Thin Layer Chromatography. *Nippon Shokuhin Kogyo Gakkaishi* **1979**, *26*, 204–208.
Ribereau-Gayon, P. The Anthocyanins of Grapes and Wine. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press, New York, 1982.
Riboh, M. Natural Colors: What Works...What Doesn't. *Food Eng.* **1977**, *49* (5), 66–72.
Sapers, G. M.; Taffer, I.; Ross, L. R. Functional Properties of a Food Colorant Prepared from Red Cabbage. *J. Food Sci.* **1981**, *46* (1), 105–109.
Sapers, G. M.; Burgher, A. M.; Phillips, J. G.; Jones, S. B.; Stone, E. G. Color and Composition of Highbush Blueberry Cultivars. *J. Am. Soc. Hortic. Sci.* **1984**, *109*, 105–111.
Sapers, G. M.; Hicks, K. B.; Burgher, A. M.; Hargrave, D. L.; Sondey, S. M.; Bilyk, A. Anthocyanin Patterns in Ripening Thornless Blackberries. *J. Am. Soc. Hortic. Sci.* **1986**, *111*, 945–950.
Shewfelt, R. L.; Ahmed, E. M. Anthocyanin Extracted From Red Cabbage Shows Promise As Coloring For Dry Beverage Mixes. *Food Prod. Dev.* **1977**, *11*, 52–62.
Shewfelt, R. L.; Ahmed, E. M. Enhancement of Powdered Soft Drink Mixes with Anthocyanin Extracts. *J. Food Sci.* **1978**, *43*, 435–438.
Shrikhande, A. J.; Francis, F. J. Anthocyanin Pigments of Sour Cherries. *J. Food Sci.* **1973**, *38*, 649–651.
Skrede, G. Color Quality of Blackcurrant Syrups During Storage Evaluated by Hunter L′, a′, b′ Values. *J. Food Sci.* **1985**, *50*, 514–525.
Skrede, G.; Naes, T.; Martens, M. Visual Color Deterioration in Blackcurrant Syrup Predicted by Different Instrumental Variables. *J. Food Sci.* **1983**, *48*, 1745–1749.
Spanos, G.; Wrolstad, R. E. Anthocyanin Pigment, Non-Volatile Acid and Sugar Composition of Red Raspberry Juice. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 1036–1046.
Suomalainen, H.; Keranen, A. J. A. The First Anthocyanins Appearing During the Ripening of Blueberries. *Nature* **1961**, *191*, 498–499.
Timberlake, C. F.; Bridle, P. Distribution of Anthocyanins in Food Plants. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press: New York, 1982.
Torre, L. C.; Barritt, B. H. Quantitative Evaluation of *Rubus* Fruit Anthocyanin Pigments. *J. Food Sci.* **1977**, *42*, 488–490.
Wallin, B. K.; Smith, B. J. Grape Anthocyanins As Food Colourings: Sources Compared. *Int. Flavor Food Addit.* **1977**, *8* (3), 102–104.
Wrolstad, R. E. Color and Pigment Analyses in Fruit Products. Oregon State University Agricultural Experiment Station Bulletin No. 624; Oregon State University: Corvallis, 1976.
Wrolstad, R. E.; Cornwell, C. J.; Culbertson, J. D.; Reyes, F. G. R. Establishing Criteria for Determining the Authenticity of Fruit Juice Concentrates. In *Quality of Selected Fruits and Vegetables of North America*; Teranishi, R., Barrera-Benitez, H., Eds.; ACS Symposium Series 170; American Chemical Society: Washington, DC, 1981.
Wulf, L. W.; Nagel, C. W. High-Pressure Liquid Chromatographic Separation of Anthocyanins of *Vitis vinifera*. *Am. J. Enol. Vitic.* **1978**, *29*, 42–49.

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Use of HPLC Separation/Photodiode Array Detection for Characterization of Anthocyanins†

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A systematic procedure for separation and characterization of anthocyanins is described. Separation of pigments was achieved by high-performance liquid chromatography (HPLC) on a polymer reversed-phase column. Methods for preparation of an anthocyanin isolate free of other interfering phenolics were developed. Photodiode array detection was employed to determine the UV–visible spectral characteristics of the pigments. Derivatives of delphinidin (delphinidin, petunidin, malvidin) can be distinguished from derivatives of cyanidin (cyanidin, peonidin), which in turn can be distinguished from pelargonidin derivatives on the basis of their different UV–visible spectra. Acylation with cinnamic acids and differentiation between 3- and 3,5-glycosidation can also be determined from the UV–visible spectrum. Auxiliary sample preparation techniques useful for pigment characterization included alkaline hydrolysis of the anthocyanins for determination of acylation. Anthocyanins not containing an o-diphenolic system can be enriched on a C_{18} reversed-phase cartridge by elution with alkaline borate buffer. With a combination of these techniques, peak assignments for the anthocyanins from sources whose anthocyanin composition is known can be readily made.

The anthocyanins are the natural pigments responsible for the red, blue, and purple colors of many plants. There are many different anthocyanins found in nature. The individual anthocyanin composition for any given plant is distinctive, and the analysis of anthocyanin and/or other flavonoids has therefore been very useful in distinguishing between species. This is especially true if there are qualitative differences (Stewart et al., 1979). With more closely related plants, such as those differing

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only at the cultivar level, the differences in the chemical profiles are mostly quantitative (Asen, 1982).

Classically, anthocyanins have been separated by a number of techniques including paper, thin-layer, and column chromatographic methods. Of these methods, paper chromatography has been the most widely used method for separation of the individual anthocyanins (Hrazdina, 1979). The mobilities (R_f values) of most of the naturally occurring anthocyanins in a number of different solvent systems have been reported (Harborne, 1967). Unfortunately, paper chromatographic methods are time-consuming and quantitation is difficult. A number of workers have reported high-performance liquid chromatographic (HPLC) methods for separation of both the anthocyanins (Wulf and Nagel, 1978; Casteele et al., 1983; Strack et al., 1980) and their aglycons (Wilkinson et al., 1977; Akavia and Strack, 1980). The advantage of HPLC is that it is fast, sensitive, and quantitative. One of the problems with the use of HPLC for analysis of anthocyanins is that absolute peak retention times can vary from worker to worker even under similar analytical conditions. This is made more complex by the fact that there is a general lack of availability of pure anthocyanin standards. The combination of a lack of standards and the problems with specification of absolute peak retention times are two factors contributing to the difficulty of using HPLC for analysis of anthocyanins. There are, however, a number of commonly available materials for which the anthocyanins have been thoroughly characterized. These materials can often be used as references.

