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Analytical Methods

Correlation of two anthocyanin quantification methods: HPLC and spectrophotometric methods

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ABSTRACT

The pH differential method and HPLC are commonly used methods by researchers and the food industry for quantifying anthocyanins in a sample. This study was conducted to establish a relationship between the two analytical methods. Seven juice samples containing an array of different individual anthocyanins were analyzed by pH differential and HPLC (two different columns and mobile phase conditions). In general, total anthocyanins were greater when expressed as malvidin-glucoside than as cyanidin-glucoside, despite the method used. This paper demonstrates the high correlation ($R \geqslant 0.925$, $p \leqslant 0.05$) between the pH differential method and HPLC (both systems) when determining the amount of anthocyanins found in samples. For laboratories that do not have the capability for HPLC analysis, the pH differential is a simple and economical method to determine total anthocyanins. This study also demonstrates the importance of reporting the standard used to express the values. There is still a need for both methods and certified anthocyanin standards.

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1. Introduction

Anthocyanins are responsible for the red, purple, and blue hues present in fruits, vegetables, and grains, as well as products made from those materials. Pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin are the six common anthocyanidins found in nature. Their structures can be varied by glycosidic substitution (glucose, galactose, rhamnose, xylose, and arabinose) at the 3 and 5 positions on the A and C rings. Additional variations occur by acylation of the sugar groups with acids. Some acylating groups commonly found are acetic acid, *p*-coumaric acid, caffeic acid, malonic acid, sinapic acid, ferulic acid, oxalic acid, and succinic acid (Giusti, Rodriguez-Saona, Griffin, & Wrolstad, 1999; Takeoka & Dao, 2002).

Most research on the quantitation, purification, separation, and identification of anthocyanins has relied on expensive equipment, and/or lengthy sample preparation. These methods include paper chromatography, thin-layer chromatography, column chromatography, solid phase extraction, counter current chromatography, UV–visible absorption spectroscopy, high performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (Skrede & Wrolstad, 2002; Takeoka & Dao, 2002).

Measurements of anthocyanin content, the major contributor to color in berries and berry products, has long been utilized as an indicator of quality of fresh and processed berry products (Wrolstad, Durst, & Lee, 2005). Interest in the beneficial effects of anthocyanins on human health has stimulated an increased demand for their use in food products and dietary supplements and created a need for an inexpensive and effective method to quantify the total anthocyanin content of a sample, while allowing results to be compared among laboratories (Lee, Durst, & Wrolstad, 2005). The pH differential method has been validated and demonstrated to be simple, quick, and accurate for measuring the total monomeric anthocyanin content of a sample (Lee et al., 2005), and it is used extensively by scientific and industry communities (Wrolstad, personal communication). In 2005, the pH differential method received first action approval from the Association of Analytical Communities (AOAC) International official methods board (AOAC method 2005.02), and will be reviewed for final action approval initiated in 2007.

Profiles of anthocyanins are distinctive for different fruits, with some varietal variation depending on the commodity. Reversed-phase HPLC coupled with photodiode array detection has been the most widely used tool for the identification, and quantification of anthocyanins. Individual anthocyanins can be separated by their polarity, which cause them to elute at different times. The anthocyanins can be quantitated with an external standard (cyanidin-3-glucoside or any purified anthocyanin standard). However, HPLC can result in an underestimation of the amount of anthocyanin

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present in samples that contain different anthocyanidins glycosides when using one standard for quantification. Typically, cyanidin-3-glucoside is selected as the external standard. The sum of the peak area at a certain wavelength (e.g. 520 nm) is used when quantifying anthocyanins by HPLC, which is generally close to the maximum wavelengths (λ_{max}) of the individual anthocyanins. Numerous studies have shown that the maximum wavelength shifts slightly dependent upon the anthocyanin chromophores. For example, the λ_{max} for malvidin-3-glucoside and cyanidin-3-glucoside in acidified (0.01% HCl) methanol are 534 and 523 nm, respectively (Durst & Wrolstad, 2005; Francis, 1982; Hong & Wrolstad, 1990).

