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High Throughput Analysis of Red Wine and Grape Phenolics—Adaptation and Validation of Methyl Cellulose Precipitable Tannin Assay and Modified Somers Color Assay to a Rapid 96 Well Plate Format

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The methyl cellulose precipitable (MCP) tannin assay and a modified version of the Somers and Evans color assay were adapted to high-throughput (HTP) analysis. To improve efficiency of the MCP tannin assay, a miniaturized 1 mL format and a HTP format using 96 well plates were developed. The Somers color assay was modified to allow the standardization of pH and ethanol concentrations of wine samples in a simple one-step dilution with a buffer solution, thus removing inconsistencies between wine matrices prior to analysis and allowing for its adaptation to a HTP format. Validation studies showed that all new formats were efficient, and results were reproducible and analogous to the original formats.

KEYWORDS: Tannins; anthocyanins; quantification; grape; red wine; wine color; assay; methyl cellulose precipitation

INTRODUCTION

Polyphenolic compounds are essential wine constituents that are responsible for major organoleptic properties including mouthfeel and color in red wine. In particular, anthocyanins, condensed tannins (proanthocyanidins), and their conjugates, pigmented polymers, are of great importance. Anthocyanins are pigments located primarily in the grape skins and are responsible for their black to red coloring (*1*). Condensed tannins are predominantly located in the skins and seeds of the fruit and are closely linked with mouthfeel and astringency (*2*). During fermentation and maceration, the skins and seeds of the grapes are held in contact with the fermenting juice, resulting in extraction of anthocyanins, tannins, and other phenolic materials. After crushing, anthocyanins begin to react with tannins and other phenolic materials to form pigmented polymers. Pigmented polymers are the most relevant pigments to the red color of older red wines (*3*, *4*). Anthocyanins, pigmented polymers, and tannins are valuable quality indicators in a wide range of plant-derived products, including fruit juices, juice concentrates, and forages (*5*−*10*).

The color of young red wine is largely due to the presence of anthocyanins in their positively charged flavylium form (colored form). However, under original wine conditions, only a small percentage of the total concentration of anthocyanins exist in this form (*4*, *11*, *12*), as the colored anthocyanins exist in equilibrium with other colorless forms. The equilibrium is medium-dependent, with the major influencing factors being wine pH and SO_2 concentration. Decreasing the pH shifts the equilibrium toward the red-colored form, while an increase in the amount of free SO_2 will cause "bleaching" of the anthocyanins and decrease the red color of the wine. During fermentation and aging, anthocyanins undergo various oxidation, condensation, and polymerization reactions with themselves and other phenolic material, in particular with condensed tannins, to form new pigmented compounds. Within the first year of aging, 50−70% of anthocyanins will have reacted to give pigmented polymers. Pigmented polymers are of major importance to long-term color stability of red wine as they are significantly more stable and less affected by changes in pH and bleaching by free SO_2 when compared to anthocyanins (*13*, *14*).

The relative concentrations of grape tannins and anthocyanins, pigmented polymers, and tannins in wines are affected by many variables including the grape cultivar, soil type, seasonal conditions, viticultural practices (i.e., irrigation, pruning), grape maturity, winemaking techniques (i.e., fermentation, maceration, pressing), and age of the wine (*12*, *14*, *15*). As discussed by Herderich and Smith (*16*), a number of established analytical methods are available for the quantitation of tannins; however, many are laborious, costly, and not suitable to large sample sets. Similar obstacles are faced with the well-known Somers and Evans wine color measurements (*11*, *17*).

Methyl Cellulose Precipitable (MCP) Tannin Assay. As recently presented in Sarneckis et al. (*18*), a method was developed for the quantification of condensed tannins by

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precipitation with methyl cellulose, referred to as the MCP tannin assay. The assay is simple and robust and permits the measurement of condensed tannins in red wine, grape extract prepared with a 50% aqueous ethanol solution, and aqueous tannin solutions. Because of the tolerance of a broad range of pH values and ethanol concentrations, the MCP tannin assay could easily be adapted for measurement of tannin in a wide variety of other plant-derived products, such as forages, black and green teas, coffee, spices, and legumes (*7*−*10*). The MCP tannin assay is based on polymer−tannin interactions, resulting in an insoluble polymer−tannin complex, which precipitates and is separated by centrifugation. It is a subtractive measure requiring the preparation of a control and treatment sample. The control sample represents the total phenolic concentration present in the matrix, whereas the treatment sample represents the phenolic concentration remaining in the supernatant solution after the tannin has precipitated. The phenolic content is monitored by measuring the absorbance at 280 nm (A_{280}) using a UV/visible spectrophotometer. By subtracting the A_{280} of the treatment sample from the A_{280} of the control sample, the A_{280} of the tannin in a solution can be determined. The A_{280} can be either used as an arbitrary value or converted to monomer equivalents (epicatechin equivalents, mg L^{-1}). The original method has been established and validated using a 10 mL final sample volume and reading of the absorbance at 280 nm in a 10 mm quartz cuvette (*18*). After performing the assay routinely in-house and monitoring its adoption within industry, the need for a format that allowed use of smaller volumes, smaller centrifuges, and a format allowing for more efficient, high throughput (HTP) measurements was quickly identified.

