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# Tannin Quantification in Red Grapes and Wine: Comparison of Polysaccharide- and Protein-Based Tannin Precipitation Techniques and Their Ability to Model Wine Astringency

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Quantification of red grape tannin and red wine tannin using the methyl cellulose precipitable (MCP) tannin assay and the Adams−Harbertson (A-H) tannin assay were investigated. The study allowed for direct comparison between the repeatability of the assays and for the assessment of other practical considerations such as time efficiency, ease of practice, and throughput, and assessed the relationships between tannin quantification by both analytical techniques. A strong correlation between the two analytical techniques was observed when quantifying grape tannin ($r^2 = 0.96$), and a good correlation was observed for wine tannins ($r^2 = 0.80$). However, significant differences in the reported tannin values for the analytical techniques were observed (approximately 3-fold). To explore potential reasons for the difference, investigations were undertaken to determine how several variables influenced the final tannin quantification for both assays. These variables included differences in the amount of tannin precipitated (monitored by HPLC), assay matrix variables, and the monomers used to report the final values. The relationship between tannin quantification and wine astringency was assessed for the MCP and A-H tannin assays, and both showed strong correlations with perceived wine astringency ($r^2 = 0.83$ and $r^2 = 0.90$, respectively). The work described here gives guidance to those wanting to understand how the values between the two assays relate; however, a conclusive explanation for the differences in values between the MCP and A-H tannin assays remains unclear, and further work in this area is required.

**KEYWORDS: Tannin quantification; tannin precipitation assay; red wine; red grape; wine astringency**

## INTRODUCTION

There is now well-established recognition of the importance of tannins in red wines to the beneficial health properties associated with red wine consumption (*1*) and their critical roles in color, taste, and mouthfeel properties (*2, 3*). However, red wine presents a challenging matrix from which to quantify and characterize tannins. Quantification and characterization of tannins remains one of the great analytical challenges in natural products chemistry. This challenge stems from the fact that tannins are inherently amphiphilic molecules with high reactivity, have a diverse range of structures, and are often found in matrices with other phenolic molecules containing similar functional groups.

While it remains essential to continue fundamental research into the structure and function of tannins using sophisticated equipment and analytical techniques, there also exists a desire for rapid, simple, and robust tools to determine total tannin concentration in grapes and wine. Many industry-focused researchers have shifted focus away from time-consuming methods such as HPLC toward methods that can be more easily adopted for routine analysis by industry practitioners. These methods are also attractive to researchers as they increase efficiency, ease of practice, and throughput. Two tannin assays in particular, the methylcellulose-precipitable (MCP) tannin assay (*4, 5*) and the Adams−Harbertson (A-H) assay (*6, 7*), have been developed and are increasingly being applied in research and industry laboratories. Both of these assays are precipitation-based and exploit the ability of tannin to complex and precipitate with polysaccharides and protein. Furthermore, both of these assays have been adapted to high throughput formats using 96 well plates, allowing for increased sample throughput and substantial reduction in time and cost (*4, 7*). Such increased throughput has made it more feasible for both researchers and industry practitioners to collate large data sets on grape and wine tannin concentration. These efficient tannin measures provide an objective measure that viticulturists and oenologists can use to better understand how tannin concentrations relate to or are affected by a number of variables including grape maturity, streaming of grape intake, monitoring of quality among vineyards, wine processing options, wine quality, and consumer preferences. Analytical validation has been previously

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reported for the MCP tannin assay (*4, 5*), but not yet for the A-H assay. The main focus of the research discussed here is not to analytically validate the A-H assay (other than to establish repeatability), but rather to compare the actual values determined using the MCP tannin assay in relation to the A-H tannin assay, which has not been reported before.

A further area of interest is the potential of tannin measures to predict astringency. Such model systems may allow prescreening of wines for studies or give an indication of wine astringency without the need for costly and time-intensive sensory studies. Tannin concentration as measured using HPLC and protein-based precipitation techniques have been shown by others to be positively correlated to sensory panel ratings of astringency. Thus, an additional focus of our research was to determine whether tannin measured using the MCP tannin assay also correlated with astringency.

Kennedy et al. have highlighted that tannins from grape and in particular red wine are a very complex, and heterogeneous mixture and therefore tannin concentration values can vary considerably depending on the analytical method applied (*8*). In order for researchers and industry practitioners to interpret data from different analytical methods, it is critical to understand how the different analytical methods relate to one another. Our research aimed to explore the similarities and differences of the two high throughput precipitation-based analytical techniques. The study allowed for direct comparison between the repeatability of the assays, for the assessment of other practical considerations such as time efficiency and ease of practice and throughput, and assessed the relationships between tannin quantification by both analytical techniques. HPLC was used to monitor the effectiveness of both precipitation-based methods on the removal of tannin sample from the sample matrix. Furthermore, as quick, easy, objective methods to evaluate wine astringency can supplement costly and time-consuming sensory studies, the correlation of tannin concentration determined by the two methods to red wine astringency was investigated.

## MATERIALS AND METHODS

**Chemicals.** All chromatographic solvents were high performance liquid chromatography (HPLC) grade, all other chemicals were analytical reagent grade, unless otherwise stated, and water was obtained from a MILLI-Q purification system. Bovine serum albumin (BSA, prepared from fraction V albumin), sodium dodecyl sulfate (SDS), triethanolamine (TEA), ferric chloride hexahydrate, methyl cellulose solution (1500 cP viscosity at 2%), (+)-catechin, and (−)-epicatechin were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia).

The purified grape seed tannin extract was isolated from Ferco Tanin vinification (SA Ferco Development, France) and purified by C18 column chromatography. Ferco seed tannin (2 g) was extracted with 90% aq. ethanol (3 × 25 mL). The extracts were combined and the solvent removed by rotary evaporation to yield 1.36 g of 90% ethanol-soluble material. This material was loaded in water onto a slurry-packed column (XK 26/40, Amersham Biosciences, Baulkham Hills, NSW, Australia) of Synergi 10 μm Hydro-RP C18 gel (Phenomenex, Lane Cove, NSW, Australia; 120 mm bed height) and eluted with 10% aq. ethanol to remove any non-tannin material and purified tannin was eluted with 100% ethanol. This material was freeze-dried, and phloroglucinolysis (*9*) indicated that it had an mDP of 6.3 and a galloylation of 13% (unpublished results).

