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Amitabh Chandra, Jatinder Rana, and Yingqin Li

Chemistry Services, Department of Analytical Services, Access Business Group, 7575 Fulton Street East, Ada, Michigan 49355, Pyxis Innovations, 7575 Fulton East, Ada, Michigan 49301, and Nutrilite Division of Access Business Group, 19600 6th Street, Lakeview, California 92567

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Separation, Identification, Quantification, and Method Validation of Anthocyanins in Botanical Supplement Raw Materials by HPLC and HPLC–MS

Amitabh Chandra,* Jatinder Rana,† and Yingqin Li‡

Chemistry Services, Department of Analytical Services, Access Business Group, 7575 Fulton Street East, Ada, Michigan 49355, Pyxis Innovations, 7575 Fulton East, Ada, Michigan 49301, and Nutrilite Division of Access Business Group, 19600 6th Street, Lakeview, California 92567

A method has been established and validated for identification and quantification of individual, as well as total, anthocyanins by HPLC and LC/ES–MS in botanical raw materials used in the herbal supplement industry. The anthocyanins were separated and identified on the basis of their respective M^+ (cation) using LC/ES–MS. Separated anthocyanins were individually calculated against one commercially available anthocyanin external standard (cyanidin-3-glucoside chloride) and expressed as its equivalents. Amounts of each anthocyanin calculated as external standard equivalent were then multiplied by a molecular-weight correction factor to afford their specific quantities. Experimental procedures and use of a molecular-weight correction factors are substantiated and validated using Balaton tart cherry and elderberry as templates. Cyanidin-3-glucoside chloride has been widely used in the botanical industry to calculate total anthocyanins. In our studies on tart cherry and elderberry, its use as external standard followed by use of molecular-weight correction factors should provide relatively accurate results for total anthocyanins, because of the presence of cyanidin as their major anthocyanidin backbone. The method proposed here is simple and has a direct sample preparation procedure without any solid-phase extraction. It enables selection and use of commercially available anthocyanins as external standards for quantification of specific anthocyanins in the sample matrix irrespective of their commercial availability as analytical standards. It can be used as a template and applied for similar quantification in several anthocyanin-containing raw materials for routine quality control procedures, thus providing consistency in analytical testing of botanical raw materials used for manufacturing efficacious and true-to-the-label nutritional supplements.

Keywords: *Anthocyanins; ES–MS; Balaton tart cherry; Prunus cerasus L.; elderberry; Sambucus nigra L.; bilberry; Vaccinium myrtillus L.; chokeberry; Aronia melanocarpa Elliot; HPLC; LC–MS; botanical supplements; raw materials*

INTRODUCTION

Anthocyanins are the glycosidic forms of anthocyanidins that impart bright and attractive colors to flowers and fruits. Major anthocyanins found in fruits are based on the cyanidin aglycon (*1*) (Figure 1). In the early 1990s, efforts were made to utilize anthocyanins as natural food colorants (*2–4*). However, the challenges for their use as natural colorants were their instability caused by enzymes, pH, temperature, and oxygen, leading to their alteration to various structural forms that were found to have a direct impact on color quality (*5, 6*). Stability of these anthocyanins is known to be increased by co-pigmentation with other natural polyphenols coexisting in plant systems (*7*). Various reports of these anthocyanins being relatively stable when stored with dextrins and in optimum pH kept alive the interest in using these pigments as natural food colorants (*6, 8*).

Recently, a great deal of renewed interest in anthocyanins has emerged because of their potential health benefits as antioxidants and antiinflammatory agents (*9, 10*). For example, there are already numerous commercial products derived from bilberry (*Vaccinium myrtillus* L.) because of its antioxidative properties (*11*). Antioxidant activities such as oxygen radical absorbance capacity (ORAC) were found to be directly related to the anthocyanin content in berries (*12*). This therapeutic benefit of anthocyanins and the respective natural sources that contain them has resulted in increasing interest in the botanical supplement industry to make products standardized on anthocyanins. Some of the most important candidates for supplying such beneficial anthocyanins are bilberry, elderberry, chokeberry and tart cherries.

