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Mark R. Smith for the degree of Master of Science in Food Science and Technology presented on August 3, 2010.

Title: Development of a Method to Measure Protein in Red Wines; A Survey of Protein, Mannan and Tannin in Pinot Noir Wines.

Abstract Approved:

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A number of methods to isolate and quantify protein in Pinot noir wines were evaluated. The combination of precipitation by acetone containing 10% w/v trichloroacetic acid followed by quantification with the Bradford assay, reported in yeast invertase equivalents yielded the most accurate results when compared to micro-Kjeldahl analysis. The technique was validated by dialysis and proteolysis experiments and was used to assay protein in 57 Pinot noir wines. These wines were found to contain protein concentrations ranging from 49 to 102 mg/L. The mannan and tannin content of the wines was also measured, but no correlation between protein content and concentrations of these components was found. The

presence of protein in red wines greater than 30 years old at concentrations typically found in white wines contradicts the notion that interactions with tannin severely reduce protein levels.

Development of a Method to Measure Protein in Red Wines;

A Survey of Protein, Mannan and Tannin in Pinot Noir Wines

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorized release of my thesis to any reader upon request.

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1. Introduction

Although protein is a minor component of wine, it can influence texture, color, flavor and aroma of wine. In spite of this, proteins in red wine have been the subject of relatively few studies, in part, because of the difficulty in isolating protein from a matrix as complex as red wine (Moreno-Arribas et al., 2002). Proteins present in wine derive mostly from the grape, *Vitis vinifera*, and to a lesser extent from the yeast, *Saccharomyces cerevisiae*, and are typically found in concentrations ranging from 15 to 230 mg/L based on a survey of white wines (Ferreira et al., 2002). Grape proteins found in wine are mostly classified as pathogenesis-related proteins, such as the thaumatin-like proteins and chitinases, while yeast proteins are primarily mannoproteins originating from the cell wall. Based on neutral sugar analysis of isolated wine protein fractions from Muscat Bailey A red wine, Yokotsuka et al. (1994) proposed that all wine proteins are glycoproteins. Various techniques are employed in the winery to either decrease protein content, such as bentonite fining, or to increase protein content, by aging wines on the lees.

Recent studies have focused on identifying proteins in wine, but have often neglected quantification. As yet, a standard method for quantifying proteins in wine has not been developed. Recently, Vincenzi et al. (2005) proposed a method for measuring protein in white and red wines by potassium-dodecyl-sulfate (KDS) precipitation followed by quantification using the BCA-Smith

assay (Smith et al., 1985). This method appeared promising, but it was later reported that the precipitation technique alone was not suitable for glycoproteins (Fusi et al., 2010). Other methods, such as Kjeldahl analysis, may require larger volumes of wine, and can be time-consuming.

Wine producers and researchers are in need of an accurate and practical method to measure protein in wine. The protein fraction of wine, although small, can have an important impact on wine quality through enhancement or prevention of haze, stabilization of foam in sparkling wines, and improved "mouthfeel," presumably through interactions with tannins.

In the present study, a number of procedures to isolate and quantify protein from Pinot noir wines were evaluated. A novel combination of an acetone/TCA precipitation followed by the Bradford assay was demonstrated to be a simple, reliable and rapid method for determining the protein content of red wines. Protein concentrations measured by this assay were nearly identical to values obtained with the micro-Kjeldahl method, and permitted many samples to be analyzed relatively rapidly, from as little as 200 µl of wine.

2. Literature Review

2.1. Wine Proteins

2.1.1. Overview. Although protein is only a minor component of wine, with concentrations ranging from 15 to 230 mg per liter (Ferreira et al., 2002), its effect on stability and sensory quality is important. The primary sources of wine protein are grapes and yeast.

2.1.2. Grape Proteins. While grape proteins found in wine vary in concentration depending on grape variety, vintage, soil, winemaking practices and climate, a similar group of proteins has been identified in a number of red and white wines (Wigand et al., 2009). Grape proteins can cause hazes that are sometimes observed in white wines. The primary proteins that have been studied from grapes in wine are chitinases and thaumatin-like (TL) proteins because they are the primary culprits in heat-induced hazes (Falconer et al., 2010). These proteins, as well as many others identified in must and wine, belong to a large group of pathogenesis-related (PR) proteins.

PR proteins encompass a large group of proteins whose expression in grapes is induced by exposure to a pathogen. These proteins have been grouped into families based on sequence similarity and related function. The families include proteins that create trans-membrane pores such as PR-1 proteins, which are thaumatin-like proteins, and PR-5 proteins, the osmotins. The PR-2 family

includes β-1, 3-glucanases. These proteins hydrolyze β-1, 3-glycosidic bonds in fungal cell walls. Chitinases also degrade the cell wall of fungi, and are classified in the PR-3, 4, 8, and 11 families (Monteiro et al., 2003). Lipid transfer proteins have also been classified as PR proteins in the PR-14 family. While these proteins are induced upon exposure to pathogens, their antimicrobial activity does not appear to be related to their ability to transfer lipids (Gomes et al., 2003).

In addition to causing hazes in wines, the PR proteins have also been identified as allergens. Proteins in the PR-2, 3, 4, 5, 8, 10, and 14 families from various plant species have demonstrated allergenic potential. The proteins in groups PR-2, 3, 4, and 8 are related to the latex allergens. PR-14 proteins have also been associated with anaphylaxis (Midoro-Horiuti et al., 2001). While proteins in these families are related between plant species, the allergenic potential of grape PR proteins is still unclear (Wigand et al., 2009).

PR proteins have been identified in a wide range of wines, throughout the world. Their presence in wine is attributed to their resistance to proteolysis and stability at the low pH of wine (Waters et al., 1996). Glycosylation may also confer stability to proteins. While it is known that many of the proteins in wine are glycosylated, it is unclear whether all wine proteins are. While VVTL1 has no potential *N*-glycosylation sites, some of the other TL proteins do (Cilindre et al., 2008). Another proposed protective mechanism is through interaction with phenolic compounds such as tannins. However, Waters et al. (1995) argued that

the resistance to proteolysis is not due to glycosylation or interaction with phenolics. Their analysis showed that all except one protein was resistant to a peptidase treatment for six weeks at 15° C, however, a control sample of BSA was digested in less than one week under the same conditions. Because the proteins contained no associated phenolics and most contained less than one mole of carbohydrate, as mannose, per mole of protein (assuming an average molecular weight of 25,000), the authors suggested that the glycosylation and association with phenolic compounds were not responsible for the resistance.

2.1.3. Yeast Proteins. It has been estimated that mannoproteins constitute around 35% of total polysaccharides in red wine (Vidal et al., 2003). These proteins are heterogeneous, ranging in size from 5000 to greater than 400,000 daltons, including a 420 kDa mannoprotein identified by Waters et al. (1994a) which was about 30% polypeptide and 70% carbohydrate, of which 98% was mannan. Most of the mannan is *N*-linked, where the glycan group is linked to an asparagine, as part of the conserved sequence Asn-Xaa-Ser/Thr where Xaa is any amino acid except proline. The *N*-linked mannan groups are comprised primarily of α-1→6 linkages and may be heavily branched, generally α-1→2 or 1→3 (Creighton, 1992). *O*-linked glycan groups on the other hand have been described as consisting primarily of small chains of one to four D-mannose resides linked α-

1→4 or 1→3 which are linked to serine or threonine residues (Waters et al., 1994a)

Mannoproteins play an important role in the colloidal stability of wines. The presence of mannoproteins can inhibit the crystallization of potassium tartrate crystals, or "wine diamonds," (Moine-Ledoux et al., 1997). Mannoproteins also inhibit tannin aggregation, as well as protein precipitation (Dupin et al., 2000), a key to the prevention of wine hazes.

2.1.3.1. Aging Wine on Yeast Lees. The process of aging wine on the lees, or 'sur lies' in French, refers to storing the wine in contact with the yeast solids, primarily *Saccharomyces cerevisiae* cells, for an extended period of time following alcoholic fermentation (Fornairon et al., 2002). This process has traditionally been used primarily for white wines, such as those with grand cru distinction, and especially sparkling wines, which may be aged in bottle with the lees for several years. However, aging wine on the lees has become more prevalent for all wine styles, including red wines (Fornairon et al., 2002), such as Pinot noir.

The specific aging process varies depending on region, wine varietal and the winemakers' preferences. Some of the processes are regulated in parts of the world, such as by EEC regulation no. 822/87, which states that a 'sur lies' wine may contain up to 5 percent fresh lees from a recent production of dry wine

(Fornairon et al., 2002). Also, for some styles, the length of time the wine is aged may be regulated, but for many wines this is controlled only by the preferences of the winemaker, and may range from only a few months to several years. The unregulated process of 'batonnage,' or stirring of the lees, is a procedure typical of Burgundian wines in which the stirring in barrel is meant to resuspend the lees to increase extraction of yeast protein, carbohydrate and other constituents as the yeast undergo autolysis (Fornairon et al., 2002).

During the process of aging wines on the lees, yeast undergo limited autolysis (below) releasing proteins, fatty acids, nucleic acids, and other cellular components that affect the quality of wine. Feuillat (2003) has also suggested using yeast strains that produce and secrete more mannoproteins, as well as strains which undergo autolysis more rapidly following fermentation. The addition of enzymes to break down yeast cell walls has also been employed to speed extraction.

2.1.3.2. Yeast Autolysis. During autolysis, hydrolytic enzymes hydrolyze cell polymers releasing internal components into the wine, including nucleotides, amino acids, peptides and fatty acids, and also components of the cell wall such as mannoproteins. This process is slow due to low cellar temperatures, which slow cell death and reduce hydrolysis rates. The basic process of yeast autolysis begins as cytoplasmic structures start to degrade releasing vacuolar

proteases into the cytoplasm. These proteases are inhibited at first, but over time, the inhibitors also degrade and the proteases can then act on other cellular components. Degradation products accumulate within the cell, but over time are extracted into the wine as a function of size, solubility and stability (Babayan et al., 1981).

