	3.0	53.4	37.3	3.2
	4	457.9	2.5	2.3	59.0	34.2	2.1

[a] One analysis on one of triplicate wines by sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE) and proteins were expressed in mg/L BSA equivalents for total protein content and in percentage among protein fractions.

Table 6. SDS-CGE analysis on juices and resultant wines from 5 vineyards with two pruning regimes in 2008[a].

Treatment[b]		Juice[b]						Wine[b]					
		Protein	Composition (%) of protein fractions (kDa)					Protein	Composition (%) of protein fractions (kDa)				
Site	Cane	(mg/L)	<19	22	28	32	>32	(mg/L)	<19	19	22	26	32
BO	2	408.9_{a}	2.7_{a}	47.4_{b}	44.5_{ab}	3.5_{a}	1.9_{a}	312.9_{a}	1.8_{a}	3.5_{a}	56.3_{bc}	35.8_{abc}	2.6_{abc}
	4	430.4_{a}	2.1_{a}	47.6_{b}	43.9_{a}	4.2_{a}	2.2_{a}	323.5_{ab}	1.8_{a}	2.7_{a}	59.3_{c}	34.3_{ab}	1.9_{a}
OY	2	532.1_{bc}	2.3_{a}	44.3_{ab}	47.2_{abc}	4.5_{ab}	1.7_{a}	361.8_{ab}	1.9_{a}	1.9_{a}	53.6_{abc}	39.0_{bc}	3.6_{cde}
	4	475.2_{ab}	2.0_{a}	45.1_{ab}	46.9_{abc}	4.6_{ab}	1.4_{a}	409.4_{b}	2.9_{a}	3.1_{a}	58.4_{c}	32.6_{a}	2.4_{ab}
SE	2	708.0_{ef}	2.5_{a}	37.0_{a}	50.5_{abc}	8.0_{b}	2.1_{a}	387.0_{ab}	2.3_{a}	3.2_{a}	50.9_{ab}	40.4_{c}	3.2_{bcd}
	4	614.3_{cde}	2.4_{a}	39.4_{ab}	52.4_{c}	4.4_{a}	1.3_{a}	400.8_{ab}	1.9_{a}	2.7_{a}	53.8_{abc}	37.8_{bc}	3.7_{cde}
SQ	2	610.4_{cd}	2.3_{a}	40.0_{ab}	50.2_{abc}	5.8_{ab}	1.8_{a}	541.6_{c}	1.9_{a}	2.5_{a}	50.0_{a}	40.7_{c}	4.4_{e}
	4	656.5_{def}	2.3_{a}	37.3_{ab}	52.4_{c}	6.0_{ab}	2.0_{a}	688.8_{d}	2.2_{a}	2.4_{a}	50.7_{ab}	39.8_{c}	4.5_{e}
VI	2	715.5_{f}	1.9_{a}	39.3_{ab}	51.0_{bc}	5.4_{ab}	2.4_{a}	566.4_{c}	2.3_{a}	1.7_{a}	54.1_{abc}	37.4_{abc}	3.9_{de}
	4	n.d.						n.d.					
Significance	Site	***	n.s.	**	***	*	n.s.	***	n.s.	n.s.	***	**	***
	Cane	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	***	n.s.	n.s.	**	**	n.s.
	Site×Cane	**	n.s.	n.s.	n.s.	*	n.s.	**	n.s.	n.s.	n.s.	n.s.	*

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). Two-way analysis excluded VI site. n.s. = not significant (> 0.05); * = significant at ≤ 0.05 level; ** = significant at ≤ 0.01 level; *** = significant at ≤ 0.001 level.

[b] Means of duplicate analyses by sodium dodecyl sulphate capillary gel electrophoresis (SDS-CGE) and proteins were expressed in mg/L BSA equivalents for total protein content and in percentage among protein fractions.

n.d. = not determined.

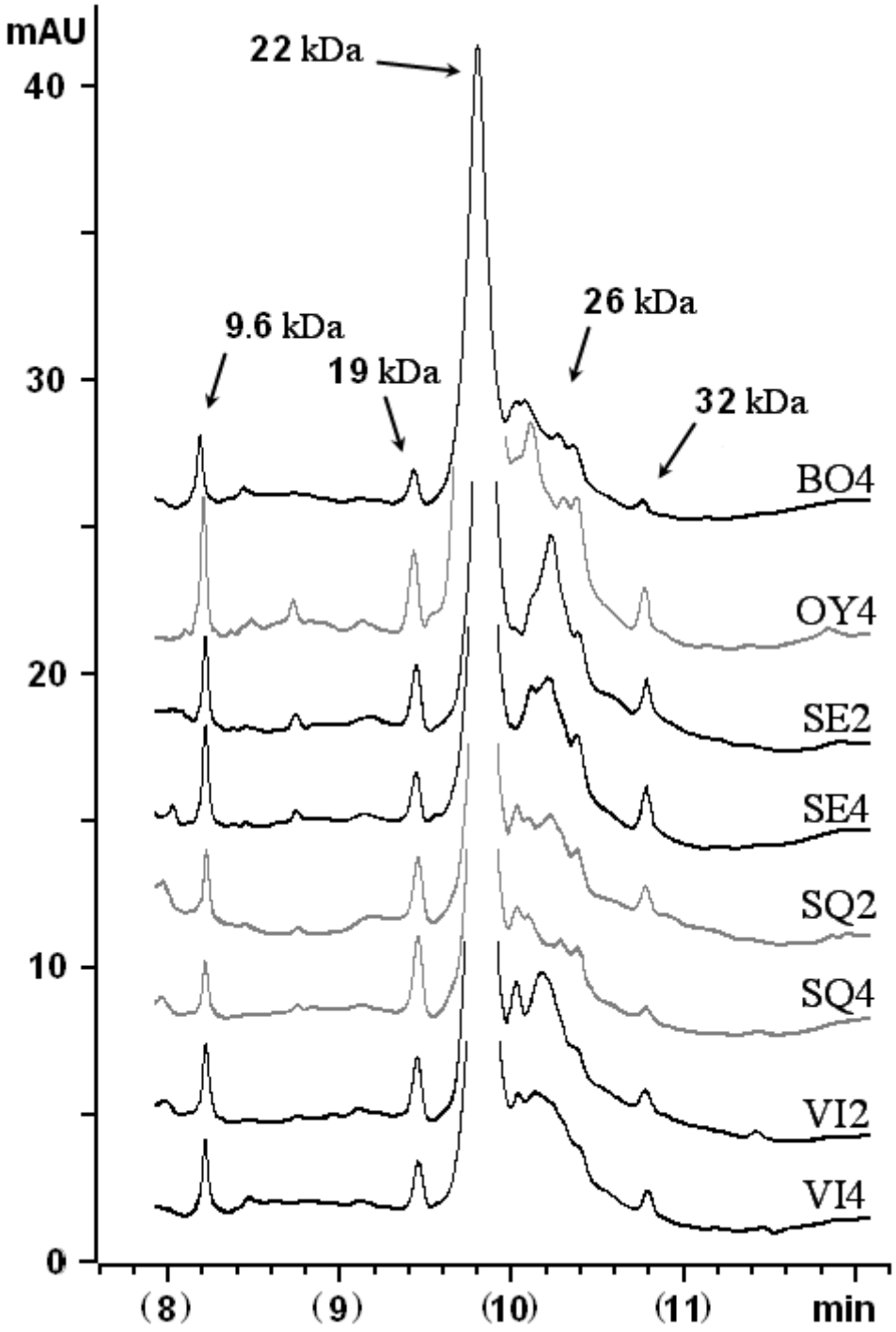

Figure 1. Electropherograms by SDS-CGE for Sauvignon blanc wines from five vineyards within Marlborough region in 2007. Vineyard abbreviations with 2 and 4 behind denoting 2-cane and 4-cane pruning treatments were present on corresponding traces. Top of identical peaks at 22 kDa were ignored for conciseness except the first trace.

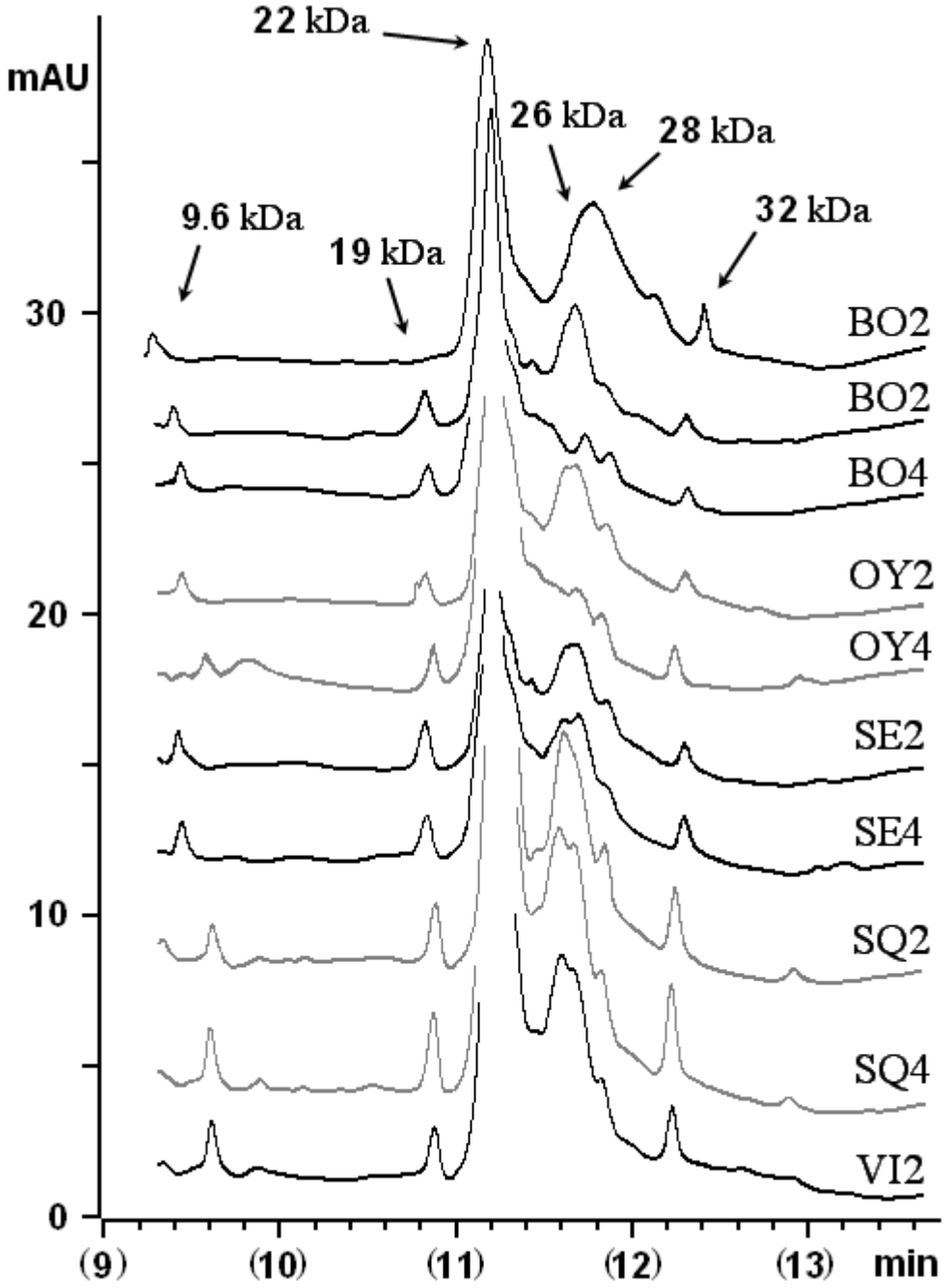

Figure 2. Electropherograms by SDS-CGE for a Sauvignon blanc juice (top trace) and wines from five vineyards within Marlborough region in 2008. Vineyard abbreviations with 2 and 4 behind denoting 2-cane and 4-cane pruning treatments were present on corresponding traces. Top of identical peaks at 22 kDa were ignored for conciseness except the first two traces.

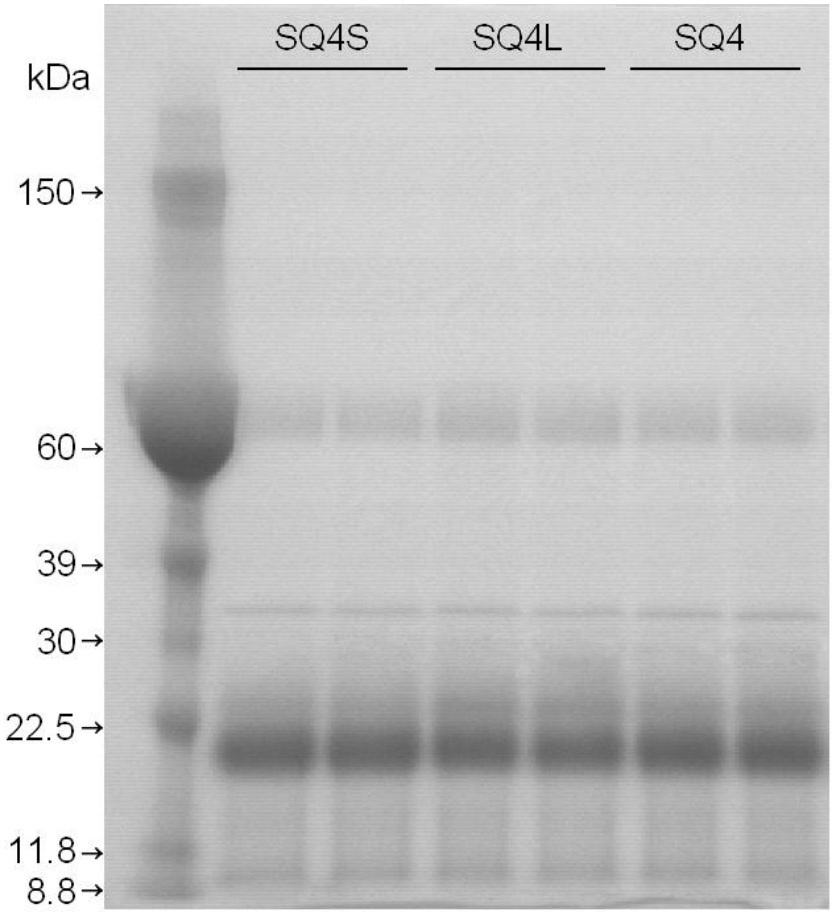

Figure 3. Comparison of electrophoretic profiles by LDS-PAGE between Sauvignon blanc wines from one vineyard (SQ) in Marlborough region in 2007. SQ4S and SQ4L denoting the 4-cane vines with trunk circumferences of extra-small (S) and extra-large size (L), respectively, with comparison to medium size vines (SQ4) were present on corresponding lanes with duplicate. Molecular weight standards were located on the left side of the gel with arrow indications.