Recently, the availability of photodiode array detection has allowed the chromatographer to determine the spectra of compounds as they elute from the HPLC column. Andersen (1985, 1987) has characterized a number of anthocyanins using this technique. This investigation describes a systematic approach for use of HPLC for the separation of anthocyanins and the use of on-line photodiode array detection for characterization of the spectral properties of the anthocyanins. The analysis of the anthocyanins in cranberry (*Vaccinium macrocarpon*), roselle (*Hibuscus sabdariffa*), and strawberry (*Fragaria anannassa*) will be described.

EXPERIMENTAL SECTION

Samples. Single-strength cranberry juice pressed from frozen cranberries was available from another study (Durst and Wrolstad, 1988). Dried roselle was obtained from General Foods Inc. (New York). Strawberry and blueberry juice concentrates were supplied by Kerr Concentrates Inc. (Salem, OR). Commercial technical-grade Malvin was obtained from the Aldrich Chemical Co. (Milwaukee, WI). Bilberry powder was supplied by Spreda (Burgdoff, Switzerland). Red cabbage extract was supplied by G. Shimizu and Co., Ltd. (Osaka, Japan). Tamarillo fruit was purchased from a local supermarket.

Extraction of Pigments. For dried samples 10 g of dried material was crushed and extracted with 100 mL of 1% HCl in methanol (1 mL of concentrated HCl in 99 mL of methanol) overnight at 0 °C. The slurry was filtered, and the solids were washed with an additional 100 mL of 1% HCl in methanol. The methanol extracts were combined and concentrated to ca. 10 mL in a rotary evaporator (30 °C). An aqueous pigment extract was prepared by evaporating the methanol extract to dryness and redissolving in 0.01% HCl (0.1 mL of concentrated HCl in 1 L of distilled water). Juice samples were diluted ca. 10-fold and used as is. Sufficient sample was used such that the final aqueous pigment extract had an absorbance of ca. 4 (1-cm path length) at 520 nm.

Paper Chromatography. Descending paper chromatography was performed with the methods described by Harborne (1967) and Du and Francis (1973). Cleaned-up samples (see below) were streaked on Whatman 3MM and chromatographed in BAW (1-butanol/acetic acid/water, 4/1/5, top layer), 1% HCl (water/concentrated HCl, 97/3), or AHW (acetic acid/HCl/water, 15/3/82). Bands were cut, eluted with methanol containing 0.01% concentrated HCl, and concentrated in a rotary evaporator. When necessary, pigments were rechromatographed in one of the other different solvent systems.

Sample Preparation for HPLC Analysis of Anthocyanidins. The hydrolysis procedure previously described by Hong and Wrolstad (1986) was used.

Sample Preparation for HPLC Analysis of Anthocyanins. Two different sample preparation methods were used. For quantitation (cleanup procedure I), an aqueous solution of the anthocyanins was adsorbed onto an activated C_{18} Sep-Pak (Waters Associates, Milford, MA). The cartridge was washed with 0.01% HCl, and the pigments were eluted with 0.01% HCl in methanol. The methanolic extract was then evaporated to ca. 1 mL. The isolate was redissolved in 4% phosphoric acid (concentrated phosphoric acid/water, 4/96, v/v) and filtered through a 0.45-μm Type HA Millipore filter (Millipore Corp., Bedford, MA).

For HPLC separation and photodiode array analysis (cleanup procedure II), an aqueous solution of the pigments was adsorbed onto hydrated polyvinylpolypyrrolidone (PVPP; GAF Corp., New York, NY), washed with 0.01% HCl until the eluate was almost colorless, and eluted with 0.01% HCl in methanol. The eluate was concentrated in a rotary evaporator to dryness and redissolved in ca. 2 mL of 1% HCl in methanol, and the pigments were precipitated with ca. 20 mL of cold (0 °C) diethyl ether. The pigments were filtered and redissolved in 1% HCl in methanol. The precipitation procedure was repeated. The final methanolic pigment extract was evaporated (in vacuo) almost to dryness, redissolved with 4% phosphoric acid, and filtered for HPLC.

Selective Elution of Anthocyanins with Alkaline Borate Buffer. Anthocyanins were adsorbed onto an activated C_{18} Sep-Pak. Alkaline borate buffer (0.1 N sodium borate, ca. pH 9) was passed through the column until the eluant was colorless (ca. 50 mL). The anthocyanins were reconverted into their red oxonium salt form by passing ca. 5 mL of 2 N HCl through the column. The procedure with borate buffer and HCl was repeated one additional time. The anthocyanins remaining on the cartridge were subsequently eluted with 0.01% HCl in methanol and concentrated to ca. 1 mL in a rotary evaporator. For HPLC, the pigments were redissolved in 4% phosphoric acid and filtered for HPLC.

Alkaline Hydrolysis of Anthocyanins. Approximately 5 mg of cleaned-up pigment was added to 10 mL of 10% aqueous KOH in a screw-cap test tube. The tube was flushed with nitrogen and capped. The pigments were hydrolyzed for 8 min at room temperature in the dark. The pigments were reconverted to their red oxonium salt form by addition of 2 N HCl. The pigments were adsorbed onto a C_{18} Sep-Pak, washed with excess 0.01% HCl, eluted with 0.01% HCl in methanol, and concentrated to ca. 1 mL. For HPLC, the pigments were redissolved in 4% phosphoric acid and filtered for HPLC.

HPLC Separation of Anthocyanidins and Anthocyanins. *Liquid Chromatograph.* Perkin-Elmer Series 400 equipped with either a Varian UV-50 variable-wavelength detector, Perkin-Elmer LCI-100 integrator, or Hewlett-Packard 1040A photodiode array detector, Hewlett-Packard 9000 computer.

Conditions for Anthocyanidin Analyses. Column: Supelcosil ODS (5 mm × 25 cm), 5-μm particle size with Bio-Rad ODS guard column. Solvent: (A) 15% acetic acid (concentrated acetic acid/water, 15/85, v/v); (B) acetonitrile. Conditions: isocratic elution at 1.5 mL/min with 85% A and 15% B at room temperature; detection at 520 nM; injection volume 25 μL.

Conditions for Anthocyanin Analyses. Column: Polymer Labs PLRP-S (4.6 mm × 25 cm), 5-μm particle size with Polymer Labs guard column. Solvent: (A) 4% phosphoric acid (concentrated phosphoric acid/water, 4/96, v/v); (B) 100% acetonitrile. Program: isocratic elution with 6% B from 0 to 10 min, linear gradient to 20% B from 10 to 55 min, isocratic elution at 20% B from 50 to 65 min; flow rate 1.0 mL/min; room temperature operation; primary detection at 520 and 260 nm (20-nM bandwidth); injection volume 20 μL. All spectral data were

Table I. Relative Percentages of Anthocyanidins Based on Total Peak Area

sample	delphinidin	cyanidin	petunidin	pelargonidin	peonidin	malvidin
cranberry	NF[a]	46	NF	NF	54	NF
roselle	59	41	NF	NF	NF	NF
strawberry	NF	24	NF	75	NF	NF

[a] NF = not found.