Because of the increasing interest in these products, there is a demand for efficient quality control measures to ensure the authenticity of the anthocyanin source and content in these products and to verify label claims. The most common method that is used in the nutritional food supplement industry is to quantify total anthocyanins in these raw materials spectrophotometrically at 520 nm at a controlled pH (*13*). Spectrophotometric determination of total anthocyanins without hydrolysis

* To whom correspondence should be addressed at Department of Analytical Services, Access Business Group. Telephone: (616) 787 5655. Fax: (616) 787 4466. E-mail: amitabh_chandra@amway.com.

† Pyxis Innovations.

‡ Nutrilite Division of Access Business Group.

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is a commonly adapted method because of its relatively milder conditions, rapidness, and cost-effective nature (*14*). This method works very well where an estimation is needed rather than an accurate quantification. Therefore, such methods are excellent tools for rapid screening of total anthocyanin contents in fruits and vegetables. Spectral similarities in the majority of anthocyanins makes this determination relatively feasible and the anthocyanins are reported as "external standard equivalents" which in most of the cases is "cyanidin-3-glucoside". Variability in sample extraction media (water, acidic water, acidic organic solvent, etc.); chemical properties of anthocyanins such as self-association, transition in structural forms (quinonoidal to flavylium), and co-pigmentation with other secondary metabolites present in the sample solution (*6, 7*); and variability in the choice of molar absorbtivity values of anthocyanins for calculation directly influences an inaccurate correlation between absorbance and actual anthocyanin concentration when measured by UV light (*15, 16*). Besides this, the spectroscopic method does not provide any specificity regarding an anthocyanin fingerprint for the determination of species identification or consistency verification for affording quality botanical supplements. Apart from this, there are excellent literature procedures available on analytical methods using HPLC and LC−MS techniques for identifying anthocyanins and, to some extent, their possible quantification (*11, 16−19*). Most of the methods are qualitative or semiquantitative, providing information on identification of anthocyanins and their respective sources. A major challenge in quantification of anthocyanins by HPLC methods is the unavailability of most standards on a commercial basis. The majority of the anthocyanins that are naturally present constitute the backbone (aglycons) usually comprising one of the six anthocyanidins such as cyanidin, delphinidin, petunidin, peonidin, malvidin, and pelargonidin. These are commercially available and can be used for quantification of total anthocyanins as anthocyanidins in fruit samples by chromatographic and spectrophotometric means. Chromatographic quantification is generally carried out by acid hydrolysis of anthocyanins to liberate their respective aglycon anthocyanidins. Such hydrolysis requiring high concentrations of mineral acids results in their degradation and thus ends up with inaccurate and highly variable results (*20*). Methods currently available are usually related to analysis of intact fruits or fruit juices. These usually tend to be very specific to the sources and cannot be adapted for a wide range of anthocyanin containing sources and sample matrixes used as raw materials in the botanical industry. They do indeed afford excellent fingerprint profiles for positive identification of the berry source (*18*). Also, because of the focus of these methods on food (intact fruits and juices) they are not validated for the analysis of finished/formulated materials (extract powders) for use in the botanical supplement industry.

This report provides an analytical procedure for positively identifying the anthocyanins and sources containing them by HPLC−MS and HPLC followed by a validated HPLC quantification method for total anthocyanins in several botanical raw materials that constitute some of the most important antioxidant supplements. The method is widely adaptable and offers a consolidated approach for total anthocyanins irrespective of the source from which they are derived.

MATERIALS AND METHODS

Standard Compounds. Cyanidin-3-rutinoside chloride (keracyanin chloride), lot number 00052510, and cyanidin-3-glucoside chloride (kuromanin chloride), lot number 00070416 (percent purity was ≥ 95% according to lot specific CoA), were obtained from Indofine (Indofine Chemical Company, Somerville, NJ). It is recommended to obtain a certificate of analysis on commercially purchased standards to obtain information such as percent purity, residual solvents, and moisture content. Corrections for standard purity may be applied if necessary.