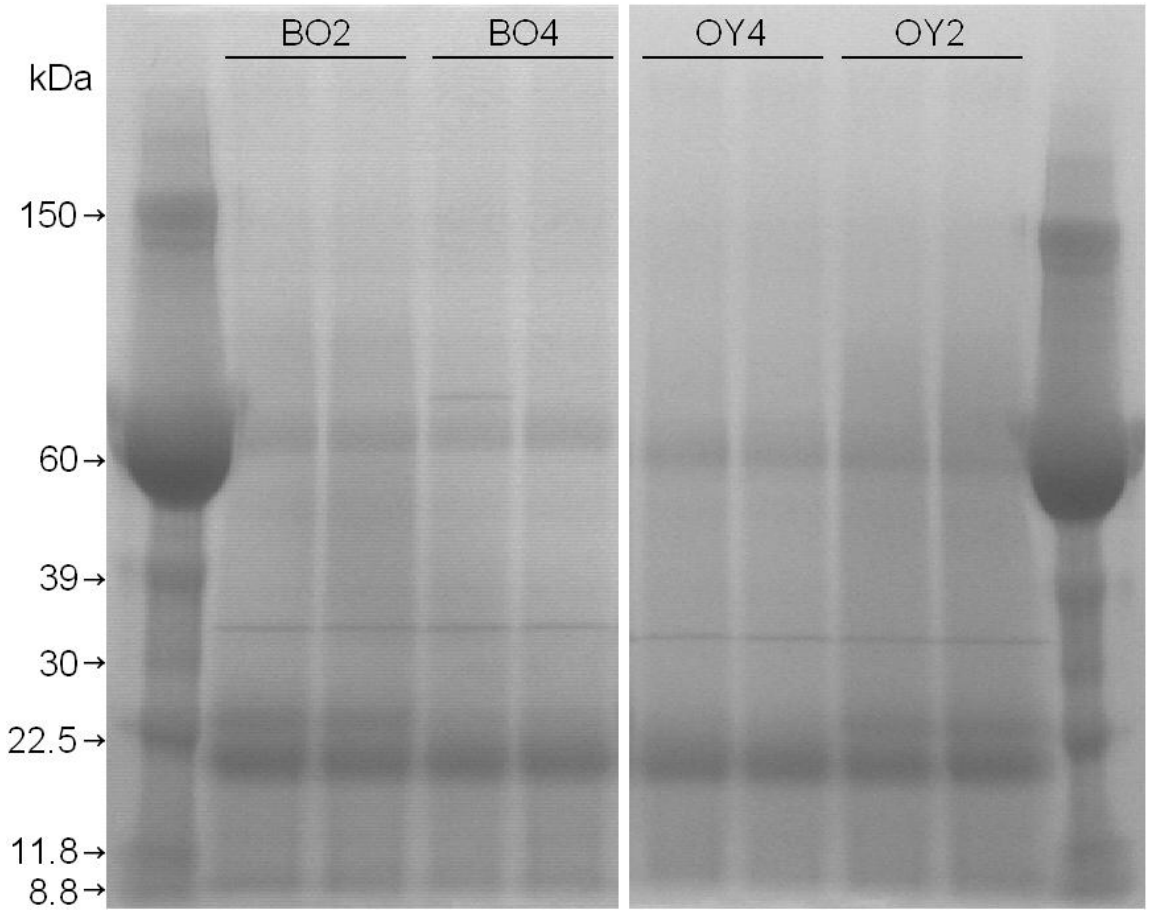

Figure 4. Comparison of electrophoretic profiles by LDS-PAGE between Sauvignon blanc wine proteins from two vineyards within Marlborough region in 2008. Vineyard abbreviations with 2 and 4 behind denoting 2-cane and 4-cane pruning treatments were present on corresponding lanes with duplicate. Molecular weight standards were located on the left side of the gel with arrow indications.

Chapter 6

(*A preparation for submission for* American Journal of Enology and Viticulture)

Effects of Juice and Wine pH Adjustments and Bentonite Addition Timing on Wine Protein Stabilization

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Short running title: **pH Effects on Wine Protein Stability**

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Abstract

Influences of juice and wine pH adjustments on Sauvignon blanc wine protein haze formation and bentonite requirement were studied by using identical juice lots. Three bentonite addition timings were also superimposed on the juice pH adjustment trial. Bentonite fining coupled with a hot/cold test (80°C for 6 hrs followed by 4°C overnight) were utilized to determine the dosage required for stability. Wine proteins were analyzed using a modified Coomassie Brilliant Blue (CBB) assay, lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE). Results indicated that lower juice pH (2.80 and 3.00) resulted in sluggish fermentation, whereas the presence or absence of bentonite during fermentation showed similar fermentation kinetics for each pH. The presence of bentonite remaining in contact with ferment improved the completion of fermentation for the sluggish ferment (pH 2.80). Lower pH wines require lower bentonite dosages for stability because of two factors: reduced wine pH improves protein adsorption efficiency by bentonite fining; decreased juice pH results in lowering wine protein content and increasing protein adsorption efficiency. Bentonite addition during fermentation was the most efficient in protein removal but fining after fermentation required the least dosage. Different fermentation scales slightly affected wine protein contents but not molecular weight (MW) profiles. Protein contents and MW profiles in stabilized wines were affected by the original juice pH with more complex patterns from high pH juice.

Key words: bentonite, Coomassie, electrophoresis, fermentation, pH, wine protein

Introduction

Protein instability as a result of excessive residual proteins remaining in white wine is a technical concern to the wine industry worldwide because of the potential for producing suspended solids in bottled wines, a fault known as haze (Ferreira et al. 2002, Waters et al. 2005). Batch-wise fining of bentonite is conventionally performed in the winery before bottling to prevent this problem. Nevertheless, quantitative and qualitative loss of wine to bentonite fining is also of concern (Høj et al. 2000).

The bentonite dosage required to achieve wine protein stability is normally determined in the winery laboratory by conducting a bentonite fining trial coupled with a procedure, commonly a thermal treatment, to force protein haze formation (Toland et al. 1996, Pocock and Waters 2006). Protein removal by bentonite adsorption occurs through a cation exchange mechanism and is hence affected by wine pH as this determines the extent of protein net charge depending on isoelectric point (pI) (Hsu and Heatherbell 1987). The pH effect may also influence other wine cations in competing with proteins for bentonite adsorption in a model wine, resulting in poor protein removal at low pH compared to higher pH (Blade and Boulton 1988). Variation in pH has been shown to influence fermentation kinetics (Kudo et al. 1998), wine protein concentration (Murphey et al. 1989) and protein haze formation (Batista et al. 2009). Addition of bentonite may occur before, during or after fermentation in the winery. Comparisons of timing of bentonite addition to achieve protein stability have been studied in terms of required dosage, lees formation and protein removal efficiency (Somers and Ziemelis 1973, Ewart et al. 1980, Miller et al. 1985, Weiss and Bisson 2002). However, conflicting results have been obtained, leading to different preferences. In addition, most studies have used standard proteins in model solutions (Blade and Boulton 1988, Siebert et al. 1996, Achaerandio et al. 2001) and further work is required on the pH effects in wine with regard to protein stability as a result of bentonite fining.

The predominant proteins found in Sauvignon blanc wines are pathogenesis-related proteins, possessing molecular weights (MW) between 20 and 30 kDa with pI values from 4.1 to 5.8 (Hsu and Heatherbell 1987, Peng et al. 1997, Esteruelas et al. 2009). Small changes in juice and wine pH within ranges of practical interest could be expected to influence Sauvignon blanc wine protein stability in terms of haze formation to heat and bentonite requirement to remove unstable proteins. In this study, both Sauvignon blanc juice and wine pH adjustments were proposed within the range encountered in commercial winemaking to attempt to determine pH effects for each. Three timings of bentonite addition were also superimposed on the juice pH adjustment trial with the aim of establishing optimal practice in protein

stabilization.

Materials and Methods

Juice and wine pH treatments

Sauvignon blanc grapes were machine harvested from a single vineyard block (M19 SBLB) Awatere Valley, Marlborough, New Zealand in late March 2008, and transported to the Brancott Winery (Pernod Ricard (NZ) Ltd.) in Blenheim.

The flow chart of experimental design is outlined in Figure 1. For the juice trial, juice samples were pH adjusted to targets of 2.8, 3.0, 3.24 (original juice) and 3.55, and cooled (5 to 8°C) overnight before racking for microvinification. After pH adjustment juice samples were fermented in 1.5 L wine bottles (1.3 L juice in each bottle) in triplicate with a starter culture (13.5 mL) of yeast suspension (giving 3 x 10^6 cell/mL in juice) of Zymaflore X5 (Laffort Oenologie, France). Three bentonite treatments were applied to each pH level: no bentonite addition before or during fermentation (control); 0.2 g/L bentonite addition to juice (with swirling) 1 hour before yeast inoculation; 0.2 g/L bentonite addition to ferment on the day soluble solids fell below 9 Brix. Fermentation temperature was regulated between 14 and 19°C in a temperature-controlled room, and fermentation progress was monitored by a DMA 35 portable density meter (Anton Parr, Austria) daily in the first three weeks and on occasion thereafter until complete (< 0.5 g/L residual sugar examined by Clinitest (Bayer, USA)). Wines from the pH 2.80 juice without bentonite treatments did not ferment completely (1.0 to 2.0 g/L residual sugar examined by Clinitest (Bayer)). Finished wines were settled overnight (5 to 8°C) and racked from lees with an addition of SO_2 (50 mg/L for wines at the three lower pHs and 100 mg/L for the higher pH wines), followed by centrifugation and bottling. Aliquots of the finished wines were frozen and bottled wines were stored in the cool room before conducting bentonite fining trials.

For the wine trial, a commercial lot (< 20,000 L) from the original juice (pH 3.24) was utilized and inoculated with Zymaflore X5 strain (Laffort Oenologie). Temperature was controlled to between 13 and 17°C, and the soluble solids content of the ferment was monitored daily. It took 46 days to complete alcoholic fermentation. Batches of the commercial wine were pH adjusted to targets of 2.80, 3.00, 3.34 (original wine), 3.65 and 3.85, cooled between 5 to 8°C overnight, vacuum filtered (glass microfiber paper, 7.0 cm, GF/C) with diatomaceous earth and bottled.

All juice and wine pH adjustments were carried out according to a standard method using potassium carbonate and tartaric acid (Iland et al. 2004). All processes mentioned above were

conducted under a covering of CO_2 except those during the fermentation. Juice and resultant wine from commercial-scale winemaking were also used in previous studies (Hung et al. 2010).

Protein recovery and analysis

Methods as reported previously were utilized (Hung et al. 2010). Briefly, total protein concentration was determined using reaction with Coomassie Brilliant Blue (CBB) dye following alkalization of the sample. Protein concentrates for LDS-PAGE and SDS-CGE procedures were prepared following recovery by ultrafiltration (10 kDa MW cut-off, Millipore) for juice samples and by precipitation with ice-cold acetone and centrifugation for wine samples. LDS-PAGE was carried out using commercial polyacrylamide gels (NuPAGE® 4-12% Bis-Tris Gel, 1.0 mm X 10 well) and SDS-CGE utilized a capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) with external pressure in CE+p mode and controlled by ChemStation system (Rev. A.10.02). Gels were run at constant voltage for 55 min at room temperature and destained gels were analyzed using Gel Doc EQ™ (Bio-Rad Laboratories, USA) gel imaging hardware and Quantity One® (Bio-Rad Laboratories, USA) imaging and analysis software. For capillary electrophoresis, samples were injected by applying pressure (50 mbar) for 2 min and constant voltage was applied for electrophoresis with detection at 214 nm. Triplicate CBB analyses were averaged for each sample. Means of one electrophoresis on two of triplicate wines were reported. Proteins in stabilized wines by SDS-CGE were from duplicate analyses on one of three stabilized wines.

Bentonite fining and protein stability test

Procedures for sodium bentonite fining and protein stability were utilized as previously reported (Hung et al. 2010). Bentonite fined wines with differences in turbidity higher than 2 NTU (Model 2100P; Hach, Loveland, CO) before and after the hot/cold test (80°C for 6 hrs followed by 4°C overnight) were considered as protein unstable. The dose rate required to stabilize a wine was defined as the quantity to achieve light scattering characterized by an increase of 2 NTU before and after hot/cold test. Bentonite requirement was determined by linear interpolation between the two closest bentonite fining rates. Measures of duplicate fining for wine pH adjustment and of wines from triplicate microvinification were averaged.

Other analysis

Soluble solids content, pH, alcohol content and titratable acidity were measured by Fourier Transform Infrared Spectroscopy (FTIR) interferometer (WineScan™ FT120; Foss, Denmark). The juice and wine pH after adjustments were also check using a Metrohm 744 pH meter (Herisau, Switzerland).

Statistical analysis

One-way analysis of variance (ANOVA) with Fisher 95% simultaneous confidence intervals was performed using MINITAB 15 statistical software to test significance for each single factor/treatment. Data for juice pH adjustment superimposed with 3 timings of bentonite addition (0.2 g/L) were further analyzed by two-way ANOVA with juice pH and addition timing as factors (Minitab 15).

Results

Fermentation kinetics

Fermentation kinetics for the various pH adjustment and bentonite addition treatments are illustrated in Figure 2. It took 50 to 60 days for pH 2.80 juices to ferment to below 0 Brix, approximately 2 to 4 times longer than the other pH treatments. The pH 3.00 juice reached 0 Brix at around 26 days and 0 Brix were achieved at 16 to 19 days for pH 3.24 and 3.55 juices irrespective of bentonite treatments. No clear effect of bentonite treatment on fermentation kinetics was found except that the pH 2.80 juice without bentonite pretreatment reached 0 Brix 10 days earlier than that with bentonite pretreatment. However, the addition of bentonite to pH 2.80 juices before or during fermentation resulted in the completion of fermentation but the pH 2.80 juice which received no bentonite treatment did not go to completion as determined by Clinitest (Bayer). Generally, juice pH has much greater influence on fermentation kinetics than bentonite treatment. Commercial-scale winemaking took nearly twice as long (36 days) to reduce soluble solids content to below 0 Brix than microvinification (18 days).

Wine composition

Negligible differences were found for each juice pH treatment in alcohol content, pH and TA between resultant wines before and after 0.2 g/L bentonite addition after microvinification (data not shown) ($p > 0.20$). The composition of wines produced by microvinification of juice after pH adjustment and receiving the same amount of bentonite at three addition timings is presented in Table 1. Juices with lower pH resulted in wines with significantly lower ethanol content and pH and higher titratable acidity irrespective of bentonite addition timing. However, no clear effects of bentonite addition timing on resultant wine ethanol content, pH and titratable acidity could be identified. Furthermore, consistent values in pH before and after microvinification were measured for pH 2.80 and 3.00 juices, whereas an increase of about 0.1 units was noted for pH 3.24 and 3.55 juices irrespective of bentonite addition timing.

The composition of wines after wine pH adjustment from commercial-scale winemaking are

presented in Table 2. Slightly lower alcohol content and higher TA in lower pH wines were noticed. An increase of about 0.1 units in pH was also observed after fermentation. In comparison with microvinification of identical juice samples (pH 3.24 with no bentonite treatment), wines produced from commercial-scale winemaking had similar pH values but were 0.7% (v/v) and 0.5 g/L higher in alcohol content and TA, respectively. The higher alcohol content was the result of addition of soluble solids content (about 1.5 Brix) during fermentation.

Protein concentration

No statistical differences were found in juice protein concentration after pH adjustment (p = 0.623). The protein content in wines produced without bentonite addition from various pH juices are presented in Table 3. Juices with lower pH had significantly lower protein concentrations after fermentation regardless of analytical method. However, wines from higher pH juices (pH 3.24 and 3.55) showed similar protein levels. In addition, protein content was lower in wines from microvinification than commercial winemaking by the CBB assay (108.5 mg/L) and SDS-CGE (532.1 mg/L) but similar when measured by LDS-PAGE (77.2 mg/L).

Protein content for wines from four juice pH adjustments in combination with an identical bentonite addition rate (0.2 g/L) at three addition timings is presented in Table 4. Again, juices with lower pH had significantly lower protein concentrations after fermentation regardless of bentonite addition timing, but to a lesser extent for wines with bentonite addition after fermentation measured by LDS-PAGE. Bentonite addition during fermentation significantly reduced wine protein concentration compared to the same addition rate at juice or finished wine at all pH values by the CBB assay but only for lower pH by LDS-PAGE.