*Grape Samples.* Forty red grape samples from the 2007 vintage were collected from several regions throughout Australia, covering a wide range of tannin concentrations from three common Australian wine varieties: Cabernet Sauvignon, Shiraz, and Merlot. Grape samples were destemmed and frozen at −20 °C on the day of harvest. Grape homogenate extracts were obtained from between 150 to 190 g of grape berries and defrosted 3 h prior to homogenization (Retsch Grindomix GM200 homogenizer) (*10*). Aqueous ethanol (10 mL, 50%) was added to 1 g (±0.04 g) of homogenate, and the samples were rotated at high speed on a Ratek suspension mixer (Stennick Scientific, Australia) for 60 min and then centrifuged at 4,000 rpm for 5 min using a Hettich Zentrifugen Universal 32 R centrifuge with a Hettich 1624 rotor (Adelab Scientific, Australia). Grape homogenate extracts were stored at −20 °C until analyzed.

*Wine Samples.* Forty-one commercially available dry red wines were selected from multiple varieties and vintages with a broad range of tannin concentrations. They included five Australian wine varieties: Cabernet Sauvignon, Shiraz, Merlot, Pinot Noir, and Durif, spanning vintages 1991 to 2006 and from a wide range of regions. After purchase, wines were stored at 17 °C until they were analyzed.

**Analytical Methods.** *Methyl Cellulose Precipitable (MCP) Tannin Assay.* Methyl cellulose solution (0.04% w/v) was prepared in accordance with the manufacturer's instructions (Sigma-Aldrich, Castle Hill, NSW, Australia).

Grape homogenate extracts were thawed at room temperature immediately prior to analysis. Wines were subsampled from freshly opened bottles and stored at room temperature.

The MCP tannin assay was performed in duplicate on wines and grape homogenate extracts using the high throughput format described in Mercurio et al. (*4*). For all samples, 300 μL of supernatant from the treatment and control samples was transferred into a 370 μL Greiner UV star 96 well plate and read at 280 nm. For all absorbance readings, water was used as a blank. Aqueous (−)-epicatechin solutions (10, 25, 50, 75, 100, 150, 200, and 250 mg/L epicatechin) were used to establish a calibration chart for reporting tannin absorbances. All $A_{280}$ (tannin) values are reported in mg/L epicatechin equivalents of the original sample (corrected for assay dilution).

*Adams−Harbertson (A-H) Tannin Assay.* All buffer solutions were prepared as described in Harbertson et al. (*6*). Buffer A: washing buffer (200 mM acetic acid and 170 mM sodium chloride, pH adjusted to 4.9 with sodium hydroxide). Buffer B: model wine (5 g $L^{-1}$ potassium bitartrate and 12% (v/v) ethanol, pH adjusted to 3.3 with 10.18 M hydrochloric acid). Buffer C: resuspension buffer (5% (v/v) triethanolamine and 5% (w/v) sodium dodecyl sulfate, pH adjusted to 3.3 with 10.18 M hydrochloric acid). Bovine serum albumin (BSA) solution: (1 mg/L BSA dissolved in buffer A). Ferric chloride reagent: (0.01 M hydrochloric acid and 10 mM ferric chloride).

Wines were subsampled from freshly opened bottles and stored at room temperature. All wine samples were diluted 1:1 with buffer B prior to analysis. Grape homogenate extracts were thawed at room temperature prior to analysis, and no dilution was required.

The A-H tannin assay was performed in duplicate on wines and grape homogenate extracts using the higher throughput format as described in Heredia et al. (*7*). Following the resolubilization of the protein−tannin pellet in buffer C, 262 μL of the mixture was transferred from the 1.5 mL microfuge tubes into wells on a 370 μL Greiner UV star 96 well plate and read at 510 nm. Next, 38 μL of ferric chloride reagent was added to each well using an Eppendorf Research Pro. (8-channel) autopipette (Crown Scientific, Australia), and plates were gently shaken on an automated flatbed plate shaker (Ratek Instruments, Australia) to mix. After a 10 min incubation, the solutions were read at 510 nm. Buffer C was used as a blank for the initial 510 nm reading, and 262 μL of buffer C with 38 μL ferric chloride solution was used as a blank for the final 510 nm reading (*6, 7*).

A calibration curve for reporting tannin absorbance was established using (+)-catechin (50, 100, 150, 200, and 250 mg/L catechin) dissolved in 10% ethanol and using a 10% ethanol blank. All $A_{510}$ (tannin) values are reported in mg/L catechin equivalents of the original sample (corrected for assay dilution).

*Spectrophotometer.* A dual beam monochromatic SpectraMax M2 UV−visible Microplate Reader (Molecular Devices, Australia) was used for all spectral analyses. Greiner UV Star 370 μL 96 well disposable plates were used, which have an optical window down to 200 nm, suitable for reading at 280 nm. The SpectraMax M2 has an inbuilt path correction function that normalized the path length of each well to 10 mm. This function allows direct comparison with a reading taken on a conventional spectrophotomer with a 10 mm path length and corrects for any variation in sample volume.

**Table 1.** Average Tannin Concentration (mg/L), and coefficient of Variation (CV %) from Triplicates of the MCP Tannin Assay and Adams−Harbertson Tannin Assay Performed on Dry Red Wines ($n = 6$)

| | MCP tannin assay | | A-H tannin assay | |
|---|---|---|---|---|
| wine sample | epicatechin equivalents (mg/L) | CV % | catechin equivalents (mg/L) | CV % |
| 2005 Cabernet Sauvignon/Merlot/Shiraz | 2300 | 1.5 | 569 | 3.4 |
| 2002 Cabernet Sauvignon/Merlot | 2241 | 5.5 | 328 | 6.9 |
| 2004 Shiraz | 2007 | 0.7 | 226 | 7.2 |
| 2004 Cabernet Sauvignon | 1935 | 1.7 | 260 | 3.8 |
| 2005 Shiraz | 1546 | 1.1 | 162 | 3.5 |
| 2005 Shiraz/Sangiovese | 1450 | 6.3 | 172 | 1.2 |

*HPLC.* HPLC was performed on an Agilent 1100 LC with DAD (Agilent, Australia) using a Phenomenex Synergi Hydro-RP column (4 $\mu$m particle size, 80 Å pore size, 150 mm × 2 mm), at 25 °C with a gradient elution as described initially in Cozzolino et al. (*11*) and later modified by Mercurio et al. (*4*). A selected set of wines and grape extract samples were analyzed by HPLC pre- and post-precipitation with methyl cellulose and BSA. For the MCP tannin assay, the supernatants from the treatment (after precipitation with methylcellulose) and control (same dilution, no methylcellulose) were injected with no further preparation. For the A-H tannin assay, the supernatants from the treatment (after precipitation with BSA in buffer A) and control (same dilution in buffer A, no BSA) were injected with no further preparation.

