

Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines by the pH Differential Method: Collaborative Study

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This collaborative study was conducted to determine the total monomeric anthocyanin concentration by the pH differential method, which is a rapid and simple spectrophotometric method based on the anthocyanin structural transformation that occurs with a change in pH (colored at pH 1.0 and colorless at pH 4.5). Eleven collaborators representing commercial laboratories, academic institutions, and government laboratories participated. Seven Youden pair materials representing fruit juices, beverages, natural colorants, and wines were tested. The repeatability relative standard deviation (RSD_r) varied from 1.06 to 4.16%. The reproducibility relative standard deviation (RSD_R) ranged from 2.69 to 10.12%. The HorRat values were ≤ 1.33 for all materials. The Study Director recommends that the method be adopted Official First Action.

Anthocyanin pigments are important to food quality because of their contribution to color and appearance. There is increasing interest in the anthocyanin content of foods and nutraceuticals because of possible health benefits. Anthocyanin pigment content can also be a useful criterion in quality control and purchase specifications of fruit juices, nutraceuticals, and natural colorants. An AOAC method is needed to rapidly and precisely determine total monomeric anthocyanin content. Our goal was to design,

organize, and conduct a collaborative study to validate the pH differential method as an AOAC method.

Anthocyanins are responsible for the red, purple, and blue hues present in fruits, vegetables, and grains. There are 6 common anthocyanidins (pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin), whose structures can vary by glycosidic substitution at the 3 and 5 positions. Additional variations occur by acylation of the sugar groups with organic acids. Figure 1 shows the basic structure of an anthocyanin pigment.

The pH differential method has been used extensively by food technologists and horticulturists to assess the quality of fresh and processed fruits and vegetables. The method can be used for the determination of total monomeric anthocyanin content, based on the structural change of the anthocyanin chromophore between pH 1.0 and 4.5 (Figure 2). The anticipated use of the method is in research and for quality control of anthocyanin-containing fruit juices, wines, natural colorants, and other beverages.

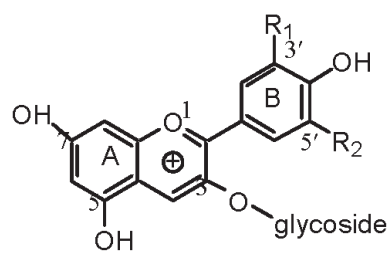
The concept of determining the amount of anthocyanin present in a material by measuring the change in absorbance at 2 different pH values (3.4 and 2.0) was first introduced by Sondheimer and Kertesz (1) in 1948. Since then, researchers have proposed using the pH values of 1.0 and 4.5 (2–5). Monomeric anthocyanins undergo a reversible structural transformation as a function of pH (colored oxonium form at pH 1.0 and colorless hemiketal form at pH 4.5; Figure 2). Thus, the difference in absorbance at the $\lambda_{\text{vis-max}}$ (ca 520 nm) of the pigment is proportional to the concentration of pigment. Figure 3 shows the spectra of huckleberry anthocyanins in buffers at pH 1.0 and 4.5. Degraded anthocyanins in the polymeric form are resistant to color change with change in pH. Therefore, polymerized anthocyanin pigments are not measured by this method because they absorb both at pH 4.5 and 1.0.

Absorbance should be measured at the $\lambda_{\text{vis-max}}$ of the pigment solution, and the pigment content should be calculated by using the molecular weight (MW) and molar

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The recommendation was approved by the Methods Committee on Commodity Foods and Commodity Products as First Action. See "Official Methods Program Actions," (2005) *Inside Laboratory Management*, July/August issue.

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$R_1=R_2=H$	Pelargonidin
$R_1=OH, R_2=H$	Cyanidin
$R_1=OCH_3, R_2=H$	Peonidin
$R_1=R_2=OH$	Delphinidin
$R_1=OCH_3, R_2=OH$	Petunidin
$R_1=R_2=OCH_3$	Malvidin

Figure 1. Basic structure of an anthocyanin pigment.

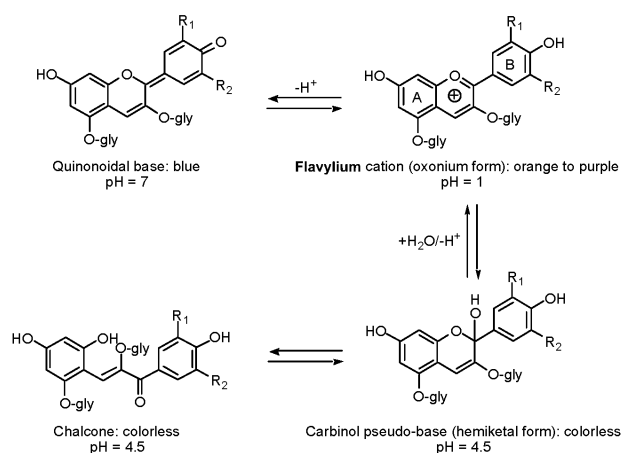


Figure 2. Predominant anthocyanin structural forms present at different pH levels.