Haze formation and bentonite requirement

In the juice pH adjustment and bentonite addition (0.2 g/L) study, juices with lower pH resulted in significantly lower wine protein haze (Tables 3 and 4) and bentonite requirement (Table 4) regardless of whether bentonite was added or the timing of addition. Significant differences in haze level were noticed among addition timings for each pH treatment ($p \leq$ 0.003) except the pH 3.55 juice (p = 0.09), and lower haze levels were measured for wines which received bentonite after fermentation except for the pH 2.80 juice. For each juice pH treatment, wines made with bentonite addition prior to or during fermentation required less subsequent bentonite than that only after fermentation ($p \leq 0.011$), whereas bentonite fining after fermentation required the least rate as far as total requirement was concerned ($p \leq 0.01$). In general, wines produced from lower pH with bentonite addition after fermentation had

lower haze formation and bentonite requirement.

Results of wine pH adjustment trial are presented in Table 2. In comparison with the original wine (pH 3.34), reducing wine pH resulted in significantly lowering bentonite requirement but small differences in haze level was retained. Increasing wine pH resulted in slightly higher bentonite requirement but much greater haze formation. In contrast, wines produced from commercial-scale winemaking required greater bentonite addition (0.27 and 0.13 g/L greater before and after filtration treatment, respectively) compared to microvinification, whereas little difference in haze level (between 42.4 and 45.6 NTU) was noted.

Protein molecular weight profile

The protein MW profile, by LDS-PAGE and SDS-CGE, for wines from microvinification of various pH juices are summarized in Table 5. A band at 21 kDa in LDS-PAGE and a peak at 22 kDa in SDS-CGE were predominant irrespective of juice pH treatment. For LDS-PAGE, a shoulder on the lower side of the 21 kDa band at 18 kDa and faint bands at 16 and 50 kDa were observed in the wines from higher pH juices (data not shown). In SDS-CGE, the 22 kDa fraction consisted of 21, 22 and 23 kDa proteins and the 26 kDa fraction represented proteins between 24 and 28 kDa (Figure 3). Proteins with MW at 19, 22 and 26 kDa in SDS-CGE contributed more than 95% of total wine protein content and are likely represented by the unresolved band at 21 kDa in LDS-PAGE (76% on average). Proportions of these main proteins in wines were little affected by juice pH adjustment. The protein at 64 kDa in LDS-PAGE was not detected in SDS-CGE. Furthermore, minor protein fractions at 33 kDa in LDS-PAGE and 32 kDa in SDS-CGE were significantly less as a proportion of the fraction in wines produced from lower pH juices, whereas proteins at lower MW (around 10 kDa in LDS-PAGE and SDS-CEG) were more consistent. In comparison, wines produced from commercial winemaking had relative low percentage of 21 kDa (58%) and high percentage of 64 kD (21%) proteins in LDS-PAGE compared to microvinification, whereas little difference in SDS-CGE was identified.

Protein MW profile characterized by LDS-PAGE for wines from juice pH adjustment and bentonite addition were summarized in Table 6. In general, the protein MW profile was similar to wines from juice pH adjustment alone but differed in relative percentage. The 21 kDa fraction decreased by 18% and the 64 kDa fraction increased by 17%, whereas the other fractions remained similar. On the basis of same bentonite addition rate (0.2 g/L) but different addition timing, wines with bentonite treatment after fermentation had higher percentage in the 21 kDa fraction and lower percentage in the 32 kDa fraction (this only detectable for wine from the pH 3.55 juice).

Proteins in stabilized wines

For wines with two extreme pH values from juice pH adjustment (pH 2.80 and 3.55) and wine pH adjustment (pH 2.80 and 3.85), differences in the composition of wines obtained at bentonite rates immediately above and below that for heat stability (0.1 g/L difference) were investigated. No significant differences in total protein content and percentage of each protein fraction by LDS-PAGE were found ($p > 0.05$) (data not shown). Thus, the composition for stabilized wine was taken to be the average of that at those fining rates and listed in Table 7. Generally, after stabilization, low pH wines contained higher total protein content and higher proportion of the 21 kDa fraction compared to high pH wines. Proteins at 21 and 64 kDa were the main proteins and contributed more than 70% of total wine protein content. Only two protein fractions at 21 and 64 kDa remained in stabilized wines from the wine pH adjustment trial following commercial-scale winemaking, whereas a more complex protein MW profile existed in stabilized wines from the microvinification of the two extreme pH juices. Furthermore, proteins in stabilized wines from microvinification of pH 2.80 and 3.55 juices were also examined by SDS-CGE and showed similar electrophoretic profiles and intensities (Figure 3). No statistical differences in wine protein content and percentage of each protein fraction were observed (data not shown) ($p > 0.09$). However, other minor peaks at lower MW (< 19 kDa) but not higher MW (40 kDa) were more noticeable for high pH wines compared to low pH wines.

Discussion

During microvinification 6 days post inoculation, the pH 2.80 ferment was noticeably darker than the others, indicating a decrease in total yeast cell population resulting in sluggish fermentation. The sluggish fermentation of lower pH juices probably results from an imbalance of potassium and hydrogen ions and also the inability of the yeast strains to tolerate low pH (Charoenchai et al. 1998, Kudo et al. 1998). There was no clear lag phase preceding initiation of sugar consumption in all microvinifications, in contrast to a similar study (Weiss and Bisson 2002), probably due to the differences in yeast strain, juice composition and fermentation size. The absence or presence of bentonite during fermentation had little effect on fermentation time and completion of fermentation for each pH juice between 3.00 and 3.55, in accord with other work (Weiss and Bisson 2002). The pH 2.80 juices without bentonite during fermentation showed a shorter but incomplete fermentation when compared to pH 2.80 juices with bentonite contact during fermentation. The presence of some insoluble solids in juice has been reported to facilitate fermentation to dryness for both laboratory and commercial winemaking (Groat and Ough 1978, Ferrando et al. 1998). This has been ascribed

to the removal of inhibitory compounds, such as short-chain fatty acids produced by the yeast, by adsorption (Lafon-Lafourcade et al. 1984, Weiss and Bisson 2002).

Lowering juice pH resulting in lower wine protein concentration is consistent with an early study that soluble protein content of wine decreased markedly at lower fermentation pH (Murphey et al. 1989) and this may occur through isoelectric precipitation (Batista et al. 2009), acid proteolysis (Manteau et al. 2003) and/or other unknown mechanisms. Thus, decreasing juice and wine pH significantly lowers bentonite requirement for the resultant wines due to two main factors. First, lower wine pH enhances positive charges of proteins and, hence, increases adsorption by bentonite (Hsu and Heatherbell 1987). Second, lower juice pH results in reduced wine protein concentration after fermentation and also improves the efficiency of protein adsorption. On the other hand, increased wine pH enhances haze formation, presumably through isoelectric precipitation as wine pH approaches protein pI values (Batista et al. 2009). Major wine proteins generally possess pI values ranging from around 4.1 up to 5.8 (Hsu and Heatherbell 1987, Esteruelas et al. 2009). Additionally, higher pH is known to accelerate wine oxidation and phenolic polymerization (Gonzáles Cartagena et al. 1994) and protein/polyphenol ratio and degree of polymerization strongly influence the amount of haze formation (Siebert 1999). Thus, increased wine pH and greater bentonite requirement may be due to enhanced protein instability to heat. Also, protein remaining in stabilized wines was higher in lower pH wines than higher pH wines as determined by the CBB assay and LDS-PAGE (but not by SDS-CGE). It can be postulated that higher pH wine required lower protein content to achieve heat stability to 2 NTU compared to lower pH wine. It has been pointed out (Hsu and Heatherbell 1987) that relatively large amounts of bentonite were required to remove small amounts of protein (1 to 2 mg/L) to achieve heat stability. Thus, pH may have an additional influence on heat instability and the determination of bentonite requirement with a hot/cold test.

On the basis of a 0.2 g/L bentonite addition rate, amendment during fermentation resulted in the lowest protein content in wine. Prolonged agitation between the bentonite and ferment for bentonite added during fermentation could be expected when compared to adding bentonite before fermentation with 1-hour settling or after fermentation. The prolonged agitation coupled with increased ethanol content as fermentation progressed could be responsible for the lowest protein content in wine. Based on model wine studies, enhanced protein adsorption capacity has been proposed due to replacement of water molecules between bentonite layers with ethanol (Blade and Boulton 1988, Achaerandio et al. 2001, Sun et al. 2007). At commercial-scale studies, bentonite addition prior to or during fermentation has also shown to

result in a lower overall bentonite dosage compared (Ewart et al. 1980, Miller et al. 1985). However, lower protein content in this study seems not to contribute to lower bentonite requirement; rather wines produced with bentonite contact during fermentation required more bentonite to reduce haze to below 2 NTU during fining trials. In contrast, other researchers (Ough et al. 1969, Somers and Ziemelis 1973) found that bentonite fining after fermentation was more effective in protein removal for stability than fining before or during fermentation. These contrasting reports may be the result of the definition of protein stability of a wine. For instance, wines made from pH 2.8 juices with bentonite addition during fermentation resulted in the least protein concentration and lower bentonite requirement to haze formation at 5.5 NTU which was visually stable. However, when the 2 NTU criterion for stability was applied, additional bentonite addition was required. At the low bentonite addition rate, other compounds and suspensions in wine were removed first rather than proteins, acting as a clarifying agent (Duncan 1992). Therefore, more bentonite dosage was required to remove the relative low protein content, in accord with a commercial-scale trial (Ewart et al. 1980).

Protein concentration and the MW profile of juice and resultant wine from the commercial-scale winemaking used in this study were previously reported (Hung et al. 2010). The slightly lower wine protein concentration and bentonite requirement but similar haze levels from the microvinification compared to commercial-scale winemaking is discussed below. Protease activity at each fermentation scale might differ as fermentation kinetics are considerably different. Although wine protein has been shown to be intrinsically resistant to proteolysis during fermentation (Waters et al. 1995), minor proteolysis has been observed (Manteau et al. 2003) and yeasts utilizing grape proteins as a sole nitrogen source have also been reported (Conterno and Delfini 1994). However, the decrease in wine protein content related to yeast secreted protease has been reported to have little influence on the haze forming potential to the hot/cold test (Dizy and Bisson 2000), in accord with results presented here. In addition, a marked decrease in bentonite requirement (0.14 g/L) but not in haze formation for wine from the commercial-scale winemaking before and after filtration was also observed. Consequently, the higher bentonite requirement for wines from commercial-scale winemaking compared to that from microvinification might be through indirect factors, such as different adsorption efficiency resulting from varied wine composition, rather than affecting the wine heat stability.

Modification of protein structure during fermentation has been reported (Waters et al. 1998, Ferreira et al. 2000). Electrophoretic differences in stabilized wines from the juice pH adjustment trial by LDS-PAGE may result from structural changes of protein in various pH

juices during microvinification, also resulting in varying adsorption affinity to bentonite. Major wine proteins and many of their hydrolyzed products with MW between 9.6 and 60 kDa have been found remaining in bentonite fined wines (Okuda et al. 2006). In addition, contribution of yeast protein to wine reported by others (Dambrouck et al. 2003) may also be involved as pH is a crucial factor affecting yeast fermentation (Kudo et al. 1998). The selectivity of bentonite fining to remove proteins as mentioned in other studies (Hsu and Heatherbell 1987, Dawes et al. 1994, Sauvage et al. 2010) is considerably influenced by the pH. For instance, trace amount of the 33 kDa fraction in LDS-PAGE, which was reported to be removed first by bentonite fining, still remained in stabilized wines for high juice pH but not for low pH treatment. Negligible differences in the SDS-CGE profile of wines with different pH values might be the result of low concentrations in stabilized wines and also different analytical mechanisms. For instance, more minor peaks (< 19 kDa) were noted for high pH wines compared to low pH wines, and the major protein of 64kDa in LDS-PAGE was not detected in SDS-CGE for all samples. Consequently, conflicting findings on bentonite selectivity for wine proteins may not only be due to pH but also the analytical methods utilized.

Conclusions

Reducing juice pH resulted in decreased wine protein content after fermentation, and increasing wine pH also enhances protein instability to heat resulting in more haze formation. The pH value significantly governed the effectiveness of protein removal by bentonite fining. The lower the wine pH the less bentonite was required for heat stability. Wines made from identical juice lot showed similar MW profile with small differences in proportion for major proteins, regardless of winemaking scales and juice pH adjustments. However, more complex MW profiles were noticed in stabilized wines from high pH juice. Bentonite addition during fermentation resulted in the lowest wine protein concentration but not the lowest total bentonite requirement. Wines produced where bentonite remained during fermentation showed less efficiency in reducing haze by subsequent bentonite fining. It is suggested that the optimal approach is to add adequate bentonite during fermentation to achieve protein stability rather than addition after fermentation.

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Table 1. Effects of juice pH adjustment and bentonite addition (0.2 g/L) timing on wine parameters[a].

Treatments		Wine composition[c]		
Addition timing[b]	Juice pH	Ethanol (% v/v)	pH	TA[d] (g/L)
Wine (Control)	2.80	11.8_{a}	2.83_{b}	13.4_{h}
	3.00	12.4_{c}	3.01_{c}	10.0_{f}
	3.24	12.9_{g}	3.35_{e}	7.5_{c}
	3.55	12.9_{fg}	3.67_{g}	6.3_{b}
Juice (Trial 1)	2.80	12.2_{b}	2.80_{a}	13.3_{h}
	3.00	12.4_{c}	3.03_{d}	9.9_{e}
	3.24	12.7_{d}	3.34_{e}	8.0_{d}
	3.55	12.9_{fg}	3.66_{g}	5.8_{a}
Ferment (Trial 2)	2.80	12.2_{b}	2.80_{a}	13.1_{g}
	3.00	12.4_{c}	3.03_{d}	9.9_{ef}
	3.24	12.8_{de}	3.34_{e}	8.1_{d}
	3.55	12.8_{ef}	3.65_{f}	6.3_{b}
Significance	pH	***	***	***
	Timing	n.s.	***	**
	pH×Timing	***	***	***

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). Two-way analysis indicated the significant levels for each factor together with interaction between factors. n.s. = not significant ($p > 0.05$).

[b] Bentonite was added to finished wine (control), juice (trial 1) or ferment at 9 Brix (trial 2).

[c] Means of triplicate wines measured by fourier transform infrared spectroscopy interferometer.

[d] TA: titratable acidity expressed as g/L tartaric acid.

Table 2. Summary of wine parameters and heat stability data after wine pH adjustment[a].

Wine pH	Resultant wine composition[b]			Protein haze[d] (NTU)	Bentonite dosage[e] (g/L)
	Ethanol (%, v/v)	pH	TA[c] (g/L)		
2.80	13.2	2.73	15.5	45.8_{b}	0.51_{a}
3.00	13.4	2.99	11.9	42.8_{a}	0.68_{b}
3.34	13.6	3.34	8.0	44.0_{ab}	0.80_{c}
3.65	13.6	3.62	6.5	54.0_{c}	0.81_{cd}
3.85	13.6	3.80	5.7	100.6_{d}	0.85_{d}
Significance		n.d.		***	***

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$).

n.d. = not determined.

[b] Means of duplicate analyses for each pH-adjusted wines measured by Fourier transform infrared spectroscopy interferometer.

[c] TA: titratable acidity expressed as g/L tartaric acid.

[d] Means of nephelometric unit of unfined wines after a hot/cold test in duplicate.

[e] Means of bentonite dosage required for protein stability in duplicate fining.

Table 3. Protein quantification and heat stability of wines from nil bentonite addition treatment of juice pH adjustment experiment[a].

Juice pH adjustment	Wine protein concentration[b] (mg/L)			Haze[e] (NTU)
	CBB assay[c]	LDS-PAGE[d]	SDS-CGE[d]	
2.80	63.6_a	52.3_a	189.4_a	20.8_a
3.00	85.1_b	67.1_b	325.3_b	46.6_b
3.24	98.5_c	77.0_c	402.8_c	45.2_b
3.55	99.2_c	75.6_c	400.1_c	77.3_c
Significance	***	**	**	***

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$).

[b] Reported in g/L BSA equivalents.

[c] Means of triplicate wines by Coomassie Brilliant Blue (CBB) dye assay.