**Statistical Analysis.** Repeatability was established by performing the MCP and A-H tannin assays in replicates of three on the same six commercial dry red wines samples. Samples were selected with the intent of including multiple varieties with varying tannin concentrations. The operator, laboratory, instruments, and reagents were held constant during each repeatability study. Statistical analysis was performed on Microsoft Excel 2003 and JMP version 5.0.1a (SAS Institute Inc. USA).

**Investigations into the Difference in Tannin Quantification between Assays.** *Methylcellulose−Tannin Complex Quantified for Iron Reactive Phenolics.* Grape homogenate extracts were thawed at room temperature prior to analysis. The MCP tannin assay was performed on three grape homogenate extracts using the 10 mL format as described in Mercurio et al. (*4*). Following analysis, the supernatant from the treatment sample was decanted, leaving the MC-Tannin pellet. Next, 8.75 mL of buffer C (A-H tannin assay) was added, and the pellet was redissolved with the aid of sonication and physical agitation with a small magnetic stirrer. Once dissolved, the absorbance of the solution was read at 510 nm. After this step, 1.25 mL of ferric chloride reagent was added and the solution mixed and incubated. Following incubation for 10 min, the solution was read at 510 nm and the first value subtracted from the second.

*Calibration Curves.* Calibration curves were established for the A-H and MCP tannin assays as per the reported methods (*4, 7*) using both catechin and epicatechin standard solutions, respectively. To investigate the influence of the specific monomer standard on the final tannin concentration result, a (−)-epicatechin calibration curve was established for the A-H tannin assay and a (+)-catechin calibration curve established for the MCP tannin assay. Both calibration curves are linear throughout the concentration range used for each.

The MCP tannin assay was prepared as described above using aqueous (+)-catechin and (−)-epicatechin at the following concentrations: 50, 100, and 200 mg/L, in replicates of four.

The A-H tannin assay was prepared as described above using (+)-catechin and (−)-epicatechin dissolved in 10% ethanol at the following concentrations: 50, 100, 150, 200, and 250 mg/L, in duplicate.

*Influence of the Monomer/Tannin Ratio of the Sample Solution on Tannin Quantification Using the MCP Tannin Assay.* Two series of aqueous standard solutions were prepared using different combinations of purified grape seed tannin extract (SA Ferco Development, France, purified by $C_{18}$ column chromatography) and the monomer (−)-epicatechin. In the first series, the purified grape seed tannin extract concentration was held constant (1200 mg/L), and the epicatechin concentration was varied (100, 500, 800, 1000, and 1500 mg/L). In the second series, the epicatechin concentration was held constant (800 mg/L), and the purified grape seed tannin extract concentration was varied (500, 1000, 1200, 1500, and 2000 mg/L). The 10 mL format of the MCP tannin assay (*4*) was performed on these solutions using a methyl cellulose/sample ratio of 3:1.

*Influence of Methyl Cellulose and Ammonium Sulfate Solution on 280 nm Absorbance.* Aqueous solutions of (+)-catechin and (−)-epicatechin (50, 100, and 200 mg/L) were prepared, and their absorbance read at 280 nm. The 10 mL format of the MCP tannin assay (*4*) was performed on the standard solutions using a methyl cellulose/sample ratio of 3:1. Absorbance readings at 280 nm for the control (ammonium sulfate addition) and treatment (MC and ammonium sulfate addition) samples were corrected for dilution (1 in 10) and compared to the absorbance reading of the aqueous monomer solutions.

*Influence of BSA on Tannin Detection Using Ferric Chloride.* The A-H tannin assay (*7*) was performed in duplicate on a 150 mg/L aqueous solution of purified grape seed tannin extract (SA Ferco Development, France, purified using a $C_{18}$ chromatography column). The same solution was analyzed for iron reactive phenolics as per the A-H tannin assay without performing the isolation step (no BSA addition). Results were compared to determine the effect of the presence of BSA on the ability of ferric chloride to cause a color change.

**Sensory Evaluation.** A subset of 20 wines from the comparison study was evaluated as part of a larger consumer preference study conducted by the Australian Wine Research Institute sensory research team (*12, 13*). The subset consisted of 10 commercial Shiraz and 10 commercial Cabernet Sauvignon wines from the 2002 and 2003 vintages, carefully selected to be broadly representative of major wine styles made in Australia. The wines were subjected to sensory descriptive analysis using a consensus method. Following 17 discussion and training sessions, a sensory panel of 12 people rated the samples for 4 appearance attributes, 25 aroma attributes, and 12 palate attributes. The attributes drying, adhesive, and surface texture were used to describe aspects of the astringent sensations of the wines. Drying was defined as feeling of lack of lubrication or desiccation, adhesive as the feeling that mouth surfaces are adhering to one another, and surface texture as the degree of sensation of particulate matter brushing against the surfaces of the mouth through the movement of wine (*14*).

Four samples (30 mL) were presented per session in glass-covered ISO wine tasting glasses. At the beginning of each session, panelists rinsed their mouths with a sample of Shiraz wine. Between samples panelists rinsed with a 2 g/L pectin solution followed by two water rinses and a 1 min forced rest. Samples were rated in triplicate using an unstructured 15 cm line scale.

## RESULTS AND DISCUSSION

**Assay Repeatability and Practical Considerations.** A study was conducted to allow for direct comparisons on the repeatability of the assays and for the assessment of other practical considerations such as time efficiency, ease of practice, and throughput. The MCP and A-H tannin assays were performed in triplicate on the same six commercial red wine samples, and the results are detailed in **Table 1**. For the MCP tannin assay, concentrations were expressed as epicatechin equivalents (mg/L) and ranged from 1450 to 2300 mg/L, and for the A-H tannin assay, tannin concentrations were expressed as catechin equivalents (mg/L) and ranged from 162 to 590 mg/L. Tannin

concentrations determined by the A-H and MCP tannin assays significantly differed in value; this was investigated further and will be discussed in greater detail. The results in **Table 1** show that in our laboratory both assays had similar repeatability (coefficient of variation (% CV) between three replicates was below 7% for both assays). The ability to perform the entire MCP tannin assay in 96 well plates greatly increased the throughput of the assay and allowed for simultaneous analysis of 48 samples in approximately 45 min. While the second phase (tannin detection step) of the A-H tannin assay can be performed in 96 well plates, the throughput of the assay is limited by the requirement to perform the first phase (tannin isolation step) in individual 1.5 mL microfuge tubes. This, coupled with multiple incubation stages, greatly reduced the throughput of the A-H tannin assay and resulted in the analysis of 10−15 samples in approximately 90 min.