2.1.3.3. Proteolysis. The primary protease involved in yeast autolysis in wine is protease A (Lurton et al., 1989). Although numerous proteases are present in yeast, a mutant lacking proteinase A was used to show that the enzyme is responsible for 60% of nitrogen release during yeast autolysis in wine (Alexandre et al., 2003). Other proteases active during autolysis in wine have been identified as yapsin proteases (Komano et al., 1999; Olsen et al., 1999). Proteolytic enzymes become much more active following sugar depletion in the wine (Alexandre et al., 2001), and the activity subsides after aging three months at pH 3 at 10° C, however at higher temperatures protease activity decreases more rapidly (Sato et al., 1997). In sparkling wines however, protease activity decreases during bottle fermentation and then increases after aging for nine months (Feuillat and Charpentier, 1982).

2.1.3.4. Degradation of Cell Wall. The yeast cell wall is also partially hydrolyzed during autolysis. Degradation is caused by the action of

glucanases (Charpentier and Freyssinet, 1989). Glucose, oligosaccharides and mannoproteins are released when endo- and exo-β-glucanases hydrolyze the β-*O*-glycosidic linkages of β-glucan chains. Proteases also act on the cell wall, releasing amino acids and peptides (Hien and Fleet, 1983). The process of cell wall degradation in wine has been described (Charpentier and Freyssinet, 1989), as beginning with glucanases hydrolyzing glucans, which releases mannoproteins that were covalently linked to the glucans. The glucans are then released from the cell wall. Finally, the protein moiety of mannoproteins is degraded by proteolysis.

2.1.4. Protein Effects on Wine Quality

2.1.4.1. Haze Protection. Wine clarity, especially that of white wines, is a key factor determining consumer acceptability. For this reason, a great deal of research has been conducted to determine what causes hazes in wine and how they can be eliminated. The most common method of removing the grape proteins that cause hazes is bentonite fining. However, use of bentonite may reduce wine quality by also removing aroma compounds (Waters et al., 1996) and other desirable components of the wine. A significant loss of wine can also occur because the bentonite does not pack tightly upon settling.

It has been found that certain mannoproteins released from yeast during fermentation and aging on the lees also help reduce the turbidity of wine. Two

high molecular weight mannoproteins have been isolated, which are able to prevent visible haziness, HPF1 and HPF2 (Waters et al., 1993 and 1994a). Other glycoproteins have also been shown to protect wine from developing a haze including a 32 kDa glycopeptide from yeast invertase (Moine-Ledoux and Dubourdieu, 1998), a glycoprotein from grapes containing arabinogalactan (Waters et al., 1994b), and an arabinogalactoprotein from apples was demonstrated to prevent hazes in wine (Pellerin et al., 1994). The common tie between all of these proteins is that they all contain a large amount of carbohydrate, either mannan in yeast-derived protein, or arabinogalactan in grape-derived protein. However, not all glycoproteins will prevent hazes, as the specific structure of the carbohydrate is important. It was shown that Hpf2p produced in *Pichia pastoris* had an altered glycan structure from that of Hpf2p produced in *S. cerevisiae* and this was associated with a reduced haze-protective function (Schmidt et al., 2009). While the mechanism of haze protection is not clear, it has been shown that mannoproteins do not prevent wine proteins from aggregating, but only reduce the average particle size of the aggregates (Waters et al., 1993).

2.1.4.2. Protein Interactions With Phenolic Compounds. A key component of red wines are phenolic compounds, responsible for pigmentation, in the form of anthocyanins and their derivatives, and also for astringency and bitterness in the form of tannins. Yeast lees interact with phenolics primarily by

adsorption on the surface of the cell wall, but also by releasing enzymes during autolysis that may alter the phenolic compounds (Mazauric and Salmon, 2005, 2006). The effect yeast lees has on proanthocyanidins, the polymers of flavanoids such as catechin, was modeled by Mazauric and Salmon (2005) by suspending lees and wine polyphenols in a model wine and monitoring interactions over time. This group found that the initial reaction is a rapid adsorption of the polyphenols on the yeast lees, followed by slower fixation reaching a maximum after about a week. In red wines, the presence of lees has the general effect of decreasing the color of wine over time, and this decrease is significant in wines aged in oak (Rodriguez et al., 2005).

Another effect of aging on lees conferred on the polyphenol content of the wine is determined by oxygen consumption. Salmon et al. (2002) studied the interaction of yeast lees with oxygen during aging. This work detected an overall decrease in oxidation in a model wine containing both yeast lees and polyphenols, as compared to either component separately. This was attributed to the interaction between the polyphenols and yeast cell membrane lipids, which are suspected to be the main targets of oxidation in yeast lees. The strong interaction between polyphenols and lipids, reduces the number of lipids exposed to oxygen, and thereby reduces lipid oxidation.

Mannoproteins from yeast are believed interact with phenolics, reducing astringency and improving color stability (Vidal et al., 2004; Fornairon-

Bonnefond, 2002). The presence of mannoproteins has been associated with slowing tannin aggregation, which delays precipitation of the aggregates (Riou et al., 2002).

2.2. Protein Analysis in Wine

2.2.1. Red Wines. Until recently, it was thought that there was little to no protein in red wines, due to the fact that proteins bind to tannins. It was assumed that all red wine protein would precipitate during the winemaking process due to the abundance of tannins in red wines (Yokotsuka and Singleton, 1997; Singleton and Trousdale, 1992; Berg, 1963). As a result, few studies have analyzed red wine proteins. Early studies neglected to report protein values for red wines, even when values were reported for white wines in the same experiments (Pilone and Berg, 1965). Analysis by SDS-PAGE has also been difficult. Marshall and Williams (1987) stated that SDS-PAGE stained with silver "is unsuitable for wine, particularly the red which gave patterns totally obscured by background stain." However, studies have identified protein in red wine in concentrations similar to whites (Yokotsuka et al., 1994). While some of the proteins precipitate due to excess tannin during vinification, heat-stable glycoproteins remain soluble (Yokotsuka and Singleton, 1997).

Because there is no standard protocol for the analysis of wine protein, a variety of different methods have been used. The results from each method are

not necessarily equivalent because a number of compounds in wine can interfere, such as phenolic compounds, which interfere with the Lowry, Bradford and Smith assays and with A_{280} measurements (Moreno-Arribas et al., 2002). Furthermore, a given protein may react to varying degrees in each of these assays resulting in apparently variable protein concentrations (Moreno-Arribas et al., 2002). For example, yeast invertase is nearly twice as reactive in the BCA assay as in the Bradford assay, when reported in BSA equivalents. In addition, because proteins are generally present in low concentrations in wine, it is often necessary to concentrate wine samples, which can lead to additional errors of under or over estimation (Moreno-Arribas et al., 2002) because different methods may differentially concentrate interfering compounds.

A number of methods have been used to concentrate and quantify protein in red wines. Ammonium sulfate precipitation has been used to concentrate protein in Muscat Bailey A, Cabernet sauvignon, Pinot noir and Merlot wines (Yokotsuka et al., 1994) and in Muscat Bailey A and Cabernet sauvignon wines (Fukui and Yokotsuka, 2003). Following precipitation in both studies, wines were dialyzed for 3 days and total nitrogen was measured by micro-Kjeldahl, with the protein then calculated as 6.25 times the nitrogen content. While the amount of wine used to quantify protein content was not specified, 73 liters of wine were used to obtain about 1,500 mg of ammonium sulfate-precipitable material. Using this method, ten vintages of Muscat Bailey A were analyzed. No correlation was

found between wine age and protein concentration (Yokotsuka et al., 1994). Within this group of red wines, protein concentrations ranged from 33 to 87 mg/L, with no significant differences observed between different varietals (Yokotsuka et al., 1994; Fukui and Yokotsuka, 2003). Of relevance to the present study, the Pinot noir tested contained 77 mg/L protein 6 years after vinification (Yokotsuka et al., 1994).

In addition to measuring the protein by Kjeldahl analysis, Yokotsuka et al. (1994) also used amino acid analysis of hydrolyzed proteins (by HPLC equipped with a ninhydrin reaction unit) to measure protein content in the same ammonium sulfate-precipitated samples and found the ratio of protein detected by micro-Kjeldahl to amino acid analysis to be 1 to 0.76. In a subsequent study, Yokotsuka et al. (1997) found that protein content of a red wine (Muscat Bailey A) estimated by amino acid analysis ranged from about 0.1 to 34% of the value measured by micro-Kjeldahl analysis. Because these were found to be glycoproteins, it is possible that Maillard reaction products generated during the amino acid analysis reduced the free amino acid content (Yokotsuka et al., 1997). Fukui and Yokotsuka (2003) also used amino acid analysis to measure protein in wines that had simply been dialyzed against distilled water for 3 days.

One study used a method that does not require any concentration steps. To determine the amount of peptide nitrogen in Tempranillo wines, Alcaide-Hidalgo et al. (2002) measured total wine nitrogen by micro-Kjeldahl analysis and

then measured free amino nitrogen by Cd-ninhydrin analysis, based on reaction of amino groups in an acid medium with a solution of $CdCl_2$ with ninhydrin as quantified by A_{507} values. Peptide nitrogen was calculated as the difference between total and free amino nitrogen. This method detected between 150 and 200 mg/L peptide nitrogen, but only about 30 mg/L of free amino nitrogen. Based on the values reported for other wines, it appears that these data, presented in figure 1 of Alcaide-Hidalgo et al. (2002), may have been reversed.

Vincenzi et al. (2005) tested a variety of methods for isolating and quantifying proteins in wine. Both red and white wines were ultrafiltered on a 1 kDa cutoff filter to remove endogenous proteins. BSA was then added to the protein-free wines prior to analysis. To isolate the BSA from the wines, 5 techniques were tested: dialysis and lyophilization, and precipitation methods using potassium dodecyl sulfate (KDS), acetone, ethanol or TCA. The isolated protein was then measured by three different assays, Bradford (Bradford, 1976), Lowry (Lowry et al., 1951) or Smith (Smith et al., 1985). The best agreement between protein added and protein measured was found with the combination of KDS precipitation and the Smith assay. Rowe et al. (2010) utilized this combination to measure protein released by yeast during fermentation of a synthetic must and during aging on the lees for 9 months following fermentation. While glycoproteins were isolated and quantified, the amounts detected were likely an underestimate because Fusi et al. (2010) has since demonstrated that the

KDS protocol precipitates glycoproteins inefficiently. They suggested that the dodecyl sulfate (DS) binds non-glycosylated proteins with greater affinity than glycoproteins, and therefore, the insolubilization of DS by addition of potassium is not adequate to precipitate the glycoproteins.