[d] Means of one electrophoresis on two of triplicate wines by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE).

[e] Means of nephelometric unit of unfined triplicate wines after a hot/cold test.

Table 4. Effects of juice pH adjustment and bentonite addition (0.2 g/L) timing on subsequent wine protein concentration and stability[a].

Treatments		Protein content (mg/L)		Heat test	
Addition timing[b]	Juice pH	CBB assay[c]	LDS-PAGE[d]	Haze[e] (NTU)	Bentonite rate[f] (g/L)
Wine (Control)	2.80	44.0_{b}	43.4_{c}	8.6_{a}	0.36_{a}
	3.00	59.3_{cd}	46.5_{d}	23.3_{b}	0.51_{d}
	3.24	82.3_{g}	46.3_{d}	32.1_{d}	0.67_{ef}
	3.55	84.9_{g}	46.4_{d}	47.9_{f}	0.72_{g}
Juice (Trial 1)	2.80	45.1_{b}	39.7_{b}	8.9_{a}	0.46_{c}
	3.00	62.2_{d}	52.3_{f}	28.2_{cd}	0.68_{f}
	3.24	75.3_{f}	57.8_{g}	40.2_{e}	0.77_{h}
	3.55	90.5_{h}	63.4_{h}	58.2_{h}	0.82_{i}
Ferment (Trial 2)	2.80	35.5_{a}	32.7_{a}	5.5_{a}	0.42_{b}
	3.00	56.3_{c}	38.7_{b}	25.7_{bc}	0.64_{e}
	3.24	68.8_{e}	48.2_{de}	42.3_{e}	0.77_{h}
	3.55	72.3_{ef}	49.4_{e}	52.4_{g}	0.84_{i}
Significance	pH	***	***	***	***
	Timing	***	***	***	***
	pH×Timing	***	***	**	*

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). Two-way analysis indicated the significant levels for each factor together with interaction between factors.

[b] Bentonite was added to finished wine (control), juice (trial 1) or ferment at 9 Brix (trial 2).

[c] Means of triplicate wines by a Coomassie dye assay and reported in g/L BSA equivalents.

[d] Means of one electrophoresis on two of triplicate wines by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and reported in g/L BSA equivalents.

[e] Means of nephelometric unit of unfined triplicate wines after a hot/cold test.

[f] Means of total bentonite dosage for triplicate wines required for protein stability.

Table 5. Proportion (%) of wine protein fractions (kDa) from juice pH adjustment and without bentonite treatment[a].

Juice pH	LDS-PAGE[b]				SDS-CGE[b]				
	10	21	33	64	<19	19	22	26	32
2.80	11.2_{a}	78.8_{a}	0.0_{a}	10.1_{c}	4.3_{b}	7.8_{c}	64.6_{a}	23.2_{a}	0.0_{a}
3.00	10.8_{a}	76.1_{a}	5.8_{b}	7.3_{ab}	2.4_{a}	6.3_{b}	60.8_{a}	28.3_{a}	2.2_{b}
3.24	11.3_{a}	73.9_{a}	8.7_{c}	6.1_{a}	2.1_{a}	4.7_{a}	63.1_{a}	26.7_{a}	3.3_{c}
3.55	9.5_{a}	75.2_{a}	7.7_{bc}	7.7_{b}	2.0_{a}	7.8_{c}	63.3_{a}	24.0_{a}	2.9_{bc}
Significance	n.s.	n.s.	**	**	*	**	n.s.	n.s.	***

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). n.s. = not significant ($p > 0.05$).

[b] Means of one electrophoresis on two of triplicate wines by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and sodium dodecyl sulfate capillary gel electrophoresis (SDS-CGE).

Table 6. LDS-PAGE analysis on subsequent wine proteins from juice pH adjustment with bentonite addition (0.2 g/L) timings[a].

Addition timing[b]	Juice pH	Proportion (%) of protein fractions[c] (kDa)			
		10	21	33	64
Wine (Control)	2.80	15.6_{de}	57.5_{c}	0.0_{a}	26.9_{fg}
	3.00	13.9_{bcd}	61.7_{d}	0.0_{a}	24.4_{de}
	3.24	12.6_{b}	64.9_{e}	0.0_{a}	22.6_{abcd}
	3.55	9.0_{a}	62.1_{d}	5.8_{c}	23.1_{bcd}
Juice (Trial 1)	2.80	16.9_{e}	56.9_{c}	0.0_{a}	26.1_{ef}
	3.00	14.3_{cd}	56.8_{c}	8.4_{f}	20.4_{a}
	3.24	13.2_{bc}	55.1_{ab}	10.6_{g}	21.0_{ab}
	3.55	12.9_{bc}	55.0_{ab}	10.3_{g}	21.8_{abc}
Ferment (Trial 2)	2.80	14.3_{bcd}	53.8_{a}	0.0_{a}	31.9_{h}
	3.00	12.7_{bc}	55.0_{ab}	3.8_{b}	28.5_{g}
	3.24	13.8_{bc}	55.3_{ab}	6.8_{d}	24.1_{cde}
	3.55	12.7_{bc}	56.2_{bc}	7.7_{e}	23.5_{cd}
Significance	pH	***	***	***	***
	Timing	**	***	***	***
	pH×Timing	**	***	***	*

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). Two-way analysis indicated the significant levels for each factor together with interaction between factors.

[b] Bentonite was added to finished wine (control), juice (trial 1) or ferment at 9 Brix (trial 2).

[c] Means of one electrophoresis on two of triplicate wines by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and data were expressed in mg/L BSA equivalents for total protein content and in percentage among protein fractions.

Table 7. LDS-PAGE analysis on proteins in stabilized wines from juice and wine pH adjustments[a].

pH adjustment		Protein (mg/L)	Proportion (%) of protein fractions[b] (kDa)					
			10	16	21	33	50	64
Juice	2.80	19.4_c	17.2	–	50.7_b	–	–	32.1_a
	3.55	16.3_b	–	9.0	42.6_a	3.2	15.6	31.7_a
Wine	2.80	14.2_b	–	–	66.3_d	–	–	33.7_a
	3.85	9.2_a	–	–	61.6_c	–	–	38.4_b

[a] Treatment means in column designated by the same letters do not differ significantly ($p > 0.05$). – means not detected.

[b] Means of one electrophoresis on four samples for each wine with closest bentonite addition rates before and after reducing haze below 2 NTU by lithium dodecyl sulfate polyacrylamide gel electrophoresis (LDS-PAGE) and data were expressed in mg/L BSA equivalents for total protein content and in percentage among protein fractions.

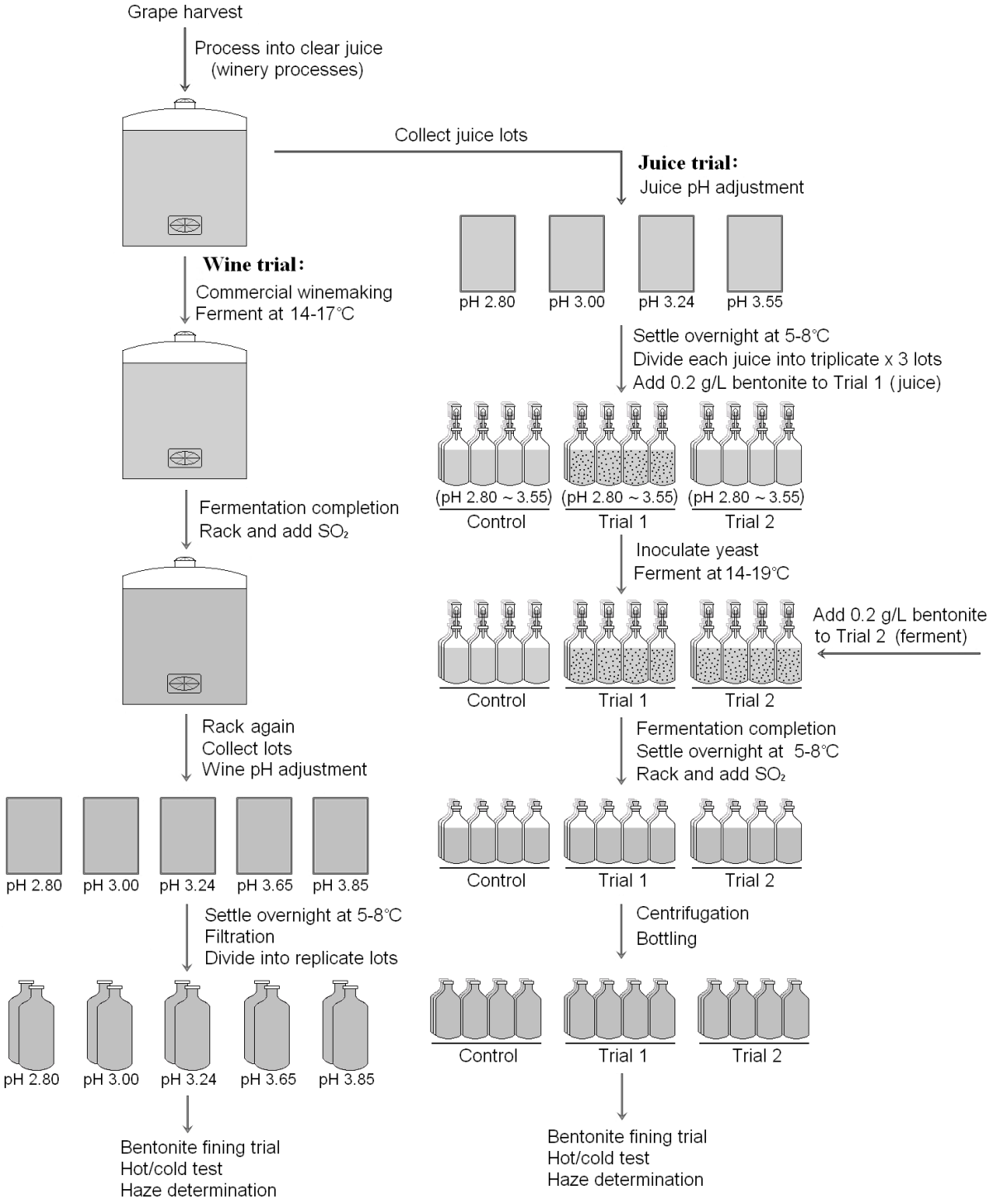

Figure 1. Diagram of the experimental design.

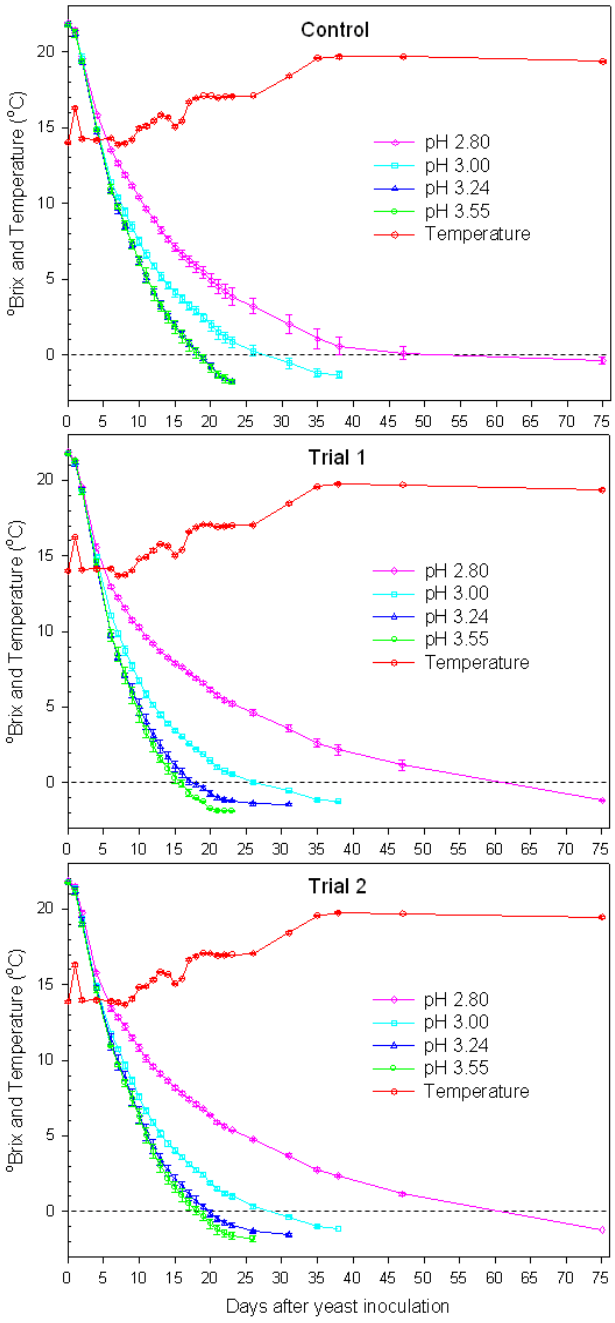

Figure 2. Kinetics of fermentation of juices adjusted to four pH levels with three bentonite treatments: control, without bentonite addition throughout fermentation; trial 1, with 0.2 g/L bentonite addition to juice; trial 2, with 0.2 g/L bentonite addition to ferment as soluble solids content fell below 9 Brix. Error bars are standard deviation of the mean of triplicate ferments and of twelve sample temperatures with the same bentonite treatment

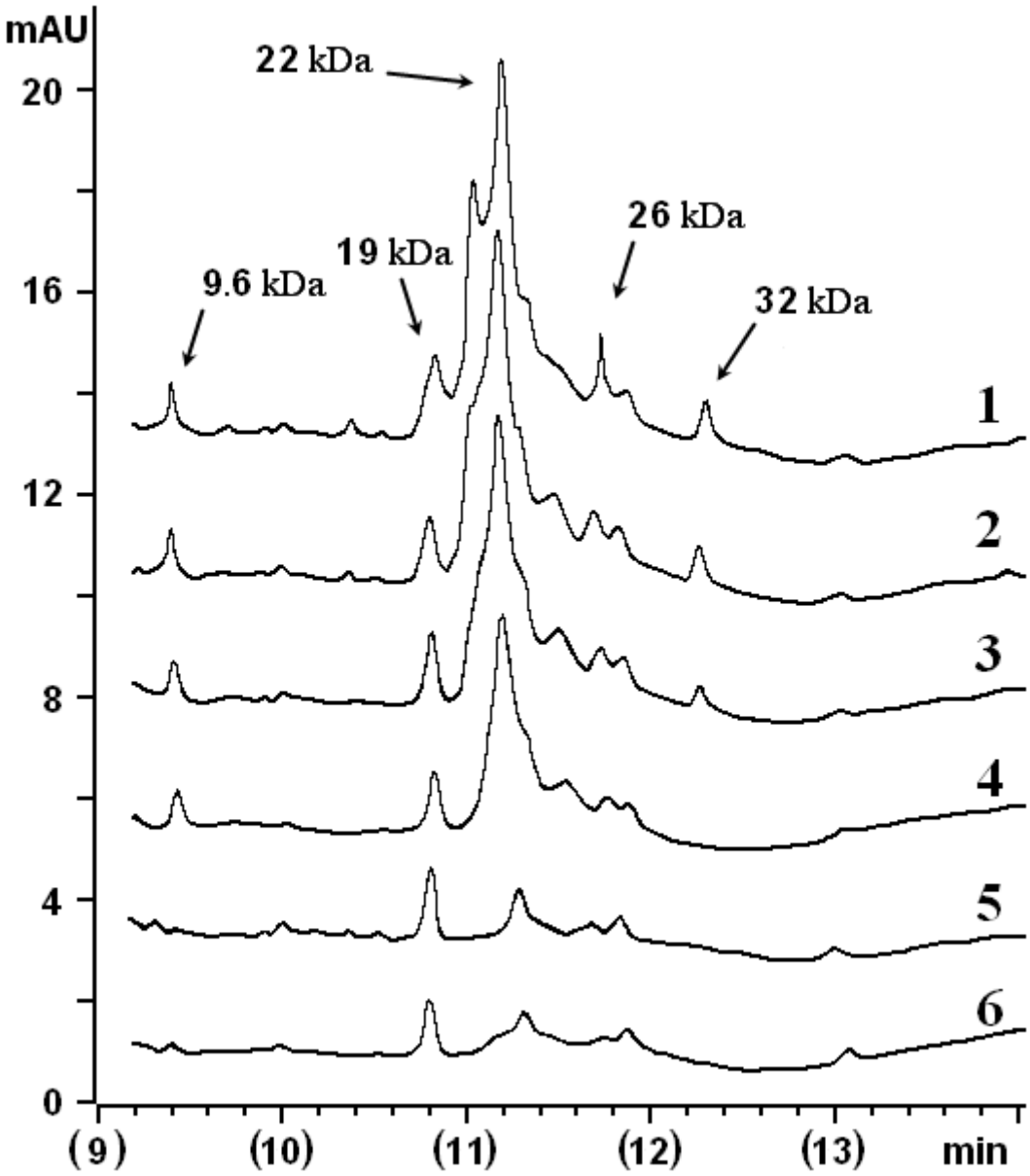

Figure 3. Electrophoretic patterns of SDS-CGE for wine proteins from microvinification of four pH juices. Trance numbers from 1 to 4 represent juice pH of 3.55, 3.24, 3.00 and 2.80, respectively. Trace 5 and 6 represent wines from pH of 3.55 and 2.80 juices, respectively, after bentonite fining to achieve protein stability to a hot/cold test.