**Comparison between Grape and Wine Tannin Concentration Determined by the MCP Tannin Assay and the A-H Tannin Assay.** Studies comparing the MCP or A-H tannin assays, respectively, with other analytical measures do exist (*5, 8, 15*); however, results directly comparing tannin concentration determined by the two precipitation-based tannin assays for winegrape varieties and red wine samples have not been published. Sarneckis et al. (*5*) have previously reported results from a comparison study between the MCP tannin assay and reverse phase HPLC using a method detailed in Cozzolino et al. (*11*). The results showed a coefficient of determination ($r^2$) of 0.62 for 50% grape homogenate extracts ($n = 54$) and an $r^2$ of 0.56 for red wines ($n = 121$). Similarly, the relationship between the A-H tannin assay and chromatography-based techniques has been investigated. Kennedy et al. (*8*) reported a study in which tannin concentrations of 40 red wines from the 2002 and 2003 vintages were quantified using the original format of the A-H tannin assay with gel permeation chromatography and phloroglucinolysis. The results showed strong correlations between the A-H tannin assay with both gel permeation chromatography ($r^2 = 0.89$) and phloroglucinolysis ($r^2 = 0.91$) (*8*). Seddon and Downey (*15*) recently reported a moderate correlation ($r^2 = 0.41$) between skin tannin extracts measured using the MCP (with 50% ethanol extracts) and the A-H (with 70% acetone extracts) tannin assays in a range of tablegrape and winegrape varieties. The use of different extraction solvents makes it challenging to determine whether any difference in the response of the assays is related to the fundamental analytical basis of the assays or related to different tannin concentrations and compositions in the samples due to the extraction protocol.

A comparative study was performed to assess the relationships between grape and wine tannin as quantified by the MCP tannin assay and the A-H tannin assay. To allow for comparison with previously reported values and to remain consistent with the reported methods, tannin concentrations were expressed as catechin equivalents for the A-H tannin assay and epicatechin equivalents for the MCP tannin assay. Grape homogenate extract results were expressed as mg of tannin per g of grape homogenate; the MCP tannin assay results were expressed as epicatechin equivalents (mg/g); and A-H tannin assay results were expressed catechin equivalents (mg/g). For wine samples, the MCP tannin assay results were expressed as epicatechin equivalents (mg/L), and for A-H, tannin assay concentrations were expressed as catechin equivalents (mg/L).

Linear regression analysis was used to investigate the relationship between the results from the two analytical techniques. **Figure 1** summarizes these analyses and shows strong correlations between the methods for grape homogenate extracts

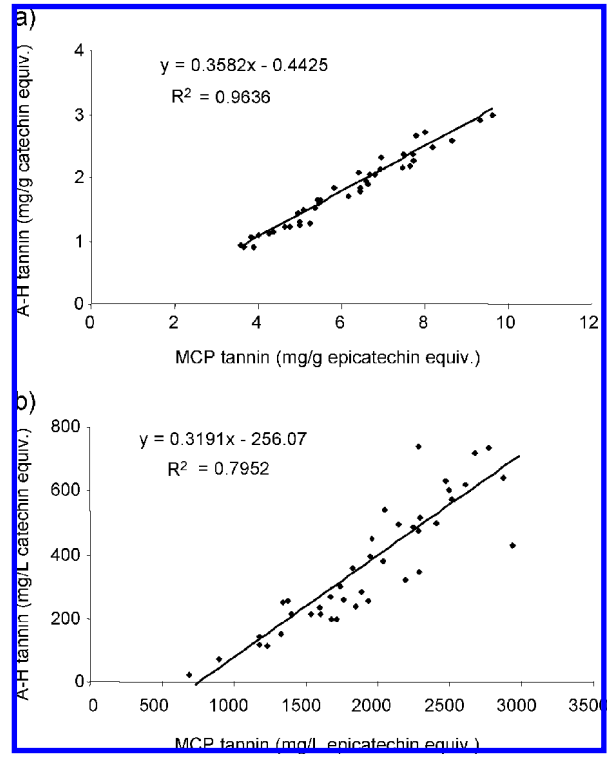


**Figure 1.** Linear regression analysis of tannin concentration determined using the MCP tannin assay and A-H tannin assay for (**a**) grape homogenate extracts ($n = 40$) and (**b**) red wine ($n = 41$).

($r^2 = 0.96$) (**Figure 1a**) and a good correlation for wine samples ($r^2 = 0.80$) (**Figure 1b**). As detailed previously, work reported by Seddon and Downey, (*15*) using the MCP and A-H tannin assays to quantify tannin in acetone extracts of the skins of some tablegrape varieties showed only a moderate correlation ($r^2 = 0.41$). While it remains unclear why this is so for acetone skin extracts of tablegrapes, our research shows that when using 50% aqueous ethanol grape extract of whole homogenized winegrapes, both assays correlate highly with each other.

Interestingly, lower correlations between the analytical methods were obtained for wines than for grape homogenate extracts. We propose that the basis for this most likely stems from the chemical structure differences between grape tannins and wine tannins and the way the two assays function. Because of the acidic and oxidative nature of vinification, wine tannins are structurally very complex and can differ greatly from grape tannins (*3, 16–18*). As a result, the interaction between wine tannins and methyl cellulose or BSA are likely to be more highly variable to that of grape tannins. The grape tannins have less structural diversity and therefore most likely interact in less diverse ways with MC and BSA; thus, they correlate more highly. In addition to general structural rearrangements that add to their diversity, wine tannins also have anthocyanins incorporated, resulting in a class of compounds called pigmented polymers, which form a subset of total tannin. The two assays differ on two levels in their approach to quantifying pigmented polymers: first in the tannin isolation (or precipitation) step and second in the detection step. In terms of pigmented polymer detection, the MCP tannin assay accounts for both the original 280 nm absorbing tannin material from the grape, as well as the 280 nm absorbing contribution from anthocyanin subunits incorporated into the tannin (which make it pigmented). The A-H measure of wine tannin may not necessarily account for the anthocyanin material incorporated into the tannin. This is because the ferric chloride used to produce the final color change

does not react with most anthocyanins, which lack the *ortho*-dihydroxy phenols required to react with ferric chloride (*19*). However, the final absorbance reading for the A-H tannin assay is taken at 510 nm, which is close to the absorbance maximum (520 nm) of anthocyanins and therefore may account for this extra phenolic material that contributes to pigmented polymers. The different approaches to the quantification of the pigmented polymer portion of total tannin may contribute not only to the reduced correlation for wines between the two assays but also partly to the difference in final tannin concentrations determined using the two assays and is discussed in more detail below.