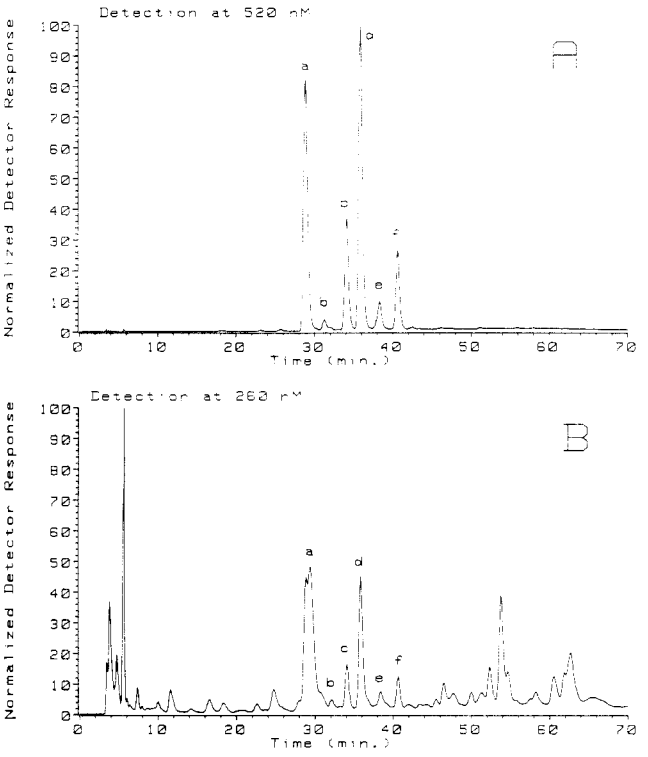

Figure 1. HPLC chromatogram of cranberry anthocyanins, minimal sample cleanup (cleanup procedure I): A, detection at 520 nm; B, detection at 260 nm. Peak identification (relative peak areas calculated with detection at 520 nM in parentheses): a, cyanidin 3-galactoside (33%); b, cyanidin 3-glucoside (2%); c, cyanidin 3-arabinoside (14%); d, peonidin 3-galactoside (38%); e, peonidin 3-glucoside (4%); f, peonidin 3-arabinoside (9%). Proportion of cyanidin glycosides: 49%. Proportion of peonidin glycosides: 51%.

smoothed with the HP system smoothing function (filter length 15).

RESULTS AND DISCUSSION

HPLC Separation of Anthocyanidins. Analysis of anthocyanidins is a good initial method in that it yields information on the nature of the aglycons. Since there are only six common anthocyanidins, identification is easily achieved. Although pure standards are difficult to obtain, suitable natural sources are readily available (Hong and Wrolstad, 1986). Table I lists the results of anthocyanidin analysis of cranberry, roselle, and strawberry. Cranberry was found to contain only cyanidin and peonidin. These results are in agreement to those previously reported (Hong and Wrolstad, 1986). Roselle was found to contain only delphinidin and cyanidin, in agreement with the results of Wilkinson et al. (1977). Strawberry was found to contain pelargonidin as the major pigment with lesser amounts of cyanidin.

HPLC Separation of Anthocyanins. The anthocyanin composition of cranberry is well characterized, both by classical paper chromatographic methods and by more recent HPLC methods. Shown in Figure 1A is an HPLC chromatogram of cranberry anthocyanins with their relative peak areas. Peak assignments were made by comparison with results reported by Durst and Wrolstad (1987), Sapers and Hargrave (1987), Hicks et al. (1985), and Hale et al. (1986). Retention is based on the relative hydrophobicity of the molecule. With the anthocyanins, the degree of glycosidation and the nature of the sugar moiety are important factors (Strack et al., 1980; Casteele et al., 1983). From these results and those of previous workers, for a given anthocyanidin, the analogous glycosides will elute in the following order (in terms of chromatographic retention in RP HPLC systems): galactoside is faster than the glucoside, which is in turn faster than the arabinoside. The results from the anthocyanidin analysis of cranberry showing the presence of only cyanidin and peonidin glycosides offer additional support for the results of the anthocyanin analysis.

Previous workers have achieved good separations using silica-based ODS HPLC columns with binary mobile phases of acetic acid/water with either acetonitrile or methanol. Acetic acid plays an important role in the separation both by acting as an organic modifier and by lowering the pH of the mobile phase. A low pH is essential to keep the anthocyanins in the red flavylium cation form, resulting in increased peak sharpness (Hale et al., 1986). Early workers did not employ mobile phases below pH 2.0 due to the possibility of damage to silica-based columns (Hale et al., 1986). There are reports, however, that there is no loss in column performance of silica columns when used with mobile phases below pH 2 (Spanos and Wrolstad, 1987; Wulf and Nagel, 1978; Bronnum-Hansen and Hansen, 1983). In this study, a polymer reversed-phase column was employed so that mobile phases at very low pH could be used without fear of column degradation. A phosphoric acid/water and acetonitrile solvent system was selected in preference to acetic acid/water/methanol as transparency in the UV was desirable for photodiode array detection.

Two different sample preparation methods were employed in this study. For quantitation of the relative amounts of the anthocyanins, samples were subjected to minimal cleanup (cleanup procedure I) to minimize changes in pigment composition. When spectra were to be taken, samples were subjected to a more vigorous cleanup procedure (cleanup procedure II) to remove interfering phenolics.

Shown in Figure 1A is a chromatogram of cranberry anthocyanins in which the sample was subjected to minimal cleanup (cleanup procedure I). When monitored at 520 nm where only the anthocyanins are detected, the chromatogram appears quite "clean". When monitored at 260 nm, however (Figure 1B), it becomes apparent that there are other compounds eluting near or coeluting with the anthocyanins. Figure 2A shows a chromatogram of cranberry anthocyanins when cleaned up with the more vigorous cleanup procedure (cleanup procedure II). It can be seen that this cleanup procedure is effective in reducing the amount of interference by UV-absorbing compounds (Figure 2B). Unfortunately, this cleanup procedure also changes the relative proportions of the anthocyanins (compare Figure 1A with Figure 2A). This demonstrates the necessity of using a minimal cleanup procedure (cleanup procedure I) when quantitation of the relative peak areas is desired.