2.2.2. Identification of red wine proteins. As of this writing, only one group has identified proteins in red wine. To isolate proteins from red wines, Wigand et al. (2009) dialyzed 200 mL of Portugieser red wine against water using a 3.5 kDa cutoff dialysis membrane for at least five days. The dialyzed wines were then lyophilized, resulting in about 220 mg of solids, which were dissolved in sodium phosphate buffer and treated twice with polyvinylpyrrolidone (PVP) to remove polyphenols. After the remaining PVP had been removed by centrifugation and filtration through a 0.22 μm polyethersulfone (PES) filter, the sample was again lyophilized to concentrate the protein before performing SDS-PAGE. Protein bands were visualized by Coommassie staining. The most intense bands were between 25 and 30 kDa in size with additional bands in the range of 60 to 70 kDa and a band at approximately 12 kDa. The bands were then digested in-gel and tryptic peptides were analyzed by Electrospray Ionization Quadrupole - Time of Flight Mass Spectrometry (ESI-Q-TOF). This analysis identified 121 tryptic peptides from the German Portugieser red wine, which were attributed to 12 grape proteins (including lipid transfer protein, VVTL1, class IV

endochitinase, and vacuolar invertase 1), and 6 yeast-derived proteins (Tos1 precursor, Cis3 precursor, Cwp1 precursor, Crh1 precursor and endochitinase precursor Cts1). Of the identified yeast proteins, several are known to be *O*-glycosylated, including Cis3 precursor, cell wall protein Cwp1 precursor, and endochitinase precursor Cts1. The proteins Tos1, the probable glycosidase Crh1 precursor and Ecm33 also have potential *N*- and *O*-linked glycosylation sites. Though this important study (Wigand et al., 2009) identified proteins for the first time in red wines, proteins were not quantified. A relatively large volume of wine was used (200 mL) and several fractionation steps were required.

2.2.3. White Wines. Proteins in white wines have generally been isolated by precipitation with ammonium salts (Waters et al., 1996; Kwon, 2004; Waters et al., 1995; Marangon et al., 2009), liquid chromatography (Van Sluyter et al., 2009; Waters et al., 1995), or by KDS precipitation (Rowe et al., 2010 using synthetic white wine; Fusi et al., 2010). Protein concentrations have been determined by measuring A_{280} of HPLC fractions (Falconer et al., 2010; Marangon et al., 2009), and use of the BCA-Smith assay (Rowe et al., 2010; Fusi et al., 2010). Due to a lack of tannins in white wines, it is even possible to simply measure protein directly in the wine with the Bradford assay. By use of this latter approach, Kwon (2004) detected 11.2 mg/L of protein, as BSA equivalents, in a Sauvignon blanc wine.

Utilizing the isolation and purification methods above, coupled with mass spectrometry, a number of studies have identified proteins in white wines including Semillon (Van Sluyter et al., 2009; Marangon et al., 2009; Falconer et al., 2010), Sauvignon blanc (Van Sluyter et al., 2009; Kwon, 2004; Falconer et al., 2010), and Muscat of Alexandria (Waters et al., 1996). The identified proteins of white wines are essentially the same as those listed earlier in the single study of red wines. The majority of the proteins are grape PR proteins including, VVTL1, chitinases, osmotin-like protein, and β-1,3-glucanase (Van Sluyter et al., 2009; Kwon, 2004; Marangon et al., 2009). Of the yeast proteins that have been identified, proteins of the cell wall predominate including Ecm33, Tos1, Crh1, β-1,3-glucanosyltransferase (Gas1) and endo-β-1,3-glucanase (Bgl2 and Exg2) (Kwon, 2004). A number of the same proteins were identified during fermentation and aging experiments of model wine (Rowe et al., 2010).

3. Materials and Methods

3.1. Protein Isolation

3.1.1. KDS Precipitation. Protein was isolated and assayed essentially as described by Vincenzi et al. (2005). Briefly, 10.1 µl of a 10% SDS solution were added to 1 ml of wine pre-filtered through a 0.45 µm filter in a 1.7 ml screw-capped tube, vortexed vigorously, and placed in a 100° C water bath for 5 minutes. Tubes were cooled quickly to room temperature on ice, and to each, 252.2 µL of 1 M KCl were added. The tubes were then mixed gently for 30 min at room temperature. The resulting mixture of wine and protein precipitate was centrifuged at 22,000 x *g* at 4°C. The pellet was washed twice with 1 M KCl at 4° C, and solubilized in 1 ml of distilled water.

3.1.2. Acetone Precipitation. Protein was isolated from wine by adding 2 volumes of ice-cold acetone to one volume of wine, which had been pre-filtered through a 0.45 µm PES syringe filter. Samples were then incubated for 45 minutes at -20° C, and centrifuged for 15 minutes at 22,000 x *g* at 4° C. The pellet was then washed once with -20° C acetone, and air-dried. The precipitate was solubilized in distilled water.

3.1.3. Acetone/TCA Precipitation. Protein was isolated from wine by adding 2 volumes of ice-cold acetone containing 10% (w/v) tricholoroacetic acid

(TCA) to one volume of wine, which had been pre-filtered through a 0.45 μm PES syringe filter. Samples were then incubated for 45 minutes at -20° C, and centrifuged for 15 minutes at 22,000 x *g* and 4° C. The pellet was then washed once with -20° C acetone, and air-dried. The precipitate was solubilized in distilled water.

3.2. Protein Quantification

3.2.1. Smith-BCA Assay. Protein was measured in the solubilized wine precipitates based on the bicinchoninic acid (BCA) assay (Smith et al., 1985), using a commercial kit (Pierce Laboratories, Rockford, IL) following the manufacturer's instructions.

3.2.2. Bradford Assay. Protein was measured in the solubilized wine precipitates based on the Bradford method (Bradford, 1976), using a commercial kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions for the micro-assay procedure.

3.2.3. Micro-Kjeldahl Assay. Total nitrogen was measured in solubilized wine precipitates following the protocol outlined in the AOAC Official Method 960.52 (AOAC, 2000). Protein was then calculated by multiplying the nitrogen content by 6.25.

3.3. Mannan Analysis

3.3.1. Immunoblotting. Mannoproteins were quantified by immunoblotting, essentially as described by Rowe et al. (2010), performed according to the manufacturer's instructions (Bio-Dot® SF Microfiltration apparatus instruction manual, Bio-Rad Laboratories, Hercules, CA). Acetone precipitates from wines were washed five times on a 10 kDa centrifugal PES membrane filters to remove low molecular weight material. By the fifth wash, BCA-reactive material in the filtrate corresponded to less than 1 mg/L BSA equivalents. After the final, wash the volume was adjusted to that equal to the initial volume of wine. Aliquots were diluted into a final volume of 200 µl and the entire 200 µl were loaded into wells in duplicate. Standards (yeast invertase, #I4504, and mannan, #M7504, Sigma-Aldrich, St. Louis, MO) were blotted onto each nitrocellulose membrane (0.45 µm Bio-Rad Laboratories Hercules, CA) in a 48-well slot blot apparatus. After the samples and standards were loaded onto the membrane by gravity flow, 250 µl per well of Tris-buffered saline (20 mM Tris, pH 7.5, 500 mM NaCl, TBS) were added under vacuum. The membrane was then removed from the apparatus, placed in a plastic box, and rinsed twice with a blocking/wash solution of Tris-buffered saline containing Tween 20 (TBS + 0.1% Tween 20, TBST) for five minutes per rinse. The membrane was then incubated for 30 minutes at room temperature with continuous mixing with 4 µg/ml of the mannose-specific, biotinylated *Narcissus pseudonarcissus* lectin (Vector Labs,

Burlingame, CA) in TBST. The membrane was washed twice with TBST and subsequently placed in TBST containing 1 µl/ml of streptavidin-conjugated alkaline phosphatase (Vector Labs) for 30 minutes at room temperature with continuous mixing. The membrane was then washed twice in TBST and rinsed once in TBS for five minutes to remove residual Tween. Following this, the membrane was equilibrated in 100 mM Tris pH 9.5 for five minutes. After equilibration, the membrane was removed, shaken to remove excess liquid, and placed blotted side up on top of plastic wrap within a dry plastic box under subdued light. The chemiluminescent alkaline phosphatase substrate DuoLuxTM (Vector Labs) was then added at a rate of 4.45 ml per 9x12 cm membrane, which was covered with plastic wrap to uniformly spread the substrate, and incubated under subdued light for 5 minutes at room temperature. The membrane was then removed and rinsed in 100 mM Tris pH 9.5 for an additional 5 minutes to reduce background exposure. Exposure to X-ray film (HyperfilmTM, Amersham Biosciences, Buckinghamshire, UK) was done in a darkroom with the membrane sandwiched between clean 8.5 x 11" plastic sheets to protect the film from moisture. The film was exposed between 30 seconds to 1 minute. After developing the film, it was scanned and the integrated densities were determined using ImageJ image analysis software (U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/) and compared to standards to determine mannan concentrations.

3.3.2. Phenol-Sulfuric Acid Carbohydrate Analysis. Carbohydrate was measured following the protocol outlined in *Current Protocols in Food Analytical Chemistry* E1.1.1-E1.1.8 (Fournier, 2001). Briefly, 25 µl of solubilized acetone precipitate was mixed with 500 µl of 4% phenol (w/v) in water. Concentrated sulfuric acid (2.5 ml) was then added down the side of the glass test tube, which was gently vortexed. Absorbance of the sample at 490 nm was measured and compared to a standard curve based on mannose.