The measured anthocyanin content of a sample can be influenced, in part, by the method used for analysis. Dossett (2007) and Lee and Finn (2007) reported the difference in the anthocyanin content determined by pH differential and the identical HPLC conditions in elderberry and black raspberry samples that will be later addressed in this paper. Others (Sáchez-Moreno, Cao, Ou, & Prior, 2003; Wu, Gu, Prior, & McKay, 2004; Wu et al., 2006) have reported that HPLC is a better method not because of its performance, but because the results indicate a higher value. The objective of this study was to compare the two commonly used anthocyanin determination methods; pH differential (AOAC method 2005.02) and HPLC analysis (two different mobile phase systems), and to aid in the understanding of reported anthocyanin values in literature based upon the method used.

2. Materials and methods

2.1. Samples

Juice samples ($n = 7$), that were not blends of different fruits, and that represented an array of anthocyanin containing products available at a local market (Nampa, ID) were purchased in 2006 (Table 1). The samples were brought to the laboratory, aliquoted into vials, immediately flushed with nitrogen gas (NorLab, Norco Inc., Nampa, ID), and then stored at −80 °C until analysis. Two purified anthocyanin standards were purchased from Polyphenols Laboratories AS (Sandnes, Norway). All samples and standards analyzed in this study are listed in Table 1.

2.2. Chemicals and reagents

All chemicals and reagents used in this study were obtained from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise. All solvents and chemicals for this investigation were analytical and HPLC grade.

2.3. Total monomeric anthocyanins (ACY) determination

ACY were determined using the pH differential method (Lee et al., 2005). Absorbance was measured at 520 and 700 nm. ACY were expressed as cyanidin-3-glucoside (cyd-glu, molar extinction coefficient of 26,900 L cm^{-1} mol^{-1} and molecular weight of 449.2 g mol^{-1}), and malvidin-3-glucoside (mvd-glu, molar extinction coefficient of 28,000 L cm^{-1} mol^{-1} and molecular weight of 463.3 g mol^{-1}). The units for ACY were mg/100 ml of sample or 100 g of standard. A SpectraMax M2 microplate reader (Molecular Devices Corp., Sunnyvale, CA) was used, as this instrument is capable of utilizing either microplates or cuvettes. Measurements of ACY on samples were replicated three times. This method is described in detail by Lee et al. (2005).

Table 1
Juice samples and standards analyzed in this study

Material	Source
Cranberry juice cocktail	Local grocery store (Nampa, ID)
Pomegranate juice	Local grocery store (Nampa, ID)
Blueberry juice	Local grocery store (Nampa, ID)
Tart cherry juice	Local grocery store (Nampa, ID)
Grape juice	Local grocery store (Nampa, ID)
Black cherry juice	Local grocery store (Nampa, ID)
Concord grape juice	Local grocery store (Nampa, ID)
Cyanidin-3-glucoside (cyd-glu)	Polyphenols Laboratories AS (Sandes, Norway)
Malvidin-3-glucoside (mvd-glu)	Polyphenols Laboratories AS (Sandes, Norway)

The materials represented a range of different anthocyanin containing products.

2.4. HPLC/DAD (HPLC coupled with a diode array detector) for anthocyanin analysis

2.4.1. System 1

A HP1100 system equipped with a DAD (Agilent Technologies Inc., Palo Alto, CA) was used to analyze the samples. A prodigy 5 μm ODS (3) 100 Å (250.0 × 4.6 mm) column fitted with 4.0 × 3.0 mm i.d. guard column, both from Phenomenex (Torrance, CA) were used. Absorbance spectra were collected for all peaks. The solvent flow rate was 1.0 ml/min. Injection volume was 25 μL. Solvent A was 100% acetonitrile. Solvent B consisted of 10% (v/v) acetic acid and 1% (v/v) phosphoric acid in water. The linear gradient was as reported earlier (Lee et al., 2005), with simultaneous detection at 280 and 520 nm. A HPLC solvent system of low concentration phosphoric acid is more commonly used than a formic acid one, but phosphate is an interference compound when utilizing a mass spectrometer detector (Wang, Race, & Shrikhande, 2003). Quantification was done by the external standard method with both cyd-glu and mvd-glu.