Shown in Figure 3 is a chromatogram of roselle anthocyanins with their relative peak areas. The delphinidin glycosides eluted before the cyanidin glycosides, and the diglycosides eluted before the corresponding monoglycosides. Identities of the roselle anthocyanins were estab-

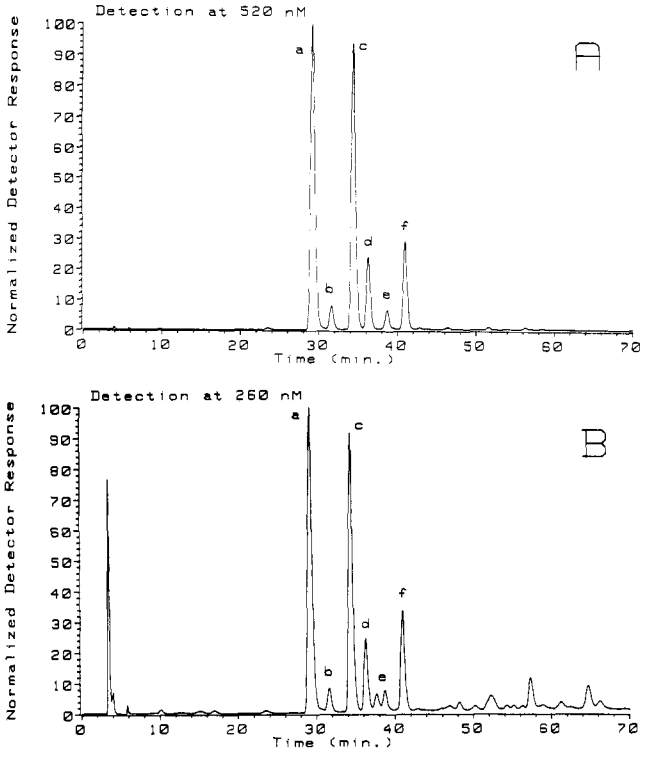

Figure 2. HPLC chromatogram of cranberry anthocyanins subjected to PVPP adsorption and precipitation by diethyl ether (cleanup procedure II): A, detection at 520 nm; B, detection at 260 nm. Peak identification: a, cyanidin 3-galactoside; b, cyanidin 3-glucoside; c, cyanidin 3-arabinoside; d, peonidin 3-galactoside; e, peonidin 3-glucoside; f, peonidin 3-arabinoside.

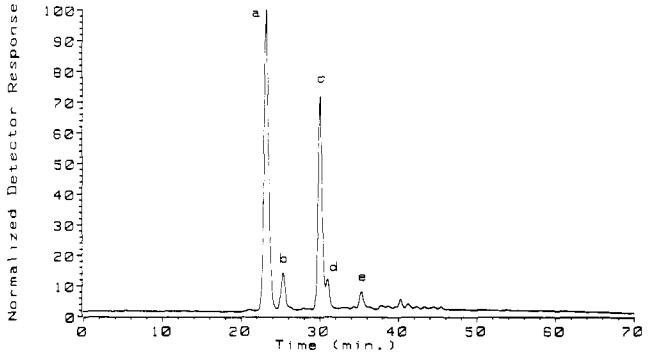

Figure 3. HPLC chromatogram of roselle anthocyanins with detection at 520 nM. Peak identification (relative peak areas calculated with detection at 520 nm in parentheses): a, delphinidin 3-sambubioside (56%); b, delphinidin 3-glucoside (4%); c, cyanidin 3-sambubioside (33%); d, cyanidin 3-glucoside (3%); e, unknown (4%). Proportion of delphinidin glycosides: 60%. Proportion of cyanidin glycosides 36%.

lished by separation with paper chromatography and identification by comparison with the R_f values reported by Du and Francis (1973). A late-eluting peak (peak e) remains unidentified.

Comparison of the relative percentages of the anthocyanidins (Table I) to the sum of relative percentages of the corresponding anthocyanins (Figure 1A for cranberry; Figure 3 for roselle) in each of the samples shows that both methods give comparable results.

Spectral Characterization of Anthocyanins. Important structural properties of the anthocyanins can be obtained from spectral data (Harborne, 1967) including the nature of the aglycon (anthocyanidin), the position of attachment of the sugar molecule (Harborne, 1958), and information regarding acylation by aromatic organic acids. Typically the spectra of anthocyanins as well as

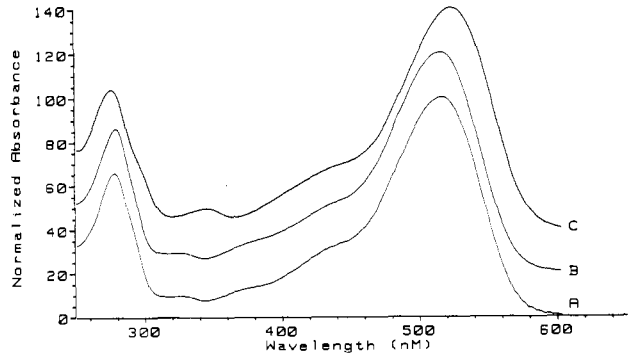

Figure 4. UV–visible spectra of cyanidin 3-galactoside (A), peonidin 3-galactoside (B), and delphinidin 3-sambubioside (C) determined by photodiode array detection.

other flavonoids are measured in methanol containing 0.01% HCl. Most of the reports in the literature employ this solvent. In measurement of spectra directly from the HPLC column, the choice of solvent to be used is limited to the mobile phase of the HPLC system. Since the spectral characteristics of the anthocyanins are dependent both on pH and on the nature of the solvent, direct comparison of spectral characteristics with those published in the literature may be inappropriate. Harborne (1958) reports that, in general, there is a 15-nm shift toward shorter wavelengths when water is substituted for methanol.

Shown in Figure 4 are the spectra of cyanidin 3-galactoside (from cranberry), delphinidin 3-sambubioside (from roselle), and peonidin 3-galactoside (from cranberry). Table II lists the visible λ_{max} and the $E_{440}/E_{\lambda_{max}}$ wavelength ratio of the anthocyanins of both cranberry and roselle as determined in this study along with values as reported in the literature. The wavelength maxima in the visible range is closely related to the hydroxylation pattern of the anthocyanin. In acidic methanol solutions, pelargonidin 3-glycosides exhibit a visible λ_{max} at about 505 nm, cyanidin and peonidin 3-glycosides have a visible λ_{max} at 520–526 nm, and delphinidin derivatives (delphinidin, malvidin, petunidin) show λ_{max} at 532–537 nm (Table II). The nature of the sugar substitution has no effect on spectra (Harborne, 1958). The visible wavelength maxima for the cyanidin and peonidin 3-glycosides from both cranberry and roselle (Table II) were similar, the values being lower than previously reported for the same pigments in acidified methanolic solvents. The two delphinidin 3-glycosides from roselle exhibited a higher wavelength maxima than for the cyanidin and peonidin 3-glycosides. These values are lower than those previously reported for the same pigments in acidified methanolic solvents. The HPLC/photodiode array results of Andersen (1985, 1987), also using a part aqueous, part organic HPLC solvent (formic acid/methanol/water), showed similar trends.