The two new assay formats proposed are a 1 mL format and a HTP format. In both cases, the formats had to be scaled down to a final volume of 1 mL. In addition to smaller sample volumes and reduced reagent costs, the main advantage of the 1 mL format is that it will allow laboratories with small bench top centrifuges to adopt the assay. The HTP format is designed to be performed in 1.1 mL 96 well deep well plates and read on a microplate reader spectrophotometer. The use of multichannel pipettes in the HTP format allows for the simultaneous preparation of 48 samples within 1 h (considering both a treatment and a control required for each sample). In addition, the HTP format permits the direct transfer of supernatant from the deep well plate used for sample preparation into a 96 well microtiter plate. Using a microplate reader spectrophotometer, a combined total of 96 treatment and control and samples can be read simultaneously, resulting in a five-fold reduction in analysis time.

Modified Somers Color Assay. Somers and Evans (*11*, *17*) established a set of spectroscopic color measurements, which not only give a measure of wine color but also give an insight into the contributing elements such as anthocyanin equilibria and phenolic composition. The original Somers assay is a four part assay, where the wine is analyzed in its original state and is then treated with excess SO_2, excess acetaldehyde, and hydrochloric acid to investigate the anthocyanin equilibrium of the wine. First, the absorbance of the young red wine sample is read at 420 (yellow/orange pigments) and 520 nm (red pigments) in its original state (with regard to wine pH and SO_2 concentration), and from these values, the wine color parameters wine color density and hue are calculated. The second reading is taken after the addition of excess SO_2 allowing for the measurement of color (A_{520}) resulting from the SO_2-resistant pigments present in the wine. Third, the original wine is treated with excess acetaldehyde, which permits the estimation of colored anthocyanins at wine pH. Finally, the wine is diluted with 1 M hydrochloric acid, lowering the pH and converting all anthocyanins and many other pigments into their colored forms. The acidified solution is then monitored at A_{520} and A_{280} to give an indication of the concentration of total red pigments and total phenolics, respectively.

The main modification to the original method, reported in this paper, is the standardization of the wine pH to pH 3.4 and the alcohol concentration to 12% v/v using a buffer solution prior to the initial analysis. As described earlier, the pH of a wine has a profound effect on color expression; therefore, comparisons between wines at differing pH values can often be misleading. Iland et al. (*19*) reported a method in which the Somers assay was performed on the wine in its original state and on the wine at pH 3.5. The Iland method involves adjustment of the pH of each wine individually by the dropwise addition of sodium hydroxide or hydrochloric acid; however, this approach is somewhat tedious. This lengthy step is overcome by adjusting the pH in a simple one in 10 dilution with a model wine buffer solution. Sodium metabisulphite or acetaldehyde can be incorporated into the buffer solution rather than added directly to individual samples, again greatly reducing preparation time. Other advantages include the ability to use a 10 mm cuvette and minimizing any residual turbidity effects through dilution. By removing the need for individually adjusting the pH of the wines and by incorporating assay reagents in the dilution buffer, this modified Somers assay has also been adapted to a HTP 96 well plate format, allowing for simultaneous analysis of large sample numbers.

As the wine industry looks toward augmenting traditional measures of grape and wine quality to meet in-house specifications and consumer demands, the need for cost-effective rapid analytical methods for quantification of grape and wine tannins and color is increasing. Access to such valuable data will allow viticulturists and enologists to make better informed decisions regarding tannin and color and to improve control over the chosen wine style. At the same time, rapid measurement of anthocyanins, pigmented polymers, and tannins as described in this paper will allow assessment of quality and other properties in a wide range of fruit concentrates, juices, and nectars (*5*, *6*).