3.4. Tannin Analysis

Tannin was measured as described by Harbertson et al. (2002). Briefly, 250 µl wine was diluted 2-fold in a buffer containing 12% ethanol (v/v), and 6 g/L potassium bitartrate, adjusted to pH 3.5. The tannin was then precipitated for 15 minutes at room temperature by addition of 1 ml of buffer containing 1 mg/ml BSA (Fraction V, VWR), 200 mM acetic acid and 170 mM NaCl, adjusted to pH 4.9. The precipitate was pelleted by centrifugation at room temperature for 5 minutes at 22,000 x *g*, and was washed once with 250 µl of the pH 4.9 buffer. The pellet was then resuspended in 875 µl of buffer containing 5% triethanolamine (TEA) (v/v) and 5% SDS (w/v). The initial absorbance was measured at 510 nm, after which 125 µl of ferric chloride reagent were added (10 mM $FeCl_3$ in 0.01 N HCl) and the absorbance was measured again after a 15-

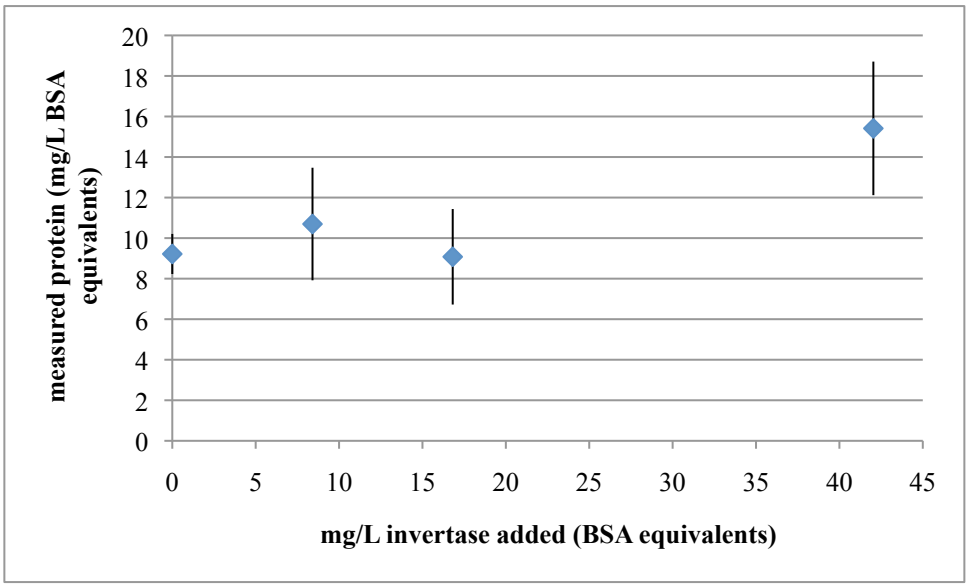
minute incubation. The difference between the two measurements was taken as the amount of tannin based on a standard curve using (+)-catechin.

4. Results and Discussion

4.1. Protein Precipitation and Quantification

A number of approaches for assaying protein in red wine were evaluated. Initially, we proposed measuring protein in Pinot noir wines by first precipitating the proteins using the KDS method and then quantifying the solubilized protein with the BCA assay, as was done previously to measure protein in model wine fermentations (Rowe et al., 2010). To verify that this method would work with red wines, various concentrations of *S. cerevisiae* invertase (Sigma #I4504) were added to wine samples prior to precipitation. As can be seen below (**Figure 1**), less than 15% of the added invertase was recovered, and the results were erratic and inconsistent. For example, when yeast invertase was added at 8, 17 or 42 mg/L BSA equivalents to a wine containing 9 mg/L endogenous protein, an additional 1.5, 0 or 6.2 mg/L was measured, respectively. These results are consistent with the report of Fusi et al. (2010) that showed the KDS precipitation method did not precipitate glycoproteins from Italian white wines, as determined by periodic acid-Schiff (PAS) staining for glycan in solubilized precipitates run on SDS-PAGE.

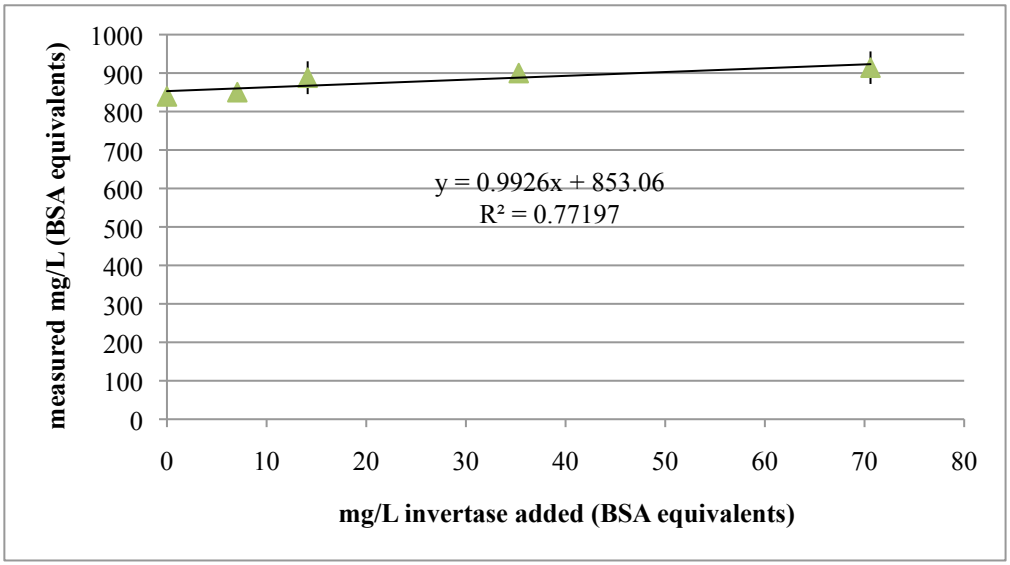
Figure 1. Protein measured by the BCA assay in wine following addition of yeast invertase, KDS precipitation and dissolution in water.



Data are means of triplicate precipitations, error bars are standard deviations.

An alternative approach for precipitating wine protein using ice-cold acetone was also evaluated (Deutscher, 1990). This method had been ruled out by Vincenzi et al. (2005) because in a wine that had been ultrafiltered through a 1 kDa cutoff filter, subsequent use of ice-cold acetone precipitated an apparent 250 mg/L protein as detected by the BCA assay. Similarly, in wine that has not been ultrafiltered, the present study measured nearly 850 mg/L BSA equivalents, which is much higher than would be expected. However, in the present study, the recovery of added invertase by use of this precipitation method was nearly 100%, as demonstrated by the slope of the linear regression equation (**Figure 2**).

Figure 2. Acetone precipitation of wine, measured by the BCA assay.

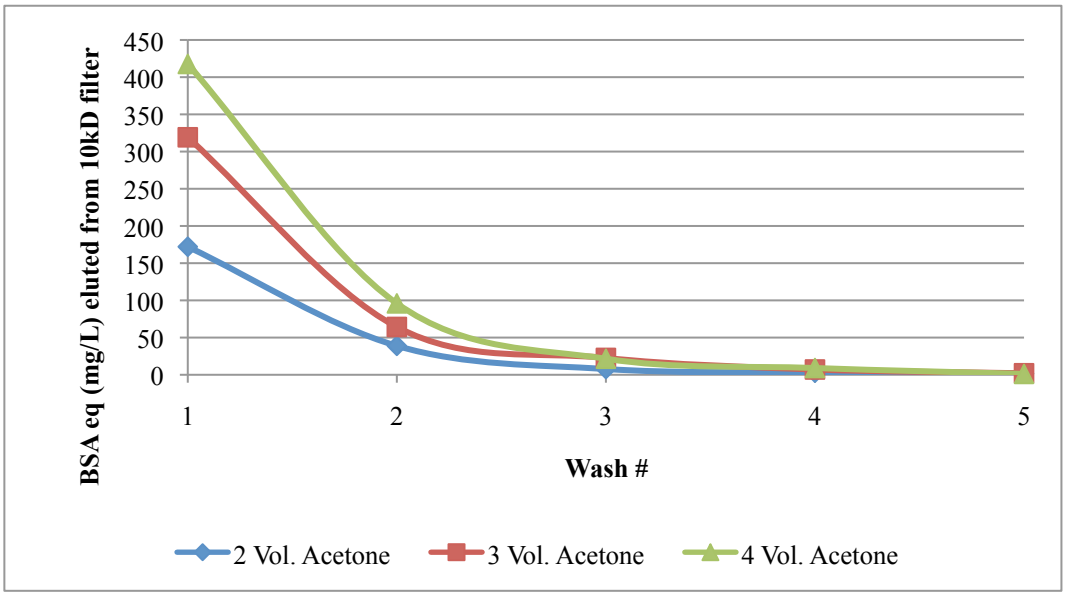


Data are means of duplicate measurements, error bars are standard deviation.

The wines tested by Vincenzi et al. (2005) had been ultrafiltered through a 1 kDa cutoff membrane, to prepare protein-free wines to which BSA had subsequently been added to allow analysis of various methods of isolating and quantifying protein. Because the filtrate was analyzed, it is likely that much of the background interference could have been due to low molecular weight phenolics, and reducing sugars, which are reactive in the BCA assay (BCA kit instructions, Pierce Laboratories, Rockford, IL). To eliminate this co-precipitated material, the solubilized protein precipitates were washed with distilled water, on 10 kDa PES centrifugal membrane filters (Acetone/10 kDa). After each concentration step, the sample was returned to its original volume with distilled

water, and the eluents were monitored to determine when all of the BCA-reactive, low molecular weight material had been eliminated. After 5 washes, the eluent contained no BCA-reactive material (**Figure 3**). The retentate was removed and the cartridge was rinsed with enough distilled water to return the sample to its original volume. Analysis of the retentates from wine samples precipitated with 2, 3 or 4 volumes of acetone to one volume of wine gave nearly identical results. However, based on the washes, it appears that less low molecular weight material was precipitated by addition of only 2 volumes of acetone. Therefore, 2 volumes of acetone were used in subsequent trials.

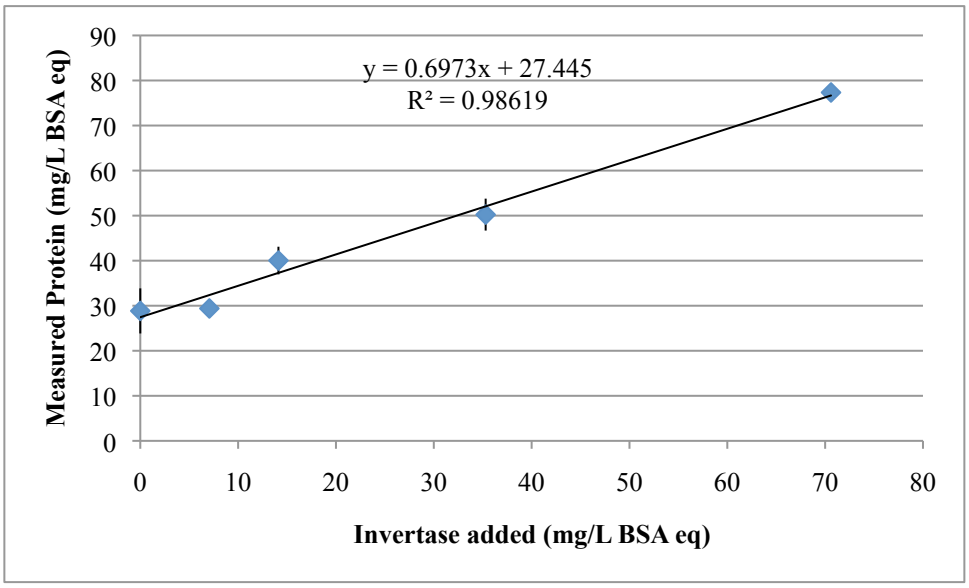
Figure 3. Eluent from Acetone/10kDa samples, measured by the BCA assay.



