EFFECT OF PROTEIN-TANNIN RATIO AND TANNIN CONCENTRATION ON THE BOVINE SERUM ALBUMIN (BSA)-BASED PRECIPITATION METHOD FOR RED WINE TANNIN CONCENTRATION

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Abstract

Aims: The main objective of the study was to ascertain whether an existing protein precipitation assay could be simply modified to determine tannin at low concentration in wines. This was achieved by mixing a greater volume of wine to a smaller, but more concentrated, volume of bovine serum albumin (BSA) to maintain the same wine-to-BSA ratio (although both the final pH and ethanol concentration varied). In addition, dilution series of each of these mixtures were prepared to investigate the effect of wine-to-BSA ratio on tannin precipitation.

Methods and results: Seven New Zealand red wines were assayed according to the BSA method using a range of protein (BSA) and wine concentrations achieved by varying wine dilutions and the volume of the model wine solution. Maximum precipitation was observed at lower wine/protein ratios in diluted wines and tannin precipitation increased as protein concentration increased. It was observed that the estimation of tannin concentration in red wine is a product of tannin/protein ratio and BSA concentration. Consequently, the methylcellulose precipitation (MCP) assay was performed to independently determine tannin concentration in red wines. Results indicate that tannin/protein ratio, BSA concentration and possibly tannin composition affect BSA-tannin precipitation.

Conclusion: For the BSA assay there appears to be a region of low tannin/protein ratio within which lower wine tannin concentrations can be determined. Overall it is suggested that tannin precipitation is linearly related to tannin concentration.

Significance and impact of the study: Results showed the limits of the BSA method for low tannin wines and the difficulty in using the method for wines with unknown tannin concentrations.

Key words: bovine serum albumin (BSA), methylcellulose precipitation (MCP), tannin, wine

Résumé

Contexte et objectifs: L'objectif principal de cette étude a été de savoir si une méthode de précipitation des protéines actuellement utilisée pouvait être simplifiée pour déterminer la concentration des tanins dans les vins qui en contiennent peu. Cette question a été abordée en mélangeant un volume important de vin à un faible volume, mais plus concentré, d'albumine de sérum de bœuf (BSA). Dans ces conditions, on maintient le même ratio vin/BSA malgré des variations du pH final et de la concentration en éthanol. Par ailleurs, des dilutions de chacune de ces solutions contenant vin et BSA ont été réalisées pour connaître l'influence du ratio vin/BSA sur la précipitation des tannins.

Méthodes et résultats: Sept vins rouges de Nouvelle-Zélande ont été testés avec la méthode utilisant la BSA. Différentes gammes de BSA et de vin ont été utilisées en faisant varier le facteur de dilution, ainsi que le volume de vin modèle. Les plus fortes précipitations ont été observées pour les faibles ratios vin/protéine dans les vins dilués, alors que la précipitation en tanins augmente lorsque la concentration en protéines augmente. On note aussi que l'estimation de la concentration en tanins dans les vins rouges dépend du rapport tanins/protéines et de la concentration en BSA. Pour cela, le test de précipitation au moyen de méthylcellulose (MCP) a été utilisé pour déterminer, de manière indépendante, la concentration en tannins dans les vins rouges. Les résultats montrent que le rapport tanins/protéines, la concentration en BSA et probablement aussi la composition en tanins jouent un rôle dans la précipitation des complexes tanins/BSA.

Conclusions: Pour le test de précipitation des tannins à la BSA, lorsque le rapport tanins/protéine est faible, on peut déterminer la concentration en tanins des vins pauvres en tanins. Plus encore, les résultats suggèrent que la précipitation des tanins est linéairement corrélée à la concentration en tanins.

Impact de l'étude: L'étude montre les limites du test à la BSA pour les vins pauvres en tanins, et la difficulté d'utiliser cette méthode pour les vins dont la concentration en tanins est inconnue.