Information regarding the glycosidation substitution pattern of the anthocyanins can also be obtained from spectra. The 3-glycosides and 3,5-diglycosides have similar spectral maxima but show differences in the 400–460-nm region (Harborne, 1967). These differences have been traditionally expressed in terms of the $E_{440}/E_{\lambda_{max}}$ ratio, the 3-glycosides exhibiting ratios that are about twice as large as for the 3,5-diglycosides. The $E_{440}/E_{\lambda_{max}}$ values obtained for all of the 3-glycosides in both cranberry and roselle (Table II) are similar, the values ranging from 29 to 35%. To examine the spectral properties of a 3,5-diglycoside, the HPLC/photodiode array properties of commercial malvin (malvidin 3,5-diglucoside) were

Table II. Spectral Characteristics of Cranberry and Roselle Anthocyanins Determined by HPLC/Photodiode Array Detection along with Spectral Values for Selected Anthocyanins As Reported in the Literature

pigment	solvent[a]	vis λ_{max}, nm	E_{440}/E_{vis}, %	ref
Values Obtained in this Study				
cyanidin 3-galactoside	M	515	33	
cyanidin 3-glucoside	M	515	33	
cyanidin 3-arabinoside	M	515	33	
cyanidin 3-sambubioside	M	517	33	
peonidin 3-galactoside	M	515	33	
peonidin 3-glucoside	M	515	33	
peonidin 3-arabinoside	M	517	35	
delphinidin 3-sambubioside	M	523	30	
delphinidin 3-glucoside	M	520	29	
Literature Values				
Cyanidin and Peonidin Glycosides				
cyanidin 3-arabinoside	b	528	23	Zapsalis and Francis, 1965
	c	520	31	Andersen, 1985
cyanidin 3-galactoside	b	526	23	Zapsalis and Francis, 1965
	c	520	30	Andersen, 1985
cyanidin 3-glucoside	a	525	22	Harborne, 1958
	c	520	22	Andersen, 1985
cyanidin 3-sambubioside	a	528	24	Du and Francis, 1973
cyanidin 3-glucoside -galactoside, and -arabinoside	c	516	30–31	Andersen, 1987
peonidin 3-arabinoside	b	527	29	Zapsalis and Francis, 1965
peonidin 3-galactoside	b	524	20	Zapsalis and Francis, 1965
peonidin 3-glucoside	a	523	26	Harborne, 1958
	a	523	23	Sakellariades and Luh, 1974
peonidin 3-glucoside	c	518	30	Andersen, 1987
Delphinidin, Petunidin, and Malvidin Glycosides				
delphinidin 3-glucoside	a	535	18	Harborne, 1958
	a	541	22	Du and Francis, 1973
	b	528	25	Andersen, 1985
delphinidin 3-sambubioside	a	542	20	Du and Francis, 1973
delphinidin 3-arabinoside, -glucoside, and -galactoside	c	524	27	Andersen, 1987
petunidin 3-glucoside	a	535	18	Harborne, 1958
petunidin 3-galactoside, -glucoside, and -arabinoside	c	524–526	24–25	Andersen, 1987
malvidin 3-glucoside	a	535	18	Harborne, 1958
malvidin 3,5-diglucoside	a	533	12	Harborne, 1958
malvidin 3-galactoside, -glucoside, and -arabinoside	c	526	26–27	Andersen, 1987
Pelargonidin Glycosides				
pelargonidin 3-glucoside	a	506	38	Harborne, 1958

[a] Key: a = 0.01% HCl in MeOH; b = 0.1% HCl in MeOH; c = HPLC solvent, formic acid/water/methanol; M = mobile phase used in this study, phosphoric acid/water/acetonitrile.

determined. The visible wavelength maxima was found to be 523 nm, which is indicative of a delphinidin derivative. The $E_{440}/E_{\lambda_{max}}$ ratio of malvin was 16%, a value about half of that for the 3-glycosides. All of the $E_{440}/E_{\lambda_{max}}$ values are higher than those previously reported for anthocyanins in methanolic solvents. These results are in agreement with those of Andersen (1985, 1987). It appears that increasing solvent polarity influences the spectral characteristics of the anthocyanins by increasing the $E_{440}/E_{\lambda_{max}}$ ratio and decreasing the visible λ_{max}.

Information regarding the presence of acylation by hydroxylated aromatic organic acids can also be obtained from spectra. This is detected by the presence of a peak in the 310-nm range. The ratio of the absorbance at the acyl λ_{max} to the absorbance at the visible λ_{max} ($E_{\lambda_{max}}$(acyl)/$E_{\lambda_{max}}$(visible) ratio) is a measure of the molar ratio of the cinnamic acid to the anthocyanin. In acidified methanolic solutions, a $E_{\lambda_{max}}$(acyl)/$E_{\lambda_{max}}$(visible) ratio of 48–71% is indicative of a 1/1 molar ratio of the cinnamic acid to the anthocyanin while $E_{\lambda_{max}}$(acyl)/$E_{\lambda_{max}}$(visible) ratio of 83–107% is characteristic of a 2/1 molar ratio of the cinnamic acid to the anthocyanin (Harborne, 1958). Both the acyl λ_{max} and the $E_{\lambda_{max}}$(acyl)/$E_{\lambda_{max}}$(visible) ratios are characteristic of the nature of the acylating acid (Harborne, 1958). None of the anthocyanins analyzed in this work exhibited a peak in this area, indicating no acylation with hydroxylated aromatic organic acids. Parts A and B of Figure 5 show the UV–visible spectra of two unidentified acylated anthocyanins from red cabbage (*Brassica oleracea*). The absorbance peak in the 310-nm range indicates acylation with a cinnamic acid. The $E_{\lambda_{max}}$(acyl)/$E_{\lambda_{max}}$(visible) ratio shows that the anthocyanin in Figure 5A is acylated with a 1/1 molar ratio of the cinnamic acid to the anthocyanin while the $E_{\lambda_{max}}$(acyl)/$E_{\lambda_{max}}$(visible) ratio for the anthocyanin shown in Figure 5B shows that it is acylated with a 2/1 molar ratio of the cinnamic acid to the anthocyanin.