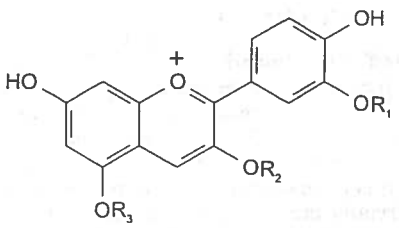
Raw Materials Containing Anthocyanin. Balaton tart cherry powder, Nutrilite lot no. 911-01, and elderberry powder, Nutrilite lot no. 006-03, were provided by the Nutrilite Division of Access Business Group (Nutrilite-Amway, Lakeview, CA). Chokeberry powder, code no. 73190100, was obtained from Artemis (Artemis International, Inc., FortWayne, IN). Bilberry extract lot no. 26831/M1 was obtained from Indena (Indena SpA, Milan, Italy). Fresh pitted Balaton tart cherries were obtained from Leelanau Fruit Company (Suttons Bay, MI).

HPLC/ES−MS Conditions. LC/ES−MS experiments were carried out on Micromass Quattro II LC−MS/MS system (Micromass, a division of Waters Corp., Beverly, MA) equipped with a Waters 2690 HPLC system and a Waters 996 PDA detector (Waters Corp., Milford, MA) and MassLynx Software version 3.3 (Micromass, a division of Waters Corp., Beverly, MA). Following are the conditions that were used. The column was an HP ODS hypersil HPLC column, 5 μm, 125/4.0 mm (Agilent Technologies, Wilmington, DE). The solvents were (A) 0.1% TFA/water (v/v) and (B) 50.4%/48.5%/1%/0.1% (v/v/v/v) water/acetonitrile/acetic acid/TFA; with a gradient of % B: initial, 20%; 26 min, 60%; 30 min, 20%; 35 min, 20% (run time 35 min). Flow rate was 0.8 mL/min, injection 10 μL (postcolumn split 10:1), and column temperature 30 °C. PDA range 200−799 nm, 520 nm as detection wavelength. Following are the MS parameters: ionization mode, ES^+; scan range, 200−1000 amu; scan rate, 1 scan/sec; and cone voltage, 20 eV.

HPLC Conditions. Experiments were carried out on a Waters 2690 HPLC system equipped with a Waters 996 PDA detector (Waters Corp., Milford, MA) and Millennium chromatography manager version 3.1 (Waters Corp., Milford, MA). Following are the operating conditions used. The column was an HP ODS hypersil HPLC column, 5 μm, 125/4.0 mm (Agilent Technologies, Wilmington, DE). The solvents were (A) 0.5% aqueous phosphoric acid (v/v), and (B) water/acetonitrile/glacial acetic acid/phosphoric acid, 50:48.5:1.0:0.5 (v/v/v/v). The solvent gradient was % B, initial, 20%; 26 min, 60%; 30 min, 20%; 35 min, 20% (run time 35 min). PDA 200−800 nm; detection wavelength 520 nm. Flow rate was 0.8 mL/min, column temperature was 30 °C, sample temperature was ambient, and injection volume was 10 μL.

Standard Preparation and Calibration Curve. Commercially available standards of cyanidin-3-rutinoside chloride (keracyanin chloride, 0.40 mg) and cyanidin-3-glucoside chloride (kuromanin chloride, 0.35 mg) were separately dissolved in 2% HCl/MeOH solution (v/v) (10 mL), and used as standard stock solutions for generating calibration curves. The stock solutions were diluted 1/5, 1/10, 1/25, and 1/100 times in 2% HCl/MeOH (v/v) to afford 0.08, 0.044, 0.018, and 0.0044 mg/mL solutions of cyanidin-3-rutinoside chloride and 0.07, 0.035, 0.014, and 0.0035 mg/mL solutions of cyanidin-3-glucoside chloride, respectively. These four standard solutions and the stock solution were injected to generate a five point calibration curve for the two standard compounds separately, using Millennium chromatography manager. Standard curve was linear with $R^2 = 0.9998$. Peak areas of the target compounds were within the linear range of the curve. Relative standard deviations for two injections per standard (for set of five standard solutions) were less than 2.0%.