Mots clés: albumine de sérum de bœuf (BSA), précipitation à la méthylcellulose (MCP), tanin, vin

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INTRODUCTION

Recently, there has been considerable interest in methods to determine tannins in grapes and wine. For example, MERCURIO AND SMITH (2008) investigated the relationship between tannins quantified by two different methods and wine astringency. Similarly, COSME *et al.* (2009) investigated the tannin profiles of a range of monovarietal wines from the Lisbon region (Portugal) and determined the proportions of polymeric flavan-3-ols in grape skins and seeds. Colorimetric methods have been widely used to quantify tannins but many of these methods do not distinguish low molecular weight phenols from the higher molecular weight compounds (SCHOFIELD *et al.*, 2001). As HERDERICH *et al.* (2006) point out, tannins are identified by their ability to complex with and precipitate proteins and this property has been used by workers as a method to selectively remove tannins from solution and hence determine their concentration (MAKKAR, 1989). The classic method of HAGERMAN and BUTLER (1978) involves the use of bovine serum albumin (BSA) and precipitation of a protein-tannin complex, followed by its separation and dissolution. The phenolics present in the dissolved complex are determined spectrophotometrically at 510 nm by the addition of ferric chloride (HARBERTSON and SPAYD, 2006). This method has been used to investigate tannins in grape berry components (HARBERTSON *et al.*, 2002, SEDDON and DOWNEY, 2008) and wines (HARBERTSON *et al.*, 2008, KENNEDY *et al.*, 2006), and a modified version has been used to estimate polymeric pigments in grapes and wines (HARBERTSON *et al.*, 2003).

Tannin-protein associations are thought to entail cross-linking of separate protein molecules by tannins that act as polydenate ligands on the protein surface involving hydrophobic effects and hydrogen bonds (CARVALHO *et al.*, 2004). The interaction with globular proteins such as BSA involves only the surface-exposed amino acid residues, whereas proline-rich linear proteins (which represent 70-80 % of human salivary proteins) involve face-to-face stacking with amino acid residues (CARVALHO *et al.*, 2004, DEAVILLE *et al.*, 2007). Additionally, precipitation by the two tannin classes found in wine differs as it is thought that hydrolysable tannins form a hydrophobic coating on the surface of the protein while condensed tannins form hydrogen-bonded cross-links between protein molecules (HARBERTSON and SPAYD, 2006, HAGERMAN *et al.*, 1998). Furthermore, DEAVILLE *et al.* (2007) reported that gallotannins bind more strongly to BSA than ellagitannins due to the aromatic rings in the hydroxydiphenyl groups of ellagitannins that are inhibited by intramolecular biphenyl linkages. Binding of polyphenols (proanthocyanidins) to protein depends on the number and location of hydroxyl groups on the aromatic ring (monophenols << meta-diphenols << ortho-diphenols < vicinal triphenols) and molecular size (SIEBERT, 1999).

A recent investigation of the influence of sample dilution on the reliability of tannin analysis by protein precipitation concluded that tannin concentrations of both diluted and concentrated samples were systematically underestimated (JENSEN *et al.*, 2008) explained by a precipitation threshold and insufficient protein for precipitation, respectively. More recently, BROOKS *et al.* (2008) presented data which indicated that one implementation of the BSA tannin precipitation assay (HARBERTSON *et al.*, 2002) does not meet criteria for acceptable precision and recovery. Other studies have indicated that all protein precipitation assays are potentially compromised by their inability to measure directly the precipitated tannin, requiring a subsequent colorimetric (or other) assay (SARNECKIS *et al.*, 2006). Consequently, an alternative assay based on precipitation of tannins with methylcellulose has been used in a number of studies (SEDDON and DOWNEY, 2008, SARNECKIS *et al.*, 2006, MERCURIO and SMITH, 2008).