Chapter 7

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Effects of Heat Test and Adsorbent Type on Wine Protein Stabilization and Their Manipulation for Practical Interpretation

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Short running title: **Heat Test and Adsorbent Affect Protein Stability**

ABSTRACT

Four heat treatments utilized for examining protein stability and four adsorbents utilized for protein removal were compared using a Sauvignon blanc wine. The 90 °C for 1 h treatment produced the most protein haze and was more sensitive in detecting haze reduction following incremental bentonite fining than 80 °C for 2, 6 or 15 h. There was a coincident point at approximately 3 nephelometric turbidity units which gave the same predicted bentonite requirement for all heat tests. Na bentonite was the most efficient adsorbent for protein removal, followed by NaCa bentonite, polymeric resin and cation-exchange resin. The polymeric resin had the advantage of regeneration capacity for use in a continuous process but suffered from recovery inefficiency. When choosing a bentonite type, NaCa bentonite is suitable for low pH wines or high bentonite requirement, whereas Na bentonite is suitable for high pH wines and low bentonite requirement with regard to less bentonite lees formation. Differences in protein removal tendency by bentonite fining were observed among wine protein molecular weight fractions but not between pH-adjusted wines fined with Na or NaCa bentonites examined by sodium dodecyl sulfate capillary gel electrophoresis.

KEYWORDS: adsorption isotherm, bentonite, pH, resin, SDS-CGE, wine protein

INTRODUCTION

Grape derived proteins which remain soluble in white wines have been considered as a fault due to the likelihood of protein denaturation with time, followed by flocculation, coagulation and eventual precipitation in the wine bottle (*1, 2*). While batch-wise fining with bentonite remains common practice for control of wine protein instability, the formation of bentonite lees and consequent losses in quantity and quality has always been a concern (*3*). Thus, alternatives to bentonite fining and/or ways to reduce bentonite use are of interest to the wine industry.

Bentonite dosage required to achieve wine protein stability is normally determined in the winery laboratory by conducting a series of bentonite fining trials coupled with a procedure to force protein haze formation and examination (*4*). High temperature is the most common treatment to induce protein haze in the wine industry and research literature (*1*) as it is less affected by other wine components (*5*) and produces haze with similar composition to natural protein precipitate (*6*). However, a standard treatment of 80 °C for 6 h utilized in the Australian wine industry and elsewhere has been regarded as resulting in over fining when compared with storage trials (*1*). The existence of various heat treatments and methods of haze determination (instrumental or visual) make it necessary to review those procedures as well as the criteria to gauge protein stability on varietal wines.

Bentonite fining has shown a tendency to remove wine proteins with higher isoelectric point (pI) and intermediate molecular weight (MW) in 2-dimensional electrophoresis (*7*), whereas no bentonite selectivity based on wine protein pI was found in chromatofocusing profiles (*8*). Recently, some preferential removal was found among wine proteins but not between adsorbents or wines in HPLC chromatograms (*9*) and that identified protein fractions were removed by bentonite at different rates as shown in 1-dimensional electrophoresis (*10*). The selectivity of bentonite fining in wine protein adsorption is still unclear and remains open to debate. Furthermore, it is generally accepted that sodium bentonite swells better and adsorbs more protein than calcium bentonite but generates a greater amount of settled lees on the basis of same addition rate (*11, 12*). Theoretically, protein adsorption efficiency by bentonite fining through cation-exchange mechanisms can be improved by lowering the wine pH as wine proteins mainly possess pI ranging from 4.1 to 5.8 (*7*). Nevertheless, adverse results have been noticed in a model wine study and it was postulated that relative exchange of hydrogen ions, protein and sodium ions could also be affected by the changes of solution pH, resulting in varied preference of the bentonite for these species (*11*). Economic use of bentonite for wine protein stabilization with respect to bentonite type, fining rate and corresponding lees

formation has not yet been quantitatively studied (*11-14*), especially in relation to the range of wine pH values encountered in winemaking. In addition, the desirability of continuous processes and regeneration ability as alternatives to batch-wise bentonite fining has been investigated with regard to finding suitable materials (*15-19*). Investigation of regenerable materials for use in continuous protein stabilization process compared to commercially used bentonites may provide more economical solutions.

An initial aim of this study was to compare the influence of various hot/cold tests on bentonite rate prediction. The next aim was to explore the potential of using regenerable resins in percolated beds; polymeric resins are able to remove wine protein but have not yet been intensively studied with respect to regeneration treatments (*19*) and a cation-exchange resin was compared with two commercial bentonites by characterizing corresponding adsorption isotherms for protein removal. Thirdly, bentonite selection was optimized with regard to minimizing lees formation within a practical pH range. Finally, the protein selectivity was examined by fining wines with extreme pH values with two bentonite types.

MATERIALS AND METHODS

Wine Samples.

A commercial Sauvignon blanc wine (alcohol 13.6% and pH 3.34) from the 2008 vintage was utilized to contrast the bentonite requirement for two bentonite types (Na and NaCa forms) as influenced by wine pH. Wines with pH at 2.80, 3.00, 3.34 (standard, not adjusted), 3.65 and 3.85 had previously been showed to require 0.51, 0.68, 0.80, 0.81 and 0.85 gL^{-1} Na bentonite, respectively, for protein stability (*20*). Wines were used after 2 weeks storage (4-8 °C) for evaluation of various thermal treatments and bentonite lees formation and after 16 months storage (4-8 °C) for determination of protein adsorption isotherms and stabilization utilizing a continuous adsorption process.

Other wines (**Table 1**), from adjacent blocks at the same vineyard (M16 SBL; Pernod Ricard (NZ) Ltd.) and produced separately in commercial-scale winemaking, were also used in bentonite requirement evaluation. Wines from this vineyard have historically shown lower bentonite requirement compared to the vineyard producing the standard wine. Wine pH, alcohol content and titratable acidity were measured using an infrared technique (WineScan™ FT120, Foss, Denmark).

Protein Recovery and Analysis.

Methods as reported previously were utilized (*21*). Briefly, total protein content was determined using reaction with Coomassie Brilliant Blue (CBB) dye following alkalization of

the sample. Wine protein concentrates for SDS-CGE procedures were prepared following recovery by precipitation with ice-cold acetone and centrifugation. An Agilent capillary electrophoresis system (Waldbronn, Germany) with external pressure in CE+p mode was utilized and controlled by ChemStation system (Rev. A.10.02). Samples were injected by applying pressure (50 mbar) for 2 min and constant voltage was applied for electrophoresis with detection at 214 nm.

Preparation of Bentonite Slurry.

A NaCa bentonite (NaCalit PORE-TEC, Erbslöh Geisenheim AG) and a Na bentonite (Volclay KWK, American Colloid Company) were used and compared. Bentonite suspensions were prepared using 60 °C deionised water and made up 5% (w/v) slurries according to a standard protocol (*22*). Newly prepared bentonite slurries were allowed to disperse completely by stirring at room temperature for > 36 h before use.

Bentonite Addition Procedure.

For the protein adsorption isotherm study, various amounts of bentonite slurries were added to standard wines (25 mL) and then made up to 27 mL with water, giving addition rates of up to 1.2 gL^{-1} for Na bentonite and up to 3.0 gL^{-1} for NaCa bentonite. After addition, wines were homogenized for 1 min and settled overnight before protein analysis. For the protein stability test, procedures for bentonite fining were utilized as previously reported (*21*).

Heat Stability Test and Bentonite Requirement.

Procedures for the standard hot/cold test (80 °C for 6 h followed by 4 °C overnight) and bentonite rate determination (2 NTU limit) were followed as in the previous study (*21*). Other thermal treatments: 80 °C for 2 h, 80 °C for 15 h and 90 °C for 1 h followed by 4 °C overnight, were used as trial tests with comparison to the standard hot/cold test. Standard wines after incremental fining of Na bentonite (0 and 0.2 to 1.1 gL^{-1} in 0.1 gL^{-1} increments) were imposed to these hot/cold tests in duplicate and followed haze measurements.

Assessment of Bentonite Lees Formation.

Standard wine samples (30 mL) were thoroughly mixed (1 min) with incremental addition of each bentonite in duplicate and then poured into KIMAX glass culture tubes (30 mL, 20 x 150 mm) and left undisturbed (4 °C). Bentonite lees formation was estimated after 1, 3, 6 and 10 days by means of visual comparison and adjusting the water level in the same type of tube which was then weighed. Bentonite lees formed in wine was expressed as percentage of wine volume (% v/v).

Preparation of Adsorbent Resin.

Polymeric resin (Amberlite® XAD16, Rohm & Haas) and cation-exchange resin (50WX8-400, Dow Chemical) were purchased from Sigma (St. Louis, MO). The polymeric resin has a nonionic, hydrophobic, crosslinked and polyaromatic surface and is suitable for adsorption of hydrophobic compounds with small to medium MW (up to 40 kDa). The strong acid cation-exchange resin has 200-400 mesh in bead size. Before first use, resins were water washed (2-3 times) before regeneration specified by the producer. This consisted of a sequence of washing steps: base (0.5% NaOH), water, acid (0.5% HCl) and water, followed by filtration (Whatman no. 541) and drying in air.

Resin Addition Procedure.

Weighed dry resins were placed in volumetric flasks (50 mL) with water additions (2 mL) for re-hydration for at least 24 h. Prior to addition to standard wines (25 mL), re-hydration was completed by ultrasonication for 1 h (Astrason™ model 9E; Branson, NY) in conjunction with occasional hydrodynamic vacuumization as suggested in another study (*9*). Wines with two resin additions (up to 12 and 42 gL^{-1} for polymeric resin and cation-exchange resin, respectively) were sealed after nitrogen purging and then remained agitated on an orbital shaker for 48 h at room temperature before protein analysis. In a preliminary test, it was determined that 30 h contact was required for maximum protein adsorption (at 6 gL^{-1} addition rate).

Continuous Protein Adsorption.

A continuous process for standard wine protein adsorption was developed using a packed column (14.5 cm x 1.5 cm, laboratory-made) and a peristaltic pump (model 502S; Watson Marlow, Falmouth, UK). Polymeric resin was packed by draining off the re-hydrated resin solution in the column. The column was pumped in up-flow mode at room temperature with a constant flow rate (3 $mLmin^{-1}$), and treated wine samples were collected at bed volume (BV, 25 mL) intervals up to 110 BV. Regeneration of the column was carried out in down-flow mode with the washing sequence as above. Base wash was conducted until the eluent became colorless. Water washing (150 mL), acid washing (150 mL) and eventual water replacement (150 mL) followed.

RESULTS

Hot/Cold Tests.

Relationships between Na bentonite addition rate, protein haze formation and protein concentration for the standard wine are illustrated in **Figure 1**. For wine which received no bentonite addition, the 90 °C for 1 h treatment produced 14% more turbidity and the 80 °C for 15 h treatment produced 22% less turbidity than the standard procedure of 80 °C for 6 h

treatment. Little difference in haze formation was observed between 80 °C for 2 and 6 h treatments (< 1%). All bentonite addition rates resulted in reductions in protein concentration and haze formation up to the addition required for protein stability (2 NTU). The 90 °C for 1 h treatment was most sensitive and the 80 °C for 15 h treatment least sensitive in detecting protein haze reduction in response to incremental rates of bentonite fining. Moreover, when the threshold for protein stability determination was set at 2 NTU, the 80 °C for 15 h treatment predicted the greatest rate (0.97 gL^{-1}) and the 90 °C for 1 h treatment predicted the least rate (0.91 gL^{-1}) for protein stability. On the other hand, the order of bentonite requirement for each treatment reverses when the threshold was set to 5 NTU. A much lower bentonite requirement (0.58 gL^{-1}) for the 80 °C for 15 h treatment was gauged when the threshold was set to 10 NTU. There was a coincident point for various heat treatments at which identical level of turbidity at approximately 3 NTU was formed in the wine of receiving the same bentonite addition rate.

Protein Adsorption Behavior.

The protein adsorption behavior of the four adsorbents was compared using batch addition. All adsorbents showed the ability to remove wine protein, but only the two bentonites and the polymeric resin showed reasonable efficiency in removing wine protein. It required around 0.8, 1.6 and 7.5 gL^{-1} to reduce wine protein content to 10 mgL^{-1} for Na bentonite, NaCa bentonite and polymeric resin, respectively. However, the cation-exchange resin achieved only 40% reduction in protein concentration at a rate of 40 gL^{-1}. The adsorption capacities of the three more efficient adsorbents to stabilize wine proteins were further examined. Protein adsorption isotherms (**Figure 2**) were derived using the Langmuir model fitted to data by the Lineweaver-Burk linear regression (*23*). The three isotherms all showed convex shapes with the one from the polymeric resin being more linear. Na bentonite had the greatest adsorption capacity, approximately two and three times higher than that of NaCa bentonite and polymeric resin, respectively.

Breakthrough Curves.

Breakthrough curves of wine protein adsorbed to polymeric resin packed column and after of regeneration treatments are depicted in **Figure 3**. For the first run, removal of more than 50% of total wine protein content was achieved in the first 30 BV and removal efficiency gradually decreased to 30% at 110 BV. However, protein adsorption capacity was gradually impaired after each regeneration cycle. At 30 BV, protein removal was 33 and 22% of total wine protein content for the second and fourth runs, respectively. At 110 BV, treated wines from

both runs showed only around 20% reduction of total wine protein concentration. In addition, progressive coloration of the resin-packed column was observed after each cycle.

Bentonite Rate and Ratio.

The standard wine (pH 3.34) required 1.53 gL^{-1} NaCa bentonite for protein stabilization to the standard hot/cold test (2 NTU), approximately 2.2 times higher than Na bentonite. When the standard wine was adjusted to pH 3.00 and 2.80, the NaCa bentonite requirements decreased dramatically by 24 and 39%, respectively, similar to Na bentonite. On the other hand, when the standard wine was adjusted to pH 3.65 and 3.85, the NaCa bentonite requirements showed much more significant increases by 30 and 67%, respectively, which was more than 10 times higher than Na bentonite. Thus, the ratio of bentonite requirement (NaCa over Na bentonite) for protein stability seem more consistent at low to medium wine pH and increased at higher wine pH values. This relationship was further evaluated using 6 commercial wines of various pH values (without adjustment). A similar trend was observed and the results from all wines are shown in **Figure 4**. A quadratic regression curve was derived describing the ratio of bentonite requirements as a function of wine pH.