In addition to acylation with cinnamic acid derivatives, anthocyanins have also been found to be acylated either with acetic acid or with malonic acid. Detection of acylation of anthocyanins with either of these acids is not possible by spectral means as they do not have a characteristic absorbance spectra in the UV. It is possible, however, to determine the presence of acylation with these acids by their HPLC elution characteristics. Acylation with acetic acid results in an increase in retention time (Wulf and Nagel, 1978). While there is little information on the HPLC elution characteristics of malonic acid acylated pigments, the polar nature of a malonic acid ester would most likely result in decreased retention when compared to the nonacylated anthocyanin. Another means of detecting any of acylated anthocyanins is that the ester bond is susceptible to alkaline hydrolysis. Hence, the disappearance of a peak after mild alkaline hydrolysis

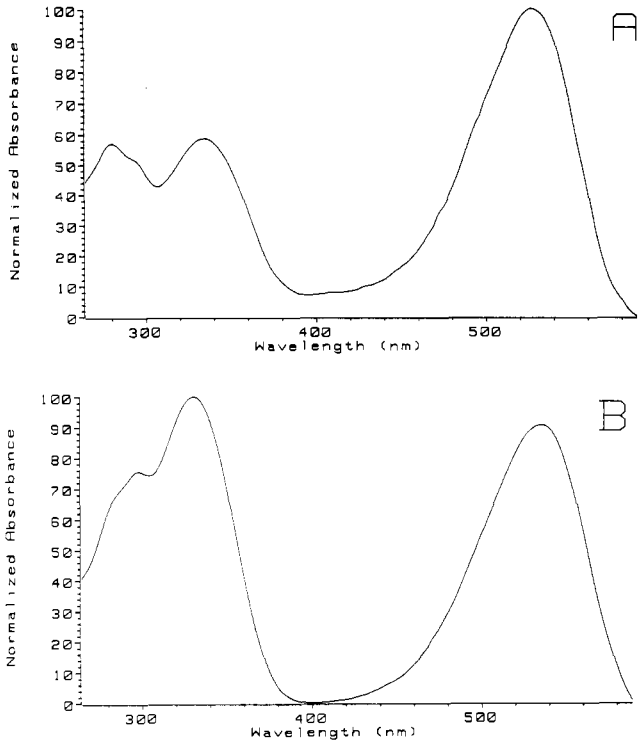

Figure 5. UV–visible spectra of two unknown anthocyanins from red cabbage. Spectral information: $\lambda_{vis\ max}$ (nm), $\lambda_{acyl\ max}$ (nm), $E_{440}/E_{\lambda_{vis\ max}}$ (%), $E_{\lambda_{acyl\ max}}/E_{\lambda_{vis\ max}}$ (%). Anthocyanin 5A: 529, 333, 21, 58. Anthocyanin 5B: 537, 320, 18, 100.

would be indicative of either an acylated anthocyanin or an alkaline labile anthocyanin.

We have also determined the spectral characteristics of the anthocyanidins and have found that the same basic spectral patterns are followed: (1) Pelargonidin derivatives have a lower visible λ_{max} (512 nm) than cyanidin derivatives (523–525 nm), which in turn have a lower visible λ_{max} than the delphinidin derivatives (529–533 nm). (2) Pelargonidin exhibits the lowest visible wavelength maxima and the highest $E_{440}/E_{\lambda_{max}}$ ratio (Table II). A pronounced shoulder in the 410–460-nm region is evident. (3) Anthocyanidins within each class have similar spectral characteristics (e.g., cyanidin and peonidin have similar spectral characteristics and delphinidin, petunidin, and malvidin have similar spectral characteristics). Comparison of the visible λ_{max} of the anthocyanidins to that of the anthocyanins (Table II) shows that, in general, the anthocyanidins exhibit a 6–10 nm higher visible λ_{max} than for the corresponding anthocyanins. These results are consistent with those of Harborne (1958) who reported a 6–12 nm larger visible λ_{max} for the anthocyanidins in 0.01% methanolic HCl. On the basis of this information and of their elution profile, anthocyanidins can be readily differentiated from anthocyanins.

Class Separation of Anthocyanins with Borate Ion. We have observed that when anthocyanins are adsorbed onto a C_{18} Sep-Pak cartridge and eluted with an alkaline borate solution that nonacylated anthocyanins containing *o*-dihydroxy systems (cyanidin, delphinidin, petunidin) are preferentially eluted while those anthocyanins not containing *o*-dihydroxy groups (pelargonidin, peonidin, malvidin) are enriched on the cartridge. Figure 6 compares the HPLC chromatogram of the fraction of cranberry anthocyanins, which remains on the Sep-Pak after elution with an alkaline borate solution, with an untreated cranberry sample. It can be seen that the peonidin glycosides are highly enriched. This technique

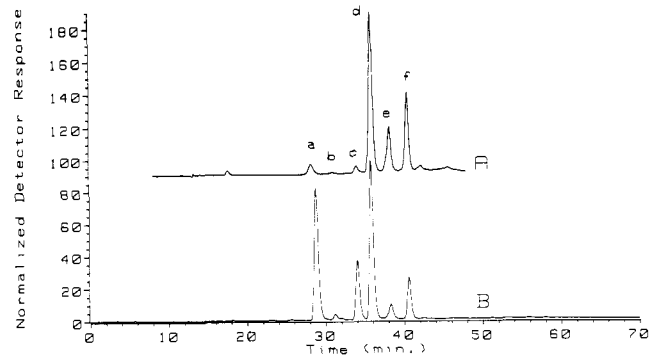

Figure 6. HPLC chromatograms: (A) fraction of cranberry anthocyanins remaining on a C_{18} Sep-Pak after elution with alkaline borate buffer; B, untreated cranberry anthocyanin chromatogram. Peak identification: a, cyanidin 3-galactoside; b, cyanidin 3-glucoside; c, cyanidin 3-arabinoside; d, peonidin 3-galactoside; e, peonidin 3-glucoside; f, peonidin 3-arabinoside.

provides an additional method for characterization of the anthocyanins. Spectral analysis can yield useful information as to the nature of the anthocyanin, the visible λ_{max} differentiating cyanidin derivatives, delphinidin derivatives, and pelargonidin. However, anthocyanins within each class cannot be differentiated. This method now allows differentiation of cyanidin from peonidin glycosides and differentiation of delphinidin and petunidin from malvidin glycosides.

Alkaline borate solutions have been used as buffers in the electrophoretic separation of phenols. The primary function of borate has been to enhance electrophoretic mobility through the formation of negatively charged complexes resulting from the reaction of borate ion with phenols possessing *o*-dihydroxy groups (Pridham, 1964). In our borate sample treatment scheme, it appears that those anthocyanins possessing the *o*-dihydroxy groups form a charged borate complex, resulting in a more hydrophilic species. The complex is preferentially eluted from the reversed-phase cartridge. Those anthocyanins not complexing with the borate ion remain in the cartridge. In our studies, we have observed that highly retained species (long retention times) such as acylated cyanidin and delphinidin glycosides are also not eluted from the cartridge. Assuming that borate complexes form, the hydrophobicities of these anthocyanins are such that any complex formed is not sufficiently hydrophilic enough to be eluted from the column. The anthocyanidins behave differently in that they are completely eliminated by the borate/Sep-Pak treatment. We believe that the very unstable anthocyanidins are completely destroyed under the alkaline conditions employed. This behavior could be applied to distinguish anthocyanidins from anthocyanins with similar retention times.

Characterization of Strawberry Anthocyanins. The potential applications of these methods can be illustrated in characterizing the anthocyanins in strawberries (*Fragaria anannassa*). Our interest in strawberries extends from the repeated occurrence of unidentified minor peaks in the strawberry HPLC chromatogram.