The purpose of this study was to investigate BSA-tannin precipitation from wines. We were prompted to undertake this research prior to analysis of wines from viticultural field trials of which, it was predicted, several would have low to medium tannin concentrations. Furthermore, observations in our laboratory noted that variable quantities of precipitate (including no precipitate) could be produced from some undiluted wines.

The study was purely an investigation into the use of an existing and widely used protein precipitation assay to assess the effect of dilution on low to medium tannin wines and also to ascertain whether the previously reported threshold effect might be overcome by the simple expedient of increasing the quantity of wine tannin relative to BSA. However, a number of factors have been found to influence protein-polyphenol interaction including : type of protein, protein concentration, tannin concentration, tannin size, degree of galloylation, pH, alcohol concentration and the ionic strength of the medium (SIEBERT *et al.*, 1996). Because a simple adaptation of an existing analytical method was sought, kinetic parameters such as reaction temperature and reaction time were not studied, and other factors, specifically final pH and ethanol concentration, were not held constant. Nevertheless, PRIGENT *et al.* (2009) found no effect of ionic strength (I = 0.023 to 0.087) on the solubility of α-lactalbumin (a globular protein) in the presence of proanthocyanidins even after an extended incubation period (3 days). Additionally, temperature in the same study only had an effect at 40 °C so our analysis was carried out at room temperature according to

HARBERTSON *et al.* (2002) although JENSEN *et al.* (2008) extended the incubation time from 10 to 30 minutes for dilution experiments. With regard to alcohol concentration, SERAFINI *et al.* (1997) found that ethanol in the range 0-22 % proportionally reduced tannin precipitation from red wine after BSA addition, although significant differences versus alcohol-free wine were only observed at higher concentrations (≥ 11%). Similarly, SIEBERT *et al.* (1996) found that the effect of alcohol concentration on haze induced in apple juice with tannin acid (hydrolysable tannin) was relatively small. HAGERMAN and BUTLER (1978) found that BSA was significantly precipitated by condensed tannins when the pH of the mixture was between 3.0 and 5.0, although they recommended a pH range of 4 to 5 for maximum precipitation. Similarly, DE FREITAS and MATEUS (2002) found that procyanidin oligomers bind extensively to BSA at around pH 4.5.

MATERIALS AND METHODS

1. Wine samples

Seven New Zealand red wines were selected to obtain a broad range of possible tannin concentrations. These were (alcohol concentration in parentheses) : Corbans Private Bin Syrah 2004 (13.8 %), Crossroads Pinot noir 2004 (13.0 %), Muddy Water Pinot noir 2004 (14.1 %), Red Rock Merlot Malbec 2006 (14.0 %), Saint Clair Pinot noir 2006 (13.5 %), Te Mata Gamay noir 2007 (13.0 %) and Vidal Cabernet-Sauvignon 2002 (14.0 %).

2. Chemicals

Bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), triethanolamine (TEA), ferric chloride, methylcellulose solution, (+)-catechin and (-)-epicatechin, and ammonium sulphate were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). All chemicals were analytical grade.

3. BSA precipitation assay

Wines were assayed for tannins according to HARBERTSON *et al.* (2002) except that a range of protein (BSA) and wine concentrations were used (table 1). Samples were centrifuged using a Heraeus Sepatech Biofuge 15 at 17,300 g for 5 minutes. Absorbance was measured at 510 nm using a Unicam Helios Alpha Spectrometer. Distilled water was used as the blank. By varying the amount and concentration of BSA and the amount and dilution of wine samples, it was possible to prepare mixtures ranging in wine/protein ratio (0.031, 0.063, 0.125, 0.250 and 0.500 mL mg^{-1} BSA) for different final BSA concentrations (0.40, 0.67, 1.00, 1.33, 1.60 and 1.80 mg mL^{-1} BSA). The amounts and concentrations in each mixture (1.5 mL total volume) are

Table 1 - Volumes used to prepare mixtures ranging in wine/protein ratio at different final BSA concentrations.