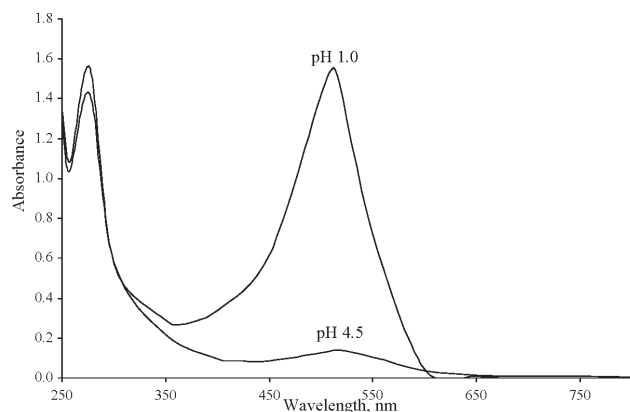


Figure 3. Spectral characteristics of huckleberry anthocyanin in buffers at pH 1.0 and 4.5.

extinction coefficient of the major anthocyanin in the matrix. For example, the anthocyanin content of wines is customarily calculated as the content of malvidin-3-glucoside (MW = 493.2) by using a molar extinction coefficient of 28 000 (6). However, natural matrixes normally contain a mixture of anthocyanins, and the proportions of these anthocyanins will vary naturally. This variation makes it unrealistic to assign specific extinction coefficients or molecular weights to routine test results. As a result, the method calls for several measurements to be obtained. For purposes of this collaborative study, analysts were instructed to measure all solutions at 520 nm, which is midrange for the different materials in the study. Results were expressed as equivalents of cyanidin-3-glucoside, which is the most common anthocyanin pigment found in nature (7). Identities of the test materials were unknown to the analysts.

Collaborative Study

A collaborative protocol was prepared by following the guidelines of AOAC INTERNATIONAL (8) and approved by the Methods Committee on Commodity Foods and Commodity Products.

Collaborating Laboratories

Eleven laboratories representing commercial laboratories, academic institutions, and government laboratories participated. Six of the laboratories were outside the United States. One analyst from each laboratory performed the analysis. Collaborators were not compensated for their participation.

Test Materials

Seven Youden pair materials were prepared; 1 member of the pair was neat (undiluted, X), and the other was diluted with distilled water (Y) to obtain a difference of $\leq 5\%$. The formula used was

$$(x_c - y_c/x_c) \leq 0.05$$

where x_c is the concentration of analyte in X, and y_c is the concentration of analyte in Y (8). Descriptions of the materials used in this study are given in Table 1. Concentrates (strawberry, raspberry, and elderberry) were initially diluted to their reported single strength °Brix (8.0, 11.0, and 9.2, respectively) as listed in 21CFR (Code of Federal Regulations), Part 101, and then diluted appropriately as Youden pairs in a 1 L volumetric flask. The standard solution was prepared by weighing 82.2 mg cyanidin-3-glucoside chloride (76.2 mg cyanidin-3-glucoside; Polyphenols Laboratories As, Sandnes, Norway), dissolving in distilled water, and diluting to a final volume of 1 L in a volumetric flask.

Two familiarization materials were included with the test sets. The materials were cranberry juice cocktail and an intensely pigmented grape juice concentrate diluted to appropriate strength. After reviewing their values,

Table 1. Materials and their sources used in the collaborative study

Material ^a	Source
Cranberry juice cocktail	Local grocery store (Corvallis, OR)
Red wine	Local grocery store (Corvallis, OR)
Natural colorant ^b	Canandaigua Concentrate (Madera, CA)
Strawberry concentrate ^c	Kerr Concentrate Inc. (Salem, OR)
Raspberry concentrate ^c	Kerr Concentrate Inc. (Salem, OR)
Elderberry concentrate ^c	Kerr Concentrate Inc. (Salem, OR)
Cyanidin-3-glucoside chloride ^d	Polyphenols Laboratories As (Sandnes, Norway)

^a Matrixes were prepared as Youden pairs.

^b A high colored grape juice concentrate.

^c Obtained as a concentrate and diluted to the appropriate °Brix.

^d Standard used in this study.

collaborators were notified to continue with the actual laboratory test samples.

The appropriate anthocyanin content of the materials ranged from 20 to 3000 mg/L (expressed as cyanidin-3-glucoside). The method required the analyst to dilute test portions with buffers to an appropriate concentration for measurements.

The materials used to prepare the laboratory samples were homogenized by stirring and then aliquots were transferred to vials (ca 25 mL). All laboratory samples were labeled with a random 3-digit number.

Laboratory samples were placed in freezable containers and stored frozen (−23°C) until packaged for shipment to collaborators. Test samples were packed with dry ice and shipped overnight via Federal Express. Shipping boxes were labeled to notify recipients to immediately store the samples frozen until analysis.

The homogeneity of the laboratory samples was evaluated in-house. Homogeneity testing was performed by selecting 2 vials from the frozen laboratory samples at random, sampling 12 times from each vial, and measuring °Brix by a digital refractometer (Auto Abbe refractometer Model 10500, Reichert-Jung, Leica Inc., Buffalo, NY). A thermal shock (transportation) was produced by placing a vial of frozen sample (stored at −23°C) at room temperature for 1 day, and then returning it to −23°C for 2 days (to simulate temperature abuse during transportation); then the pH differential was measured and duplicated. Long-term stability testing was done by measuring the pH differential of random vials after 0 and 3 months of storage at −23°C.