Data are measurements from pooled washes from duplicate samples.

To validate the method, yeast invertase was added to wine samples, and assayed for recovery. Although the recovery was only about 70% (**Figure 4**), the results were consistent, with variance less than 20% of means, for several representative wines.

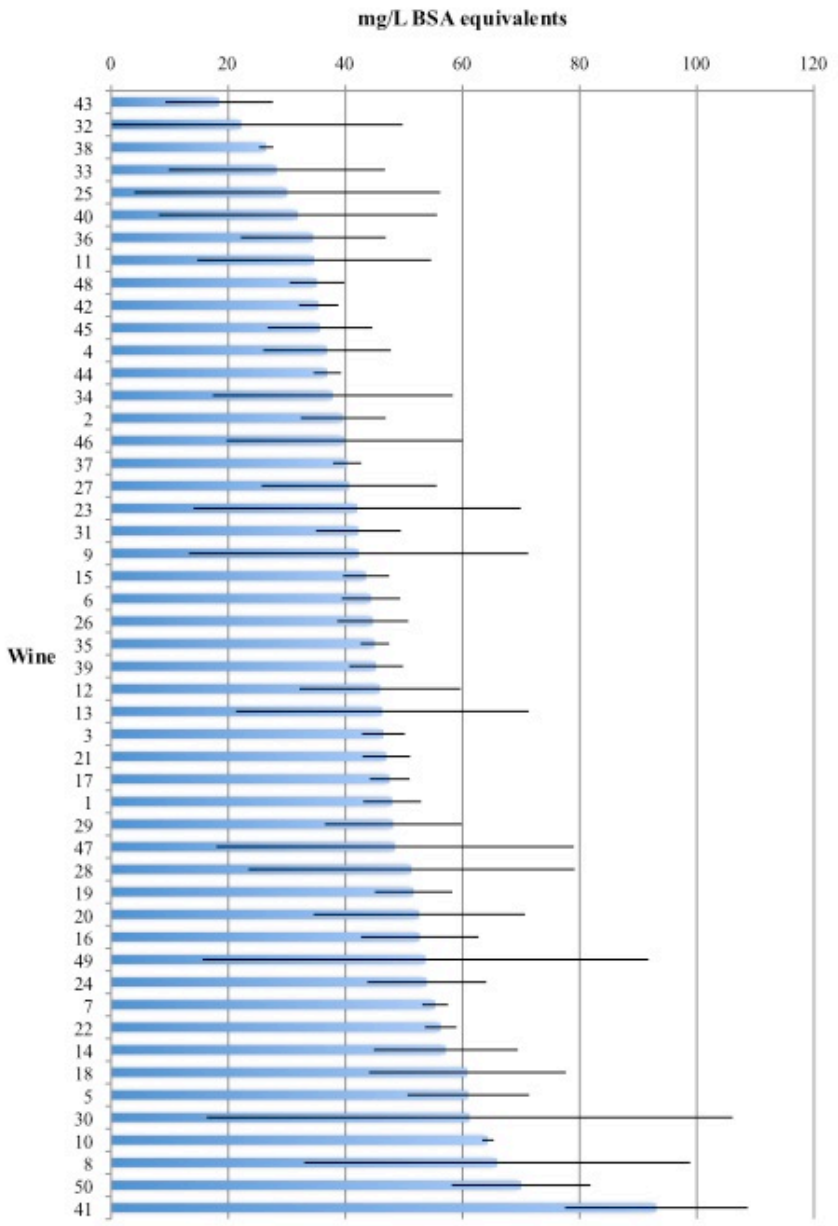
Figure 4. Protein measured in acetone/10 kDa samples from wine spiked with invertase as measured by the BCA assay.



Data are means of duplicate analyses, error bars are standard deviations.

Because this method appeared promising, a set of 50 Pinot noir wines was assayed by the acetone/10 kDa procedure. Unfortunately, significant variance between repetitions was apparent. Only half of the samples had variances less than 20% of their means (**Figure 5**). Because of this high variance, a more reliable method for assaying protein in red wines was sought.

Figure 5. Protein measured in acetone/10 kDa wine samples measured by the BCA assay.



Data are means of triplicate measurements, error bars are standard deviations.

Upon assaying the same acetone-precipitated samples using the Bradford method, it was evident that the material that interfered with the BCA assay did not interfere with the Bradford assay. While consistent results were obtained, the values were only a quarter as high as those obtained by the BCA assay of the same acetone/10 kDa wines in BSA equivalents. It was also apparent that invertase, a yeast mannoprotein, was less reactive in the Bradford assay than in the BCA assay. For example, if a solution of 100 μg/ml invertase was measured with the BCA assay, it corresponded to approximately 18 μg/ml BSA equivalents but only 9 μg/ml BSA equivalents, as measured by the Bradford assay. Because different proteins react differently in different protein assays, it was concluded that it would be better to report measured proteins as invertase equivalents rather than BSA equivalents, because as a glycoprotein, invertase is more representative of actual wine proteins. To confirm these findings, solubilized acetone precipitates of the wines were then assayed by the micro-Kjeldahl assay to measure total nitrogen. The values obtained by micro-Kjeldahl most closely matched those of the invertase equivalents measured in the acetone/10 kDa wines measured by the BCA assay (**Table 1**). Because much of the earlier variance may have stemmed from washing the acetone precipitates on the centrifugal filters, further options were explored to find a more reliable protein precipitation method.

Table 1. Comparison of protein quantification methods using acetone-precipitated wine samples.

Coded Wine	Protein by micro-kjeldahl (mg/L)	BSA eq (mg/L) by BCA	BSA eq (mg/L) by BCA[a]	Invertase eq (mg/L) by BCA[a]	BSA eq (mg/L) by Bradford	Invertase eq (mg/L) by Bradford
1	221.05	587.61± 16.20	47.99 ± 4.95	271.91 ± 28.05	10.20 ± 0.47	113.35 ± 5.23
2	214.48	517.43 ± 17.34	39.63 ± 7.26	224.54 ± 41.14	8.85 ± 0.60	98.29 ± 6.65
3	203.54	546.2 ± 48.83	46.49 ± 3.68	263.41 ± 20.85	9.94 ± 1.30	110.42 ± 14.40
4	223.24 ±6.19	508.69 ± 26.72	36.87 ± 10.90	208.91 ± 61.76	9.91 ± 0.48	110.09 ± 5.31

Data are means of 2 or 3 replicates ± standard deviations.
[a] protein measured in solubilized acetone/10 kDa precipitates.

By precipitating the proteins in wine with acetone, then solubilizing the pellet in water and performing additional precipitations, much of the BCA reactive material could also be eliminated. Following the fourth precipitation, protein values measured by the BCA assay (**Table 2**) were similar to the values obtained by the acetone/10 kDa procedure (**Table 1**).

Table 2. Effect of repeated acetone precipitations on protein measured by the BCA assay.

Coded Wine	1st Precipitation	2nd Precipitation	3rd Precipitation	4th Precipitation
1	552.55 ± 94.09	131.72 ± 0.46	66.16 ± 3.81	50.74 ± 4.74
2	499.53 ± 5.74	115.55 ± 0.43	65.27 ± 11.83	43.32 ± 1.26

Data are means ± standard deviations of duplicate measurements. Values are reported in mg/L BSA equivalents.

While the method using repeated acetone precipitations appeared promising, a third precipitation technique was evaluated. The acetone/TCA method, in which two volumes of ice-cold acetone acidified with 10% TCA (w/v) were used to precipitate one volume of wine pre-filtered through a 0.45 μm PES filter, also yielded consistent results, with variance less than 20% of means. Protein concentrations measured using the BCA assay were similar to the values measured with acetone/10 kDa processed wine samples, ranging from 40 to 60 mg/L BSA equivalents. Micro-Kjeldahl analysis was used to validate the method. The values that most closely agreed with micro-Kjeldahl analysis were those measured in invertase equivalents by the Bradford assay rather than the BCA assay (**Table 3**). For example, the solubilized acetone/TCA pellet from wine #1 contained about 81 mg/L protein based on micro-Kjeldahl analysis, and about 80 mg/L invertase equivalents by the Bradford assay.