Wine/protein (mL mg^{-1})		0.03			0.06			0.13			0.25			0.50			Final solution pH
Final BSA (mg mL^{-1})	Stock BSA (mg mL^{-1})	Wine (µL)	Model wine (µL)	Stock BSA (µL)	Wine (µL)	Model wine (µL)	Stock BSA (µL)	Wine (µL)	Model wine (µL)	Stock BSA (µL)	Wine (µL)	Model wine (µL)	Stock BSA (µL)	Wine (µL)	Model wine (µL)	Stock BSA (µL)	
0.40	0.50	19	281	1200	38	262	1200	75	225	1200	150	150	1200	300	0	1200	4.9
0.67	1.0	31	469	1000	63	437	1000	125	375	1000	250	250	1000	500	0	1000	4.9
1.00	2.0	47	703	750	94	656	750	188	562	750	375	375	750	750	0	750	4.8
1.33	4.0	63	937	500	125	875	500	250	750	500	500	500	500	1000	0	500	4.7
1.60	8.0	75	1125	300	150	1050	300	300	900	300	600	600	300	1200	0	300	4.5
1.80	18	84	1266	150	169	1181	150	338	1012	150	675	675	150	1350	0	150	4.2

given in table 1. BSA stock solutions were prepared in a buffer consisting of 0.20 M acetic acid and 0.17 M NaCl, adjusted to pH 4.9. Wine samples were diluted as appropriate in a model wine consisting of 5 g L^{-1} potassium bitartrate in 12 % v/v ethanol and adjusted to pH 3.3. Thus, the row labelled 0.67 mg mL^{-1} BSA (table 1) corresponds to a wine dilution series utilising standard conditions from HARBERTSON *et al.* (2002). Results from such a dilution series have recently been reported by JENSEN *et al.* (2008). In our study, results for dilution series at other BSA concentrations were achieved although this entailed varying the volume of the pH 4.9 buffer solution which was combined with the (appropriately diluted) wine ; i. e., from 1.20 to 0.15 mL (in a total volume of 1.5 mL) for BSA concentrations of 0.4 and 1.8 mg mL^{-1}, respectively. The pH levels of the solutions (table 1) were found to be in the recommended range (4.2 to 4.9) for protein precipitation. Ethanol concentrations in the final mixtures varied from 2.4 % (depending on the wine) for the row labelled 0.40 mg mL^{-1} BSA (table 1) to 10.8 % for the row labelled 1.80 mg mL^{-1} BSA (data not shown).

4. Methylcellulose precipitation (MCP) assay

The MCP assay was performed according to MERCURIO *et al.* (2007) on five of the seven wines (the Crossroads Pinot noir 2004 and Muddy Water Pinot noir 2004 were not analysed). MCP analyses were carried out twice in triplicate.

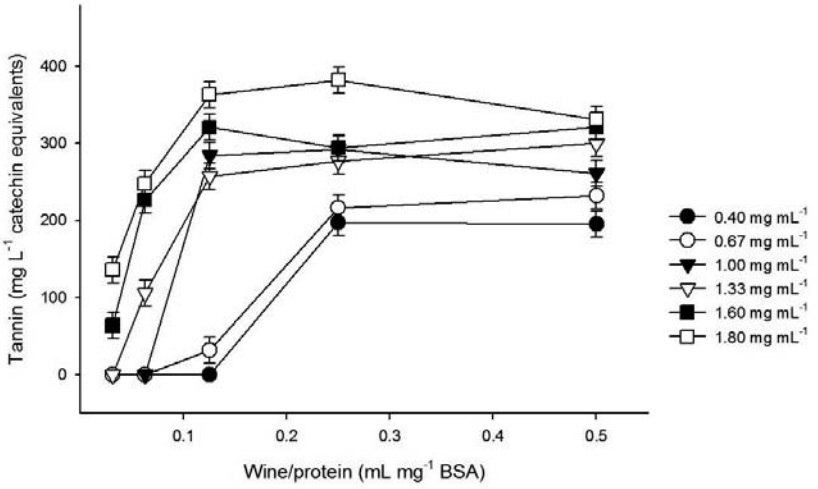

Figure 1 - The effect of wine/protein ratio on tannin concentration determined with the BSA precipitation assay using a range of BSA concentrations for a 2004 New Zealand Syrah wine.