MATERIALS AND METHODS

MCP Tannin Assay. The assay was performed on wine and grape homogenate extracts as described in Sarneckis et al. (*18*) with the exception of the volume of methyl cellulose solution added, which was increased by 50%. Subsequent to the original publication, a broader range of samples were surveyed, and it was observed that in a few samples, 2 mL of 0.04% methyl cellulose solution (as used in the original method) was insufficient to achieve the complete removal of tannin from the matrix. Following a second series of optimization trials in which variations to the methyl cellulose:tannin solutions ratio were investigated, optimal ratios of 3:0.25 and 3:1 were determined for wine and grape homogenate extracts, respectively. The efficiency of the MCP tannin assay was monitored both spectroscopically (by recording the absorbance at 280 nm) and by high-performance liquid chromatography (HPLC). For a method description in the form of a standard operating protocol, please refer to Mercurio et al. (*20*).

Methyl cellulose solution (0.04% w/v; Sigma M-0387, 1500 cP viscosity at 2%) was prepared in accordance with the manufacturer's instructions. Grape homogenate extracts were generated using between 150 and 190 g of grape berries (destemmed and frozen) that were defrosted 3 h prior to homogenization (Retsch Grindomix GM200 homogenizer) (*21*). Aqueous ethanol (10 mL, 50%) was added to between 0.96 and 1.04 g of homogenate, and the sample was spun at high speed on a Ratek suspension mixer (Stennick Scientific, Australia) for 60 min and then centrifuged at 4000 rpm for 5 min using a Hettich

Zentifugen Universal 32 R centrifuge with a Hettich 1624 rotor (Adelab Scientific, Australia). Wine samples were subsampled from freshly open bottles and stored at room temperature; no further preparation was required.

Treatment Sample. To a 10 mL centrifuge tube, 3.00 mL of methyl cellulose solution was added to the required volume of tannin-containing solution (0.25 mL of wine or 1.00 mL of grape homogenate extract), and the tube was capped, inverted several times, and allowed to stand for 2−3 min. Following the addition of 2.00 mL of saturated ammonium sulfate solution, the sample was made up to final volume with deionized water, mixed again, and allowed to stand for 10 min at room temperature before centrifugation. Centrifugation was performed at 4000 rpm for 5 min on a Hettich Universal 32 R centrifuge with a Hettich 1624 rotor (Adelab Scientific).

Control Sample. The same volume of tannin-containing solution was added as per the treatment sample. Following the addition of 2.00 mL of saturated ammonium sulfate solution, the sample was made up to final volume with deionized water (in place of the methyl cellulose polymer solution), mixed, and allowed to stand for 10 min at room temperature before centrifugation as above.

The 1 mL and HTP formats of the assay were performed in the same sequence as the 10 mL format described above but were downsized to a final volume of 1 mL. **Table 1** shows the required reagent volumes for all formats of the assay. The 1 mL assay was performed in 1.5 mL microfuge tubes. Centrifugation was performed at 10000 rpm for 5 min on a Thermo Electron Corp. IEC Micromax microcentrifuge (Biolab, Australia). Again, the HTP format was based on the 10 mL assay; however, sample preparation and tannin precipitation were performed in Axygen 1.1 mL 96 well deep well plates. The addition of all reagents to the respective wells was performed using an eight-channel autopipet (Eppendorf Research Pro., Crown Scientific, Australia). Mixing was achieved by gently shaking the plate, ideally on an automated flatbed plate shaker. Centrifugation was performed at 2000 rpm for 5 min on a Hettich Universal 32 R centrifuge with a Hettich 1645 rotor (Adelab Scientific). For all three formats, 300 μL of supernatant from the treatment and control samples was transferred into a 370 μL Greiner UV star 96 well plate and read using a SpectraMax M2 Microplate Reader (Molecular Devices, Australia).

Calculations for MCP Tannin Assay. The arbitrary $A_{280\ \mathrm{Tannin}}$ value was converted into epicatechin equivalents (mg L^{-1}) with the aid of a calibration curve that should be established for each spectrophotometer/plate reader. Wine samples had a dilution factor of 40, and extract samples had a dilution factor of 10.

$$\text{Tannin concentration in homogenate (mg/g)} = \frac{[\text{Tannin}]e \times Ve}{Wh}$$

where
[Tannin]e = tannin concentration in extract (mg/L epicatechin eq.)
Ve = final volume of extract (L)
Wh = initial weight of homogenate sample (g)

Precision. Precision was established by performing the 10 mL, 1 mL, and HTP formats in replicates of eight on four dry red wines and four grape homogenate extract samples. Samples were selected to include multiple varieties and a broad range of tannin concentrations. The following conditions were held constant during each precision study: operator, laboratory, instruments, and reagents. Statistical analysis of the data was performed on Microsoft Excel 2003 and Systat 10 (SPSS, Chicago, IL).

