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## Determination of Total Anthocyanins in Grapes

### Version Control

Version	Changes made in this version
1.0	First version of this method
1.1	Changes based on reference group feedback
1.2	Changes made to sections 12, 13 and 14
1.3	Changes based on reference group feedback

It should be noted that this procedure represents the current industry best practice for the measurement of colour in grapes expressed as total anthocyanins. It is expected that this method will be open to modification and improvement as experience within industry dictates, as technology improves or the understanding of the science behind grape chemistry improves.

### 1. Introduction/Foreword

The purpose of this method is to define the measurement of colour in grapes, expressed as total anthocyanins. It is intended to be a reference method that other industry-based methods can be validated against and to act as the method of choice when arbitrating disputes. It does not necessarily replace in house methods, but any such method needs to be suitably validated against this method. The approaches for cross validation of in house methods which use the same principle as this method are outlined in section 17 of this procedure.

Where a different analytical principal is used to determine colour in grapes (i.e. a secondary method such as NIR spectroscopy) a more extensive form of validation against this reference method is required. This is outlined in a separate procedure (ref. IESP4.0, Development and validation of secondary analytical methods for the determination of grape parameters).

### 2. Scope

The principle of this spectroscopic analysis is based on the methods published by Iland et al. (1996). This analysis applies to the measurement of total anthocyanins in red grapes.

### 3. Terminology

UV/visible (UV-Vis or UV/Vis) spectroscopy refers to absorption or reflectance in part of the ultraviolet and the adjacent visible spectral regions of the electromagnetic spectrum.

A UV/visible spectrophotometer is a scientific instrument that measures the amount of light that is absorbed by a sample.

Spectrophotometric measurement is defined as the quantitative measurement of the reflection or transmission properties of a material as a function of wavelength.



#### 4. Measurement Principle

Anthocyanins are extracted from the skins of black grapes during fermentation, during which ethanol levels can increase from 0 to 14% or greater. However, as different fermentation conditions can extract different proportions of anthocyanins, a “exhaustive extraction” is required to ensure consistent and meaningful measurements. Extraction with a 50% v/v ethanol solution at pH 2 has been shown to yield at least 94% extraction of anthocyanins, which is described as the “potentially available anthocyanins”.

The quantification of anthocyanins in the extraction solution is governed by the application of the Beer-Lambert Law, which describes the linear relationship between the absorbance and concentration of an absorbing species. Total anthocyanin concentration is determined using absorbance measurement at 520 nm. Light is passed through the sample and the amount that is transmitted through the sample is measured. From this, the amount of light absorbed by the sample at that wavelength is calculated and reported as the absorbance at that wavelength.

The calculation for the concentration of anthocyanins is based on the extinction coefficient of malvidin-3-glucoside, the major anthocyanin in grape skins, which is nominally 500 as reported by Somers and Evans (1974).

#### 5. Units of Measurement

Total anthocyanins are expressed as milligrams of anthocyanins per gram of fruit (mg/g berry weight).

#### 6. Metrological/Technical Requirements

N/A

#### 7. Health and Safety Considerations

Hydrochloric acid is harmful if swallowed, inhaled, or in contact with skin. Preparation and use of 1M hydrochloric acid from concentrated hydrochloric acid should be carried out in a fume cupboard while wearing safety glasses, gloves, and a lab coat.

Ethanol is highly flammable and irritating to eyes and skin. Hydrochloric acid is harmful if swallowed, inhaled, or in contact with skin. Preparation and use of 50% acidified ethanol from absolute ethanol and concentrated hydrochloric acid should be carried out in a fume cupboard while wearing safety glasses, gloves, and a lab coat.

#### 8. Materials/Apparatus

- UV/Visible spectrophotometer capable of measuring in the range 250-520nm
- 10 mm path length quartz cuvette
- Fixed volume pipettes (calibrated only) to deliver volumes of 1 mL and 10 mL
- High speed homogenisation unit



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- Centrifuge capable of maintaining speeds of approximately 3,500 rpm
  - Rotating wheel mixer (if available)
  - Centrifuge tubes, 10 mL, with caps
  - Volumetric flasks of 1 L capacity
  - Analytical balance with minimum scale reading 0.01 g
  - Plastic 250 mL containers with lids
  - Kimwipes