Error bars show the average standard error, calculated as the harmonic mean of the pooled variance from replicate analyses.

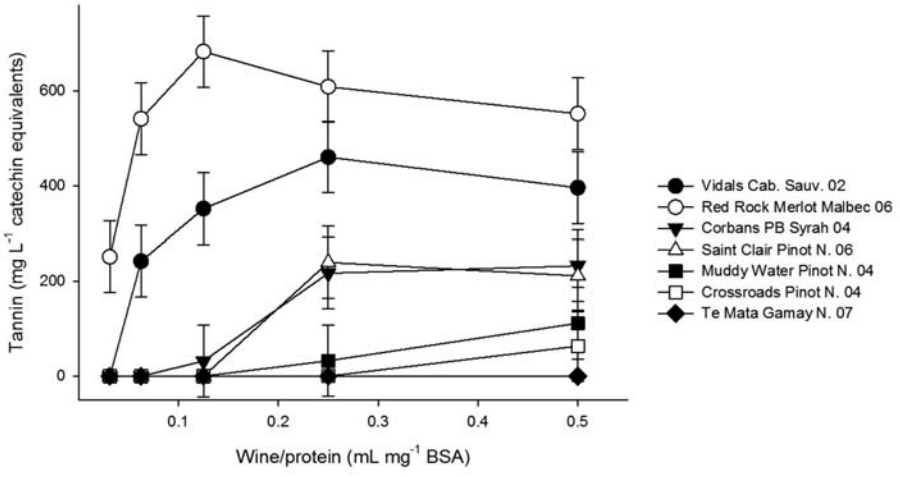

Figure 2 - The effect of wine/protein ratio on tannin concentration determined using the BSA precipitation assay for a range of New Zealand red wines. [BSA] = 0.67 mg mL^{-1}.

Error bars show the average standard error, calculated as the harmonic mean of the pooled variance from replicate analyses.

5. Statistical analyses

A variable number of replicates (up to 6) were used in the determination of tannin by the BSA precipitation assay. Consequently, average standard errors were calculated as the harmonic mean of the pooled variance from replicate analyses using Excel 2003 for Windows XP (Microsoft, USA). Graphs were produced using SigmaPlot version 2002, Windows version 8.02 (SPSS Inc, Cranes Software International Pty Ltd, Melbourne, Australia).

RESULTS AND DISCUSSION

Data showing the effect of wine/protein ratio and the apparent effect of BSA concentration on assayed tannin concentration for one of the wines (Corbans Private Bin Syrah 2004) are presented in figure 1. Results varied from 0 to 380 mg catechin equivalents (CE) L^{-1} wine across the full range of solution conditions. With a BSA concentration of 0.67 mg mL^{-1}, corresponding to the standard condition of the HARBERTSON *et al.* (2002) assay, the results ranged from 0 to 230 mg CE L^{-1} wine. The other wines showed similar patterns (data not shown): Red Rock Merlot Malbec 2006, a wine with a high tannin concentration, gave results of 0 to 860 and 250 to 680 mg CE L^{-1}, across the full range of solution conditions and with a BSA concentration of 0.67 mg mL^{-1}, respectively; and Te Mata Gamay noir 2007, the wine with the lowest tannin concentration, gave results of 0 to 100 and 0 mg CE L^{-1}, respectively.