Statistical Analyses

AOAC Interlaboratory Statistical Program 2001 for Youden pairs/split levels (Joanna M. Lynch, Ithaca, NY, copyright 2001, Version 1.1) was used to calculate method performance statistics and identify outliers according to the

guidelines of AOAC INTERNATIONAL (8). The outliers were determined at $\alpha = 0.025$, and the *t*-statistics for equivalence of variance were tested at $\alpha = 0.05$. To assess the homogeneity of the splitting of each material, a 1-way analysis of variance (ANOVA), performed with the PROC ANOVA software of the Statistical Analysis System (SAS; 9), was used to determine if the mean °Brix values for the 2 randomly selected vials for each material varied significantly between themselves. Homogeneity was evaluated by using a significance level of $\alpha = 0.05$.

Methods of Standard Analyses

To investigate recovery issues for the standard used in the collaborative study, additional cyanidin-3-glucoside chloride was purchased from 2 vendors, Polyphenols Laboratories As and Extrasynthese (Lyon, France). Percent purity by liquid chromatography (LC), percent purity by molar absorptivity, percent moisture content, and hygroscopicity at 1 relative humidity were determined. Only moisture determination and hygroscopicity were duplicated because of the high cost of the standards.

Standards were prepared by weighing 17.00 ± 0.01 mg into a glass weighing bottle (with lid) that had been cleaned, dried in an oven, and cooled overnight in a desiccator over phosphorus pentoxide (P₂O₅) under vacuum.

Determination of Molar Absorptivity and Percent Purity

The purity of each standard was investigated by determining its molar absorptivity at 520 nm (λ_{max} used in the collaborative study) and at its true λ_{max} (510 nm) obtained in pH 1.0 buffer. This test was conducted by using the same standard material used in the collaborative study 2 months after the purchase date, and the 2 newly purchased standards (as received). The values obtained were compared with the literature value for the extinction coefficient for cyanidin-3-glucoside of $26\,900 \text{ L} \times \text{cm}^{-1} \times \text{mol}^{-1}$ (10), by using molecular weights of $484.5 \text{ g} \times \text{mol}^{-1}$ for cyanidin-3-glucoside chloride and $449.2 \text{ g} \times \text{mol}^{-1}$ for cyanidin-3-glucoside; also, percent purity was determined by LC (monitored at 520 and 280 nm, respectively). Anthocyanins were separated by reversed-phase LC using a Hewlett-Packard 1090 liquid chromatograph (Agilent Technologies Inc., Wilmington, DE), equipped with a photodiode array detector. Absorbance spectra were recorded for all peaks. The flow rate was 1 mL/min, and the injection volume was 20 μL . Column temperature was maintained at 40°C. A Prodigy 5 μm ODS (3), 100 Å (250 \times 4.6 mm) column from Phenomenex was used (Torrance, CA). Solvent A was 100% acetonitrile. Solvent B was 10% (v/v) acetic acid and 1% (v/v) phosphoric acid in water. All solvents were LC grade. The program used a linear gradient from 2 to 20% solvent A in 25 min; then a linear gradient of solvent A from 20 to 40% in 5 min, with simultaneous detection at 520 and 280 nm (11). Peak areas were used to calculate percent purity based on LC, at both wavelengths.

Moisture Determination

Standards were weighed and dried in a desiccator in the presence of phosphorus pentoxide (P_2O_5) under vacuum until a constant weight was reached. Weight loss after drying over phosphorus pentoxide was used to determine % moisture content (unbound water). Percent moisture content (true % mass loss) was reported as % moisture on a dry-weight basis.

Determination of Hygroscopicity

Hygroscopicity, % equilibrium moisture content (EMC), was determined by using the method reported by Callahan et al. (12). Hygroscopicity was evaluated by storing the standards in a desiccator containing a saturated potassium bromide (KBr) solution and placing it in a 25°C precision incubator (Fisher Scientific Isotemp), which maintained a constant 83% relative humidity. The humidity of the chamber was measured by using a Taylor hygrometer (Model 5522E, Fletcher, NC). Standards were stored in the humidity chamber for several weeks, while the weight was monitored until a static weight was reached; hygroscopicity (% EMC at 25°C) was then reported.

AOAC Official Method 2005.02
Total Monomeric Anthocyanin Pigment
Content of Fruit Juices, Beverages, Natural
Colorants, and Wines
pH Differential Method
First Action 2005

(Applicable to the determination of monomeric anthocyanins in fruit juices, beverages, natural colorants, and wines within the range of 20–3000 mg/L as cyanidin-3-glucoside equivalents.)

See Table 2005.02 for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Monomeric anthocyanin pigments reversibly change color with a change in pH; the colored oxonium form exists at pH 1.0, and the colorless hemiketal form predominates at pH 4.5. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. Results are expressed on a cyanidin-3-glucoside basis. Degraded anthocyanins in the polymeric form are resistant to color change regardless of pH and are not included in the measurements because they absorb at pH 4.5 as well as pH 1.0.

B. Apparatus

(a) *pH meter*.—Standardized with pH 4.0 and 7.0 standard buffer solutions.

(b) *Visible spectrophotometer*.—Performance of the spectrophotometer at 520 nm should be verified with reference standards for wavelength accuracy, photometric accuracy, photometric linearity, and stray light.