**Investigation into the Difference in Tannin Quantity Determined Using the Two Analytical Techniques.** Kennedy et al. highlighted that wine tannins are a very complex and heterogeneous mixture and therefore reported that tannin concentration values can vary considerably depending on the analytical method applied (*8*). Regression analyses of the MCP and A-H tannin assays (**Figure 1**) showed slopes of 0.36 and 0.32 for grape and wine samples, respectively, revealing a systematic, almost 3-fold, difference in tannin concentration. Importantly, this difference exists for both grape and wine tannin and therefore cannot be due primarily to structural differences between grape tannins and wine tannins. The intercepts of the line seen in **Figure 1** are also of relevant. The intercept of the line seen for the grape extracts was close to zero, indicating that although the two methods gave different tannin concentration values, they were removing very similar amounts of tannin material from the sample. This was not the case for wine samples. The *x*-axis intercept of the line for wine samples was relatively large, indicating that the MCP tannin assay was removing more tannin material than the A-H tannin assay. According to the equation of the line shown in **Figure 1b**, a wine sample analyzed with the MCP tannin assay giving a result of 802 mg/L epicatechin equivalents would have contained no tannin material when analyzed by the A-H tannin assay.

The reasons for the difference in slopes (grape and wine) and intercept (wine) between the two assays were not immediately apparent; therefore, further investigations were undertaken. As mentioned previously, the MCP and A-H tannin assays essentially have two parts to them: a tannin isolation step (or precipitation step) and a tannin detection step. A range of experiments were performed in order to investigate the influence of the isolation and detection steps on the difference in reported tannin concentration values.

*Isolation Step.* Reverse phase HPLC was used as a reference tool to monitor tannin removal from the sample matrix in both the MCP and A-H tannin assays. While the majority of condensed tannins elute by HPLC as a single, broad peak (or hump) at 28 min (after an increase in the acetonitrile organic phase at 26 min), additional tannin does also elute throughout the chromatogram as a broad bleed, causing a raised baseline (*20–24*). This bleed is more pronounced for wines because of their increased structural diversity.

*Grape Extracts.* Chromatograms in **Figure 2** demonstrate the efficiency of both assays in removing tannin material from a Cabernet Sauvignon grape homogenate extract (**Figure 2a** and **b**) and a 2005 shiraz wine (**Figure 2c** and **d**). **Figure 2a** shows the removal of tannin by the A-H tannin assay using three overlaid chromatograms (280 nm): the whole extract in black (extract diluted with buffer A with no BSA), the post-precipitation in red (extract after precipitation with BSA in buffer A), and the chromatogram of BSA in buffer A solution in blue. As shown in the overlays, BSA coelutes with the tannin peak at 28 min and therefore contributes to the area of the tannin peak in the treatment sample shown in red. **Figure 2b** shows the removal of tannin by the MCP tannin assay using two overlaid chromatograms at 280 nm: the whole extract in black (extract sample diluted with no methylcellulose) and the postprecipitation sample in red (extract after precipitation with methylcellulose). The expanded windows in **Figure**s **2a** and **b** highlight the removal of tannin from the sample for each assay. The ability of both assays to remove the tannin material present in the bleed was observed by a drop in the baseline in both treatment samples. It is evident that both assays are equally and highly proficient at removing tannin from the grape extract and that neither assay is removing individual, early eluting phenolic compounds such as monomers and anthocyanins from the sample matrix. This observation is supported by the near zero intercept seen with the linear regression analysis of grape extracts (**Figure 1a**).

*Wines.* The overlaid chromatograms in **Figure 2c** and **d** show the removal of tannins from a 2005 Shiraz wine after precipitation with BSA and methyl cellulose respectively. As with the grape homogenate extracts, the chromatograms of the treatment samples showed no removal of individual early eluting phenolic compounds, and the equally effective ability of both assays to remove the tannin material present in the bleed section was observed by a drop in the baseline. The expanded window in **Figure 2d** shows that wine tannins were completely removed from a 2005 Shiraz wine after precipitation with methyl cellulose; however, **Figure 2c** shows that a portion of tannin remains after precipitation with BSA, indicating incomplete removal of tannin from the wine sample. Although only one wine sample is shown, this was observed for all wine samples analyzed with the A-H tannin assay. The intercept from the regression analysis performed on wine samples shown in **Figure 1b** supports the observation demonstrated in the chromatograms that the MCP tannin assay is removing more tannin material than the A-H tannin assay.

The basis for the above observation may lie in the different approaches to isolation of pigmented polymers. Methyl cellulose complexes and precipitates all tannins and pigmented polymers observable by HPLC in the wine sample, whereas BSA does not. The A-H tannin assay differentiates pigmented polymers into two classes: polymeric pigments that *do* precipitate with BSA and polymeric pigments that *do not* precipitate (*25*). Adams and Harbertson (*26*) previously reported that dimeric and trimeric procyanidins do not complex with BSA; therefore, the two classes of pigments were labeled as large polymeric pigments (LPP) (precipitate with BSA) and small polymeric pigments (SPP) (do not precipitate with BSA) (*25, 26*). However, LPP and SPP have not been chemically characterized, and it remains unclear whether the ability to precipitate with BSA is determined by size alone, or if other physicochemical properties of the pigments are important. Our data suggests that the portion of the tannin peak not removed by precipitation with BSA may be representative of the compounds referred to as SPP, although this cannot be confirmed until this material is characterized. Although less material appears, by HPLC, to be isolated from wines by the A-H tannin assay, and the A-H tannin assay values are lower than the MCP tannin assay values, this cannot be the primary reason for the differences in values between the assays. This is because for grape extracts, both assays appear, by HPLC, to remove the same amount of material, and yet the two assay values are still substantially different, indicating that isolation differences are not the primary reason for the difference. Thus, we continued to investigate factors that could account for the differences.