Modified Somers Assay. For both the 10 mL and the HTP formats, after incubation, 300 μL of each treatment was transferred into 370 μL Greiner UV star 96 well plates and read using the SpectraMax M2 Microplate Reader. All wines were subsampled from freshly opened bottles and centrifuged at 4000 rpm for 5 min using a Hettich Universal 32 R centrifuge with a Hettich 1624 rotor (Adelab Scientific).

Treatment A: one in 10 dilution of wine in buffer 1 (model wine, 0.5% w/v tartaric acid in 12% v/v ethanol adjusted to pH 3.4 with 5 M NaOH). Absorbance was read at 420 and 520 nm immediately after mixing.

Treatment B: one in 10 dilution of wine in buffer 1 plus 0.375% w/v sodium metabisulphite. Samples were mixed and incubated at room temperature for 1 h. Absorbance was read at 520 nm.

Treatment C: one in 10 dilution of wine in buffer 1 + 0.1% v/v acetaldehyde. Samples were mixed and incubated at room temperature for 1 h. Absorbance was read at 420 and 520 nm.

Treatment D: one in 50 dilution of wine in 1 M HCl. Samples were mixed and incubated at room temperature in the dark for 3 h. Absorbance was read at 280 and 520 nm.

The 10 mL format was performed in 10 mL centrifuge tubes. Tubes were capped and inverted several times to allow for mixing prior to incubation. The HTP assay was performed in 1.1 mL 96 well deep well plates. Treatments A−C were performed in the same plate. However, as treatment D required storage in the dark for 3 h, it was essential that this treatment be performed in a separate plate. Apart from 10-fold downsizing, the major difference between the two assay formats was that the original 10 mL assay required the addition of the wine sample to the specific buffer solution; however, to allow for better mixing, the sequence was reversed in the HTP format, where the buffer was added to the wine sample. The plates were sealed with the appropriate sealing mat and shaken gently, ideally on an automated flatbed plate shaker to allow for mixing prior to incubation.

Calculations for Modified Somers Color Parameters.

Chemical age 1 (no units):
$A_{520\mathrm{sulfite}}/A_{520\mathrm{acetal}}$

Chemical age 2 (no units):
$A_{520\mathrm{sulfite}}/(5 \times A_{520\mathrm{HCl}})$

Degree of ionization of anthocyanins (%):
$$\left\{ \frac{(10 \times A_{520\mathrm{buffer1}}) - (10 \times A_{520\mathrm{sulfite}})}{(50 \times A_{520\mathrm{HCl}}) - [1.6667 \times (10 \times A_{520\mathrm{sulfite}})]} \right\} \times 100$$

Total anthocyanins (mg/L):
$20 \times [(50 \times A_{520\mathrm{HCl}}) - 1.6667 \times (10 \times A_{520\ \mathrm{sulfite}})]$

Color density (au):
$(A_{420\mathrm{buffer1}} + A_{520\mathrm{buffer1}}) \times 10$

***Color density, SO_2-corrected (au):**
$(A_{420\mathrm{acetal}} + A_{520\mathrm{acetal}}) \times 10$

Hue (no units):
$A_{420\mathrm{buffer1}}/A_{520\mathrm{buffer1}}$

***SO_2-resistant pigments (au)**
$A_{520\mathrm{sulfite}} \times 10$

Total phenolics (au):
$(A_{280\mathrm{HCl}} \times 50) - 4$

where an asterisk indicates that it is not an original Somers parameter.

Validation. Precision was established by performing the 10 mL format in triplicate and the HTP format performed in replicates of eight on four commercial dry red wines samples. Samples were selected with the intent of including multiple varieties with varying colors. The following conditions were held constant during each precision study: operator, laboratory, instruments, and reagents. Statistical analysis was performed on Microsoft Excel 2003 and Systat 1 (SPSS).