The range of results obtained in this study appears to be much greater than the one of JENSEN *et al.* (2008), who reported underestimation of up to 27 % for their dilution series. Nevertheless, we believe that results from the two studies are compatible. It is apparent from the data of JENSEN *et al.* (2008) that estimated tannin concentrations declined most markedly at high dilution factors and that this effect was more apparent and occurred at lower dilutions for lower tannin concentration wines. The same pattern was observed in our study but wines had generally lower tannin concentrations and the maximum dilution was greater (16-fold compared to 10-fold). Figure 2 shows data for our range of wines at a BSA concentration of 0.67 mg mL^{-1} and can be compared with figure 2 of JENSEN *et al.* (2008). In our study, tannin concentrations determined with the standard conditions of the HARBERTSON *et al.* (2002) assay were in the range 0-600 mg CE L^{-1}, which is similar to results reported by MERCURIO and SMITH (2008) for a range of Australian red wine varieties. It is interesting to note that BSA-precipitable tannin for the three wines with clearly the lowest tannin concentrations (Muddy Water Pinot noir 2004, Crossroads Pinot noir 2004 and Te Mata Gamay noir 2007) increased continually with increasing wine/protein ratio (up to 0.5, undiluted wine) in contrast to the other wines for which maximum or plateau values were obtained at lower wine/protein ratios (diluted wines). These results indicate that low tannin wines such as Pinot noir may not require dilution if the BSA method is used to analyse total tannin concentration.

When the protein precipitation assay was carried out using a range of BSA concentrations at a defined wine/protein ratio, it appeared that tannin precipitation by protein increased as the concentration of protein increased although it should be noted that pH decreased

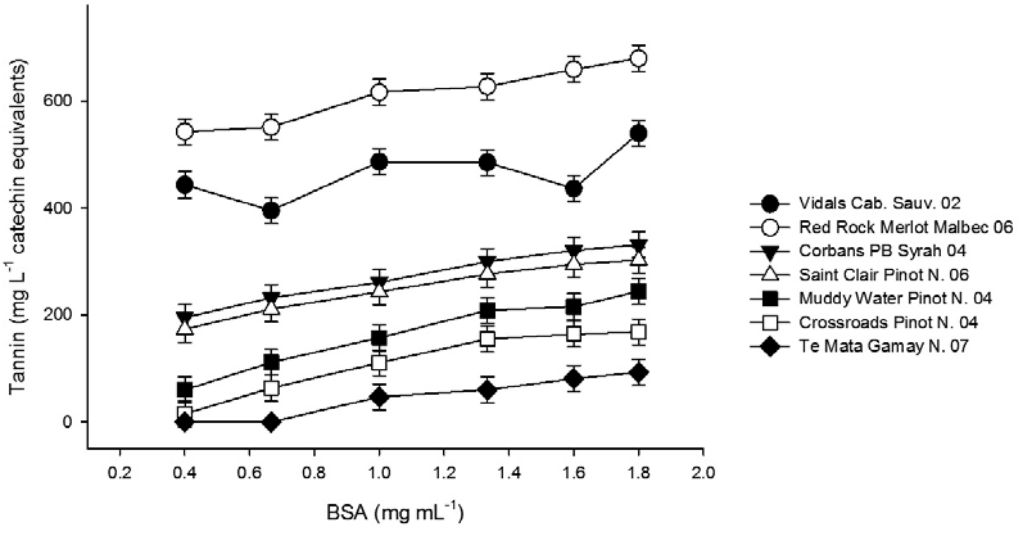

Figure 3 - The effect of protein concentration on tannin concentration determined using the BSA precipitation assay for a range of New Zealand red wines. Wine/BSA = 0.5 mL mg^{-1}.

Error bars show the average standard error, calculated as the harmonic mean of the pooled variance from replicate analyses.

and ethanol concentration increased across this series. However, as indicated above, other work has found that increasing ethanol concentration decreases the precipitation of tannin by BSA (SERAFINI *et al.*, 1997), and all pH values were within the range required for maximum precipitation (HAGERMAN and BUTLER, 1978). Figure 3 shows the results obtained with the undiluted wines (column labelled 0.5 mL wine mg^{-1} BSA in table 1), but equivalent data were obtained for the range of wine dilutions investigated (data not shown). It seems that increasing protein concentration increases the precipitation of tannin from solution.