(c) *Glass or disposable cuvetts for spectrophotometer*.—1 cm pathlength.

(d) *Volumetric flasks*.—50 mL.

C. Reagents

(a) *pH 1.0 buffer (potassium chloride, 0.025M)*.—Weigh 1.86 g KCl into a beaker and add distilled water to ca 980 mL. Measure the pH, and adjust pH to 1.0 (± 0.05) with HCl (ca 6.3 mL). Transfer to a 1 L volumetric flask, and dilute to volume with distilled water.

(b) *pH 4.5 buffer (sodium acetate, 0.4M)*.—Weigh 54.43 g $CH_3CO_2Na \cdot 3H_2O$ in a beaker, and add distilled water to ca 960 mL. Measure the pH, and adjust pH to 4.5 (± 0.05) with HCl (ca 20 mL). Transfer to a 1 L volumetric flask, and dilute to volume with distilled water.

Table 2005.02 Interlaboratory study results for the determination of total monomeric anthocyanin pigment content by the pH differential method

Material	Mean, mg/L ^a	No. of labs, a (b) ^b	s_r^c	RSD _r , % ^d	s_R^e	RSD _R , % ^d	r^f	R^g	HorRat
Cranberry juice cocktail	13.6	10 (1)	0.57	4.16	1.09	8.00	1.59	3.05	0.74
Red wine	201.6	11 (0)	5.29	2.62	15.99	7.93	14.81	44.76	1.10
Natural colorant	640.8	11 (0)	11.97	1.87	36.52	5.70	33.52	102.25	0.94
Strawberry juice	63.6	10 (1)	2.43	3.82	6.44	10.12	6.81	18.03	1.18
Raspberry juice	336.7	11 (0)	10.80	3.21	17.62	5.23	30.24	49.32	0.79
Elderberry juice	3006.8	10 (0)	31.78	1.06	191.84	6.38	88.97	537.15	1.33
Standard	44.8	11 (0)	0.53	1.19	1.20	2.69	1.49	3.37	0.3

^a Expressed as cyanidin-3-glucoside equivalents.

^b a = Number of laboratories retained after removal of outliers; (b) = number of laboratories removed as outliers.

^c s_r = Repeatability standard deviation.

^d RSD = Relative standard deviation.

^e s_R = Reproducibility standard deviation.

^f r = Repeatability value.

^g R = Reproducibility value.

D. Preparation of Test Solution

Perform all dilutions in 50 mL volumetric flasks, **B(d)**. Use volumetric pipets for addition of the test portion. The maximum test portion added should be ≤ 10 mL (1 part test portion, 4 parts buffer) so as not to exceed the buffer capacity of the reagents.

Determine the appropriate dilution factor by diluting the test portion with pH 1.0 buffer, **C(a)**, until absorbance at 520 nm is within the linear range of the spectrophotometer. (For most spectrophotometers, the absorbance should be between 0.2 and 1.4 AU.) Using this dilution factor, prepare 2 dilutions of the test sample, one with pH 1.0 buffer and the other with pH 4.5 buffer.

E. Determination

Determine absorbance of test portion diluted with pH 1.0 buffer, **C(a)**, and pH 4.5 buffer, **C(b)**, at both 520 and 700 nm. The diluted test portions are read versus a blank cell filled with distilled water. Measure absorbance within 20–50 min of preparation.

Note: The reason for measuring the absorbance at 700 nm is to correct for haze. However, if the diluted test portion is excessively turbid, clarify by centrifuging or filtering before measurement. Use a filter (e.g., Millipore™ membrane filter, ≤ 1.2 μm pore size, Millipore Corp., Bedford, MA) that will not absorb the anthocyanins.

F. Calculations

Calculate anthocyanin pigment concentration, expressed as cyanidin-3-glucoside equivalents, as follows:

$$\text{Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L)} = \frac{A \times \text{MW} \times \text{DF} \times 10^3}{\epsilon \times l}$$

where $A = (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 1.0}} - (A_{520\text{nm}} - A_{700\text{nm}})_{\text{pH 4.5}}$; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in **D**; l = pathlength in cm; $\epsilon = 26\,900$ molar extinction coefficient, in $\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$, for cyd-3-glu; and 10^3 = factor for conversion from g to mg.

Note: In some cases, the predominant anthocyanin in a material may be known and different from cyanidin-3-glucoside. It is critical that the wavelength, molecular weight, and absorptivity used be specified if results are not expressed as cyanidin-3-glucoside equivalents. Report results as monomeric anthocyanins, expressed as cyanidin-3-glucoside equivalents in mg/L.

G. Limitations and Restrictions

The presence of ethanol in test samples does not interfere with the assay at the levels typically encountered in wine (10–14%). Although determination of total anthocyanin pigment is useful in assessing the quality of fruit juices and beverages, it is of limited value by itself in authenticity investigations and should be used in conjunction with analyses for individual anthocyanins. FD&C Red No. 40,

cochineal, and beet powder did not interfere at concentrations of $<10\%$ of total color, but they did lead to a reduction in measured anthocyanin content at higher concentrations.

Reference: *J. AOAC Int.* **88**, 1269 (2005).