Analytical Methods. *Spectrophotometer.* A dual beam monochromatic SpectraMax M2 UV−visible Microplate Reader (Molecular Devices) was used for all spectral analysis. Greiner UV Star 370 μL 96 well disposable plates were used, which have an optical window

Table 1. Optimized Volumes of Sample and Reagents for the All Three Formats of the MCP Tannin Assay for Wine and Grape Extract Samples

sample type	assay format	treatment				control			
		tannin solution	polymer	salt	water	tannin solution	polymer	salt	water
wine	10 mL	0.25 mL	3 mL	2 mL	4.75 mL	0.25 mL	0 mL	2 mL	7.75 mL
	1 mL	25 μL	300 μL	200 μL	475 μL	25 μL	0 μL	200 μL	775 μL
	HTP	25 μL	300 μL	200 μL	475 μL	25 μL	0 μL	200 μL	775 μL
extract	10 mL	1 mL	3 mL	2 mL	4 mL	1 mL	0 mL	2 mL	7 mL
	1 mL	100 μL	300 μL	200 μL	400 μL	100 μL	0 μL	200 μL	700 μL
	HTP	100 μL	300 μL	200 μL	400 μL	100 μL	0 μL	200 μL	700 μL

down to 200 nm, and therefore did not interfere with the reading at 280 nm. The SpectraMax M2 has a built-in path-correction function that normalized the path length of each well to 10 mm and corrected for any variation in sample volume. For the MCP tannin assay, water was used as a blank. Aqueous (−)-epicatechin solutions (10, 25, 50, 75, 100, 150, 200, and 250 mg L^{-1} epicatechin) were used to establish a calibration chart for reporting tannin absorbances. All A_{280} (tannin) values are reported in mg L^{-1} or g L^{-1} epicatechin equivalents of the original sample (i.e., corrected for assay dilution). For the modified Somers assay, buffer 1 was used as a reference for treatments A−C and 1 M HCl was used as a reference for treatment D.

HPLC. HPLC was performed on an Agilent 1100 LC (Agilent, Australia) using a gradient elution based on the method described in Cozzolino et al. (*22*). To improve integration and quantification of the tannin peak, the gradient was modified to achieve separation of the quercetin and tannin peaks. The column was a Phenomenex Synergi Hydro-RP (4 μm particle size, 80 Å pore size, 150 mm × 2 mm), at 25 °C. Solvents were (A) 1% acetonitrile and 1.5% phosphoric acid in water and (B) 20% solvent A and 80% acetonitrile for gradient elution at a flow rate of 0.4 mL/min: 0 (14.5% solvent B), 18 (27.5% solvent B), 24 (27.5% solvent B), 25 (50.0% solvent B), 26 (50.0% solvent B), 30 (100% solvent B), 32 (100% solvent B), 32.01 (14.5% solvent B), and 40 min (14.5% solvent B).

RESULTS AND DISCUSSION

MCP Tannin Assay. Optimization trials were conducted initially on the 10 mL format and then repeated on the 1 mL and HTP formats. The efficiency of the assay to remove the tannin from each matrix was monitored by HPLC. It was evident that for wine (250 μL) and grape homogenate extract (1000 μL) samples, 3 mL of polymer solution and 2 mL of saturated ammonium sulfate solution resulted in the complete removal of tannin.

For the development and validation trials, HPLC was employed as a reference tool to assess the removal of tannin from the matrix. **Figure 1** illustrates the removal of tannin from a grape homogenate extract using the 10 mL, 1 mL, and HTP formats of the MCP tannin assay. It shows four overlaid chromatograms (280 nm), including the supernatants of treatment samples from all three formats of the assay and the supernatant of the control sample from the 1 mL assay format. The chromatograms demonstrate the efficiency of the MCP tannin assay to remove tannin (eluting at 28 min) from the sample, while all other phenolic compounds remained unchanged. The expanded window highlights the removal of tannin in all three formats. A small dip in the baseline coincides with the tannin peak; this is due to the solvent gradient system used and has minimal effect on the quantification. It should be noted that the peak identified as the tannin peak (eluting at 28 min) represents a wide range of condensed tannins. While some 4,8-linked dimers and trimers have been shown to elute earlier as distinct peaks, the majority of the condensed tannins from wine and grape samples typically coelute in this tannin peak (*28*).