Sample Preparation. *Balaton Tart Cherry Fruits.* Fruits were thawed at room temperature if cherries were frozen. Approximately 25 to 30 cherries were blended in a food mill to produce a thick puree. A 10-g sample was placed into a 50-mL flat-bottom centrifuge tube, and 20 mL of DI water was



Tart Cherry Anthocyanins:

1. Cyanidin-3-glucosyl-rutinoside (R_1=H, R_2= hex+rut, R_3= H)
2. Cyanidin-3-arabinosyl-rutinoside (R_1= H, R_2= pent+rut, R_3= H)
3. Cyanidin-3-rutinoside (R_1= H, R_2= rut, R_3= H)
4. Peonidin-3-rutinoside (R_1= OMe, R_2= rut, R_3= H)

Elderberry Anthocyanins:

5. Cyanidin-3-sambubioside-5-glucoside (R_1= H, R_2= sam, R_3= hex)
6. Cyanidin-3-sambubioside (R_1= H, R_2= sam, R_3= H)
7. Cyanidin-3-glucoside (R_1= H, R_2= hex, R_3= H)
8. Cyanidin (R_1= R_2= R_3= H)

(hex = hexose, pent = pentose, rut = rutinose, sam = sambubiose)

Figure 1. Anthocyanins identified in Balaton tart cherry and elderberry.

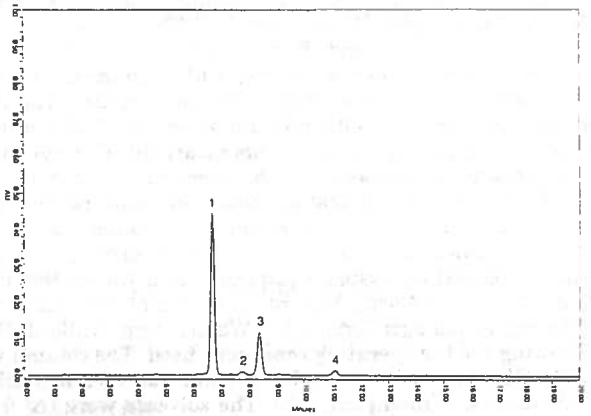


Figure 2. Typical HPLC profile of anthocyanins in Balaton tart cherry fruits. Compounds: **1**, cyd-3-glucosyl-rutinoside, M^+ = 757; **2**, cyd-3-arabinosyl-rutinoside, M^+ = 727; **3**, cyd-3-rutinoside, M^+ = 595; **4**, peo-3-rutinoside, M^+ = 609; (cyd = cyanidin; peo = peonidin).

added. The tube was capped, and the sample was sonicated for 15 min with occasional swirling. The sample was vortexed, then centrifuged at 3000 rpm for 10 min. An aliquot of 5 mL was weighed in a 10-mL volumetric flask, and then the volume (10 mL) was made up with 2% HCl/MeOH. The sample was then mixed thoroughly and filtered through a 0.45-μ PTFE filter into an HPLC vial, and then analyzed by HPLC (Figure 2). Five replicates of samples were prepared between two analysts (total of 10 samples).

Balaton Tart Cherry, Elderberry, Chokeberry, and Bilberry Powders. Sample weights of 200 mg, 25 mg, 40 mg, and 15 mg of the Balaton tart cherry, elderberry, chokeberry, and bilberry powders, respectively, were weighed into 20-mL capped glass vials, respectively. A 10-mL portion of 2% HCL/MeOH solution was accurately pipeted into each of the vials. Sample solutions were capped, placed in a sonicator, and extracted by sonication for 15 min with occasional shaking. They were mixed well and filtered through a 0.45-μ PTFE filter directly into a HPLC vial. Each sample was analyzed by HPLC (Figures 3–6). Five replicates of samples were prepared between two analysts (total of 10 samples) for Balaton tart cherry and elderberry powders, and chokeberry and bilberry were analyzed in duplicate.