Results and Discussion

The total monomeric anthocyanin results from the collaborative study are provided in Table 2. Two Cochran outliers were identified (Collaborator 3: cranberry juice cocktail; Collaborator 11: strawberry juice). For each set of Youden pairs, there was no significant difference ($\alpha = 0.05$) between the variances for the undiluted and diluted members of each pair, except for the strawberry juice, possibly because of the high polymeric anthocyanin (resulting in a high absorbance value with pH 4.5 buffer) present in strawberry concentrates.

The HorRat values for the materials ranged from 0.30 (standard material) to 1.33 (elderberry juice), which were well below the acceptable range (HorRat value of ≤ 2.0). The repeatability relative standard deviations (RSD_r) ranged from 1.06% for elderberry juice to 4.16% for cranberry juice cocktail. The reproducibility relative standard deviations (RSD_R) ranged from 2.69% for the standard to 10.12% for strawberry juice (Table **2005.02**). The HorRat value for the standard was noticeably lower than those for the test materials, probably because it was a single anthocyanin pigment rather than a mixture and it lacked the background complexities of a natural matrix.

For the homogeneity tests (Table 3), the mean °Brix values for the 2 randomly selected vials for each material did not vary significantly among themselves ($P > 0.05$). There were insufficient data to perform statistical analyses after the thermal shock and for the long-term study. However, the results for 0–3 months indicate little change (Figure 4).

Because the major objective of the collaborative study was to evaluate the performance of the method between laboratories, it was appropriate for all participants to use the same λ_{max} and a common molecular weight and extinction coefficient for calculating total monomeric anthocyanin content. Materials were not identified for the analysts; therefore, the pigment on which the calculation is based needed to be assigned. Cyanidin-3-glucoside was selected because it is the most common anthocyanin in nature (7) and several investigators have determined its extinction coefficient, with values ranging from 18 800 to 34 300 $\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$, depending on the solvent, wavelength of maximum absorbance, and “purity” (Table 4). The extinction coefficient value of 26 900 $\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ selected for this collaborative study may not be the “true” value, but it is reasonable in light of the values reported in the literature for cyanidin-3-glucoside in similar aqueous systems (10, 14, 15, 17, 19). In usual practice, the λ_{max} of the matrix of interest can be determined with the test portion diluted in the pH 1.0 buffer, and the absorptivity and molecular weight of the predominant anthocyanin present can be used to calculate the final values. Anthocyanin absorptivities have been

determined in acidic aqueous or alcohol solutions (5). Absorptivities that have been determined in acidic alcohol should not be used in the pH differential method because of solvent effects. If the absorptivity of the major anthocyanin present in the material has not been determined, or if the major anthocyanin is unknown, it is appropriate to calculate total monomeric anthocyanin content by using the absorptivity and molecular weight of cyanidin-3-glucoside ($\epsilon = 26\,900 \text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ and $\text{MW} = 449.2 \text{ g/mol}$). It is critical that the absorptivity, wavelength, and molecular weight used in the determination be reported so that results among laboratories can be compared on an equivalent basis.

The pH differential method is based on the assumption that monomeric, or "pure," anthocyanins have little or no absorbance in pH 4.5 buffer, and that polymeric or degraded anthocyanins will absorb at this pH. Although nearly all monomeric anthocyanins are in the hemiketal form at pH 4.5, a small proportion are in the quinoidal form or the flavylum form, which will make a small contribution to the absorbance. The cyanidin-3-glucoside standard used in the collaborative study exhibited a low absorbance in pH 4.5 buffer.

Presently, there is no quantitative certified reference material for cyanidin-3-glucoside. The standard used in the collaborative study was purchased as a chromatographically pure form of cyanidin-3-glucoside chloride (>97% pure, as determined by LC, with UV-Vis detection at 520 and 280 nm, according to the datasheet provided by the supplier). Possible impurities listed on the datasheet were other anthocyanins, flavonoids, or polyphenols. When the total monomeric anthocyanin content of the standard solution (82.2 mg cyanidin-3-glucoside

chloride/L = 76.2 mg cyanidin-3-glucoside/L) was measured, the average measured content was much lower (45.8 mg/L, expressed as cyanidin-3-glucoside). This value gave an estimated "recovery" of only 60% ($45.8/76.2 \times 100$). Although the standard was chromatographically pure, it may have contained bound and unbound water, and/or other materials not absorbing at 520 and 280 nm. Despite using extreme care when purifying anthocyanins, researchers have reported the presence of water of crystallization (19–21), which would not be accounted for when purity is determined by LC. Efforts to determine the true molar absorptivity of a purified anthocyanin are hindered by the formidable problem of obtaining the pure crystalline anthocyanin in adequate quantities. Anthocyanins are highly hygroscopic (22–24) and difficult to obtain in a pure crystalline form.

The purity of the same "standard" material used in the collaborative study 2 months after the date of purchase was found to be 74.6% (by using $A_{510 \text{ nm}}$) or 71.0% (by using $A_{520 \text{ nm}}$) by molar absorptivity. Thus, measuring anthocyanin content at 520 nm rather than at the true λ_{max} would contribute to the low recovery in the collaborative study.