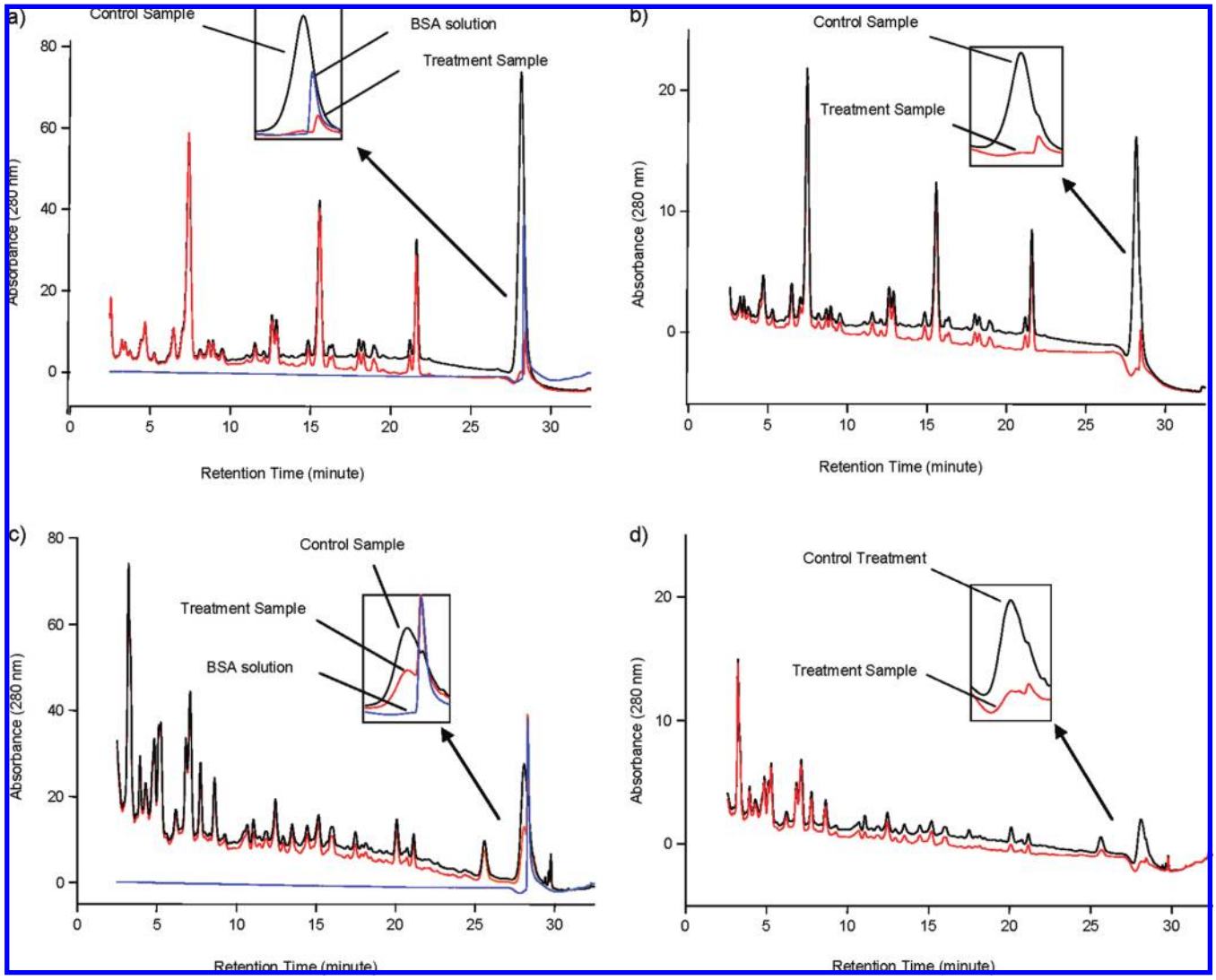


**Figure 2.** Overlaid chromatograms at 280 nm of supernatants from the treatment (in red) and control (in black) samples of a grape homogenate extract analyzed by (**a**) the A-H tannin assay and (**b**) the MCP tannin assay, and of a wine sample analyzed by (**c**) the A-H tannin assay and (**d**) the MCP tannin assay. Chromatograms of the BSA solution (in blue) used in the A-H tannin assay are overlaid in (**a**) and (**c**).

*Methylcellulose−Tannin Complex Quantified for Iron Reactive Phenolics.* An experiment was therefore designed to further investigate whether the MCP and A-H tannin assays were capable of isolating and detecting the same amount of tannin from the sample matrix (**Figure 3**). To remove the complication of pigmented polymer detection, grape homogenate extract samples were used during this experiment. The MCP and A-H tannin assays were performed on the same three grape homogenate extracts and the results compared. Then, to confirm that the two assays were capable of isolating and detecting the same amount of tannin material, the methyl cellulose−tannin (MC−tannin) complexes were redissolved and analyzed for iron reactive phenolics as per the final detection step of the A-H tannin assay. As shown in **Table 2** and **Figure 3**, when the MC−tannin complexes were analyzed for iron reactive phenolics, the actual numerical values were very similar to that of the A-H tannin assay. This demonstrates that although the two assays use different complexation principles in their isolation steps the amount of grape tannin isolated is very similar. Reinforcing what was shown in the HPLC analysis, these results imply that the near 3-fold difference in numerical values is a function of the different detection steps used for each assay and does not reflect the tannin isolation step.

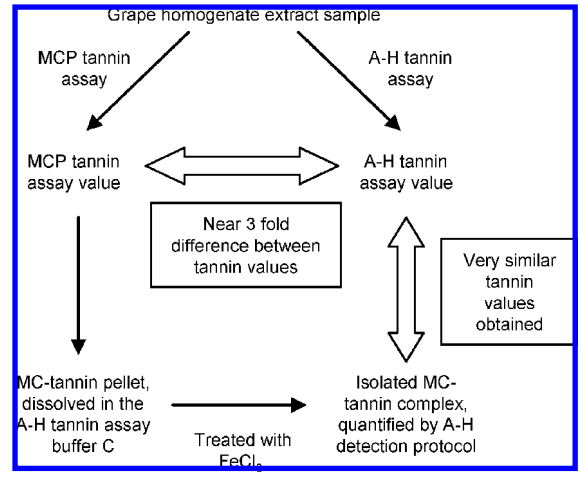


**Figure 3.** Experimental design and summarized results for the methyl cellulose−tannin complex quantified for iron reactive phenolics.

