The Australian Wine Research Institute

Fact Sheet
ANALYSIS

Measuring total anthocyanins (colour) in red grape berries

Scope

This method describes the measurement of total anthocyanins in red grape berries based on the methods described by Iland et al. (1996, 2000). It involves extraction of these compounds from a homogenised grape sample, expression of their colour at low pH and quantification based on their absorbance in the visible region of the light spectrum.

Malvidin-3-glucoside is the major anthocyanin in *Vitis vinifera* grapes but is not the only anthocyanin and the results are expressed in malvidin-equivalents for comparative purposes only. A minimum sample size of 50 g (approximately 50 berries) is required for this determination.

Equipment and apparatus

1. Calibrated spectrophotometer (traditional, plate reader, or other type) capable of measuring absorbance at 520 nm with an accuracy of ±2 nm.

   Note that if using a style other than traditional, the calculation may differ from that presented here and must be validated before use.

2. Cuvettes suitable for the instrument being used, for example, 10 mm path length quartz, optical glass or acrylic cuvettes (4 mL capacity). For large sample numbers, disposable acrylic cuvettes offer convenience, as the final dilutions can be made directly into the cuvettes.

3. Liquid handling devices for accurate delivery of 200 µL, 3.8 mL and 10 mL volumes.

4. Analytical balance with minimum scale reading of 0.001 g.

5. Homogeniser appropriate to the sample size:
    - For small sample sizes (50-100 g) an *Ultra-Turrax T25* high-speed homogeniser with an S25N dispersing head (Janke & Kunkel GmbH & Co., Germany) can be used.
    - For intermediate sized samples (200-250 g), a *Retsch Grindomix GM200* homogeniser (Retsch GmbH & Co. KG, Germany), fitted with a floating lid, can be used.
    - For larger samples (200-400 g), a *Waring blender, Model 38BL41* (Waring Commercial, USA) can be used.
6. Centrifuge capable of a radial centrifugal force (RCF) of at least 1800 g (i.e. 4,000 rpm with a 10 cm rotating radius).
7. Mixing device such as a rotary suspension mixer, shaker table or roller mixer.

It is noted that other types and brands of equipment can be used, for example to automate the procedure; however, such changes must always be validated against the standard procedures presented here.

Reagents

1. 1.0 M hydrochloric acid.
2. 50% v/v ethanol in *Milli-Q* (or equivalent) water.

Procedure

1. Samples can be analysed fresh or can be frozen (-20°C) prior to analysis. Fresh samples must be stored cool (approximately 4°C) and analysed within 24 hours of collection. For field samples, this can be achieved, for example, by storing freshly collected grapes in cooler boxes with ice packs until delivery to the laboratory. Short-term freezing of whole berries (i.e. overnight) does not significantly affect the colour of grapes, although there might be some loss of colour with grapes that have an anthocyanin concentration of greater than 1.9 mg/g. There is no significant colour loss during storage of frozen whole grapes for at least three months. On the other hand, homogenates of red grapes lose colour (approximately 0.1 mg/g) after even overnight freezing (Cynkar et al. 2004).
2. If the sample is large, a representative sub-sample must be taken. This is best done with frozen grapes to avoid juice loss, using the following procedure:
    - if the sample contains bunches, remove all berries from the rachis by hand and place into a tray or container. If the berries are loose, just place all berries into a tray or container;
    - gently mix the berries by hand; and
    - randomly select berries from different areas within the container to make up the required sample size (e.g. 200 berries, or approximately 200 g). Further replicates can be taken if required and stored frozen for reference.
3. Usually the determination is reported on a mass basis (i.e. per gram of grapes), but if the determination is required to be expressed on a 'per berry ' basis, a sample of a defined number of berries (e.g. 50 berry sample) is weighed to determine the average berry weight. Record the berry weight to at least two decimal places or as appropriate to the balance being used.

4. If frozen, berries are thawed (usually overnight in a refrigerator at 4°C) and processed cold (less than 10°C) to minimise oxidation of colour components.

5. Berry samples are homogenised according to the following settings:

   - *Ultra-Turrax:*

     Process at the maximum speed setting (24,000 rpm) for approximately 30 seconds, scrape the homogenate from the homogeniser shaft into the vessel, and then homogenise for a further 30 seconds. Ensure that all seeds are thoroughly macerated and all homogenate is scraped from the homogeniser shaft and collected in the homogenising vessel.

   - *Retsch:*

     Process the sample at a speed of 8,000 rpm for 20 seconds.

   - *Waring:*

     Process for 60 seconds on high speed.

Comparative trials with these three blenders have shown no significant differences with total anthocyanin measurement, but the Waring blender did show reduced recovery of total phenolics, possibly due to the fact that it does not homogenise grape seeds as effectively as the other types.

Please note that the homogenisation step can give rise to a significant safety hazard. Please ensure that adequate attention to safety and all recommendations of safety precautions and maintenance provided by the manufacturer is considered when using these types of equipment.

6. Once homogenised, samples must proceed to the extraction step within four hours.

7. Mix the homogenate well, then transfer approximately 1 g to a pre-tared plastic 10 mL centrifuge tube. Record the weight (to at least two decimal places or as appropriate to the balance being used) of the homogenate portion for use in calculations.

8. Add 10 mL of 50% v/v aqueous ethanol to the portion of homogenate, cap the tube and agitate on a mixing device for one hour. Alternatively, mix by inverting the tube regularly (approximately every 10 minutes) over a period of one hour. Ensure that mixing is efficient and that the pellet does not become lodged in the bottom of the tube.