Precision studies were conducted on dry red wine and 50% ethanol grape homogenate extracts by performing the three optimized formats of the assay in replicates of eight on four wine and grape extract samples (**Tables 2** and **3**). Wine samples were selected to include several varieties covering a broad range of tannin concentrations from the 2004 and 2005 vintages. Wine tannin concentrations were expressed as epicatechin equivalents (g L^{-1}) and ranged from 1.6−3.0 g L^{-1}. The coefficient of variation (CV) between the eight replicates was below 10% for all assay formats. Grape homogenate extracts were prepared from 2006 vintage fruit. Tannin concentrations were expressed as epicatechin equivalents (mg g^{-1}) of homogenate and ranged

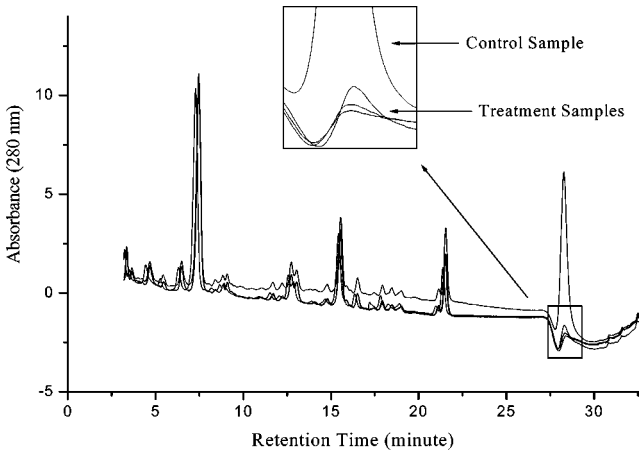

Figure 1. Chromatogram at 280 nm of supernatant from treatment samples using the 10 mL, 1 mL, and HTP formats on the same grape extract overlaid with the supernatant of the respective 1 mL control sample. The expanded window shows complete removal of tannin with each assay format.

Table 2. Average A_{280} Tannin, Epicatechin Equivalents (mg L^{-1}), and CV (%) for the 10 mL, 1 mL, and HTP Formats of the MCP Tannin Assay on Dry Red Wines ($n = 8$)

	assay format	average A_{280} tannin	average epicatechin equivalents (mg L^{-1})	CV % of epicatechin equivalents
Cabernet Sauvignon/ Shiraz/Merlot blend 2004	10 mL	0.499	1858	4.1
	1 mL	0.502	1870	4.9
	HTP	0.529	1974	6.6
Shiraz 2004	10 mL	0.424	1564	4.5
	1 mL	0.436	1610	9.3
	HTP	0.447	1653	8.5
Cabernet Sauvignon 2004	10 mL	0.735	2784	2.8
	1 mL	0.803	3051	6.0
	HTP	0.775	2940	4.2
Merlot 2005	10 mL	0.532	1988	3.8
	1 mL	0.528	1972	9.8
	HTP	0.493	1835	4.7

Table 3. Average $A_{280\ Tannin,}$ Epicatechin Equivalents (mg g^{-1}) of Homogenate, and Respective CV (%) for the 10 mL, 1 mL, and HTP Formats of the MCP Tannin Assay on Grape Homogenate Extracts ($n = 8$)

	assay format	average A_{280} tannin	average epicatechin equivalents (mg g^{-1})	CV % of epicatechin equivalents
Cabernet	10 mL	0.357	3.42	2.5
Sauvignon	1 mL	0.357	3.42	1.4
2006	HTP	0.349	3.35	6.4
Shiraz	10 mL	0.607	5.98	1.5
2006	1 mL	0.605	5.96	2.1
	HTP	0.597	5.88	4.3
Cabernet	10 mL	0.413	4.00	4.3
Sauvignon	1 mL	0.451	4.39	4.3
2006	HTP	0.413	3.99	4.3
Shiraz	10 mL	0.394	3.82	3.6
2006	1 mL	0.417	4.07	4.8
	HTP	0.370	3.58	2.4

Table 4. Results from Comparative Statistical Analysis of the MCP Tannin Analysis using the 10 mL, 1 mL, and HTP Formats[a]

	ANOVA			
	format		format × sample	
	F ratio	*P* value	*F* ratio	*P* value
dry red wine	3.3	0.044	4.3	0.001
50% grape homogenate extract	22.0	<0.001	5.0	<0.001

[a] ANOVA was conducted considering the format type only and the interaction of format type and sample.

from 3.4 to 5.9 mg g^{-1}, with CV between the eight replicates from 1.4 to 6.4%. Additional validation parameters such as linearity, drift, and recovery had been previously determined and demonstrated robustness of the tannin precipitation step of the assay (*18*).

Table 4 displays results from analysis of variance (ANOVA) tests performed on the precision data. While the ANOVA results indicate statistically significant differences between the formats, due in part to the low standard errors, the magnitude of the differences in relation to the total magnitude of the measure is small. There was significant method and sample interaction, but the differences were small. We consider the differences to be within the margin anticipated for such physically different formats but nonetheless recommend selecting just one format for comparison throughout a study.