Other studies, albeit involving hydrolysable tannins, have suggested that there exists a critical point for precipitation of the tannin-BSA complex from solution which is strongly dependent on the tannin/BSA ratio in the reaction solution (SILBER *et al.*, 1998). Studies with condensed tannins suggest that the quantity of insoluble precipitate from the formation of complexes with protein increases rapidly up to a maximum and remains relatively constant thereafter (DE FREITAS and MATEUS, 2002; HAGERMAN and BUTLER, 1978). Since wines contain different concentrations of tannin, these would occur at different BSA concentrations. In order to investigate whether our data conformed to such a model, we have plotted the quantity of tannin precipitated by BSA as a function of MCP tannin and BSA concentrations in the solution mixture (figure 4) for five of the wines (i. e., those assayed by both BSA and methylcellulose precipitation (MCP) methods). This allowed all the data from these wines to be plotted on a tannin concentration as opposed to a wine dilution basis. Because the estimated concentration of tannin in each wine appears to be a function of a number of factors, we used MCP tannin as an independent measure of the tannin concentration.

Figure 4 indicates that the relationship between tannin precipitated by BSA and MCP tannin (an independent measure of concentration) is similar for all the wines, although there is some variation in the amount of tannin precipitated by BSA at seemingly equal MCP tannin concentrations and for different wines (figure 4, insert) which could indicate some variability in tannin/protein stoichiometry and might be related to tannin composition. A recent report regarding the validity or otherwise of the HARBERTSON *et al.* (2002) assay ascribes significant inter-laboratory variation to non-stoichiometric protein-tannin precipitation (BROOKS *et al.*, 2008). Recently, MERCURIO and SMITH (2008) found that methylcellulose complexes and precipitates all tannins and pigmented polymers observable by HPLC whereas BSA does not. HARBERTSON *et al.* (2003) previously reported that dimeric and trimeric procyanidins do not complex with BSA; therefore, two classes of pigments are labelled as large polymeric pigments (LPP: precipitate