9. Centrifuge the homogenate/ethanol mixture at 1800 g for 10 minutes. The supernatant is now termed the 'extract'. (This same extract can also be used for the determination of total phenolics.)

10. Centrifuge the homogenate/ethanol mixture at 1800 g for 10 minutes. If approximately 1 g of homogenate was used (i.e. 0.95 to 1.05 g), the final extract volume is estimated as 10.5 mL. Alternatively, this volume can be measured with a suitable volume measuring container.

11. The extract can be stored in the freezer (at -20°C) for up to 3 months without significant loss of colour.

12. Transfer 200 µL of extract to an acrylic cuvette (10 mm path length), add 3.8 mL of 1.0 M HCl, cover with Parafilm then mix by inverting. Incubate at room temperature for at least 3 hours, but not

longer than 24 hours. This step is critical in allowing full expression of colour.

The incubation time can be reduced if the 50% ethanol solution (i.e. that used in step 9) is adjusted to pH 2.0 with HCl, however if the extract is also required for a glycosyl-glucose assay, the 50% ethanol solution must not be acidified. Note that some industry practitioners have reduced the incubation time using other alternative procedures, but these must be validated before use. It may also be necessary to adjust the dilution to ensure that the absorbance is within the working range of the spectrophotometer, and any such change to the procedure must be validated before use.

13. Measure absorbance of the acidified diluted extract at 520 nm using a 1.0 M HCl blank.

Calculations

$$\text{Anthocyanins (mg/g)} = \frac{{}^{a}A_{520} \times {}^{b}DF \times {}^{c}\text{final extract volume (mL)} \times 1000}{{}^{d}500 \times 100 \times \text{homogenate weight (g)}}$$

$$\text{Anthocyanins (mg/berry)} = \frac{{}^{a}A_{520} \times {}^{b}DF \times {}^{c}\text{final extract volume (mL)} \times \text{berry weight (g)} \times 1000}{{}^{d}500 \times 100 \times \text{homogenate weight (g)}}$$

Notes:

[a] absorbance at 520 nm, 10 mm path length (Procedure step 13)

[b] dilution factor (DF) at procedure step 12 (dilution of extract in 1 M HCl): 20 in this case

[c] if the weight of homogenate sample taken at procedure step 8 is kept within a 5% range (0.95 - 1.05 g), a standard figure of 10.5 mL can be used as the final extract volume

[d] absorbance of a 1% w/v (1 g/100 mL) solution of malvidin-3-glucoside, 10 mm path length (Somers and Evans, 1974) - this is a value traditionally used within the Australian wine industry.

Using a molecular weight of 529 for malvidin-3-glucoside equates to a molar absorbance of 26,455. Literature values for this vary due to many confounding factors such as non-linearity resulting from copigmentation at high concentrations, temperature, pH effects and the difficulty in obtaining stable reference material of high purity. Malvidin-3-glucoside is the major anthocyanin in *Vitis vinifera* grapes but is not the only anthocyanin and the results are expressed in malvidin-equivalents for comparative purposes only.

Interpretation of results

Generally speaking, the amount of coloured material in red grapes will depend on the

variety and regional source of the fruit and the climatic conditions encountered during the growing season. Analysts should be aware of the variable nature of grapegrowing and the following data (Table 1) are provided for indicative purposes only.

The data are sourced from a set of analytical data for grape berry samples of four varieties sourced from a range of regions across Australia over the period 1996 to 2003. The data are grouped in sugar maturity ranges to reflect colour maturation.

TSS range (ºBrix)	Colour (mg/g)	Shiraz	Cabernet Sauvignon	Merlot
20.1-22.0	minimum maximum average n	0.37 2.35 1.22 404	0.21 2.09 1.15 455	0.37 1.73 1.00 55
22.1-24.0	minimum maximum average n	0.46 2. 47 1.38 567	0.52 2.69 1.37 519	0.29 2.45 1.26 106
24.1-26.0	minimum maximum average n	0.47 2.73 1.59 492	0.55 2.81 1.51 251	0.53 2.22 1.46 56

Note: n =number of samples

Table 1. Concentration of anthocyanins in red grapes (segregated by variety and sugar maturity collected from various growing regions in Australia (1996-2003).

Estimation of uncertainty

Repeated sampling and analysis of homogenates by one operator on one instrument at the AWRI has shown a coefficient of variation (CV; standard deviation relative to mean) of 2%, but this should be verified for each individual laboratory's situation. Note that analyses from replicate samples taken from a given batch of grapes will tend to have a higher CV than those from replicate samples taken from homogenate of the same batch of grapes, due to the higher intrinsic error in sampling grapes.

For a single operator on a single day, the estimation of uncertainty in this determination (for a 95% confidence interval) is 2 x CV= 4%.

Therefore, allowing for other variations, such as operator and daily operation, it is reasonable to use 5% as an estimate of the uncertainty of measurement.

Quality assurance

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

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 5%.

2. A potassium dichromate standard (0.01 M in 1 mM sulfuric acid) or some other stable coloured solution, can be included in every batch of spectral readings to check on the instrument performance. The reading should be within 5% of the established expected value.

3. During initial method validation, the extraction efficiency must be checked by secondary extraction and colour measurement of the pellet from step 9 of the procedure, taking care to remove any coloured extract from the pellet first, by washing with cold water before the second extraction. The extraction efficiency must be greater than 95% and typical values would be approximately 98%.

Please contact the AWRI for details of this validation step if uncertain.

4. 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.

Other critical equipment such as balances and volume measuring devices must be calibrated as described in standard procedures (e.g. Morris and Fen 2002; Prowse 1985; NATA 1995; AS 2162.2-1998).

References and further reading

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