

# Measuring red wine colour using the Modified Somers assay

## Scope

Somers and Evans (1974, 1977) established a set of spectroscopic colour measurements, which not only give a measure of wine colour 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 analysed in its original state and is then treated with excess SO<sub>2</sub>, 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<sub>2</sub> concentration), and from these values, the wine colour parameters wine color density and hue are calculated. The second reading is taken after the addition of excess SO<sub>2</sub> allowing for the measurement of colour (A520) resulting from the SO<sub>2</sub>-resistant pigments present in the wine. Third, the original wine is treated with excess acetaldehyde, which permits the estimation of coloured 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 coloured forms. The acidified solution is then monitored at A520 and A280 to give an indication of the concentration of total red pigments and total phenolics, respectively.

The main modification to the original method, reported in Mercurio et al. (2007), is the standardisation 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.

## Equipment and apparatus

1. A calibrated spectrophotometer (traditional, plate reader, or other type) capable of measuring absorbance at 280, 420 and 520 nm with a wavelength accuracy of  $\pm 2$  nm. Note that if using a style other than traditional, the calculation might differ from that presented here and must be validated before use.
2. Cuvettes suitable for the instrument being used (see below).
3. Centrifuge capable of a radial centrifugal force (RCF) of at least 1800g (i.e. 4,000 rpm with a 10 cm rotating radius).
4. Mixing device such as a rotary suspension mixer, shaker table or roller mixer.

If required, wines can be clarified by centrifugation e.g. Clements 2000, 10 mL tubes, 15 minutes at 3,500 rpm, 20 cm radius. Sample volumes required with this modified method are small so if available, a microfuge is very efficient in clarification, as the g forces are high and large numbers of samples can be done simultaneously.

### 10 mL Assay

1. 10 mm pathlength cuvettes - either quartz or acrylic disposable cuvettes with optical window below 280 nm.
2. 10 mL centrifuge tubes.
3. Pipettes for accurate dispensing of 0.25 mL to 10 mL volumes.
4. Centrifuge suitable for 10 mL centrifuge tubes.



### High Through-put (HTP) Assay (Plate Reader)

1. 96-well deep well plates and corresponding aluminium sealing foil and silicone sealing mats (e.g. Axygen 1.1 mL) for performing the assay.
2. 96-well plates (e.g. Greiner UV Star 370  $\mu$ L) for reading absorbance.
3. Multi-channel pipette for accurate dispensing from 25  $\mu$ L to 775  $\mu$ L volumes.
4. Centrifuge and rotor suitable for deep well 96-well plates.

### Reagents

1. Buffer 1 (model wine, 0.5 % w/v tartaric acid in 12 % v/v ethanol adjust to pH 3.4 with 5M NaOH)
2. 0.375 % w/v sodium metabisulfite
3. 0.1% v/v acetaldehyde
4. 1M HCl

### Procedure

Below is a general procedure for performing the Modified Somers colour assay.

For both the 10 mL and HTP formats, after incubation, 300  $\mu$ L of each treatment was transferred into 370  $\mu$ L Greiner UV star 96 well plates and read using the SpectraMax M2 Microplate Reader. All wines were sub-sampled from freshly opened bottles and centrifuged at 4,000 rpm for 5 minutes using a Hettich Universal 32 R centrifuge with a Hettich 1624 rotor (Adelab Scientific, Australia).

- Treatment A: 1 in 10 dilution of wine in Buffer 1. Absorbance to be read at 420 nm and 520 nm immediately after mixing.
- Treatment B: 1 in 10 dilution of wine in Buffer 1 plus 0.375 % w/v sodium metabisulfite. Samples were mixed and incubated at room temperature for 1 hour. Absorbance to be read at 520 nm.
- Treatment C: 1 in 10 dilution of wine in Buffer 1 + 0.1 % v/v acetaldehyde. Samples were mixed and incubated at room temperature for 1 hour. Absorbance to be read at 420 nm and 520 nm.
- Treatment D: 1 in 50 dilution of wine in 1M HCl, samples were mixed and incubated at room temperature in the dark for 3 hours. Absorbance to be read at 280 nm and 520 nm.