Table 5 summarizes the properties of the cyanidin-3-glucoside standards. Moisture contents were 12.9% for standard 1 (used in the collaborative study), 3.5% for standard 2, and 10.5% for standard 3. (Standards 1 and 3 were obtained from the same manufacturer.) When a portion of the standard used in the collaborative study (standard 1, Table 5) was analyzed by LC (regardless of test portion size or dilution), the resulting chromatogram had one large peak (cyanidin-3-glucoside) and numerous small peaks (impurities,

Table 2. Total monomeric anthocyanin (mg/L, cyanidin-3-glucoside equivalents) levels of all Youden pair samples analyzed by collaborators

Collaborator	Cranberry juice cocktail		Red wine		Natural colorant		Strawberry juice		Raspberry juice		Elderberry juice		Standard	
	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b
1	15.0	13.8	218.8	210.9	693.3	672.8	69.0	66.1	364.2	356.7	3113.5	2948.2	46.6	44.5
2	14.2	13.8	225.0	215.8	688.0	664.6	72.8	70.3	356.1	346.5	3193.7	3030.8	46.3	44.1
3	16.3 ^c	12.0 ^c	220.0	225.0	724.7	673.0	78.5	69.3	367.4	347.8	1527.9 ^d	2187.6 ^d	47.9	45.8
4	12.5	13.7	184.0	192.0	623.7	640.8	57.7	56.5	321.9	332.7	3181.1	3093.5	45.0	42.2
5	13.0	13.1	211.7	195.2	652.1	621.2	65.1	62.6	345.7	325.6	3147.7	2955.7	44.8	43.6
6	13.2	12.8	209.7	200.6	657.3	634.2	64.5	64.5	353.2	325.1	3208.3	3076.8	45.8	43.3
7	14.4	15.5	173.9	172.1	598.0	584.5	61.1	58.8	359.7	314.7	3101.5	2931.4	44.8	44.0
8	13.4	12.9	199.6	200.2	648.8	628.8	60.6	58.0	326.5	322.3	3248.2	3202.2	44.3	41.9
9	12.4	11.4	202.7	200.7	641.2	625.1	51.9	56.7	308.1	302.3	2941.5	2782.5	44.6	42.8
10	13.4	13.4	182.9	180.5	600.3	563.6	65.8	62.3	331.5	325.6	3019.2	2848.8	46.4	45.4
11	15.0	15.4	213.7	200.8	637.9	622.9	92.7 ^c	66.8 ^c	350.7	322.3	2615.0	2496.5	47.3	44.1

^a Undiluted member of the Youden pair sample.

^b Member of the Youden pair sample diluted to make $\leq 5\%$ difference.

^c Cochran outlier.

^d Invalid data (laboratory sample vial was cracked upon arrival).

Table 3. °Brix values for the homogeneity of the test materials (12 measurements from 2 randomly selected vials)

Measurements	Cranberry juice cocktail		Red wine		Natural colorant		Strawberry juice		Raspberry juice		Elderberry juice		Standard ^c																
	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b	X ^a	Y ^b															
1	13.70	13.69	13.20	13.21	9.55	9.49	9.13	9.18	1.65	1.66	1.56	1.56	8.19	8.20	7.88	7.87	9.17	9.17	8.82	8.82	10.46	10.49	9.94	9.99	0.03	0.03	0.03	0.03	
2	13.71	13.70	13.20	13.20	9.54	9.49	9.13	9.17	1.65	1.67	1.56	1.59	8.18	8.18	7.87	7.87	9.17	9.16	8.82	8.82	10.49	10.51	9.97	10.02	0.03	0.03	0.03	0.03	
3	13.73	13.71	13.20	13.19	9.54	9.54	9.21	9.21	1.67	1.67	1.59	1.59	8.18	8.18	7.86	7.87	9.18	9.18	8.82	8.82	10.51	10.55	10.01	10.07	0.03	0.03	0.03	0.03	
4	13.70	13.70	13.20	13.19	9.53	9.55	9.21	9.14	1.68	1.67	1.59	1.56	8.18	8.18	7.86	7.87	9.18	9.18	8.80	8.82	10.54	10.57	10.04	10.06	0.03	0.02	0.03	0.03	
5	13.73	13.70	13.19	13.19	9.52	9.57	9.14	9.21	1.60	1.73	1.57	1.56	8.18	8.18	7.86	7.87	9.17	9.16	8.81	8.82	11.53	10.48	10.10	10.13	0.03	0.03	0.02	0.03	
6	13.73	13.70	13.19	13.20	9.52	9.57	9.14	9.13	1.62	1.73	1.56	1.57	8.18	8.18	7.86	7.87	9.17	9.16	8.82	8.81	11.54	10.48	10.13	10.13	0.03	0.03	0.02	0.03	
7	13.69	13.70	13.19	13.19	9.52	9.56	9.21	9.13	1.67	1.63	1.58	1.59	8.19	8.19	7.85	7.86	9.16	9.16	8.81	8.81	10.49	10.54	10.20	10.25	0.02	0.03	0.02	0.03	
8	13.70	13.71	13.19	13.19	9.52	9.56	9.17	9.13	1.67	1.62	1.59	1.60	8.19	8.19	7.86	7.85	9.17	9.16	8.81	8.81	10.52	10.52	10.26	10.26	0.03	0.03	0.03	0.03	
9	13.70	13.70	13.33	13.19	9.56	9.58	9.17	9.20	1.69	1.63	1.60	1.63	8.18	8.17	7.86	7.87	9.16	9.17	8.82	8.82	10.56	10.58	10.34	10.23	0.02	0.03	0.03	0.02	
10	13.69	13.69	13.33	13.19	9.52	9.55	9.17	9.19	1.69	1.62	1.62	1.62	8.18	8.18	7.87	7.87	9.16	9.16	8.81	8.82	10.57	10.58	10.35	10.28	0.03	0.03	0.02	0.03	
11	13.69	13.69	13.21	13.31	9.52	9.56	9.18	9.14	1.65	1.65	1.63	1.63	8.17	8.17	7.86	7.86	9.17	9.17	8.82	8.82	10.61	10.62	10.30	10.20	0.03	0.03	0.02	0.02	
12	13.70	13.69	13.20	13.30	9.52	9.56	9.17	9.12	1.65	1.63	1.62	1.65	8.17	8.18	7.86	7.86	9.17	9.17	8.82	8.82	10.61	10.66	10.31	10.15	0.02	0.03	0.03	0.03	
P-value	0.15	NSD ^d	0.74	NSD	0.06	NSD	0.62	NSD	0.90	NSD	0.57	NSD	0.79	NSD	0.26	NSD	0.41	NSD	0.3	NSD	0.19	NSD	0.77	NSD	0.30	NSD	0.19	NSD	
Mean (SD ^e)	13.70 (0.01)	13.22 (0.05)			9.54 (0.02)		9.17 (0.03)		1.66 (0.03)		1.59 (0.03)		8.18 (0.01)		7.86 (0.01)		9.17 (0.01)		8.82 (0.01)		10.63 (0.28)		10.16 (0.12)		0.03 (0.00)		0.03 (0.00)		0.03 (0.00)