2.4.2. System 2

A routinely used gradient elution, coupled to the ion trap MS was the second solvent system. The HPLC condition was as previously described in detail by former publication (Lee & Finn, 2007), where solvent A was acetic acid:trifluoroacetic acid:acetonitrile:water = 10%:0.2%:5.0%:84.8% (v/v/v/v), and solvent B was acetonitrile. A Synergi Hydro-RP 80 Å (150.0 × 2.0 mm, 4 μm) column fitted with a guard column (Phenomenex Inc., Torrance, CA) was used. Anthocyanins were quantified as cyd-glu and mvd-glu. Injection volume was 25 μL.

2.5. Statistical analysis

Correlation was determined on the anthocyanin values obtained by pH differential method and HPLC. *t*-Test calculation was done to compare the variables analyzed by the different methods ($\alpha = 0.05$). Statistica for Windows version 7.1 was used (StatSoft Inc., Tulsa, OK).

3. Results and discussion

Table 2 summarizes all total anthocyanin values obtained from the methods and standards used. All measurements of total anthocyanins, regardless of the analytical method, were greater when expressed as mvd-glu, than when expressed as cyd-glu. Microplate read values were slightly elevated when compared to cuvette read values except for either grape juice reading (where the opposite was observed), and all values were not significantly different from one another (*t*-test, $p > 0.05$). Total anthocyanins expressed as mvd-glu were 5.2% larger than cyd-glu expressed values, which was due to the difference to which extinction coefficient and molecular weight values affected calculations. This finding emphasizes the importance of indicating which individual anthocyanin, and its molar extinction coefficient and molecular weight values

Table 2
Total anthocyanin content (mg of anthocyanins/100 ml) of all samples, from both HPLC systems and the pH differential method (with cuvettes and microplates)

	Method used							
	pH differential conducted with cuvettes		pH differential conducted with microplates		HPLC system 1		HPLC system 2	
	Expressed as cyd-glu	Expressed as mvd-glu	Expressed as cyd-glu	Expressed as mvd-glu	Expressed as cyd-glu	Expressed as mvd-glu	Expressed as cyd-glu	Expressed as mvd-glu
Codes	pHcc	pHcm	pHmc	pHmm	S1c	S1m	S2c	S2m
Cranberry juice cocktail	1.31 (0.01)	1.38 (0.01)	1.33 (0.01)	1.40 (0.01)	1.08 (0.11)	1.86 (0.19)	0.92 (0.04)	1.65 (0.07)
Pomegranate juice	5.92 (0.09)	6.25 (0.09)	5.94 (0.03)	6.27 (0.03)	25.19 (2.69)	43.54 (4.65)	23.63 (0.23)	42.60 (0.42)
Blueberry juice	3.56 (0.03)	3.76 (0.04)	3.82 (0.02)	4.04 (0.02)	14.09 (1.37)	24.35 (2.37)	9.97 (0.21)	17.98 (0.37)
Tart cherry juice	2.09 (0.02)	2.20 (0.02)	2.11 (0.04)	2.23 (0.04)	4.14 (0.21)	7.16 (0.37)	4.41 (0.07)	7.96 (0.13)
Grape juice	1.64 (0.04)	1.73 (0.04)	1.60 (0.02)	1.69 (0.02)	4.67 (0.40)	8.08 (0.69)	4.55 (0.06)	8.20 (0.10)
Black cherry juice	4.39 (0.03)	4.64 (0.03)	4.74 (0.02)	5.01 (0.02)	12.54 (0.65)	21.67 (1.12)	11.79 (0.15)	21.25 (0.27)
Concord grape juice	3.12 (0.06)	3.29 (0.07)	3.05 (0.03)	3.22 (0.03)	9.23 (0.56)	15.95 (0.97)	9.18 (0.11)	16.55 (0.19)

Cyd-glu = cyanidin-3-glucoside; mvd-glu = malvidin-3-glucoside; and values in parenthesis are standard errors.

were used for calculating total anthocyanins by the pH differential method.