The 10 mL format is to be performed in 10 mL centrifuge tubes. The tubes capped and inverted several times to allow for mixing prior to incubation.

The HTP assay is to be performed in 1.1 mL 96 well deep well plates.

Treatments A, B and C can be performed in the same plate. However, as treatment D requires storage in the dark for 3 hours it is essential that this treatment be performed in a separate plate.

The plates are sealed with the appropriate sealing mat and shaken gently, ideally on an automated flatbed plate shaker to allow for mixing prior to incubation.

## Calculations

### Chemical Age 1 (no units):

$$A_{520 \text{ sulfite}}/A_{520 \text{ acetal}}$$

### Chemical Age 2 (no units):

$$A_{520 \text{ sulfite}}/(5 \times A_{520 \text{ HCl}})$$

### Degree of ionisation of anthocyanins (%):

$$\left\{ \frac{(10 \times A_{520 \text{ buffer 1}}) - (10 \times A_{520 \text{ sulfite}})}{(50 \times A_{520 \text{ HCl}}) - [1.6667 \times (10 \times A_{520 \text{ sulfite}})]} \right\} \times 100$$

### Total anthocyanins (mg/L):

$$20 \times [(50 \times A_{520 \text{ HCl}}) - (1.6667 \times (10 \times A_{520 \text{ sulfite}}))]$$

### Colour density (au):

$$(A_{420 \text{ buffer 1}} + A_{520 \text{ buffer 1}}) \times 10$$

### \*Colour density: SO<sub>2</sub> corrected (au):

$$(A_{420 \text{ acetal}} + A_{520 \text{ acetal}}) \times 10$$

### Hue (no units):

$$A_{420 \text{ buffer 1}} / A_{520 \text{ buffer 1}}$$

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

### \*SO<sub>2</sub> Resistant Pigments (au):

$$A_{520 \text{ sulfite}} \times 10$$

\* not an original Somers parameter

## Interpretation of results

A dual beam monochromatic SpectraMax M2 UV-Visible Microplate Reader (Molecular Devices, Australia) was used for all spectral analysis. Greiner UV Star 370  $\mu\text{L}$  96 well disposable plates were used, which have an optical window down to 200 nm, therefore did not interfere with the reading at 280 nm. The SpectraMax M2 has an in-built path-correction function that normalised the pathlength of each well to 10 mm and corrects for any variation in sample volume. For the modified Somers assay *buffer 1* was used as a blank for treatments A, B and C and 1M HCl was used as a reference for treatment D.

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 1). Samples were selected to include several varieties with varying colour, from the 2005 and 2006 vintages. Absorbance values were used to calculate a series of colour parameters as described in methods and materials and results underwent

statistical analysis. The coefficient of variation 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 ionisation of anthocyanins for the 2005 Cabernet Sauvignon sample.

**Table 1.** Colour parameters and their coefficient of variation (CV %) for the 10 mL and HTP formats of the modified Somers assay of four young commercial dry red wines.

Colour parameter		Shiraz 2005		Shiraz 2006		Cabernet Sauvignon 2005		Cabernet Sauvignon 2006	
		10 mL	HTP	10 mL	HTP	10 mL	HTP	10 mL	HTP
Chemical Age 1 (au 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 (au 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 Ionisation 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 <sup>-1</sup> )	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
Colour 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
Colour Density SO <sub>2</sub> 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 <sub>2</sub> Resistant Pigments (au)	Results	0.395	0.401	0.289	0.286	0.334	0.341	0.263	0.267
	CV %	1.0	2.4	0.5	1.4	1.8	0.9	0.3	0.9