Bentonite Lees Formation.

It was observed that, lees formation fluctuated for the Na bentonite at the low to medium addition rates during the early setting period before day 6, whereas higher Na bentonite and all NaCa bentonite addition rates showed gradual decreases in lees volume precipitation before day 6. Steady bentonite lees volumes were achieved after 6-day setting regardless of addition rate and bentonite type. Differences in bentonite lees formation in response to incremental addition rates between Na and NaCa bentonites with 4 settling periods are illustrated in **Figure 5**. The derived quadratic equation for Na bentonite was around y axis, while the quadratic equation for NaCa bentonite was around x axis. At the same addition rate, the Na bentonite deposited several times higher lees volume compared to the NaCa bentonite.

Selection for Bentonite Types.

Because the relationship between the amount of lees formed and bentonite fining rate differed markedly between bentonite types (**Figure 4** and **5**), it was possible to derive a curve which delineated regions where the two bentonite types would be economically most appropriate as a function of pH (**Figure 6**). Results indicated that the Na bentonite is more suitable for wines with lower bentonite requirement (≤ 0.5 gL^{-1}) or wines with medium to high pH (> 3.30), especially for wines with higher pH and higher bentonite requirement, whereas the NaCa bentonite is more suitable for wines with low to medium pH (< 3.30), especially for wines with lower pH and higher bentonite requirement.

Protein Removal Profile.

Electropherograms of wines of low and high pH (2.80 and 3.85) after fining with 2 types of bentonite are illustrated in **Figure 7** and summarized in **Table 2**. It should be noted that there was a greater reduction in total wine protein concentration for the pH 2.80 wine (26%) compared to pH 3.85 wine (10%) as a result of storage (frozen for 1 year), but similar MW profiles and relative percentages were maintained except for a reduction in the 32 kDa fraction in the pH 2.80 wine. Common proteins were at 9.6, 13, 19, 22, 26, 32 and 40 kDa with 22 and 26 kDa contributing 90% of total wine protein. Generally, there was little difference in protein removal tendency based on protein MW between the two bentonites, but there were apparent differences between protein fractions in response to bentonite fining. Fractions at 9.6 and 32 kDa were low in concentration and removed first at lower bentonite addition rates, whereas fractions at 13, 19 and 40 kDa were resistant to bentonite fining and remained in stabilized wines. Proteins at 24.6, 26.5 and 27.6 kDa, which constituted the 26 kDa fraction, were removed gradually with the 24.6 kDa peak decreasing more in response to bentonite fining. The 22 kDa fraction could be resolved into three peaks at 21, 22 and 23 kDa with increasing bentonite addition rates except that no 21 kDa peak was observed in the pH 2.80 wine. The 23 kDa peak appeared to be more resistant to bentonite fining compared to the 21 and 22 kDa peaks.

DISCUSSION

Heated haze formation in wine depends on the temperature and duration of heating. Generally, increasing temperature and/or thermal duration also increased haze formation (*1, 24*). Pocock and Rankine (*25*) investigated the combined effect of the two factors (temperatures up to 80°C with various heating times up to 24 h) and concluded that the 80 °C for 6 h procedure produced the most haze. As a consequence, these authors suggested the 80 °C for 6 h procedure for wine protein stability test, and it has been used as a standard procedure in many wineries worldwide. Recently, this procedure has been considered as too strict, resulting in over fining when compared with storage trials (*1*). Two aspects regarding the over fining can be discussed from the present work with regard to the criteria for protein heat stability test. Firstly, the 90 °C for 1 h procedure produced the most haze and was the most sensitive to bentonite fining compared to the other heat treatments. This procedure has shown to be less affected by other wine components and result in closer precipitate composition to natural haze over various methods such as ethanol test, tannin test and commercial kits (*5, 6*). Additionally, thaumatin-like proteins were found to be the principal protein in Sauvignon blanc wine (70%)

and natural precipitate (*26, 27*). The thaumatin-like protein has also shown to be susceptible only at high temperature and precipitated incompletely even at 80 °C for 30 min (*10*). Therefore, higher temperature processes, like the 90 °C for 1 h procedure, may be a suitable test in prediction of bentonite requirement. Secondly, the threshold setting in the assessment of haze formation is crucial in determining bentonite requirement. The nephelometric threshold utilized in the literature is mostly around 2 NTU (*1*) but may be up to 14 NTU (*4*). It was observed that many samples in glass cells (60 x 25 mm, 15 mL) were visually clear when the measured turbidity was below 10 NTU in the open laboratory environment, in accord with others (*13, 28*). In addition, differences in sample wine coloration after thermal treatment was noticed. Coloration of wines is likely to affect the visual and spectrophotometric turbidity (*1, 28*). Also, the intensive browning of wines resulted from severe heat treatment could possibly contribute browning pigment precipitation to wine as described in other studies (*29*). For example, wines with bentonite addition at 1.0 and 1.1 gL^{-1} and subjected to the 80 °C for 15 h treatment had nephelometric readings at 1.30 (stable) and 2.15 NTU (unstable), respectively. The coincident point of nephelometric reading at 3 NTU may be a suitable threshold setting for heat stability test because at this reading of bentonite rate prediction was less affected by the temperature and duration of heating, which was not found when using 2 NTU in other studies (*1*). When choosing a method for wine heat stability determination using turbidity assessment, the factors discussed above need to be considered. Differences in haze formation as a result of thermal treatment could be ascribed to differences in phenolic oxidation and polymerization for each heated wine (*29, 30*), changing the protein/polyphenol ratio and thus affecting haze formation profile (*31*). It has been claimed that it is the wine matrix, rather than protein concentration and composition, that determines haze formation response to heat treatments (*24*). However, differences in thermal susceptibility between protein fractions as observed by Sauvage et al. (*10*) may also be important.

Among the four adsorbents studied, bentonites were the most efficient at reducing wine protein. Similar results from comparisons of various types of adsorbent in removing proteins from model wine and/or real wine systems have also been reported (*15-17*). Although bentonite and the polymeric resin showed similar convex protein adsorption isotherms, only the polymeric resin could be used in a percolated bed due to its low swelling property, adequate particle size and regeneration capability (*15*). The polymeric resin packed column was able to remove wine proteins in a continuous process as other researchers found with the same material (*19*). However, the protein binding capacity of this resin gradually reduced

after each regeneration cycle. This is presumably due to the adsorption of wine pigments as indicated by progressive coloration of the resin as well as other wine components which were not completely removed during regeneration. The macroreticular characteristics and aromatic nature of this polymeric resin surface seems to have the ability to adsorb many wine components. Adsorption of phenols and heavy metals and reduction of color intensity (420 nm absorbance) in treated wine by the identical resin has been reported (*19*). In addition, the much shorter contact time between polymeric resin and wine in the continuous treatment (< 9 min) compared to that done to be required in batch trials (30 h) was insufficient to maximize protein adsorption. The importance of adequate residence time for wine in continuous protein stabilization processes has been demonstrated (*32*).

The two to four-fold increase in NaCa bentonite required to stabilize wine compared with Na bentonite in the present work is in accord with an Australian report for 8 commercial bentonites evaluated using a pH 3.29 white wine (*12*); and similar to a model wine study (*11*) in which Na bentonite showed a higher BSA adsorption capacity at around 2 and 2.7 fold compared to NaCa and CaNa bentonites, respectively, and 3.6 fold compared to Ca bentonite. The general phenomena described above has been ascribed to the fact that Na form bentonites swell more completely than Ca form bentonites, resulting in greater interlayer spacing and effective surface area for protein adsorption (*11-13*). More complicated mechanisms are required if the effect of wine pH is to be explained. It was found in a model wine study (*11*) that lower wine pH (3.2) has lower protein adsorption capacity than higher wine pH (3.8) using bovine serum albumin (BSA; 66 kDa and pI = 4.3 to 4.6) as a standard protein. It was proposed that the higher concentration of hydrogen ions at lower pH competed with proteins for available binding sites. In contrast with a real wine system in the present work, the decreased wine pH correlated with reduced bentonite requirement for both Na and NaCa bentonites, implying that the protein adsorption by bentonite was more effective at lower wine pH due to electrostatic attraction. These different results may be attributed to the different protein characteristics and the complex compounds in the real wine system. Wine proteins with MW between 60 and 65 kDa and pI values from 4.1 to 8.0, similar size and pI value to BSA, have been shown to be highly resistant to bentonite fining (*7*). The major wine proteins with MW between 20 to 30 kDa and pI value from 4.1 to 5.8, which are the most heat unstable proteins and need to be removed for stability, are highly resistant to proteolysis compared to BSA (*7*, *33*), implying different structure and composition between these wine proteins and BSA. Protein characteristics (pI, MW and dimension) have been shown to influence adsorption behavior by a metal adsorbent in the identical model wine (*34*).

Differences in adsorption behavior in trisacryl-packed column were also observed between a real wine and standard proteins (BSA, ovalbumin or haemoglobin) in model wines (*17*). Moreover, in the higher wine pH range (> 3.34), bentonite requirement was less affected by increasing wine pH for Na bentonite but not for NaCa bentonite. This may be due to the characteristics of the Na bentonite amplifying other adsorption mechanisms at higher wine pH when compared to NaCa bentonite. For instance, highly dispersed and swollen Na bentonite could directly and indirectly interact with protein through other wine compounds, like polyphenols and polysaccharides, forming a large net and trapping impurities. Adsorption of protein, polyphenol and polysaccharide by bentonite has been reported (*17*), and complex formation between protein, polyphenol and polysaccharide also possibly occurred in wine (*6*). Furthermore, although Na bentonite had a higher protein adsorption capacity than NaCa bentonite, Na bentonite produced much greater bentonite lees on the basis of the same addition rate. Similar results were noted in a comparison of bentonite types and fining rate required for stability on lees formation (*12*, *13*), although no clear guidelines for economic use under practical conditions were indicated. The derived equivalent lees formation for NaCa bentonite and Na bentonite in the present work gives a clear pattern. Apparently, the NaCa bentonite is suitable for low to medium pH wines with high bentonite requirement, whereas the Na bentonite is suitable for lower bentonite requirement or for medium to high pH wines with higher bentonite requirement. This quantitative outcomes support the general claim that Ca bentonite is more effective in removing protein at lower wine pH than Na bentonite (*11*).

Selective removal of some protein fractions by bentonite but no effect of bentonite type is in agreement with a study using HPLC fractionation (*9*). These results imply that bentonite type and wine pH only influence wine protein adsorption efficiency and capacity rather than modifying the wine protein adsorption preference. In addition, small differences in MW profile (13 and 21 kDa peaks) in un-fined wines of different pH values were observed. The undetected 21 kDa peak is likely the result of pH adjustment. Although proteins were recovered from wines before analysis, the effect of modifying wine pH may carry over to the final sample and influence the electrophoretic behavior since the sample pH is a crucial factor in optimizing protein separation by SDS-CGE (*35*). The 13 kDa peak that appeared in high pH wine and remained after stabilization is similar to findings of smaller hydrolyzed proteins resulting from the hydrolysis of other proteins in stabilized wine (*36*). High pH conditions probably contribute new protein fractions as mentioned in a previous study (*20*). Total wine protein content but not relative percentage of MW profile in the pH 2.80 wine was significantly lower than that in the pH 3.85 wine in SDS-CGE, presumably due to partial

precipitation under the conditions of low pH and long storage time (-20°C for 1 year) coupled with freezing and thawing treatments. The effects of pH and 1-year storage had been shown to reduce wine protein content but had little impact on relative percentage in the MW profile (*5*, *20*).

ABBREVIATIONS USED

BSA, bovine serum albumin; BV, bed volume; CBB, Coomassie Brilliant Blue; MW, molecular weight; pI, isoelectric point; SDS-CGE, sodium dodecyl sulfate capillary gel electrophoresis; NTU, nephelometric turbidity unit.

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Table 1. Composition of commercial wines and bentonite requirement

Vintage	Alcohol (%, v/v)	pH	TA (g/L)	Na bentonite rate[a] (g/L)	NaCa bentonite rate[a,b] (g/L)
2007	12.62	3.40	6.8	0.63	1.73 ± 0.02
	12.84	3.30	6.8	0.47	1.01 ± 0.00
2008	13.8	3.17	8.6	0.58	1.32 ± 0.04
	13.9	3.15	8.5	0.53	1.03 ± 0.04
	13.4	3.15	8.4	0.53	0.99 ± 0.02
	14.2	3.10	8.6	0.49	1.00 ± 0.01

[a] Determination through a hot/cold test (80 °C for 6 h followed by 4 °C overnight) and interpolation with turbidity threshold set at 2 nephelometric turbidity units.

[b] Means ± SD of duplicate bentonite fining.

Table 2. Wine protein removal profile base on MW by SDS-CGE analysis with incremental fining of two bentonite types

pH	Bentonite type	Addition rate (g/L)	Protein (mg/L)	Proportion (%) of protein fractions (kDa)					
				<19	19	22	26	32	>32
2.80	Na	0.0	392.0	2.7	6.9	64.6	25.1	0.8	0.0
		0.2	254.7	1.9	8.6	63.8	25.7	0.0	0.0
		0.4	140.0	0.0	14.5	60.0	25.4	0.0	0.0
		0.6	89.9	0.0	23.6	42.7	33.7	0.0	0.0
	NaCa	0.25	285.0	2.9	7.6	63.9	24.2	0.0	1.4
		0.70	181.2	1.9	10.8	60.2	25.0	0.0	2.2
		1.00	114.7	0.0	16.9	53.8	25.6	0.0	3.7
		1.30	78.9±2.3	0.0	22.3	35.3	36.3	0.0	6.0
3.85	Na	0.0	479.7	2.4	6.4	65.0	23.7	2.5	0.0
		0.4	266.3	1.0	7.8	63.9	27.3	0.0	0.0
		0.7	126.2	2.5	15.2	44.3	38.0	0.0	0.0
		0.9	91.4	3.4	21.5	34.6	40.5	0.0	0.0
	NaCa	0.5	426.2	1.5	5.9	60.9	27.1	3.0	1.5
		1.5	226.1	1.1	10.0	60.9	26.0	0.0	2.0
		2.5	115.2	1.7	13.2	47.4	34.5	0.0	3.2
		3.4	72.8±0.6	3.4	22.0	30.6	37.3	0.0	6.8

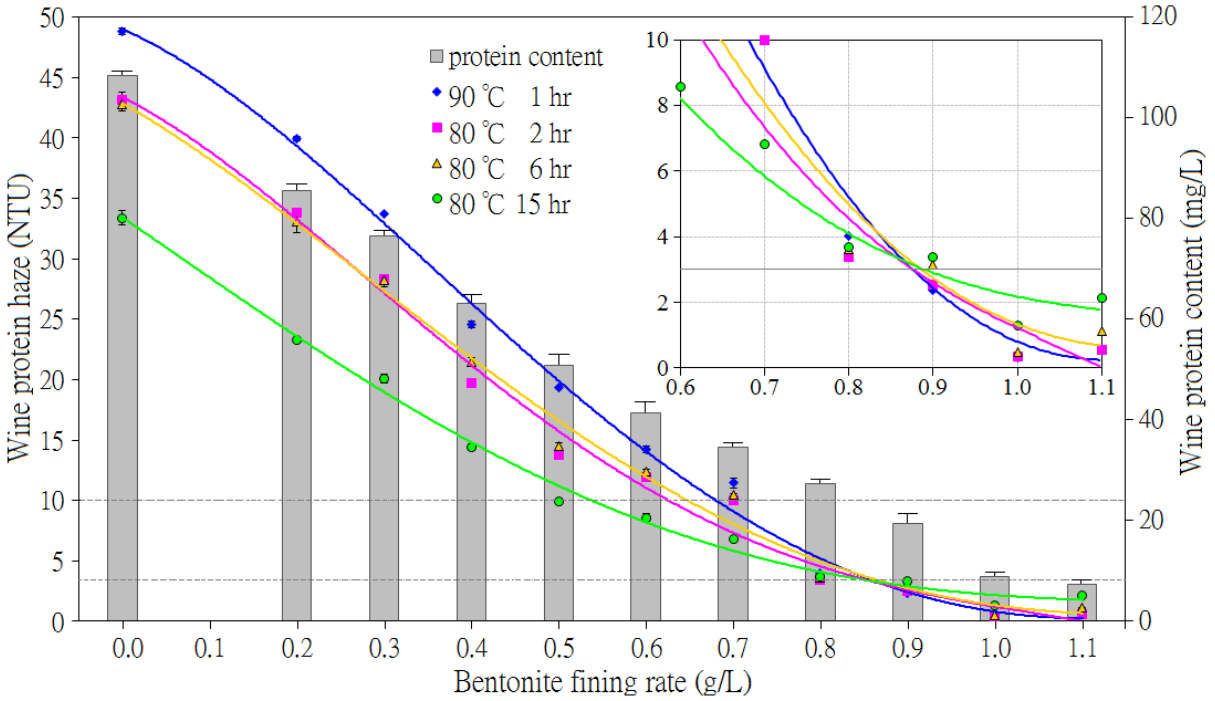

Figure 1. Comparison of wine protein concentration after a series of bentonite fining trials and corresponding wine haze formation in 4 hot treatments. Quadratic curves were drawn for visual comparison. Two dash lines indicated the turbidity threshold settings at 3 and 10 NUT.