Overall, values from the HPLC were higher than anthocyanin content obtained by the pH differential method (Table 2), except for cranberry juice cocktail expressed as cyd-glu. This discrepancy observed with cranberry juice cocktail (cyd-glu equivalent results) may be due to the individual anthocyanins within this sample compared to other juice samples, or due to variation that occurred from analyzing a sample with low anthocyanin content. This trend has been observed before (Dossett, 2007; Lee & Finn, 2007), where samples were analyzed using identical methods and conditions used in this study (pH differential method and HPLC by system 2). Elderberry samples (110 measurements) examined by HPLC were 2.0–2.3 times greater than the values from pH differential method (Lee & Finn, 2007), which was also the tendency seen in black raspberry samples (data from 205 measurements) (Dossett, 2007). Both studies reported anthocyanins as cyd-glu and used the same microplate reader conditions. The samples examined in those studies primarily contained cyanidin derived anthocyanins, so this study was conducted to examine the method performance for an array of anthocyanin containing samples. The trends of all the values were the same (samples with high values from HPLC were also high from the pH differential method) and these values were highly correlated ($R \geqslant 0.925, p \leqslant 0.05$) to one another despite the difference in individual anthocyanins found in the analyte tested (Table 3).

Values obtained from the two HPLC systems had a correlation (R) value greater than 0.926 ($p \leqslant 0.05$) quantified by both standards (Table 3). Despite the difference in column and mobile phase used, as long as the values were expressed as the same external standard, the anthocyanin levels found in the samples were similar (difference between the two systems were of 0.05–4.12 mg cyd-glu equivalent/100 ml and 0.12– 6.37 mg mvd-glu equivalent/100 ml in anthocyanin levels). Samples expressed as mvd-glu were 42% higher than when expressed as cyd-glu for both HPLC systems. Values expressed as mvd-glu from systems 1 and 2 were not significantly different from one another (*t*-test, $p > 0.05$), which was also the case for cyd-glu expressed values.

Table 3
Correlations (*R*) between the anthocyanins obtained from the different methods and standards

Codes	pHcc	pHcm	pHmc	pHmm	S1c	S1m	S2c	S2m
pHcc	–	–	–	–	–	–	–	–
pHcm	0.999	–	–	–	–	–	–	–
pHmc	0.978	0.978	–	–	–	–	–	–
pHmm	0.978	0.978	0.999	–	–	–	–	–
S1c	0.938	0.925	0.925	0.925	–	–	–	–
S1m	0.938	0.925	0.925	0.925	0.999	–	–	–
S2c	0.948	0.948	0.948	0.948	0.926	0.926	–	–
S2m	0.948	0.948	0.948	0.948	0.926	0.926	0.999	–

Method and standard used for the codes are in Table 2. All *R* values indicated a strong positive correlation.

Comparisons between the cuvette readings to 96-well microplate readings were made for the pH differential method (Table 3), because, while the AOAC method specifies using a 1-cm cuvette for the absorbance readings, microplate readers are a convenient alternative to spectrophotometers, especially when running a large number of samples. All samples were diluted with the appropriate buffers in cuvettes or microplates and the absorbances were taken with 1-cm pathlength cuvettes and with 96-well microplates. The two values obtained had a correlation value of 0.985 (for both cyd-glu and mvd-glu expressed values, respectively; Spearman rank correlation, $p \leqslant 0.05$) when the two total anthocyanin values of the purchased standards were included in the correlation calculation. The correlation values of total anthocyanins without the two standards were greater than 0.978 ($p \leqslant 0.05$), so *R* slightly decreased. From these results, a microplate reader is an acceptable alternative to reduce preparation time, reagent usage, and chemical waste compared to individual cuvettes when processing a large number of samples.

The elution order of the individual anthocyanins was similar for both mobile phase and column systems used for the HPLC (Fig. 1). Fig. 1 shows the HPLC profiles of cranberry juice cocktail (A and B) and blueberry juice (C and D). Peaks eluted out earlier in system 2 than they did in system 1. While the response factor was different (system 2 produced a larger peak area) for each system, this was not a problem because the standard used to calculate these values was run in the same system as the sample. Peak assignments for the different juices were not done in this study. However, the identities of the anthocyanins in these juices are well established in the literature. Cranberry juice cocktail contains cyanidin and peonidin derivatives, cherry juice contains cyanidin derived pigments, concord grape juice contains cyanidin, delphinidin, peonidin, petunidin, and malvidin derived anthocyanins, and pomegranate juice contains cyanidin, delphinidin, and pelargonidin derived anthocyanins (Gonçalves et al., 2004; Hong & Wrolstad, 1990; Pérez-Vicente, Gil-Izquierdo, & Garcia-Vuguera, 2002; Wang et al., 2003; Wu & Prior, 2005; Wu et al., 2006). Individual anthocyanidin derived pigments will respond differently to the conditions used to analyzed them, while the degree of acylation, degree of glycosylation, various acid and glycoside moieties, absorbance not measured at individual peak's λ_{max}, and solvent effect from changing gradient will further the variation of the response of individual anthocyanins to the each method and conditions used in the analysis of choice