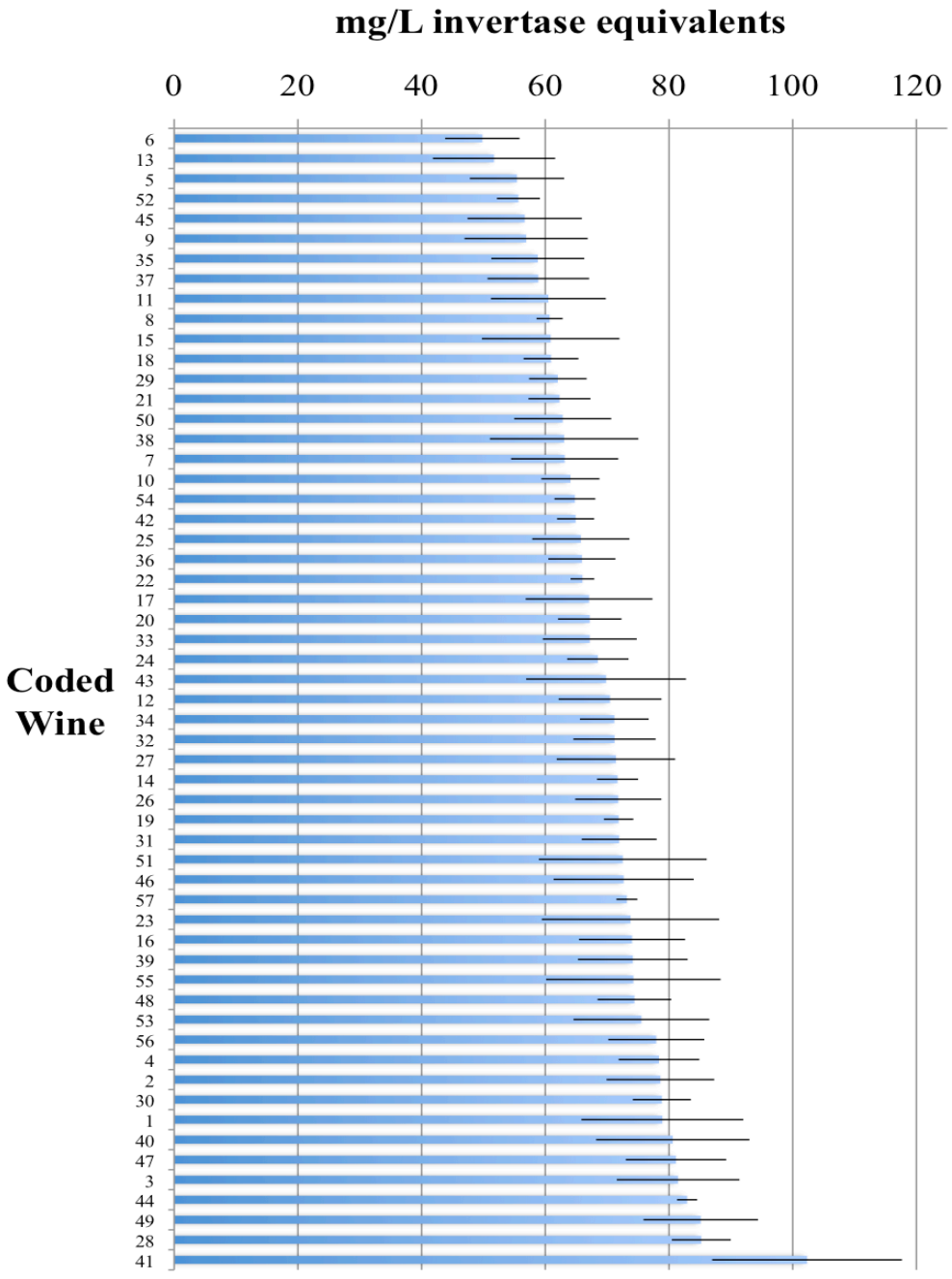
Table 3. Comparison of protein quantification methods using acetone/TCA precipitation.

Coded Wine	Protein by micro-kjeldahl (mg/L)	BSA eq (mg/L) by BCA	Invertase eq (mg/L) by BCA	BSA eq (mg/L) by Bradford	Invertase eq (mg/L) by Bradford
1	80.98 ± 9.29	53.44 ± 4.51	302.47 ± 25.53	7.10 ± 1.18	78.88 ± 13.11
2	89.73	49.29 ± 4.62	278.98 ± 26.15	7.08 ± 0.78	78.66 ± 8.67
3	nd	60.01 ± 0.79	339.66 ± 4.47	7.33 ± 0.89	81.44 ± 9.89
4	nd	45.48 ± 0.70	257.42 ± 3.96	7.06 ± 0.59	78.44 ± 6.55

nd, not determined.

Because the combined acetone/TCA precipitation and Bradford quantification assay were more consistent and rapid than previous methods tested, the set of 50 Pinot noir wines were re-assayed for protein content with 7 additional Pinot noir wines included (**Figure 6, Table 8**).

Figure 6. Bradford measured protein in acetone/TCA-precipitated wine samples.



Data are means of triplicate measurements, error bars are standard deviations.

To ensure that all of the nitrogen being measured in the solubilized acetone/TCA precipitates by micro-Kjeldahl analysis was high molecular weight nitrogen, total nitrogen was measured in wine #1, the acetone/TCA pellet, and in the pellet wash solution. Subsequently, nitrogen was analyzed in dialyzed wine #1, the acetone/TCA pellet from dialyzed wine, and the pellet wash solution. The total wine nitrogen decreased 90% due to dialysis, representing loss of low molecular weight nitrogen. Prior to dialysis, the nitrogen in the pellet only accounted for 4% of the total nitrogen in the wine (**Table 4**). After dialysis, the same amount of nitrogen was recovered, 13-14 mg/L, but it accounted for nearly 40% of total wine nitrogen (**Table 5**). The 60% of nitrogen in the dialyzed wine that did not precipitate was likely low molecular weight nitrogen that did not diffuse out of the dialysis tubing (15 ml of wine were dialyzed in 3.5 kDa tubing at 4° C for 30 hours with stirring against 6 changes of 2 L distilled water). The amount of nitrogen detected in the acetone/TCA pellet after a single acetone wash (**Table 4**) was the same as detected in the pellet following dialysis (**Table 5**), which indicates that the nitrogen in the pellet is associated with high molecular weight material. Significantly, the experiment also demonstrated that the acetone/TCA precipitation protocol was a much faster and more efficient means of obtaining the protein fraction than dialysis.

Table 4. Nitrogen content of wine #1, supernatant and pellet.

	Wine #1	Supernatant/Wash Acetone/TCA	Pellet from wine #1
mg/L nitrogen	314.81 ± 27.24	309.38 ± 34.91	12.96 ± 1.49
Percent of total	100%	98.3%	4.1%

Data are means and standard deviations of duplicate measurements.
Percent of total value calculated based on means.

Table 5. Nitrogen content of dialyzed wine #1, supernatant and pellet.

	Dialyzed Wine #1	Supernatant/Wash Acetone/TCA	Pellet from dialyzed wine #1
mg/L nitrogen	37.12 ± 4.95	21.71 ± 0.50	14.01 ± 2.48
Percent of total	100%	58.5%	37.7%

Data are means and standard deviations of duplicate measurements.
Percent of total value calculated based on means.

Because it was presumed that the nitrogen detected in the acetone/TCA pellet was protein nitrogen, the effect of proteolysis was evaluated. While nucleic acids may account for some nitrogen in wine, Charpentier et al. (2005) found a maximum nucleic acid content of 3 mg/L in wine, which is insignificant compared to protein concentrations detected in the present study, accounting for less than five percent of the average nitrogen found in these Pinot noir wines. Fifteen milliliters of wine #1 was initially precipitated with acetone/TCA to generate a pellet. The pellet was then solubilized in 30 mM Tris buffer, pH 8.5. The solubilized pellet was incubated with 500 µg proteinase K for 14 hours at 50 °C. Samples of proteinase K alone and the solubilized pellet alone were incubated in parallel under the same conditions. Following incubation, all

samples were precipitated with acetone/TCA, and protein in the acetone-washed precipitates was determined by micro-Kjeldahl analysis, where nitrogen content was multiplied by 6.25 to calculate protein, yielding 1,683.9 μg protein in the initial sample containing proteinase K and solubilized pellet. Following the 14 h incubation, the sample containing both solubilized pellet and added proteinase K, 604.1 μg of protein were measured whereas 696.0 and 415.8 μg were measured in the solubilized pellet only and proteinase K only incubations, respectively (**Table 6**). In the sample containing only the solubilized pellet, nearly half of the starting protein did not precipitate after incubation. While some of this loss might be explained by partial hydrolysis of protein in an alkaline environment (pH 8.5) at elevated temperature (50° C), other as-yet undetermined factors are likely to contribute to the observed loss of protein. Because proteinase K incubated alone did not undergo significant self-digestion, where it is more likely to do so than in a sample containing other protein substrates, it is likely that an equal amount, 415.8 μg or more, was contained in the protein recovered from the sample containing both the solubilized pellet and proteinase K. Therefore, while 604 μg of protein was recovered in the acetone/TCA pellet following proteinase K treatment, about 416 μg, or 70%, is likely to be proteinase K, and 188 μg, or 30%, is likely to be non-hydrolyzed wine protein. It follows therefore, that about 85% of the initial wine protein added to the reaction was hydrolyzed.

Table 6. Kjeldahl protein in proteinase K-digested sample.

	Proteinase K alone	Wine #1 precipitate alone	Wine #1 precipitate with proteinase K
Initial	469.2 ±18.6	1214.7 ± 139.4	1683.9[a]
Protein precipitated by acetone/ 10% TCA following 14 hour 50° C incubation	415.8 ± 6.2	696.0 ± 43.3	604.1 ± 99.0
Wine protein digested by protease or hydrolyzed	--	42.7%	84.5%

[a] initial protein estimated based on measured values of individual components.

Protein was assayed by micro-Kjeldahl in the acetone/TCA pellets of four Pinot noir wines covering the range of protein concentrations measured by the Bradford assay among the set of 57 wines subjected to analysis. Protein measured by micro-Kjeldahl analysis and by Bradford assay of the solubilized acetone/TCA pellets are shown in **Table 7.** It is apparent that the protein values measured by the two methods are nearly the same.

Table 7. Kjeldahl validation of Acetone/TCA precipitated protein measurements.

Coded Wine	Kjeldahl Protein (mg/L)	Invertase eq (mg/L) by Bradford Assay
1	80.98 ± 9.29	78.92 ± 13.09
6	62.37 ± 4.64	49.83 ± 6.01
35	77.70 ± 1.55	58.78 ± 7.52
41	103.96 ± 10.83	102.32 ± 15.34

Table 8. Protein, mannan and tannin measured in set of 57 Pinot noir wines.