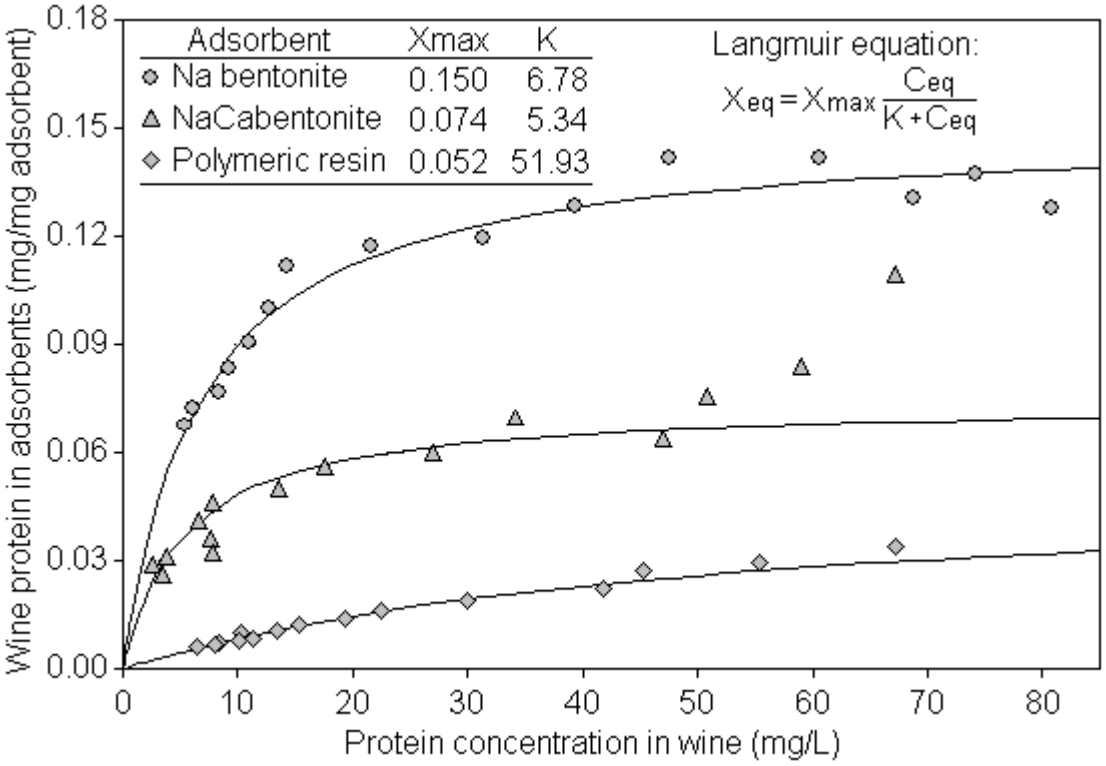

Figure 2. Wine protein adsorption behaviors for three adsorbents. Adsorption isotherms were derived using the Langmuir model fitted to data by the Lineweaver-Burk linear regression.

X_{eq}: Adsorption capacity (mg protein mg^{-1} adsorbent).

X_{max}: Maximum adsorption capacity (mg protein mg^{-1} adsorbent).

C_{eq}: Equilibrium protein concentration in wine (mgL^{-1}).

K: Langmuir constant (mgL^{-1}).

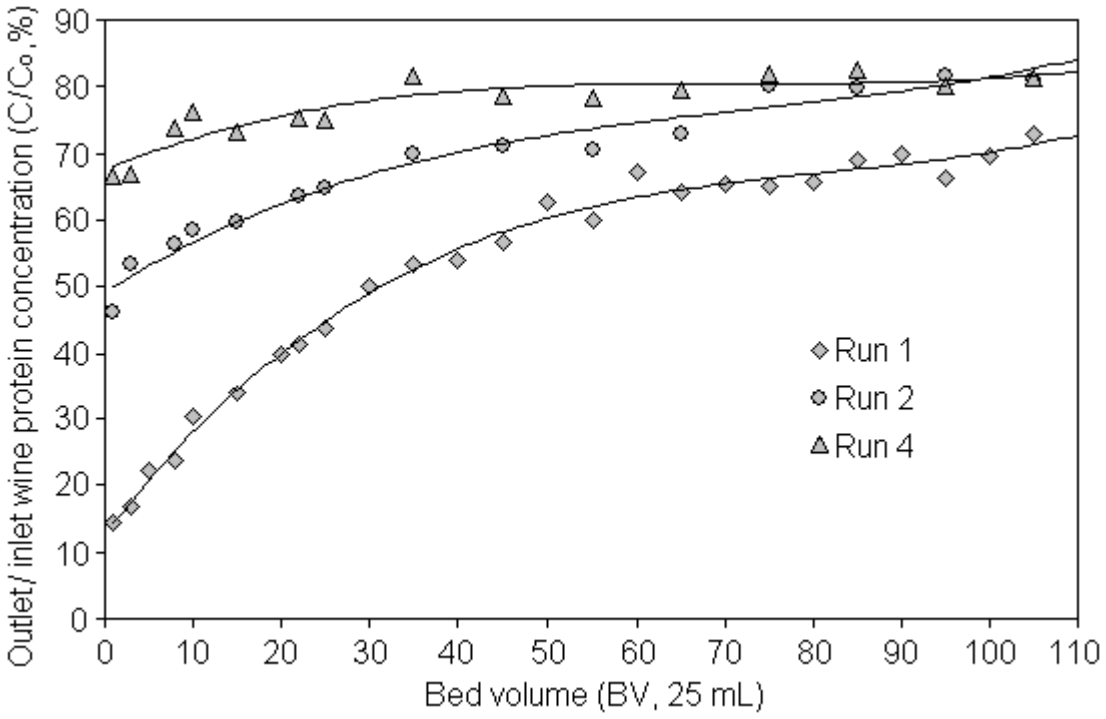

Figure 3. Breakthrough curves of wine protein adsorption in a polymeric resin packed column (14.5 cm x 1.5 cm) with flowing rate at 3 $mLmin^{-1}$ in up-flow mode, showing quadratic curves for the first run and runs after regeneration cycles.

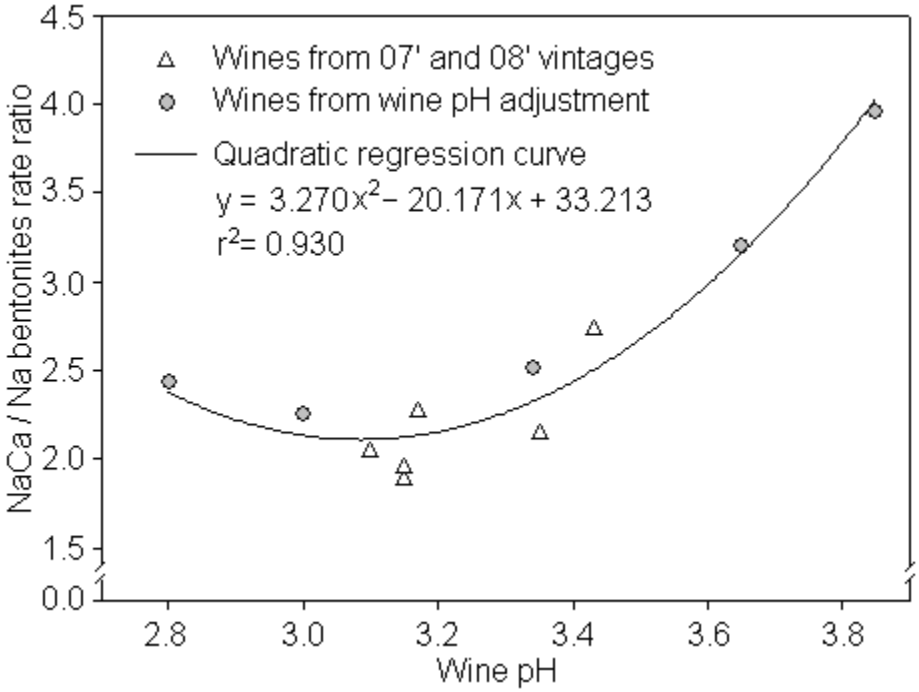

Figure 4. Relationships of required dosage ratio between two bentonites (NaCa and Na forms) and wine pH examined.

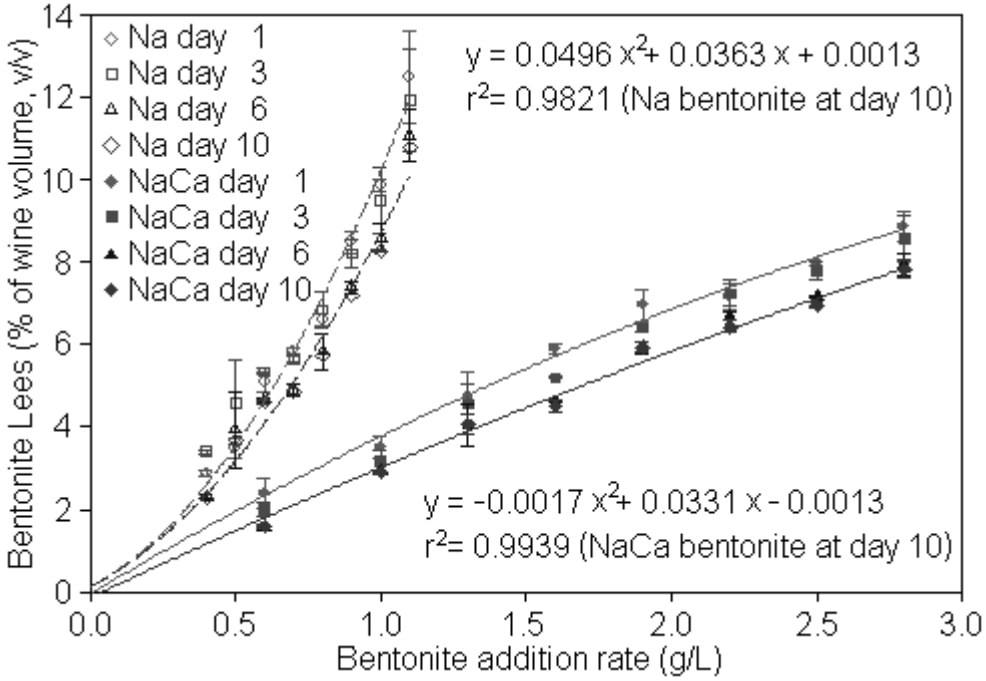

Figure 5. Relationships of bentonite lees formation with incremental fining rates and settling periods of 1, 3, 6 and 10 days for two bentonite types. Quadratic curves were derived for bentonite lees formation after 1 and 10 days settling as well as corresponding equations for the 10 days settling.

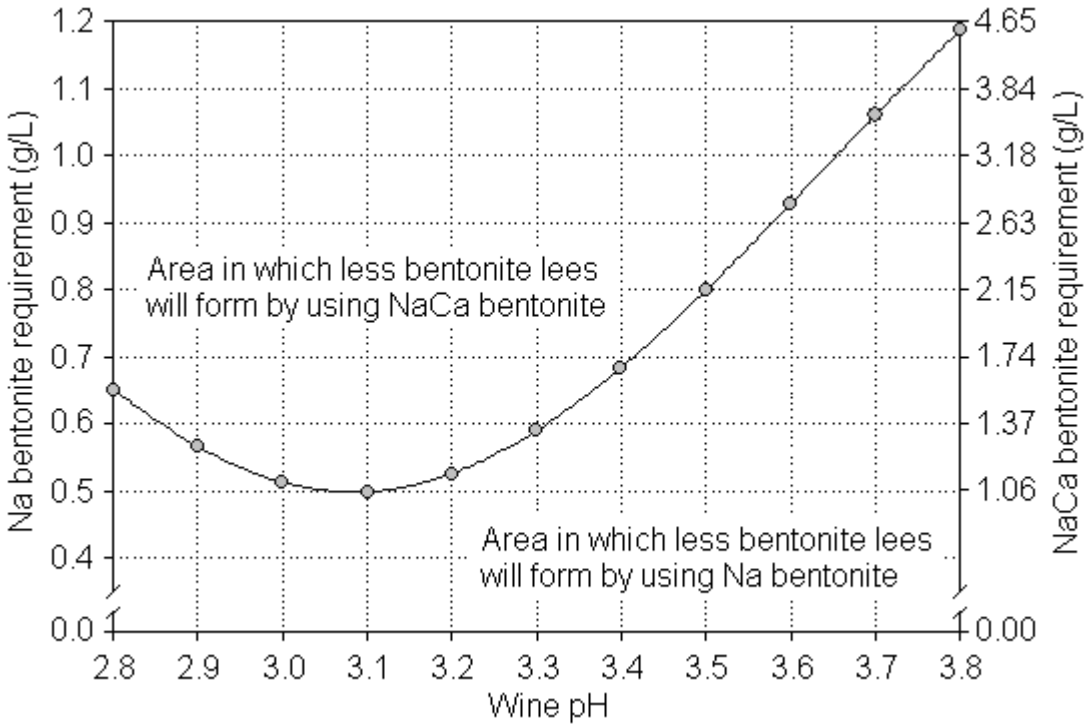

Figure 6. A curve of equivalent lees formation between two bentonite fining to various pH wines (Sauvignon blanc), indicating the suitable application conditions for both bentonite.