^a Undiluted member of the Youden pair sample.

^b Diluted member of the Youden pair sample to make ≤5% difference.

^c Standard = Cyanidin-3-glucoside chloride.

^d NSD = Not significantly different at $\alpha = 0.05$.

^e SD = Standard deviation within-tube variation.

detected by LC, that absorbed 520 and 280 nm). On the basis of the absorbance at 520 and 280 nm, the purity values for standard 1 were 98.2 and 93.8%, respectively. Purity values for standard 2 were 99.6% (monitored at 520 nm) and 98.9% (monitored at 280 nm). Purity values for standard 3 were 95.2% (monitored at 520 nm) and 94.1% (monitored at 280 nm). The purities based on molar absorptivity were 74.6, 99.1, and 80.3% for standards 1, 2, and 3, respectively. Hygroscopicity values for standards 1, 2, and 3 at 83% relative humidity were 22.4, 10.0, and 22.2%, respectively.

There was no uniformity regarding each company's instructions on how to store and handle the anthocyanin standards. One datasheet advised storage in darkness at low temperatures ($<-5^{\circ}\text{C}$) for no more than a few days. Another firm advised storing the standard at -20°C in a dry and dark place, and also placing the standard in a desiccator under vacuum for 48 h before use. Neither company provided an expiration date, but both recommended using the standard soon after reception, and to never store the product in solution. They further stated that the stability of the products was not always known and very difficult to measure.

The validity and basic principles for determining the anthocyanin pigment concentration by the pH differential method have been widely accepted by natural product chemists for years. A combination of the following factors may account for the low recovery or purity of the standard: moisture content, hygroscopicity of anthocyanins, presence of impurities (polyphenolics and/or polymeric anthocyanins), the possibility

that the molar extinction coefficient of $26\,900\text{ L} \times \text{mol}^{-1} \times \text{cm}^{-1}$ is not the "true" value, measurement of the absorbance at 520 nm rather than at 510 nm, and the minor contribution of quinoidal and flavylium forms to absorbance at pH 4.5. This collaborative study has demonstrated that total monomeric anthocyanin pigment content can be measured with excellent agreement between laboratories. A further advantage of the method is that it does not require the purchase of costly standards, as is the case when anthocyanin content is measured by LC by the external standard method. The experiments concerning the moisture content, purity, and hygroscopicity of anthocyanin standards in this investigation call attention to the importance of taking these properties into consideration when experiments are conducted with anthocyanin standards.

Collaborators' Comments

Collaborators made the following comments during the study: (1) several "samples" produced spectra whose maximum was not 520 nm; (2) "sample" absorbance was measured with a 2 mm cell; (3) the use of 100 mL volumetric flasks or serial dilution for the dark juices should be allowed; and (4) "sample" container arrived cracked.