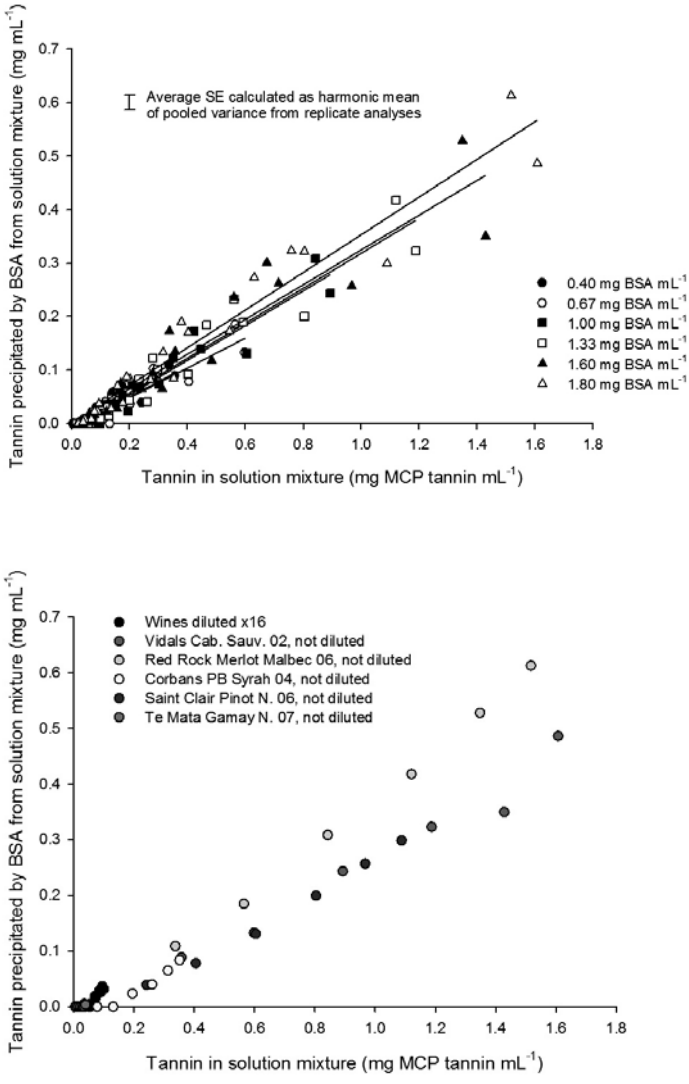

Figure 4 - Plot of tannin precipitated using a range of BSA concentrations for five New Zealand red wines. Lines are regressions at each BSA concentration. Insert (below): Subset of data for two wine dilutions.

with BSA) and small polymeric pigments (SPP: do not precipitate with BSA) (MERCURIO and SMITH, 2008). This observation would suggest that the MCP assay could be better suited to low tannin wines that might have a higher proportion of SPP than other wines, although HARBERTSON and SPAYD (2006) suggested that if protein is available in extreme excess then it can remove all phenolics rather than selectively removing polymers (tannins). It may also be that methylcellulose has a higher binding affinity for condensed tannins than the globular protein, BSA, which could marginally affect the tannin concentration results in both assays. There is also some evidence (from the small but consistent increase in slope, indicated by the fitted lines in figure 4) that at higher BSA concentrations a greater proportion of the tannin in solution is precipitated (in line with figure 3). In contrast to the results of JENSEN *et al.* (2008), figure 4 shows no evidence,

over the range of tannin concentrations investigated, for a situation in which tannin concentration exceeds the capacity of BSA for precipitation of that tannin (i. e., there is no maximum in precipitated tannin with increasing tannin concentration in the solution mixture). Overall, it seems that tannin precipitation from these mixtures is linearly related to tannin concentration since protein is in excess (for the range 0.40-1.80 mg BSA mL^{-1}).

It is clear from our data that both wine/protein (i. e., tannin/protein) ratio and BSA concentration affect estimated wine tannin concentration. In particular, there appears to be a region at low tannin/protein ratio within which lower wine tannin concentrations are determined (Figures 1 and 2) although overall figure 4 suggested that tannin precipitation from these mixtures is linearly related to tannin concentration. Nevertheless, it seems that when the quantity of tannin in the solution mixture is < 0.1 mg MCP tannin mL^{-1}, either no precipitate with BSA is formed or it is not possible to recover it adequately with the techniques used in this study (figure 4). This was exemplified by the linear regression between tannin measured by BSA and that measured by MCP (BSA tannin concentration mg catechin equivalents L^{-1} = 0.31 x MCP tannin concentration mg epicatechin equivalents L^{-1} - 82 ; R^2 = 0.832, P < 0.05), which is also very similar to that determined by MERCURIO and SMITH (2008) for a range of Australian dry red wines, except for a difference in the intercepts. Indeed, that on the x-axis correspond to 800 mg epicatechin equivalents L^{-1} in the Australian study and to 260 mg L^{-1} for the New Zealand wines, although it should be noted that a total of forty one wines were analysed in the MERCURIO and SMITH (2008) study compared to only five here. These observations support the suggestion made by JENSEN *et al.* (2008) of a threshold tannin level for precipitation with BSA to occur (260 mg epicatechin equivalents L^{-1} MCP tannin from our regression between BSA and MCP assays, or approximately 100 mg L^{-1} from figure 4, insert).

Our results have shown that tannin/protein ratio (figure 2), BSA concentration (figure 3) and possibly tannin composition (figure 4, insert) affect tannin precipitated by BSA although overall there is a strong relationship between tannin concentration in the solution mixture and tannin precipitated by BSA.

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