Coded Wine	Vintage	Protein (mg/L invertase equivalents)	Mannan equivalents (mg/L)	Tannin (mg/L catechin equivalents)
1	2004	78.92 ± 13.09	643.7 ± 191.2	0.0 ± 0.0
2	2004	78.61 ± 8.71	397.2 ± 209.2	0.0 ± 0.0
3	2004	81.47 ± 9.92	699.1 ± 210.7	0.0 ± 0.0
4	2004	78.38 ± 6.53	476.5 ± 236.4	0.0 ± 0.0
5	2004	55.42 ± 7.63	643.8 ± 255.7	13.9 ± 7.3
6	2004	49.83 ± 6.01	546.5 ± 226.3	27.4 ± 8.3
7	2004	63.13 ± 8.67	526.2 ± 292.4	19.3 ± 17.0
8	2004	60.70 ± 2.12	414.3 ± 269.7	28.6 ± 5.4
9	2003	56.90 ± 9.96	347.1 ± 424.6	394.8 ± 11.3
10	2004	64.06 ± 4.71	534.4 ± 330.2	171.9 ± 8.0
11	2003	60.49 ± 9.29	731.5 ± 155.5	425.4 ± 7.3
12	2004	70.47 ± 8.32	685.8 ± 103.6	173.1 ± 3.4
13	2004	51.71 ± 9.89	803.6 ± 69.3	165.7 ± 1.6
14	2004	71.68 ± 3.32	692.3 ± 118.0	151.2 ± 3.5
15	2003	60.87 ± 11.12	761.7 ± 80.1	413.0 ± 6.7
16	2004	74.03 ± 8.58	660.7 ± 143.6	157.8 ± 9.4
17	2004	67.07 ± 10.26	628.3 ± 96.4	176.4 ± 6.2
18	2004	60.93 ± 4.44	534.3 ± 165.6	159.7 ± 7.1
19	2004	71.86 ± 2.39	491.3 ± 192.4	198.5 ± 9.3

20	2004	67.19 ± 5.13	275.4 ± 172.4	162.2 ± 1.9
21	2004	62.29 ± 5.01	567.0 ± 103.9	256.0 ± 4.0
22	2004	66.00 ± 1.92	446.4 ± 139.5	203.0 ± 5.3
23	2004	73.77 ± 14.34	470.0 ± 174.4	226.6 ± 9.7
24	2004	68.49 ± 4.96	529.2 ± 55.9	173.6 ± 6.1
25	2004	65.74 ± 7.86	393.2 ± 321.7	177.8 ± 8.9
26	2004	71.80 ± 6.97	525.5 ± 88.8	161.6 ± 5.1
27	2004	71.42 ± 9.58	480.6 ± 24.9	232.5 ± 5.9
28	2004	85.22 ± 4.76	389.9 ± 106.6	236.0 ± 13.6
29	2004	62.03 ± 4.66	480.5 ± 136.3	204.8 ± 5.8
30	2004	78.84 ± 4.69	404.2 ± 266.0	198.7 ± 1.3
31	2004	71.94 ± 6.06	552.9 ± 23.5	161.8 ± 15.4
32	2004	71.19 ± 6.65	289.9 ± 140.7	196.7 ± 5.4
33	2004	67.19 ± 7.60	536.2 ± 57.2	113.0 ± 2.8
34	2004	71.16 ± 5.55	517.2 ± 143.9	139.6 ± 11.0
35	2004	58.78 ± 7.52	509.3 ± 78.6	121.3 ± 1.9
36	2004	65.91 ± 5.43	515.0 ± 67.1	113.8 ± 2.7
37	2004	58.87 ± 8.21	387.4 ± 82.2	107.7 ± 6.2
38	1979	63.05 ± 12.00	307.3 ± 55.4	12.0 ± 8.8
39	1980	74.15 ± 8.86	228.0 ± 58.1	113.3 ± 9.8
40	1983	80.61 ± 12.40	310.3 ± 142.1	255.3 ± 9.7
41	1978	102.32 ± 15.34	562.2 ± 38.2	583.0 ± 6.8

42	1980	64.90 ± 3.00	414.6 ± 99.3	15.5 ± 4.0
43	2001	69.83 ± 12.93	294.3 ± 129.5	69.9 ± 4.0
44	2002	82.93 ± 1.63	564.1 ± 111.0	177.3 ± 6.2
45	unknown	56.67 ± 9.24	382.7 ± 135.0	43.4 ± 8.3
46	1999	72.67 ± 11.32	232.6 ± 139.2	0.0 ± 0.0
47	1999	81.13 ± 8.12	241.0 ± 172.8	0.0 ± 0.0
48	1999	74.41 ± 5.96	147.0 ± 159.4	0.0 ± 0.0
49	1999	85.13 ± 9.26	80.2 ± 69.7	0.0 ± 0.0
50	1999	62.81 ± 7.85	123.8 ± 103.0	0.0 ± 0.0
51	1983	72.52 ± 13.57	335.7 ± 38.4[a]	140.0 ± 6.8
52	1985	55.65 ± 3.48	354.7 ± 34.5[a]	86.9 ± 1.5
53	1993	75.54 ± 10.98	263.7 ± 59.8[a]	165.3 ± 2.9
54	1996	64.78 ± 3.30	319.3 ± 45.1[a]	71.1 ± 5.8
55	1998	74.23 ± 14.11	290.4 ± 21.6[a]	56.2 ± 5.7
56	2001	77.94 ± 7.76	370.9 ± 36.9[a]	148.7 ± 2.6
57	2002	73.19 ± 1.70	351.7 ± 39.3[a]	153.2 ± 1.4

Data are means ± standard deviations of triplicate measurements.

[a] For samples 51-57, mannan concentrations were measured in solubilized acetone/TCA precipitates. For all other samples, mannan was measured in solubilized acetone/10 kDa precipitates.

4.2. Mannan

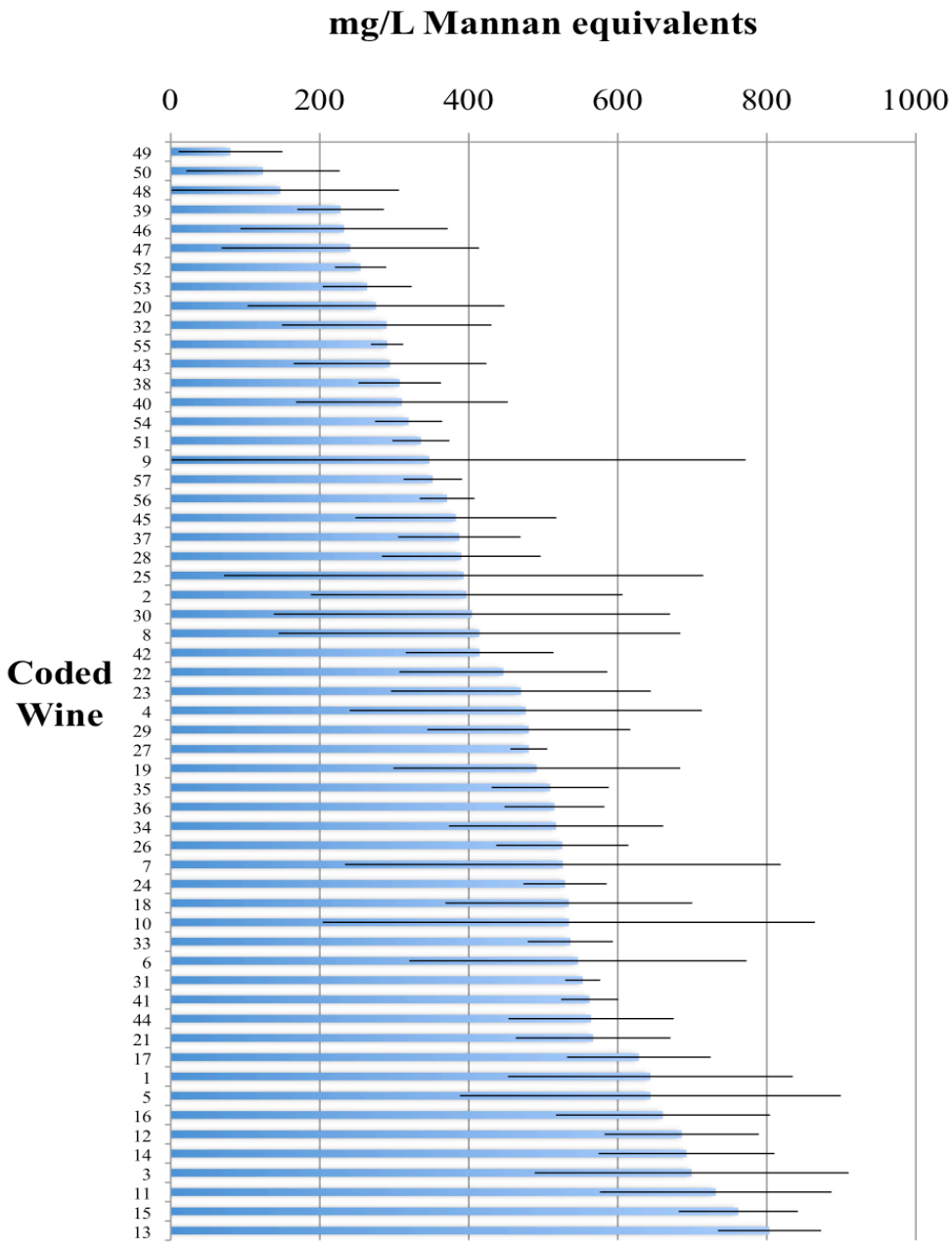
In addition to measuring protein, mannan concentrations were measured in the acetone pellet washed on a 10 kDa centrifugal PES filter by immunoblotting (**Figure 7, Table 8**). Mannan concentrations in the wines ranged from 80 to over 800 mg/L mannan equivalents. This method resulted in high variance, including some standard deviations greater than the mean mannan concentration. However, total carbohydrate measured in wines #1-4, by the phenol-sulfuric acid analysis yielded very similar results (**Table 9**). Considering that most identified wine proteins are grape-derived, and would not contain mannan, the values measured by immunoblot are likely an overestimate.

Table 9. Phenol-sulfuric acid carbohydrate analysis.

Coded Wine	mg/L mannan equivalents by phenol-sulfuric acid	mg/L mannan equivalents by immunoblot
1	623.40 ± 41.13	643.72 ± 191.15
2	508.43 ± 24.49	397.24 ± 209.23
3	663.66 ± 30.22	699.15 ± 210.67
4	587.64 ± 32.73	476.47 ± 236.35

No apparent trends were observed between mannan concentration, by immunoblotting of acetone/10 kDa samples, and protein concentration, measured by the Bradford assay in solubilized acetone/TCA pellets, or mannan concentration and wine age. In studies on the binding affinity of the mannan-specific daffodil lectin used here, Barre et al. (1996) observed greater binding capacity to complex glycans. Stronger binding capacity could explain why invertase reacted more intensely in the immunoblotting assay. For example, the integrated density calculated for 100 ng of invertase is equivalent to nearly 200 ng of mannan. While the mean ratio of protein to mannan was 1 to 6.75 is on the upper limit of what would be expected, if the mannan concentration were reported in invertase equivalents, the protein to mannan ratio of 1 to 3.38 which equates to a holoprotein composed of 22% peptide, and 78% glycogen. This is similar to the 30% to 70% ratio reported for the 420 kDa protein identified by Waters et al. (1994a).