*Do the Monomers Used to Establish the Calibration Curves Affect the Final Assay Values?* To explore the difference in tannin detection steps, a study was conducted to investigate

**Table 2.** Tannin Concentrations As Determined by the MCP Tannin Assay (Epicatechin Equivalents (mg/g)), the A-H Tannin Assay (Catechin Equivalents (mg/g)), and MC−Tannin Complex Quantified for Iron Reactive Phenolics (Catechin Equivalents (mg/g)) for the Same Three Grape Homogenate Extracts

| | MCP tannin assay | A-H tannin assay | MC−tannin pellet quantified for iron reactive phenolics |
|---|---|---|---|
| grape extract | epicatechin equivalents (mg/L) | catechin equivalents (mg/L) | catechin equivalents (mg/L) |
| Shiraz | 524 | 109 | 122 |
| Cabernet Sauvignon 1 | 338 | 51 | 48 |
| Cabernet Sauvignon 2 | 373 | 64 | 55 |

**Table 3.** Comparison of Equations of the Lines for Epicatechin and Catechin Calibration Curves Established for the MCP Tannin Assay and the A-H Tannin Assay

| monomer standard | MCP tannin assay calibration curve | A-H tannin assay calibration curve |
|---|---|---|
| (−)-epicatechin | $y = 0.010x + 0.082$ | $y = 0.007x - 0.289$ |
| (+)-catechin | $y = 0.010x + 0.068$ | $y = 0.007x + 0.359$ |

whether the monomer (catechin or epicatechin) used to establish the calibration curves has any influence on the final values from the assays.

Grape and wine tannins are very structurally diverse and comprise a large number of chemically unique structures (*16, 27*). As explained by Adams and Scholz, tannin composed of only catechin and epicatechin subunits with oligomers only as large as eight subunits could contain more than 500 chemically unique structures (*27*). For this reason, a representative tannin standard is not available, and materials that only approximate the properties of tannins must be used. The diversity of chemical structures that make up tannin and their variability from one product to the next is also the reason that although repeatability can be established, accuracy cannot because there is no right answer. Therefore, in order to approximate the mass of tannin material and facilitate the comparison of results, readily available monomer standards, such as catechin and epicatechin, are often used when quantifying tannins. As they constitute the major subunits of grape tannins, these monomers are the most widely available materials that most closely approximate the spectral properties of tannins. The choice of which monomer standard to use is relativity arbitrary; catechin is regularly used for the A-H tannin assay and epicatechin for the MCP tannin assay (chosen because of its low hygroscopicity (*5*)). It is important to reinforce that neither of these standards precipitate with MC or BSA; they are only used to approximate the spectral properties of tannins. Throughout this study, results were expressed according to the reported methods, epicatechin equivalents for the MCP tannin assay and catechin equivalents for the A-H tannin assay. To investigate the influence of the monomer standard on the final reported tannin value, calibration curves were established using both epicatechin and catechin as per the reported methods, and the equations of the lines are detailed in **Table 3**. The results indicate that there is minimal difference in the slope and intercept of the calibration curves. Thus, it can be concluded that the choice of monomer standard does not account for the near 3-fold difference seen in the tannin concentration.

*Does the Matrix of Each Assay Influence the Final Value?* Given that MC−tannin pellets isolated from grape extracts react with ferric chloride to give values very similar to the values determined by performing the A-H tannin assay directly on the grape extracts, there must be a reason for the difference in values other than the isolation step or the monomers used to report the values. One hypothesis is that interactions may exist between the different chemicals in the respective matrices of the assays, which result in substantial differences in the spectral properties of the phenolics. To test this hypothesis, investigations into the effect of chemical interactions on the final value of each assay were undertaken. Experimental design and summarized findings are outlined in **Figure 4**. For the MCP tannin assay, variations in the monomer to tannin ratio of the sample solution were explored as it is well established that phenolics can interact with each other to alter the extinction coefficients of each of the interacting molecules (*28–30*) (**Figure 4a**). In addition, for the MCP tannin assay, the influence of buffer salts and methyl cellulose on the relative 280 nm absorbance of the phenolics in solution was investigated (**Figure 4b**). Variations in the monomer and tannin ratio had no effect on the absorbance at 280 nm and therefore no influence on tannin quantification. An increase in absorbance at 280 nm was observed with the addition of ammonium sulfate and methyl cellulose; however, this increase was not sufficient to account for the near 3-fold difference seen between the two assays. For the A-H tannin assay, the effect of BSA on the detection of iron reactive phenolics was examined as described in **Figure 4c**. It was shown that the presence of BSA had no effect on the absorbance reading of the ferric chloride−tannin complex and thus did not influence the quantification of tannin.

In summary, we have shown that the difference in reported tannin concentration between the MCP and A-H tannin assays is not primarily caused by differences in isolation of tannin material, but rather a function of the different detection methods used. As discussed earlier, no tannin standards will ever be available that accurately represent the massive diversity of molecules described as tannin, and tannin quantification is largely dependent on the method employed (*8, 31*). Therefore, direct comparisons between tannin concentrations quantified by various analytical techniques should continue to be made with consideration of the operating principles of the particular assay used and an understanding of how the value determined using one method compares to those determined using other methods. The work described here gives guidance to those wanting to understand how the values between the two assays relate. A conclusive explanation for the differences in values between the MCP and A-H tannin assays remains unclear, and further work in this area is required.

**Correlation of Analytical Techniques with Astringency Perception.** Wine matrix variables such as pH, acidity, viscosity, and alcohol concentration as well as the structure of wine tannins can modulate the perception of wine astringency (*2, 32, 33*). However, many studies have shown that tannin quantified using several diverse analytical methods correlates reasonably well with wine astringency, independent of the wine matrix (*8, 34, 35*). Much of the research on tannin quantification and tools for evaluating wine astringency has focused on the quantification of tannins by chromatography and protein-based precipitation assays. Monteleone et al. (*35*) and Condelli et al. (*34*). have successfully developed predic-