The statement about analytes having a different maximum absorbance at a different wavelength is well taken and discussed above. We believe that the statement to "determine the appropriate dilution factor" is sufficient for the analyst to make the decision about whether to use a 2 mm cell,

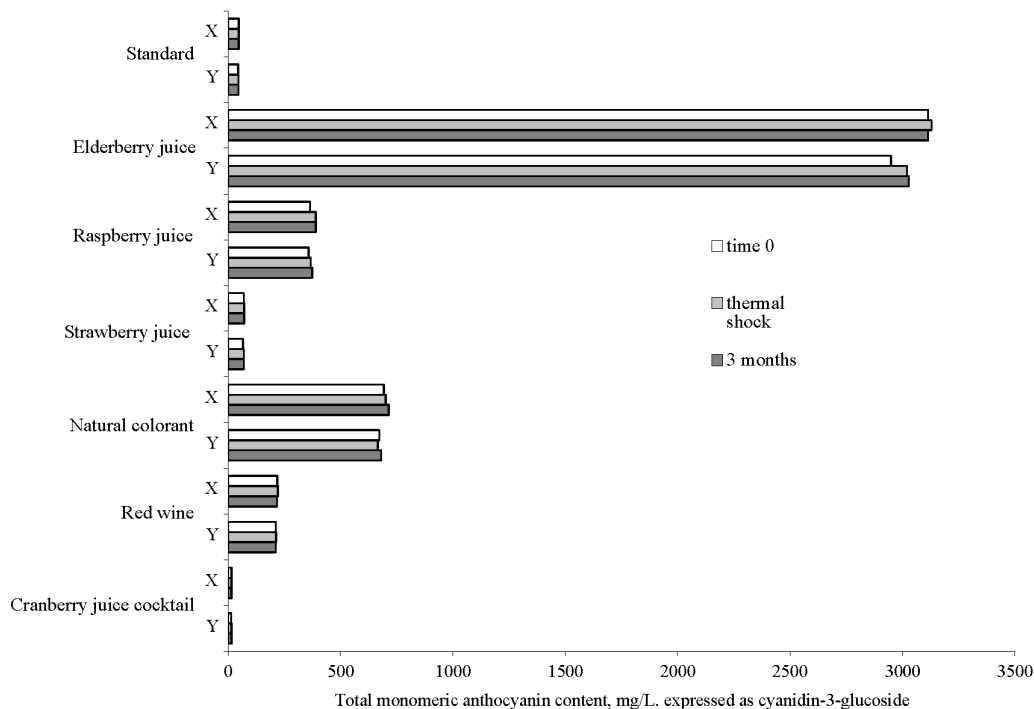


Figure 4. Values for total monomeric anthocyanin content, obtained after the thermal shock and during the long-term stability study of the test samples (X = undiluted member of the Youden pair sample, Y = member of the Youden pair sample diluted to make $\leq 5\%$ difference).

Table 4. Molar absorptivity (ϵ) values of cyanidin-3-glucoside reported in the literature

Solvent system	$\lambda_{\text{vis-max}}$, nm	Molar absorptivity (ϵ) ^a	Ref.
Aqueous buffer, pH 1.0	510	26900	10
1% HCl in methanol	530	34300	13
0.1N HCl	520	25740	14
Buffer, pH 1.0	510	24800	15
10% Ethanol, pH 1.5	512	18800	16
Buffer, pH 1.0	510	20000	17
0.1M HCl in various concentrations of aqueous ethanol	530	27876–32678	18
0.1N HCl	510	26300	19

^a Units are $\text{L} \times \text{mol}^{-1} \times \text{cm}^{-1}$.

100 mL volumetric flasks, or serial dilutions. The collaborators' containers that arrived cracked were replaced (except in one case).

Recommendations

On the basis of the results from the collaborative study, the Study Director recommends that the pH differential method for determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines be adopted as a First Action Official Method of AOAC INTERNATIONAL.

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Table 5. Results from the investigation of cyanidin-3-glucoside standards

	Standard 1 ^a	Standard 2 ^b	Standard 3 ^c
% Moisture ^d	12.9	3.5	10.5
Hygroscopicity ^e , % EMC	22.4	10.0	22.2
% Purity by molar absorptivity (as received) by using $A_{510\text{nm}}$	74.6	99.1	80.3
Extinction coefficient determined by using $A_{510\text{nm}}$ ^f	20072	26672	21606
% Purity by molar absorptivity (as received) using $A_{520\text{nm}}$	71.0	93.2	76.3
Extinction coefficient determined by using $A_{520\text{nm}}$ ^g	19103	25076	20526
% Purity by LC reported in datasheet	>97	99.3	>97
% Purity by LC conducted in-house and monitored at 520 nm	98.2	99.6	95.2
% Purity by LC conducted in-house and monitored at 280 nm	93.8	98.9	94.1
Cost ^h	\$290.00 for 100 mg	\$806.68 for 50 mg	\$416.08 for 100 mg

^a Standard used in the collaborative study; purchased from Polyphenols Laboratories As.

^b Additional cyanidin-3-glucoside chloride purchased from Extrasynthese.

^c Additional cyanidin-3-glucoside chloride purchased from Polyphenols Laboratories As.

^d Determined by placing cyanidin-3-glucoside chloride over phosphorous pentoxide under vacuum.

^e Standards were placed in an 83% relative humidity chamber (saturated potassium bromide solution placed in a 25°C incubator).

^f Extinction coefficient determined by dissolving cyanidin-3-glucoside chloride in pH 1.0 buffer and measuring the absorbance at 510 nm.

^g Extinction coefficient determined by dissolving cyanidin-3-glucoside chloride in pH 1.0 buffer and measuring the absorbance at 520 nm, which is the λ_{max} used in the collaborative study.

^h Cost of standard does not include shipping and handling.

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