## 9. Reagents

- 1.0 M Hydrochloric Acid. Prepare by adding 83 mL of concentrated HCl (approximately 37 %) to approximately 600 mL Milli-Q water in a 1 L volumetric flask, temperate to 20 °C, then fill to the 1 L mark with Milli-Q water and mix thoroughly. Prepare fresh every 3 months.
- 50 %v/v ethanol in Milli-Q water. Prepare by adding 500 mL of absolute ethanol to approximately 400 mL of Milli-Q water in a 1 L volumetric flask, temperate to 20 °C, then fill to the 1 L mark with Milli-Q water. Prepare fresh every 3 months.
- Acidified 50 % v/v ethanol in Milli-Q water. Prepare by adding 4.4 mL of concentrated HCl (37%) to approximately 600 mL 50 % v/v ethanol in a 1 L volumetric flask, temperate to 20 °C, then fill to the 1 L mark with 50% v/v ethanol. Prepare fresh every 3 months.

## 10. Verification/Calibration

### Spectrophotometer

The actual verification and calibration steps will be defined by the type of instrumentation being employed. In general terms, instrumentation stability and performance should be evaluated by using a suitable standard reference material against the following criteria:

- Wavelength accuracy
- Wavelength repeatability
- Response repeatability
- Photometric linearity
- Photometric noise

Depending on the instrument these performance aspects are typically evaluated using internal diagnostic checks built into the instruments' application software, or they can be evaluated using an accredited external reference material. These checks must be carried out and compliance recorded on a 6 monthly schedule, or immediately before use if the instrument has not been used for an extended period of time.

Daily the instrument must be zeroed against a water blank.



## Homogeniser

The homogenisation process is a significant requirement for achieving consistent results. It is highly recommended that a laboratory grade instrument is used. However, other equivalent homogenisers are acceptable if they can be shown to meet the validation protocols below. It should be noted that studies have shown that most typical domestic blenders are not able to provide consistent results in line with the requirements of this protocol.

An internal validation of the homogenisation process should be carried out to understand the time required to achieve optimum and reproducible results using a process as follows.

1. Source and randomise 1kg of grape berries.
2. Randomly spit the sample into 5 x 200g lots.
3. Homogenise each lot of samples for 5 different increasing time periods (e.g., 30 seconds, 60 seconds, 90 seconds, 120 seconds and 150 seconds).
4. Inspect sample ensuring all seeds are thoroughly macerated with the homogenate having a smooth consistent appearance. Discard any samples where this not the case. If more than 3 time points are discarded begin the process again using longer time intervals or different equipment.
5. Determine the anthocyanin content in each sample as per the procedure in section 12 below from step 4.
6. The minimum homogenisation time is that in which there is no significantly increased anthocyanin content at longer homogenisation times
7. Using a new batch of randomised berries and the homogenisation time from step 6 above, homogenise 3 separate sets of berries and separately determine the anthocyanin concentration as per section 12 below.
8. The anthocyanin results should not vary by more than 10% from the mean result of the replicates. If they do repeat the procedure with longer homogenisation time until consistency is achieved.
9. Document all results from the process in an appendix to the procedure validation.
10. The process should then be repeated before the start of each season or if a change is made to the homogenisation equipment.

## 11. Environmental Conditions

A dark, enclosed space such as a cupboard is required to incubate the samples at room temperature.

## 12. Measurement Procedure

1. Ensure the UV/Visible spectrophotometer is turned on and is given sufficient time to warm up as per the manufacturers recommendations.
2. A representative grape sample is taken just prior to homogenisation using the following procedure:
  - a. If the sample contains bunches, remove all berries from the rachis by hand and place into tray or container. If the berries are loose, just place all berries into a tray or container.
  - b. Gently mix the berries by hand being careful not to split the skins of any of them.
  - c. Randomly take berries from different areas within the tray or container until you have approximately 200 berries (or approximately 200 g) and place into a clearly labeled 250 mL container.
  - d. Any berries that are left after taking the random sample can be disposed of in an appropriate



manner.