Modified Somers Validation. Precision was established by performing the 10 mL format in triplicate and the HTP format in replicates of eight on four dry red wine samples (**Table 5**). Samples were selected to include several varieties with varying colors from the 2005 and 2006 vintages. Absorbance values were used to calculate a series of color parameters as described in the Materials and Methods, and results underwent statistical analysis. The CV between the eight replicates using the HTP assay format was between 0.1 and 6.4% for all parameters. For the 10 mL format, the CV % for all parameters was less than or equal to 3.6%, with the exception of degree of ionization of anthocyanins for the 2005 Cabernet Sauvignon sample. **Table 6** shows results from ANOVA tests and linear regression analysis for the same data. ANOVA showed some statistically significant differences between the two formats; however, these differences were small and in part a result of low standard errors for both formats. For many of the parameters, no statistically significant differences were observed when considering the interactions of the assay format type and sample, indicating that the observations were robust and independent of sample matrix. Linear regression analysis was conducted to determine the relationship between the results gained from the two assay formats. **Table 6** demonstrates that all parameters showed a good relationship between the two assay formats with eight out of nine of the parameters showing an R^2 value of greater or equal to 0.99. The probability values (p) were calculated for the intercept; for all parameters other than hue, the intercepts for the linear regression were not significantly different, indicating no systematic bias between the formats. Although a significant difference was seen with hue, the actual difference was small and again magnified due to the low standard errors for this measure.

Previously, validation studies were conducted by Walkenhorst (*23*) in which the 10 mL format of the modified Somers method was compared to the original Somers method using 70 Australian dry red table wines from a range of regions, vintages, and varieties; this work showed a strong positive relationship between all parameters when comparing the two methods.

The modifications described allow the analysis of red wine samples using the entire suite of Somers parameters as reported earlier (*11*) with no effect on the integrity or reproducibility of the data. Color density is routinely used throughout the wine industry and research facilities alike to quantify the visual appearance of wine. As reported by Somers (*17*) and subsequently in a number of papers (*24*−*27*), strong positive correlations have been made between wine color density and wine quality. In addition to the originally reported parameter, color density SO_2-corrected has been included, which is a measure of the wine color density under excess acetaldehyde conditions. The addition of acetaldehyde allows for the restoration of colored SO_2-bleached pigments, which can be a valuable tool when comparing wines with varying free SO_2 concentrations. This is demonstrated in **Table 5** where SO_2 bleaching had minimal effect in the two 2006 Shiraz; however, addition of acetaldehyde to the two Cabernet Sauvignon samples resulted in an increase of up to 17% (1.8 au) of the original wine color density.

The concentrations of total anthocyanins and pigmented polymers as calculated by the Somers assay have been shown to positively correlate with the concentration resulting from HPLC analysis. Studies conducted by Peng et al. (*28*) showed that the estimation of SO_2-resistant pigments calculated using Somers measures strongly correlated with the concentration of pigmented polymers resulting from HPLC analysis. Similarly, regression analysis of total anthocyanins calculated using Somers measures and the quantification of anthocyanins via HPLC revealed a positive correlation (unpublished data). While HPLC is an important research tool, its application in industry is limited due to lengthy analysis times and expensive equipment and maintenance costs. The modified method of the Somers assay can therefore be employed as a HTP, low-cost alternative to monitor anthocyanins and stable pigments and is suitable to determine the "monomeric index" in anthocyanin-containing products such as fruit juices and juice concentrates (*5*).

While the modification to the Somers assay does result in numerous advantages, the dilution of the wine will alter any copigmentation effects. Copigmentation is a solution phenomenon in which the pigments in wine form molecular associations

Table 5. Color Parameters and Their CVs (%) for the 10 mL and HTP Formats of the Modified Somers Assay of Four Young Commercial Dry Red Wines