A survey of the anthocyanins in strawberry shows that the major pigment is pelargonidin 3-glucoside, with smaller amounts of cyanidin 3-glucoside (Timberlake and Bridle, 1982). A third pigment, possibly a isomer of pelargonidin 3-glucoside, was reported by Wrolstad et al. (1970). The anthocyanidin profile (Table I) shows that the major anthocyanidin is pelargonidin with lesser amounts of cyanidin. Figure 7A shows the HPLC chromatogram for strawberry anthocyanins. The spectral characteristics of the individual peaks are included in Table III. Figure

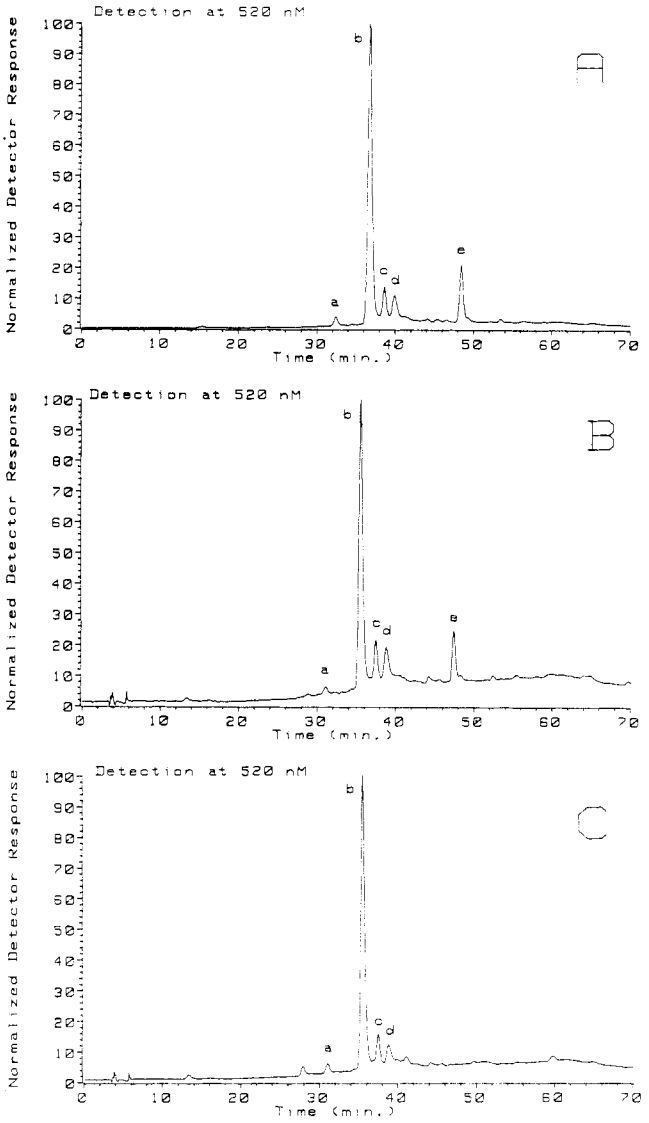

Figure 7. HPLC chromatograms of strawberry anthocyanins: (A) untreated strawberry sample; (B) fraction remaining on a C_{18} Sep-Pak after elution with alkaline borate buffer; (C) strawberry anthocyanins after alkaline hydrolysis. Peak identification: a, cyanidin 3-glucoside; b, pelargonidin 3-glucoside; c, pelargonidin 3-rutinoside (tentative); d, pelargonidin derivative (tentative); e, pelargonidin 3-glycoside acylated with acetic acid (tentative).

Table III. Spectral Characteristics of Strawberry Anthocyanins As Determined by HPLC/Photodiode Array Detection[a]

peak no. (Figure 7)	vis λ_{max}, nm	E_{440}/E_{vis}	acyl peak
a	ND	ND	N
b	501	45	N
c	501	50	N
d	509	61	N
e	501	51	N

[a] Key: ND = not determined; N = no acylation with hydroxy aromatic acid detected.

7B shows a typical HPLC chromatogram of the strawberry anthocyanins remaining on a C_{18} Sep-Pak after elution with alkaline borate buffer. This treatment had little effect on the major strawberry anthocyanins (peaks b–e). The conclusion, therefore is, that they are most likely to be pelargonidin derivatives and not cyanidin derivatives. Peak a, which has the same retention time as cyanidin 3-glucoside, is reduced in its relative peak area but is not completely eliminated. The major peak (peak b) is identified as pelargonidin 3-glucoside. Its spectral characteristics are similar to that of its aglycon in that a pronounced shoulder in the 410–450-nm region is present. This results in a higher E_{440}/E_{vis} ratio than for the other common anthocyanidin glycosides (Table II). These results are consistent with those of Harborne (1958). The visible wavelength maxima (Table III) are lower than for cyanidin glycosides.

The visible λ_{max}, E_{440}/E_{vis} ratio (Table III), and the appearance of a shoulder in the 400–460-nm region for peaks c and e indicate that these unknown pigments are also pelargonidin 3-glycosides. Both peaks elute later than pelargonidin 3-glucoside, indicating that the sugar substituents exhibit greater hydrophobicity than glucose. Some possibilities are (1) glycosidation with a more hydrophobic sugar group, e.g., rutinose, arabinose, and xylose; (2) acylation of the sugar moiety; and (3) formation of polymerized pigments. No UV absorbance maxima were found in the 310-nm range for any of the strawberry anthocyanins, indicating no acylation with hydroxy aromatic organic acids.

Peak c is tentatively identified as pelargonidin 3-rutinoside on the following basis: (1) Its spectral characteristics show that it is a pelargonidin derivative. (2) This peak coelutes with pelargonidin 3-rutinoside from Tamarillo (*Cyphomandra betaceae*) fruit (Wrolstad and Heatherbell, 1974). Figure 7C shows an HPLC chromatogram of strawberry anthocyanins after mild alkaline hydrolysis. The disappearance of peak e indicates that it is acylated. The UV spectrum eliminates the possibility of acylation with an aromatic acid. The late retention time of peak e is consistent with the possibility of acylation with acetic acid. From this information, we speculate that peak e is an acetic acid acylated pelargonidin derivative. For definitive identification, further studies will need to be performed on purified fractions of these anthocyanins. Peak d remains unidentified. The visible λ_{max} is higher than that for a pelargonidin derivative and lower than that of a cyanidin derivative. A shoulder in the 400–460-nm region is evidence that it may be a pelargonidin derivative. It is not hydrolyzed under alkaline conditions, indicating that it is not acylated, it does not exhibit any characteristics to indicate that it is pelargonidin (aglycon), and it is not eluted from a C_{18} Sep-Pak with borate buffer, supporting the hypothesis that is is a glycosylated pelargonidin derivative.