3. Process each berry sample at the appropriate speed and time in the homogenising unit. Ensure that all seeds are thoroughly macerated and all homogenate is scraped from the homogeniser and collected in the storage container. All samples should be thoroughly remixed by stirring immediately before subsampling to ensure no settling out of material occurs before subsampling.
4. Weigh  $1.0 \text{ g} \pm 0.1 \text{ g}$  into a 10 mL centrifuge tube and record the weight.
5. Add 10 mL of acidified 50% v/v ethanol/Milli-Q water and cap the tube.
6. Rotate on the rotating wheel mixer for 1 hour.
7. Centrifuge the homogenate/ethanol mixture at approximately 3,500 rpm for 5 minutes.
8. Pour the supernatant liquid into an appropriate sized measuring cylinder to and measure the volume of the extract to the nearest 0.2 mL. This will be the final extract volume required for the calculation step as outlined in Section 13.
9. Add 1 mL of the extract to 10 mL of 1M HCl (total volume will be 11 mL).
10. Incubate for at least 1 hour and no longer than 4 hours in a dark place (eg. cupboard) at room temperature.
11. Set instrument for measurement at 520 nm.
12. Zero with 1M HCl in 10 mm quartz cuvette.
13. Measure the absorbance of the extract at 520nm.

### 13. Calculations/Corrections

Total anthocyanins (mg/g fruit);

$$= \frac{A_{520}^{\text{HCl}}}{500} \times \text{dilution factor}^* \times \frac{\text{final extract volume (mL)}}{100} \times \frac{1000}{\text{weight of homogenate taken for extraction (g)}}$$

\* the dilution factor is the dilution of a portion of the extract into 1M HCl (Note, if 1.0mL of 'the extract' is added into 10mL of 1M HCl, the dilution factor is 11.)

Note: As a general guide, 1g of homogenate extracted with 10 mL of 50% v/v aqueous ethanol will result in a 'final extract volume' of approximately 10.5 mL, but this value can vary depending on berry weight.

### 14. Uncertainty of Measurement

Uncertainty of measurement (UoM) is calculated by determining the average, standard deviation and the coefficient of variation of reproducibility data of 7 replicates. From this it can be estimated that the MU at the 95% confidence interval is equal to  $2 \times \text{SD}$  and  $2 \times \text{CV} (\%)$ . The maximum UoM for total anthocyanins (mg/g) is for this method as described should be in the region of  $\pm 5 \%$  for an individual laboratory on replicate homogenates. Each laboratory must demonstrate its typical UoM as part of its validation procedure.



## 15. Limits of Detection

For the method as outlined within this procedure the validated limit of quantification is 0.01 mg/g.

## 16. Reporting Results

After the calculation is complete, results are reported as milligrams of anthocyanins per gram of berry weight to two decimal places.

## 17. Validation Requirements

The determination of anthocyanins can be significantly impacted by small differences in procedure or equipment. For this reason, it is important that, prior to the method being put into use, the laboratory validates its results against an independent source.

If using a variation of the procedure outlined above (but still reliant on the extraction of a homogenised sample followed by UV/Visible spectroscopy) the procedure can be validated as follows:

1. Source 10 separate samples of black grapes.
2. From each set randomise 400 grams of berries and then randomly split the sample into 2 x 200g sample sets.
3. Samples should then be immediately refrigerated.
4. One replicate of each sample should be submitted to an accredited independent laboratory for analysis using the reference method as outlined in this procedure to determine anthocyanin content of each sample.
5. Within a one-week timeframe the samples should also be analysed in the source laboratory using the in-house procedure.
6. For each sample calculate the difference in result from the source laboratory and the reference laboratory and then average these differences.
7. This average should not vary by exceed 10% of the mean of the sample results from the source laboratory.
8. At the beginning of each season this procedure should be repeated to ensure no changes have been introduced that may affect the results.
9. Document all results from the process in an appendix to the procedure validation.

If the in-house methodology for the determination of anthocyanins uses a different technology such as NIR spectroscopy, then a much more rigorous validation procedure is required as set out in a separate procedure (ref. IESP4.0, Development and validation of secondary analytical methods for the determination of grape parameters)

## 18. Quality Assurance

The reliability of this method is monitored using the following procedure:

1. First and every tenth determination is performed in duplicate. Duplicates should agree to within the stated UoM values determined in the section 14.



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## 19. References

Iland, P. G., Cynkar, W., FRANCIS, I. L., Williams, P. J., Coombe, B. G. (1996), Optimisation of methods for the determination of total and red-free glycosyl glucose in black grape berries of *Vitis vinifera*. *Aust J Grape Wine R* 2, 171–178.

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Somers, T.C. Evans, M.E. (1974) Wine quality: correlations with colour density and anthocyanin equilibria in a group of young red wines. *J. Sci. Food Agric.* 25: 1369-1379.



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