Previously, validation studies were conducted by Walkenhorst (2001) 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 (1977) with no effect on the integrity or reproducibility of the data. *Colour Density* is routinely used throughout wine industry and research facilities alike, to quantify the visual appearance of wine. As reported by Somers (1974) strong positive correlations have been made between wine colour density and wine quality. In addition to the originally reported parameter, *Colour Density SO<sub>2</sub> corrected* has been included, which is a measure of the wine colour density under excess acetaldehyde conditions. The addition of acetaldehyde allows for the restoration of coloured SO<sub>2</sub>-bleached pigments, which can be a valuable tool when comparing wines with varying free SO<sub>2</sub> concentrations. This is demonstrated in Table 5 where SO<sub>2</sub>-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 colour 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. (2002) showed that the estimation of SO<sub>2</sub> 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 high through-put, 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 (Bonerz et al. 2006).

While the modification to the Somers assay does result in numerous advantages, the dilution of the wine will alter any co-pigmentation effects. Co-pigmentation is a solution phenomenon in which the pigments in wine form molecular associations or complexes with other phenolic material resulting in deviations from Beer's law (Boulton 2001). Maintaining a constant pH and alcohol level in the diluents minimises these co-pigmentation effects and is an important aspect of the Modified Somers method.

### Estimations of uncertainty

Table 2 shows results from analysis of variance tests (ANOVA) and linear regression analysis for the same data. Analysis of variance 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 2 demonstrates that all parameters showed a good relationship between the two assay formats with eight out of nine of the parameters showing an R<sup>2</sup> 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.

**Table 2:** Results from comparative statistical analysis of the colour parameters from the 10 mL and HTP formats. Analysis of Variance (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 HTP formats ( $n=4$ ).

Colour parameter	Analysis of Variance				Linear Regression				
	Format		Format*Sample		$R^2$	Slope		Intercept	
	F-ratio	P value	F-ratio	P value		Value	Std error	Value	Std error
Chemical Age 1 (au units)	0.3	NS	3.4	0.028	0.99	0.83	0.05	+ 0.08	0.02
Chemical Age 2 (au units)	12.3	0.001	1.3	NS	1.00	1.06	0.01	- 0.00	0.00
Degree of Ionisation of Anthocyanins (%)	14.4	0.001	0.5	NS	0.99	1.08	0.06	- 0.00	0.01
Total Anthocyanins (mg L <sup>-1</sup> )	18.2	<0.001	0.2	NS	1.00	0.99	0.01	- 9.99	3.69
Colour Density (au)	8.7	0.006	0.7	NS	1.00	1.02	0.04	+ 0.02	0.46
Colour Density SO <sub>2</sub> 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 <sub>2</sub> Resistant Pigments (au)	4.239	0.047	1.557	NS	1.00	1.04	0.05	- 0.01	0.02

NS - not significant ( $p > 0.05$ )

\* - ( $p < 0.05$ ) calculated for intercept

It must be stressed that the uncertainty of measurement should be determined and validated for each individual laboratory's situation using standard recognised practices that take into account all possible variables including operators and equipment used in the conduct of the assay. In practical terms, the uncertainty of measurement can often be estimated as  $2 \times CV$  (for a 95% confidence interval) as determined by the laboratory. Therefore, allowing for other variations, such as operator and daily operation, it might be reasonable to use up to 15% as an estimate of the uncertainty of measurement.

## Quality assurance

1. Duplicate determinations should be run according to the degree of confidence required by the laboratory. This can range from as frequently as one duplicate in every five samples, or as little as one sample every week depending on the situation. Where duplicates are used, the average of the results should agree to within the defined limits of uncertainty of measurement.
2. The performance of the spectrophotometer must be checked according to standard procedures (e.g. Australian Standard AS 3753-2001), and should include as a minimum:
  - ensuring adequate instrument warm-up to avoid excessive drift; and
  - routine instrument and calibration performance checks to ensure wavelength accuracy, absorbance accuracy and repeatability.
3. Other critical equipment such as balances and volume measuring devices must be calibrated as described in standard procedures (e.g. Morris and Fen 2002; NATA 1995; AS 2162.2-1998).

4. It is recommended that a solution is prepared, frozen and used as a standard for monitoring assay performance over time.

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