CONCLUSION

In addition to HPLC retention order, the use of online photodiode array detection is a powerful tool to aid in the characterization of anthocyanins. Removal of interfering phenolic compounds by sample preparation procedures results in a sufficiently clean anthocyanin extract such that spectra obtained by photodiode array detection can be used for identification. Derivatives of delphinidin, cyanidin, and pelargonidin can be distinguished from each other by their visible λ_{max} spectral means while anthocyanins acylated with hydroxy aromatic organic acids can be identified by their characteristic spectral absorption maxima in the 310-nm range. The presence of different glycosidic substituents can be differentiated due to different retention characteristics by reversed-phased HPLC. Nonacylated anthocyanins without *o*-dihydroxy groups (pelargonidin, malvidin, peonidin) can be selectively concentrated on a C_{18} Sep-Pak with an alkaline borate buffer as an elutant, thus serving as a means of distinguishing those anthocyanins from cyanidin, petunidin, and delphinidin derivatives. Acy-

lated anthocyanins can be eliminated from a chromatogram by mild alkaline hydrolysis.

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LITERATURE CITED

Akavia, N.; Strack, D. High Performance Liquid Chromatography of Anthocyanidins as a New Approach to Study Flower Pigment Genetics. *Z. Naturforsch.* **1980**, *35C*, 16–19.

Andersen, O. M. Chromatographic Separation of Anthocyanins in Cowberry (Lingonberry) *Vaccinium vites-idaea* L. *J. Food Sci.* **1985**, *50*, 1230–1232.

Andersen, O. M. Anthocyanins in Fruits of *Vaccinium uliginosum* L. (Bog Whortleberry). *J. Food Sci.* **1987**, *52*, 665–666.

Asen, S. Identification of Flavonoid Chemical Markers in Roses and their High Pressure Liquid Chromatographic Resolution and Quantitation for Cultivar Identification. *J. Am. Soc. Hortic. Sci.* **1982**, *107* (5), 744–750.

Bronnum-Hansen, K.; Hansen, S. High-Performance Liquid Chromatographic Separation of Anthocyanins of *Sambucus nigra* L. *J. Chromatogr.* **1983**, *262*, 385–392.

Casteele, K. V.; Geiger, H.; De Loose, R.; Van Sumere, C. Separation of Some Anthocyanidins, Anthocyanins, Proanthocyanins and Related Substances by Reversed-Phase High-Performance Liquid Chromatography. *J. Chromatogr.* **1983**, *259*, 291–300.

Du, C. T.; Francis, F. J. Anthocyanins of Roselle (*Hibiscus sabdariffa*, L.). *J. Food Sci.* **1973**, *38*, 810–812.

Durst, R. W.; Wrolstad, R. E. Compositional Analysis of Authentic Cranberry Juice: Anthocyanins, Anthocyanidins, Sugars and Nonvolatile Organic Acids. Presentation No. 163 at the 101st Annual Meeting of the AOAC, San Francisco, CA, Sept 14, 1987.

Francis, F. J.; Harborne, J. B.; Barker, W. G. Anthocyanins in the Lowbush Blueberry, *Vaccinium angustifolium*. *J. Food Sci.* **1966**, *31*, 583–587.

Hale, M. L.; Francis, F. J.; Fagerson, I. S. Detection of Enocyanin in Cranberry Juice Cocktail by HPLC Anthocyanin Profile. *J. Food Sci.* **1986**, *51* (6), 1511–1513.

Harborne, J. B. Spectral Methods of Characterizing Anthocyanins. *Biochem. J.* **1958**, *70*, 22–28.

Harborne, J. B. *Comparative Biochemistry of the Flavonoids*; Academic Press: London, 1967.

Hicks, K. B.; Sondey, S. M.; Hargrave, D.; Sapers, G. M.; Bilyk, A. Automated Preparative HPLC of Anthocyanins. *LC* **1985**, *3* (11), 981–984.

Hong, V.; Wrolstad, R. E. Cranberry Juice Composition. *J. Assoc. Off. Anal. Chem.* **1986**, *69* (2), 199–207.

Hrazdina, G. Recent Techniques in the Analysis of Anthocyanins in Fruits and Beverages. *Analysis of Food and Beverages*. Academic Press: London, 1979, Vol. 1, pp 141–159.

Pridham, J. B. Paper Electrophoresis of Phenolic Compounds. *Methods of Polyphenol Chemistry*; Pergamon Press: New York, 1964.

Sakellariades, H. C.; Luh, B. S. Anthocyanins in Barbera Grapes. *J. Food Sci.* **1974**, *39*, 329–333.

Sapers, G. M.; Hargrave, D. L. Proportions of Individual Anthocyanins in Fruits of Cranberry Cultivars. *J. Am. Soc. Hortic. Sci.* **1987**, *112*, 100–104.

Spanos, G.; Wrolstad, R. E. Anthocyanin Pigment, Non-Volatile Acid and Sugar Composition of Red Rasberry Juice. *J. Assoc. Off. Anal. Chem.* **1987**, *70*, 1036–1046.

Stewart, R. N.; Asen, S.; Massie, D. R.; Norris, K. H. The Identification of Poinsettia Cultivars by HPLC Analysis of their Anthocyanin Content. *Biochem. Syst. Ecol.* **1979**, *7*, 281–287.

Strack, D.; Akavia, N.; Reznik, H. High Performance Liquid Chromatographic Identification of Anthocyanins. *Z. Naturforsch.* **1980**, *35C*, 533–538.

Timberlake, C. F.; Bridle, P. Distribution of Anthocyanins in Food Plants. In *Anthocyanins as Food Colors*; Markakis, P., Ed.; Academic Press: New York, 1982.

Wilkinson, M.; Sweeny, J. G.; Iacobucci, G. A. High-Pressure Liquid Chromatography of Anthocyanidins. *J. Chromatogr.* **1977**, *132*, 349–351.

Wrolstad, R. E.; Heatherbell, D. A. Identification of Anthocyanins and Distribution of Flavonoids in Tamarillo Fruit (*Cyphomandra betaceae* (Cav.) Sendt.). *J. Sci. Food Agric.* **1974**, *25*, 1221–1228.

Wrolstad, R. E.; Hildrum, K. I.; Amos, J. F. Characterization of an Additional Anthocyanin Pigment in Extracts of Strawberries, Fragaria. *J. Chromatogr.* **1970**, *50*, 311–318.

Wulf, L. W.; Nagel, C. W. High-Pressure Liquid Chromatographic Separation of Anthocyanins of *Vitis vinifera*. *Am. J. Enol. Vitic.* **1978**, *29*, 42–49.

Zapsalis, C.; Francis, F. J. Cranberry Anthocyanins. *J. Food Sci.* **1965**, *30*, 396–399.

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