Figure 7. Mannan analysis of Pinot noir wines by immunoblotting.

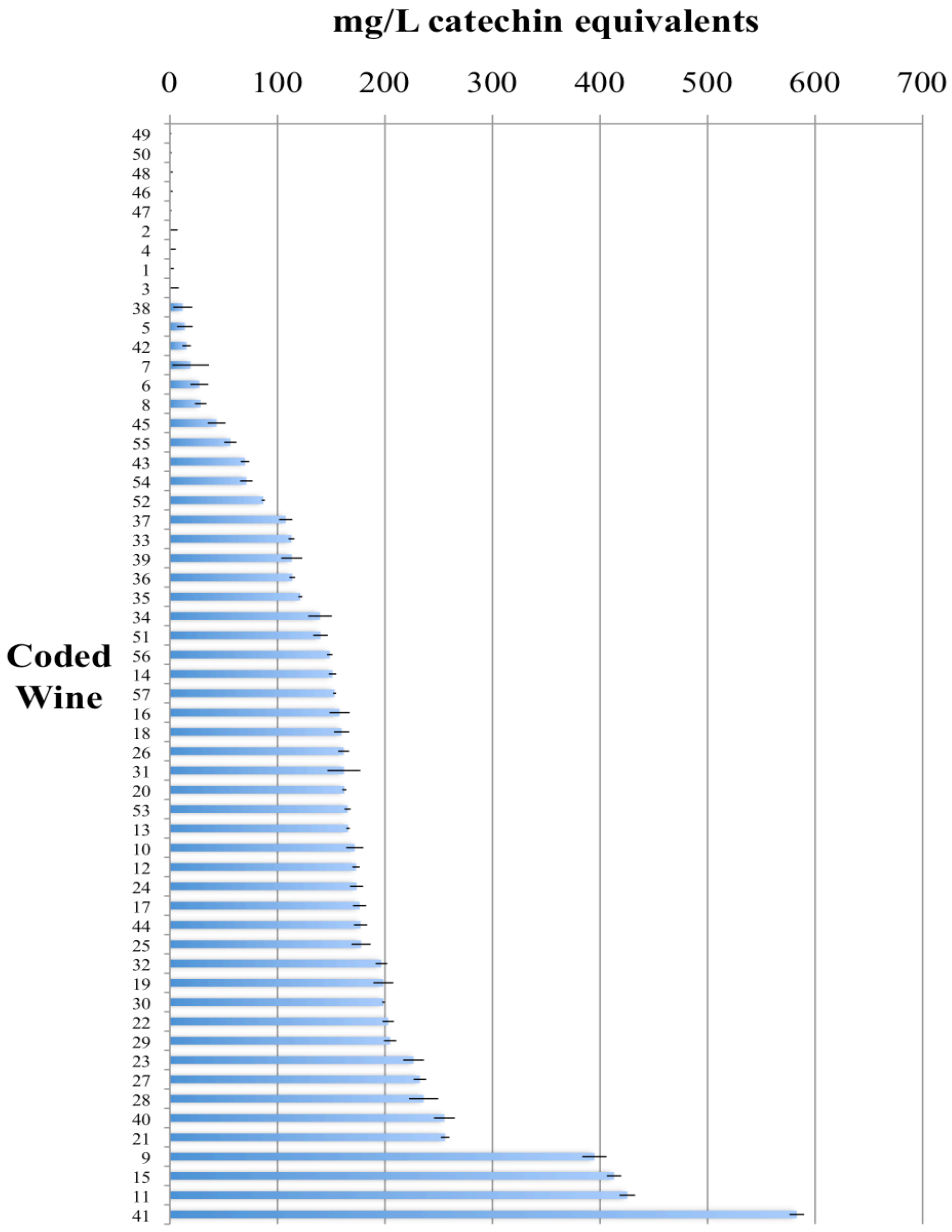


Data are means of triplicate measurements, error bars are standard deviations.

4.3. Tannin

Tannin was measured in the set of 57 wines (**Figure 8, Table 8**). In a study of tannin concentrations measured in Oregon Pinot noir wines, Harbertson et al. (2008) found concentrations ranging from 32 to 918 mg/L catechin equivalents, with a mean ± sd concentration 382 ± 202 mg/L. The majority of wines tested had similar tannin concentrations, ranging from 0 to 583 mg/L catechin equivalents, with a mean ± sd of 142 ± 126 mg/L. No correlation was observed between the concentration of tannin and wine age, or between tannin and protein concentration.

Figure 8. Tannin measured in Pinot noir wines.



Data are means of triplicate measurements, error bars are standard deviations.

5. Conclusions

A new and simple method for the isolation and quantification of protein in red wines was developed. Protein measured in 57 Pinot noir wines, ranging in age from five to thirty-two years old were found to contain 49 to 102 mg/L of protein. No correlation was evident between protein, mannan, tannin or wine age.

It is significant that the protein concentrations measured are comparable to those typically found in white wines (Fukui and Yokotsuka, 2003). Considering that until recently it was believed that there was little to no protein present in red wines (Singleton, and Trousdale, 1992), it is impressive to see protein remaining in Pinot noir wines for more than 30 years. Presence of mannan in these aged wines also indicates that a portion of the long-lived protein is yeast-derived mannoproteins.

This new assay should facilitate study of protein in red wine. The lack of an extensive study on proteins in red wine attest to the difficulties of measuring this wine component in a tannin-rich matrix. The ease of the developed assay will allow a relatively rapid and accurate measurement of wine protein and may allow future detailed study of important tannin-protein interactions that are presumed to contribute to the sensory quality of red wine.

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Appendices

Appendix 1. List of Pinot noir Wines.

Wines listed with volumes of lees, or enzyme additions, are on a per barrel bassis. In wines 46-50, the yeast strain is also given.

Wine Code	Description
1	Chehalem CC- 4L lees
2	Chehalem CC- 4L lees / 10g ECF
3	Chehalem CC- 4L lees / Stirred
4	Chehalem CC- 4L lees / 10g ECF / Stirred
5	Chehalem St- 4L lees
6	Chehalem St- 4L lees / 10g ECF
7	Chehalem St- 4L lees / Stirred
8	Chehalem St- 4L lees / 10g ECF / Stirred
9	Willakenzie - 2003 Control
10	Willakenzie - 2004 Control
11	Willakenzie - 2003 100g artificial lees
12	Willakenzie - 2004 100g artificial lees / Pectenzym
13	Willakenzie - 2004 100g artificial lees
14	Willakenzie - 2004 200g artificial lees
15	Willakenzie - 2003 4L lees
16	Willakenzie - 2004 4L lees

17	Willakenzie - 2004 4L 2003 lees
18	Willakenzie - 2004 4L lees / Pectenzym
19	Willakenzie - 2004 8L 2003 lees
20	Willakenzie - 2004 8L lees
21	Bethel Heights - 2004 10g ECF
22	Bethel Heights - 2004 Control
23	Bethel Heights - 2004 15g ECF
24	Bethel Heights - 200412L lees / 10g ECF
25	Bethel Heights - 2004 12L lees
26	Bethel Heights - 200412L lees / 15g ECF
27	Bethel Heights - 2004 4L lees / 15g ECF
28	Bethel Heights - 2004 4L lees
29	Bethel Heights - 2004 4L lees / 10g ECF
30	Bethel Heights - 2004 8L lees
31	Bethel Heights - 2004 8L lees / 10g ECF
32	Bethel Heights - 2004 8L lees / 15g ECF
33	Bethel Heights #2 - 2004 Control
34	Bethel Heights #2 - 2004 12L 2003 lees
35	Bethel Heights #2 - 2004 2L 2003 lees
36	Bethel Heights #2 - 2004 4L 2003 lees

37	Bethel Heights #2 - 2004 8L 2003 lees
38	Eyrie - 1979
39	Eyrie - 1980
40	Ellendale - 1983
41	Amity - 1978
42	Alpine - 1980
43	Erath - 2001
44	Benton Lane - 2002 L-block barrel sample
45	Cameron
46	Byron - 1999 BRL97
47	Byron - 1999 D254
48	Byron - 1999 BGY
49	Byron - 1999 BM45
50	Byron - 1999 RC212
51	Amity – 1983 Sunyside
52	Amity – 1985 Wadensvil
53	Amity – 1993 Winter's Hill Farm
54	Amity – 1996
55	Amity – 1998 Zielinski
56	Kramer – 2001 Rebeca's Reserve

57	Kramer – 2002 Estate

Appendix 2. BCA measured protein in acetone/10 kDa treated wines.

Proteins were precipitated with acetone. The solubilized pellets were washed 5 times with water on 10 kDa PES centrifugal filters to remove low molecular weight, BCA-reactive material. Protein was measured in the solubilized, washed pellets in triplicate with the BCA assay. Although this method appeared to give accurate measurements, the results had high variance, likely stemming from the wash procedure.

Coded Wine	Protein (mg/L BSA equivalents)	Standard Deviation
1	47.99	4.95
2	39.63	7.26
3	46.49	3.68
4	36.87	10.90
5	60.99	10.39
6	44.37	5.00
7	55.33	2.21
8	65.94	32.97
9	42.26	28.97
10	64.34	0.99
11	34.68	19.98
12	45.93	13.75
13	46.30	25.00

14	57.15	12.30
15	43.53	3.94
16	52.70	10.05
17	47.56	3.42
18	60.85	16.81
19	51.65	6.61
20	52.62	18.06
21	47.00	4.08
22	56.28	2.71
23	42.02	27.94
24	53.90	10.17
25	30.08	26.11
26	44.69	6.07
27	40.64	14.97
28	51.29	27.86
29	48.18	11.70
30	61.23	44.91
31	42.22	7.26
32	22.22	27.56
33	28.29	18.48
34	37.85	20.50
35	45.03	2.45

36	34.51	12.39
37	40.30	2.42
38	26.48	1.21
39	45.25	4.60
40	31.90	23.76
41	93.11	15.61
42	35.44	3.37
43	18.44	9.20
44	36.91	2.36
45	35.66	8.94
46	39.83	20.16
47	48.49	30.52
48	35.15	4.69
49	53.69	38.07
50	70.00	11.86