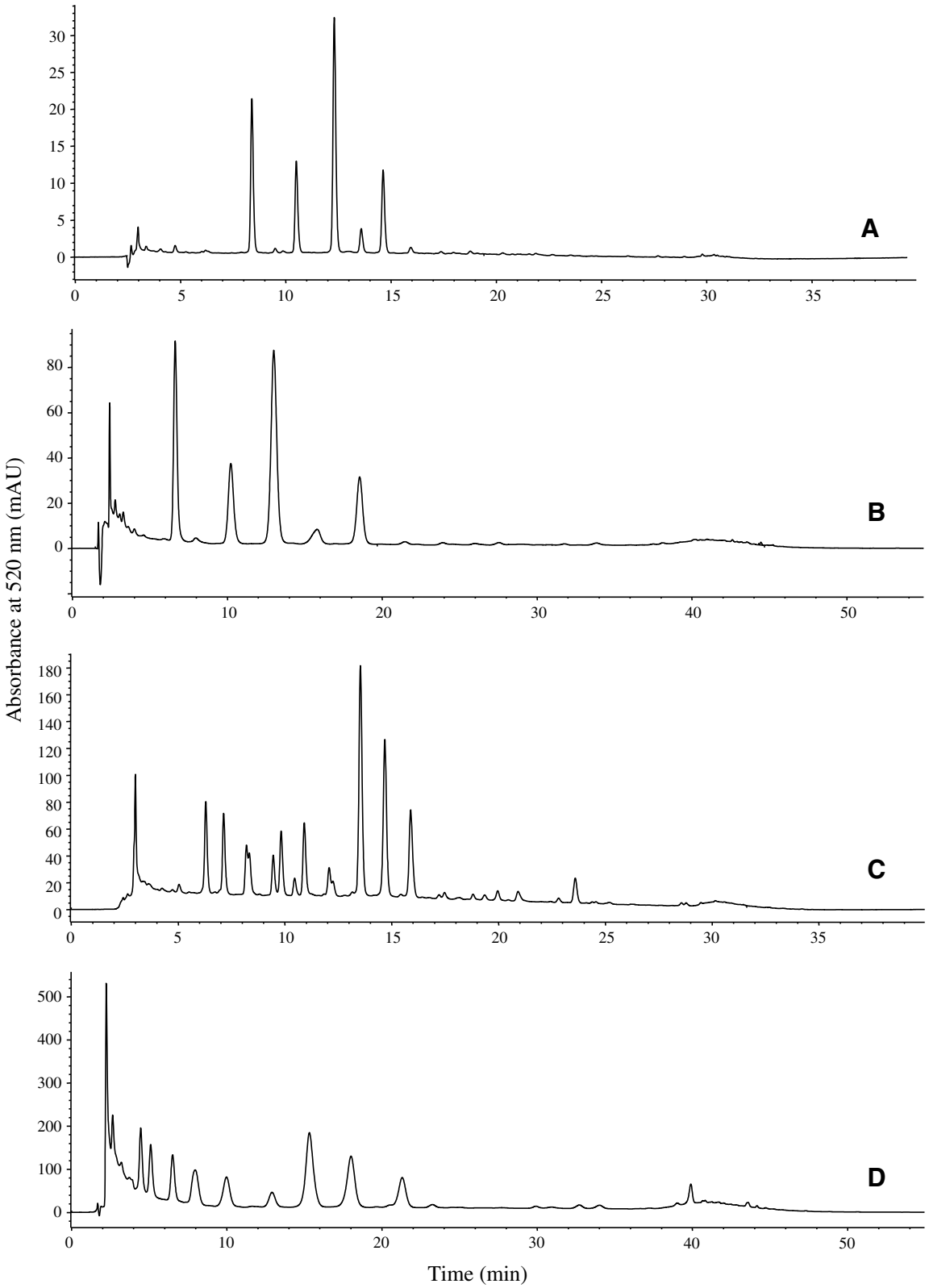

Fig. 1. Chromatograms of cranberry juice cocktail (A and B) and blueberry juice (C and D) run with the two mobile phases and columns (system 1: A and C & system 2: B and D).