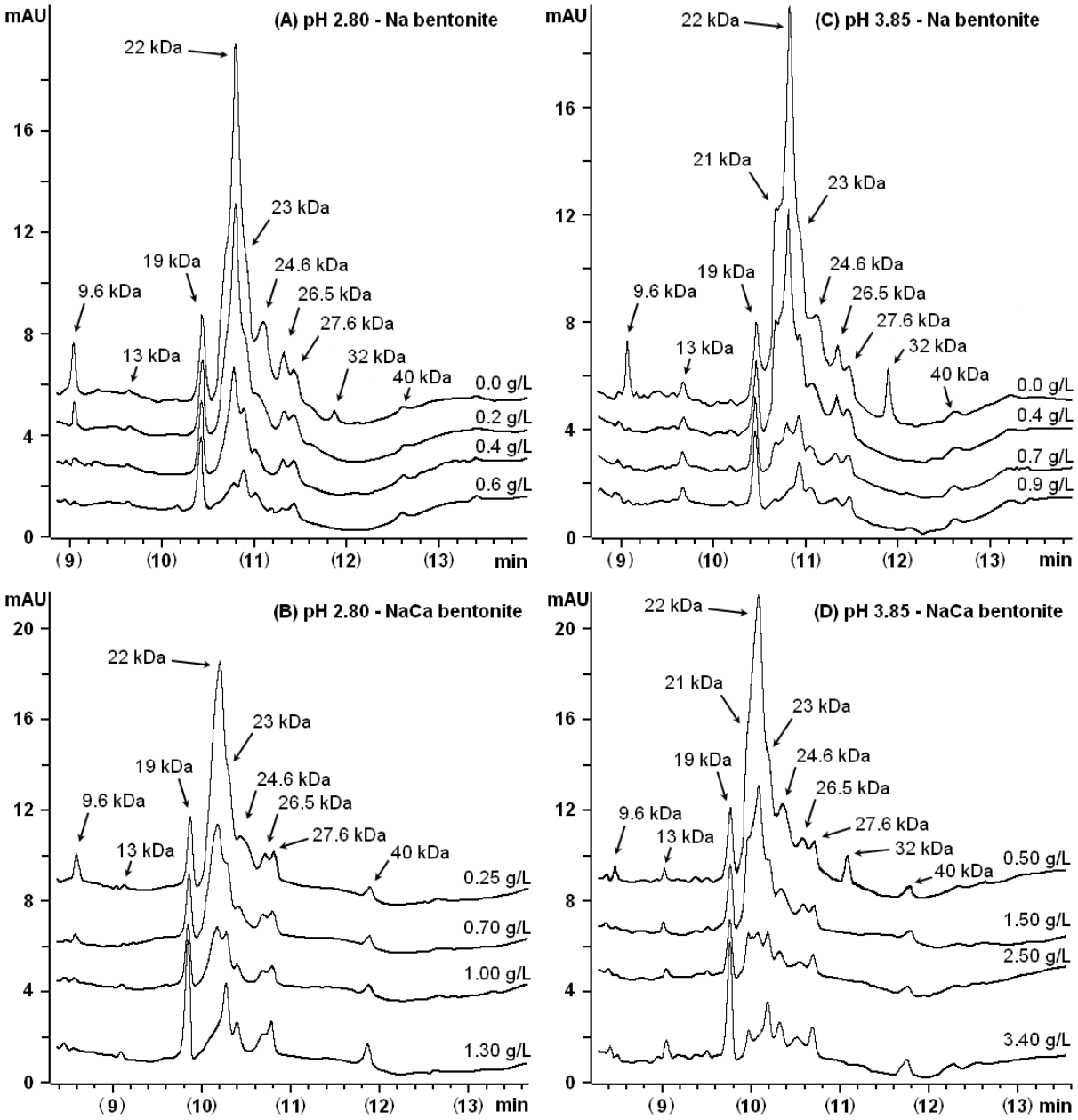

Figure 7. Electropherograms of protein MW profiles of wines receiving increment bentonite fining rate as indicated on each trace. (A)(B): Wine was pH adjusted to 2.80 and fined with Na and NaCa bentonite, respectively; (C)(D): Wine was pH adjusted to 3.85 and fined with Na and NaCa bentonite, respectively. The lowest traces in each electrophoregram were the wine attaining protein stability to a hot/cold test. Proteins were recovered from 300 μL wines for analysis except the lowest traces in (B)(D) in which 600 μL wines were applied.

Chapter 8
General Discussion and Conclusions

8.1 Protein analyses of Marlborough Sauvignon blanc

In this study, methodologies for the analysis of proteins in Marlborough Sauvignon blanc are demonstrated. The protein content in juice and wine varied depended on recovery procedures and/or analytical methods (Moreno-Arribas et al. 2002, Vincenzi et al. 2005a). Acetone precipitation recovered the most wine proteins, whereas ultrafiltration (10 kDa molecular weight cut-off) is suitable for juice protein recovery without interference from sugar precipitation (Yokotsuka et al. 1991, Hung et al. 2010b). The CBB assay resulted in wine protein concentration (n = 18) about 42% higher compared to LDS-PAGE and about 4.2 times lower compared to SDS-CGE (Hung et al. 2010b). However, the range in wine protein content determined by the CBB assay (average 113.3 mg L^{-1}) and LDS-PAGE (average 79.5 mg L^{-1}) is in the common range described in the literature (Ferreira et al. 2002, Waters et al. 2005). Nevertheless, wine protein concentration determined by the SDS-CGE (average 483.6 mg L^{-1}) might be more accurate as Coomassie dye assays have been reported to underestimate juice/wine proteins by a factor of 2-5 compared to protein amino acid summation (Waters et al. 1991, Duncan 1992).

The electrophoretic patterns for LDS-PAGE and SDS-CGE were similar in respect of molecular weight but differed in relative intensity. These techniques utilise essentially the same separation mechanism based on molecular size but different detection mechanisms; dye-protein interaction and ultraviolet absorption, respectively (Hung et al. 2010b). The molecular weights of major wine proteins in Marlborough Sauvignon blanc, found at 19-33 kDa (Hung et al. 2010c), is in accord with other reports worldwide regardless of variety (Hsu and Heatherbell 1987a, Waters et al. 1991, Yokotsuka et al. 1991, Marchal et al. 1996, Ferreira et al. 2000) and have been demonstrated to be pathogenesis-related proteins causing haze in wine (Waters et al. 1996, Esteruelas et al. 2009b). The 21/22 kDa and 23/26 kDa fractions have similar molecular weight to those reported as thaumatin-like proteins and chitinases, respectively (Peng et al. 1997, Pocock et al. 2000). In total these two major proteins averaged at 62% and 90% of wine protein content by LDS-PAGE and SDS-CGE, respectively (Hung et al. 2010c). Other proteins consistently observed in this study were at 32/33 kDa, likely to be β-1,3-glucanase which was also heat unstable (Sauvage et al. 2010) and at 9.6/10 kDa, likely the lipid transfer protein (Okuda et al. 2006) which is speculated to

occur depending on variety (Wigand et al. 2009). The 64 kDa fraction detected by LDS-PAGE but not by SDS-CGE has a similar molecular weight to a grape vacuolar invertase (Jégou et al. 2009).

In general, Marlborough Sauvignon blanc wine protein content varied depending on vineyard site, growing conditions and vintage (Hung et al. 2010c) and also subsequent winemaking treatments (Hung et al. 2010a), whereas the protein molecular weight profile between wines only varied slightly (Hung et al. 2010a, c, d).

8.2 Stable and unstable wine proteins

Waters et al. (1991) suggested ideal processes to demonstrate heat unstable proteins by using back-addition of specific wine proteins to protein-free wine to form haze and/or by removal of specific proteins from wine to achieve heat stability. Although proteins with molecular weight at 19-33 kDa are the predominant proteins at over 97% (on average) of wine protein content by SDS-CGE (Hung et al. 2010c), there exist differences in susceptibility to thermal/bentonite treatments or natural precipitation among observed protein fractions (Esteruelas et al. 2009a, Sauvage et al. 2010).

Proteins with similar molecular weight to the 32/33 kDa fraction have been shown to be the most heat unstable protein (Sauvage et al. 2010) and readily removed by bentonite fining (Hsu and Heatherbell 1987b), in accord with the present work showing ease of loss during processing and vulnerable characteristics to low pH fermentation and bentonite adsorption (Hung et al. 2010a, b). However, trace amounts remaining in stabilised wine from pH 3.55 juice were detected by LDS-PAGE (Hung et al. 2010a). The 9.6/10 kDa fraction was unaffected by juice pH adjustment prior to fermentation irrespective of its low concentration (Hung et al. 2010a). The 9.6/10 kDa fraction, like the 32/33 kDa fraction, was generally removed by bentonite fining at lower addition rates (Hung et al. 2010d). However, trace amounts remaining in stabilised wine from pH 2.80 juice were also detected by LDS-PAGE (Hung et al. 2010a). Proteins with similar molecular weight to our 32/33 and 9.6/10 kDa fractions were recently reported to be involved in natural, heat- or chemical-induced haze in Sauvignon blance wine (Esteruelas et al. 2009a, b). Results from our studies and the literature indicate that these proteins are unstable proteins but trace amounts might remain in stabilised wines after bentonite fining resulting from various pH juices.

Differences in susceptibility to heat denaturation between thaumatin-like protein and chitinase

have been observed with a portion of thaumatin-like protein being very stable to heat or heavy bentonite treatments (Sauvage et al. 2010). This might partially explain why bentonite requirement correlates better with the 26 kDa fraction than the 22 kDa fraction (Hung et al. 2010c). However, trace amounts of both 22 and 26 kDa fractions remained in stabilised wines (Hung et al. 2010a, d). These two protein fractions would be expected to contribute the most heat instability to Marlborough Sauvignon blanc wine due to their high concentration and haze formation ability (Pocock et al. 2007, Hung et al. 2010c). Furthermore, the 64 kDa fraction was also found in natural, heat- or chemical-induced wine haze (Esteruelas et al. 2009a). This protein has been shown highly resistant to bentonite fining resulting in remaining large proportion in stabilised wine after bentonite fining (Hsu and Heatherbell 1987b, Hung et al. 2010a).

Concentrations of stable proteins in wine during storage trials have been measured in the range 27-72 mg L^{-1} depending on variety (Yokotsuka et al. 2007). Stable proteins after bentonite fining to heat stability ranged up to 35 mg L^{-1} (Fukui and Yokotsuka 2003, Hung et al. 2010b). The composition of stable proteins in stabilised wine included common proteins observed in wine (invertase, thaumatin-like proteins and lipid transfer proteins) and a large number of their hydrolysis products (Okuda et al. 2006). The more complex protein profile found in stabilised wine from the high pH fermentation compared to the low pH fermentation in the present work (Hung et al. 2010a) might be a result of protein hydrolysis and/or yeast autolysis. Results were also influenced by the methods utilised. For instance, wine natural precipitate contained major proteins at 18-22 kDa, whereas the high and low temperature denaturation resulted in major wine protein precipitate at 18-25 and 22-25 kDa, respectively (Esteruelas et al. 2009a).

It seems that unstable proteins are not entirely specific and each fraction contains a portion of stable and unstable proteins as evident from protein analyses based on molecular weight by SDS-CGE (Hung et al. 2010a, d), isoelectric point by chromatofocusing (Dawes et al. 1994), protein size/charge ratio by capillary zone electrophoresis (Dizy and Bisson 2000) and separation by anion exchange HPLC (Sarmento et al. 2000a).

8.3 Wine protein stabilisation

As plant pathogenesis-related proteins are important agents against fungal pathogens (Ferreira et al. 2006), the accumulation of PR proteins during vegetative growth may be necessary to secure grapevine health and grape productivity and preferable to genetically modifying vines

to underexpress pathogenesis-related proteins to reduce wine protein instability (Ferreira et al. 2004, Martinez-Esteso et al. 2009). It has been found that environmental conditions during vegetative growth govern the grape protein content and profile (Monteiro et al. 2003a) and wine protein haze formation and bentonite requirement could vary from vintage to vintage (Hung et al. 2010c). Thus, control of protein instability and maximising wine quality during winemaking practices remains an important challenge.

Bentonite fining remains the most common and effective treatment for protein stabilisation in the wine industry (Waters et al. 2005). Timing, type and mode of bentonite use could possibly be optimised. It has been suggested that minimum bentonite use and lees formation could be achieved when bentonite addition occurs during fermentation for microvinification and commercial winemaking (Ewart et al. 1980, Hung et al. 2010a). Also, bentonite addition through an in-line dosing system has been demonstrated to recover a greater volume of quality wine compared to traditional settling (Nordestgaard et al. 2007). A strategy for the selection of bentonite type (Na or NaCa) was proposed through studying lees formation and effects of wine pH by Hung et al. (2010d). Na bentonite produced much greater lees volume (more than 2 times) than NaCa bentonite on the same addition rate basis, whereas NaCa bentonite required much greater addition rate (more than 2 times) to achieve protein stability compared to Na bentonite, in accord with other reports (Rankine and Emerson 1963, Leske et al. 1995). It was possible to derive a curve showing equivalent lees formation for Na and NaCa which indicates that NaCa bentonite is suitable for low pH wines, whereas Na bentonite is suitable for high pH wines with regard to less bentonite lees formation (Hung et al. 2010d).

The effect of pH on protein adsorption by bentonite addition has often been studied using model wine systems but rarely in real winemaking situations (Blade and Boulton 1988, Waters et al. 2005). Because of the complexity of wine composition and the diversity of wine proteins (Monteiro et al. 2001), model wine studies may not adequately represent wine conditions (Pachova et al. 2002, Bruijn et al. 2009). Reducing wine pH resulted in more effective use of bentonite for protein removal using a Sauvignon blanc wine in the present work (Hung et al. 2010d) and as is generally believed through electrostatical attraction (Ferreira et al. 2002), but in contrast to model wine studies using standard proteins (Blade and Boulton 1988, Sun et al. 2007). Reducing wine pH by addition of organic acids, such as tartaric, malic and citric acids, was recently reported to enhance wine protein stability to heat treatment through the electrostatic interaction between proteins and organic acids (Batista et

al. 2010). As a consequence, the slightly higher protein concentration that remained in lower pH wine compared to higher pH wine after protein stabilisation (Hung et al. 2010a) may be explained. Additionally, the lower the juice pH the lower the resultant wine protein concentration after fermentation and this might be expected to contribute to a lower bentonite requirement (Murphey et al. 1989, Hung et al. 2010a). However, the mechanism is not fully elucidated and might involve proposed protein isoelectric precipitation (Batista et al. 2009) and/or acid proteolysis (Manteau et al. 2003).

It is possible to utilise findings in this study to minimise bentonite use to achieve wine protein stability through a combination of treatments including pH adjustment, timing of bentonite addition and selection of bentonite type.

It is necessary to review methodologies used to predict wine protein instability as well as the definition of protein stability to match the real wine conditions. The 80 °C for 6 hours procedure was considered to overestimate bentonite requirement in predicting short to medium term stability compared to 80 °C for 2 hours procedure (Pocock and Waters 2006). The 90 °C for 1 hour procedure coupled with a threshold setting at 3 nephelometric turbidity units may be most appropriate due to maximum haze induction, ability to precipitate thaumatin-like protein, induced-precipitate close to natural haze composition and it is less affected by temperature, heating duration and other wine non-proteinaceous parameters compared to other procedures (Sarmento et al. 2000a, Esteruelas et al. 2009a, Hung et al. 2010d, Sauvage et al. 2010). Alternatively, proposed protein quantification assays coupled with setting of protein concentration for stability threshold might be a simple, fast and reliable methodology to predict wine protein stability and bentonite requirement (Weiss and Bisson 2001, Hung et al. 2010b).

8.4 Recommendations for future study

In this study, the quantification and characterisation of proteins based on molecular weight of Marlborough Sauvignon blanc, together with the optimisation of practical treatments such as pH adjustment, timing of bentonite addition and selection of bentonite type for wine protein instability control has been carried out. Although pathogenesis-related proteins are presumably the dominant proteins in Sauvignon blanc, they were not unambiguously identified nor compared with other varieties. Some aspects derived from this study might be investigated.

1. To further identify wine proteins from various varieties in respect of their molecular weight distribution by SDS-CGE, isoelectric point, amino acid sequence and identity in order to compare with the present work.

2. To specifically remove the 26 kDa fraction (presumably chitinases) by chitin adsorbents or proteolytic treatments as this protein seems more susceptible to proteolysis than the 22 kDa fraction and correlates well with bentonite requirement to heat stability.

3. To use the developed CBB assay to predict bentonite requirement and compare this with the commonly used hot/cold test as well as storage trials.

4. To examine the combined treatments of pH adjustment and bentonite addition during fermentation in commercial-scale winemaking as a method to remove proteins for stability.

5. To identify the differences between stable and unstable proteins to elucidate mechanisms why these proteins differ in their ease of removal by bentonite fining or heat treatment.

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