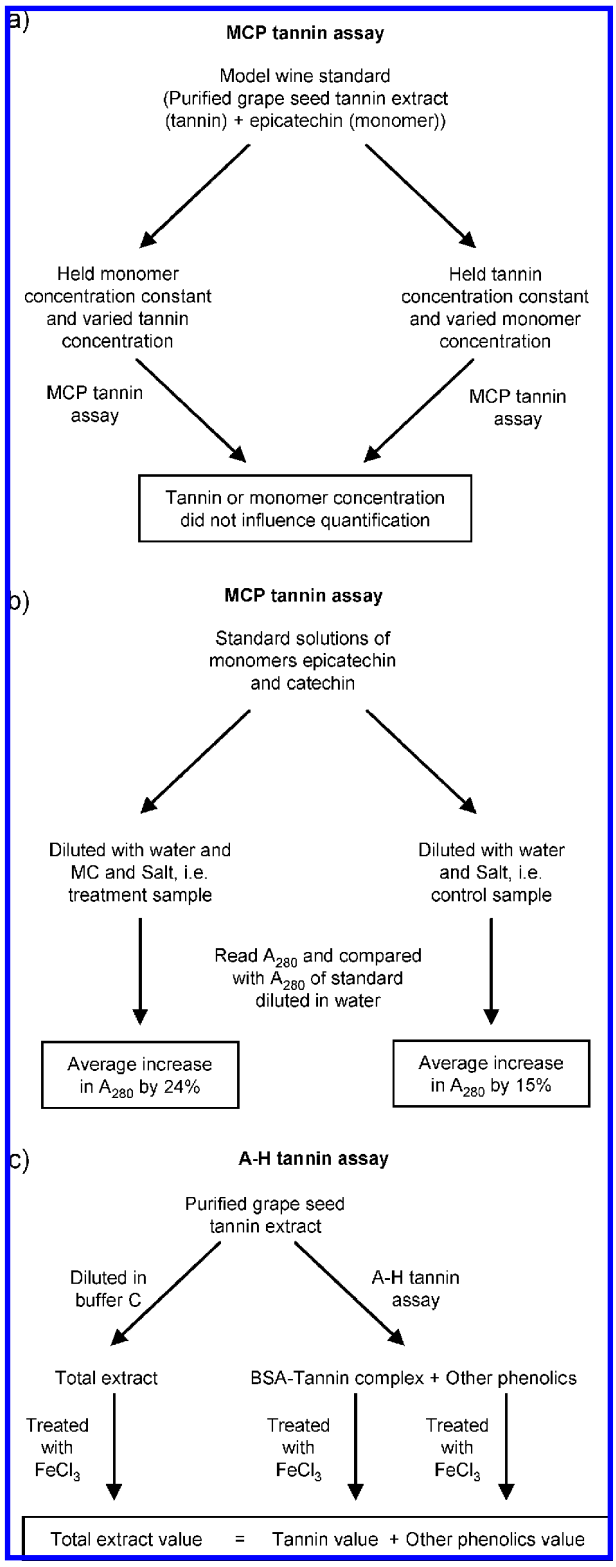


**Figure 4.** Experimental design and summarized results from the investigation into matrix influence of the qualification of tannin using the MCP and A-H tannin assays. (**a**) Influence of the monomer/tannin ratio of the sample solution on tannin quantification using the MCP tannin assay. (**b**) Influence of methyl cellulose and ammonium sulfate solution on 280 nm absorbance. (**c**) Influence of BSA on tannin detection using ferric chloride.

tion models ($r^2 = 0.95$) for perceived wine astringency by assessing the ability of wine to develop turbidity by reacting with the protein mucin. Of particular relevance is a study by

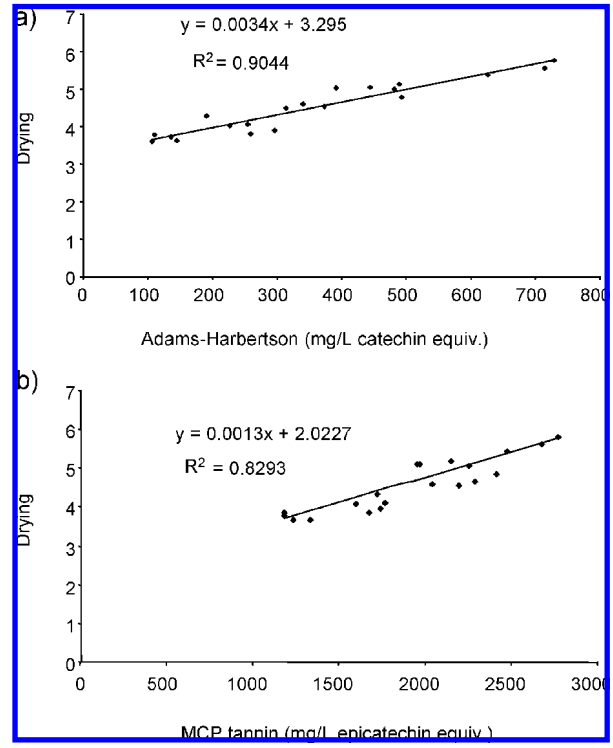


**Figure 5.** Linear regression analysis between wine ($n = 20$) tannin concentration as determined by the (**a**) Adams−Harbertson tannin assay (mg/L catechin equivalents) and (**b**) MCP tannin assay (mg/L epicatechin equivalents)) with the sensory descriptor drying as perceived by a trained panel.

Kennedy et al. in which 40 red wines were evaluated for astringency by five wine industry professionals and compared to results from several tannin quantification methods including the original format of the A-H tannin assay (*8*). Of the analytical methods investigated, the A-H tannin assay showed the strongest correlations with wine astringency ($r^2 = 0.82$), followed by gel permeation chromatography ($r^2 = 0.74$) and phloroglucinolysis ($r^2 = 0.73$).

One focus of this study was to assess the utility and relevance of the MCP tannin assay to the wine industry; therefore, it was pertinent to investigate the correlation of the assay with wine astringency ratings. Higher throughput methods such as the MCP tannin assay have the potential to provide an objective, cost-effective, rapid analytical method to evaluate wine astringency as an alternative to costly and time-consuming sensory studies. Wine astringency data for 10 Shiraz and 10 Cabernet Sauvignon wines from the 2002 and 2003 vintage (*12, 13*) was collected using sensory descriptive analysis with a trained sensory panel. Among other sensory attributes, the following astringency related descriptors were quantified in triplicate: surface texture, adhesiveness, and drying. These sensory descriptors correlated very strongly with each other ($r^2 = 0.96$); therefore, only results for the drying attribute are shown. **Figure 5** shows the results of linear regression analyses between tannin concentration (as determined using both the MCP tannin assay and the A-H tannin assay) and drying as rated by the trained panel. Strong correlations with drying were observed with the MCP tannin assay values, with an $r^2$ value of 0.83. Interestingly, with this set of wines an $r^2$ value of 0.90 was observed for the A-H tannin assay, which was higher than that reported by Kennedy et al. ($r^2 = 0.82$) (*8*). Thus, the MCP tannin assay models wine astringency with reasonable

confidence. The simplicity and efficiency of this assay coupled with its ability to objectively predict wine astringency could prove very useful for both researchers and wine industry practitioners.

## ABBREVIATIONS USED

MCP, methyl cellulose precipitable; A-H, Adams−Harbertson; HPLC, high performance liquid chromatography; CV, coefficient of variation; au, absorbance units; BSA, bovine serum albumin; MC, methyl cellulose.

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