(Durst & Wrolstad, 2005; Francis, 1982; Giusti & Wrolstad, 2005; Hong & Wrolstad, 1990).

Past studies conducted in this laboratory that used the same methods and conditions were also compared (Fig. 2) with values obtained from this study. Anthocyanin contents in Fig. 2 were from pH differential values using microplates, and HPLC values from the condition of system 2. Values from this study, elderberry samples (Lee & Finn, 2007), black raspberry samples (Dossett, 2007), along with Cabernet Sauvignon (*Vitis vinifera* L.) grape samples were used to calculate a correlation value (R) of 0.931 ($n = 517$, $p \leqslant 0.05$) for

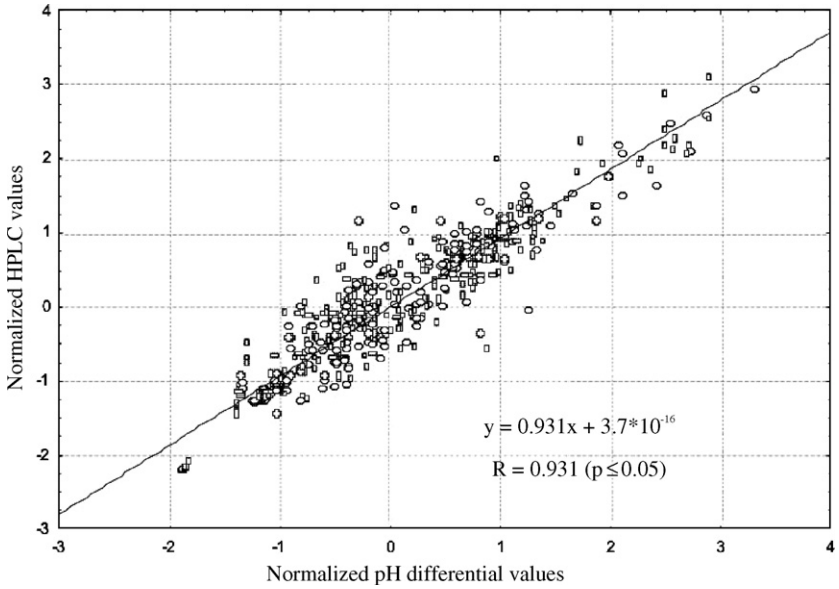

Fig. 2. Anthocyanin contents obtained from four studies. Juice, elderberry, black raspberry, and Cabernet Sauvignon grape samples (values obtained from this study, Lee and Finn, 2007; Dossett, 2007; unpublished data, $n = 517$) were used. All values were normalized. R value indicates correlation.

Fig. 2. This again illustrates the high correlation between the two methods for measuring total anthocyanins. It should be noted, however, that values for Cabernet Sauvignon grape samples had similar pH differential and HPLC determined values, in contrast to the greater HPLC values found in this paper. The only difference for these two analyses was the source of the mvd-glu external standard. But despite that difference, there was a high correlation between the two methods, which might be due to the impurity present in the standard (Lee et al., 2005).

4. Conclusion

In conclusion, values acquired from all methods (pH differential and the two different HPLC solvents system) were highly correlated with one another for quantifying anthocyanins. HPLC is an invaluable tool for identifying and quantifying different individual anthocyanin in a sample. But, the pH differential method is a simple, rapid, and economical means for determining the amount of anthocyanins in a sample, and this method has been verified by AOAC's strict validation guidelines. The pH differential method is a good alternative for laboratories that do not have access to a HPLC. The use of a microplate reader in place of a spectrophotometer can greatly increase the throughput of samples processed by the pH differential method. This study demonstrates the importance of reporting methods and the standards used when discussing total anthocyanins. Comparison of total anthocyanin values should be conducted with caution since values reported in the literature were dependent upon the method and standard used. Until a certified anthocyanin standard is available, this study should aid in the ability of different laboratories to compare their values with each other.

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