		Shiraz				Cabernet Sauvignon			
		2005		2006		2005		2006	
color parameter		10 mL	HTP	10 mL	HTP	10 mL	HTP	10 mL	HTP
chemical age 1 (no units)	results	0.501	0.501	0.346	0.360	0.552	0.0527	0.325	0.347
	CV %	1.6	3.4	1.1	3.8	1.7	3.3	3.6	6.4
chemical age 2 (no units)	results	0.183	0.192	0.100	0.102	0.203	0.213	0.077	0.080
	CV %	0.8	4.7	1.3	2.0	1.3	2.9	1.0	2.3
degree of ionization of anthocyanins (%)	results	24	25	23	25	17	18	16	17
	CV %	1.2	4.7	1.1	2.8	10.0	6.3	2.3	2.3
total anthocyanins (mg L^{-1})	results	299	284	483	465	218	207	595	580
	CV %	1.0	4.7	1.0	2.2	0.2	4.1	1.2	2.1
color density (au)	results	13.1	13.1	13.7	13.9	9.1	9.3	12.1	12.4
	CV %	0.7	2.0	0.3	1.5	3.2	1.9	1.0	1.3
color density SO_2 corrected (au)	results	13.6	13.9	13.6	13.2	10.4	11.1	13.2	13.0
	CV %	0.8	3.0	0.6	2.8	0.4	3.1	3.0	4.8
hue (no units)	results	0.740	0.743	0.630	0.628	0.756	0.761	0.655	0.655
	CV %	0.0	0.2	0.2	0.1	0.1	0.1	0.1	0.3
total phenolics (au)	results	54.9	53.2	54.1	52.3	45.0	43.4	64.1	62.8
	CV %	0.6	2.8	0.8	2.0	0.4	2.8	1.1	1.9
SO_2 resistant pigments (au)	results	3.95	4.01	2.89	2.86	3.34	3.41	2.63	2.67
	CV %	1.0	2.4	0.5	1.4	1.8	0.9	0.3	0.9

Table 6. Results from Comparative Statistical Analysis of the Color Parameters from the 10 mL and HTP Formats[a]

	ANOVA				linear regression				
	format		format × sample		R^2	slope		intercept	
color parameter	*F* ratio	*P* value	*F* ratio	*P* value		value	SE	value	SE
chemical age 1 (no units)	0.3	NS	3.4	0.028	0.99	0.83	0.05	+0.08	0.02
chemical age 2 (no units)	12.3	0.001	1.3	NS	1.00	1.06	0.01	–0.00	0.00
degree of ionization of anthocyanins (%)	14.4	0.001	0.5	NS	0.99	1.08	0.06	–0.00	0.01
total anthocyanins (mg L^{-1})	18.2	<0.001	0.2	NS	1.00	0.99	0.01	–9.99	3.69
color density (au)	8.7	0.006	0.7	NS	1.00	1.02	0.04	+0.02	0.46
color density SO_2 corrected (au)	0.5	NS	3.7	0.021	0.93	0.72	0.14	+3.65	1.76
hue (no units)	10.3	0.003	16.0	<0.001	1.00	1.05	0.01	–0.03*	0.01
total phenolics (au)	14.9	<0.001	0.3	NS	1.00	0.98	0.03	–0.37	1.79
SO_2 resistant pigments (au)	4.239	0.047	1.557	NS	1.00	1.04	0.05	–0.01	0.02

[a] ANOVA was conducted considering the format type only and the interaction of format type and sample. The coefficient of determination (R^2) was calculated for each parameter from the regression analysis between the 10 mL and the HTP formats ($n = 4$). NS, not significant ($p > 0.05$); *$p < 0.05$ calculated for intercept.

or complexes with other phenolic material resulting in deviations from Beer's law (*29*). Maintaining a constant pH and alcohol level in the diluents minimizes these copigmentation effects and is an important aspect of the modified Somers method.

It is well-accepted that the tannin concentration is critical to the color and astringency of red wines; however, it is not regularly monitored within the vineyard or winery. While there are a range of established analytical methods available for the quantification of tannins, these are often laborious, indirect, and lack specificity toward tannins (*16*). The method presented herein offers a rapid and robust method to quantify tannins in grape homogenate extracts and red wine samples. The modified Somers assay allows the standardization of pH and ethanol concentration of wine samples in a simple one-step dilution with a buffer solution, thus removing inconsistencies such as copigmentation and SO_2 bleaching in the wine matrix prior to analysis and allowing for its adaptation to a HTP format.

The adaptation of the MCP tannin assay and modified Somers assay to a HTP format significantly reduced the time requirements for the assays without compromising efficiency or reproducibility. It is anticipated that the new formats will be valuable tools for monitoring the concentrations of tannin and anthocyanins in grape and wine samples but also in other fruit concentrates, beverages, and plant-derived products.

ABBREVIATIONS USED

MCP, methyl cellulose precipitable; HPLC, high-performance liquid chromatography; HTP, high throughput; CV, coefficient of variation; au, absorbance units.

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