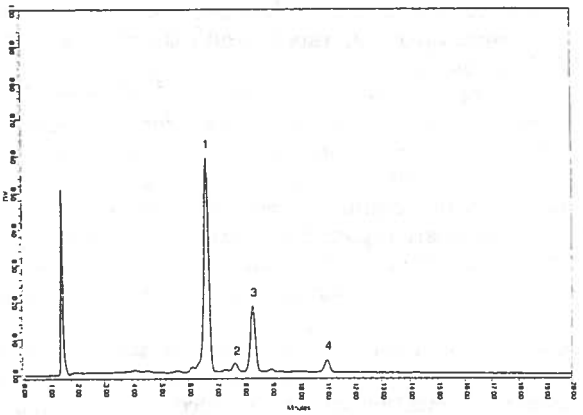


Figure 3. Typical HPLC profile of anthocyanins in Balaton tart cherry powder. Compounds: 1, cyd-3-glucosyl-rutinoside, M^+ = 757; **2**, cyd-3-arabinosyl-rutinoside, M^+ = 727; **3**, cyd-3-rutinoside, M^+ = 595; **4**, peo-3-rutinoside, M^+ = 609; (cyd = cyanidin; peo = peonidin).

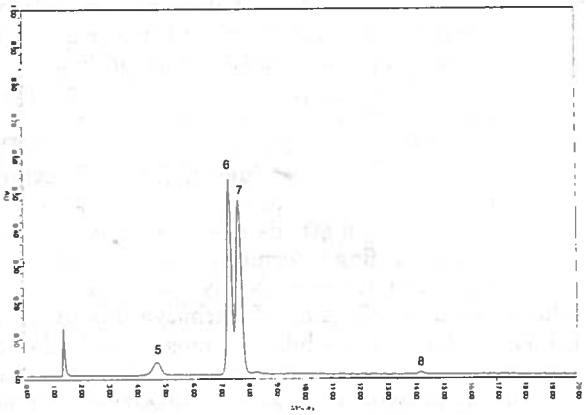


Figure 4. Typical HPLC profile of anthocyanins in elderberry powder. Compounds: **5**, cyd-3-sambubioside-5-glucoside, M^+ = 743; **6**, cyd-3-sambubioside, M^+ = 581; **7**, cyd-3-glucoside, M^+ = 449; **8**, cyd, M^+ = 287; (cyd = cyanidin).

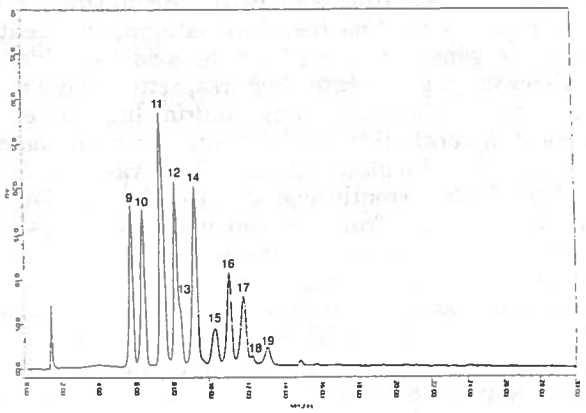


Figure 5. Typical HPLC profile of anthocyanins in bilberry powder. Compounds: **9**, dpd-3-galactoside, M^+ = 465; **10**, dpd-3-glucoside, M^+ = 465; **11**, cyd-3-galactoside, M^+ = 449; **12**, cyd-3-glucoside, M^+ = 449; **13**, pet-3-galactoside, M^+ = 479; **14**, pet-3-glucoside, M^+ = 479; **15**, pet-3-arabinoside, M^+ = 449; **16**, peo-3-galactoside, M^+ = 463; **17**, malv-3-galactoside, M^+ = 493; **18**, malv-3-glucoside, M^+ = 493; **19**, malv-3-arabinoside, M^+ = 463; (dpd = delphinidin, pet = petunidin, peo = peonidin, cyd = cyanidin, malv = malvidin).

Calculations. The amounts of anthocyanins (mg/g) identified in the samples were individually expressed as ($A \times DF$) $\times$ $CF/S\ Wt$; where A = mg/mL amount of anthocyanin expressed as the external standard equivalent (either cyanidin-

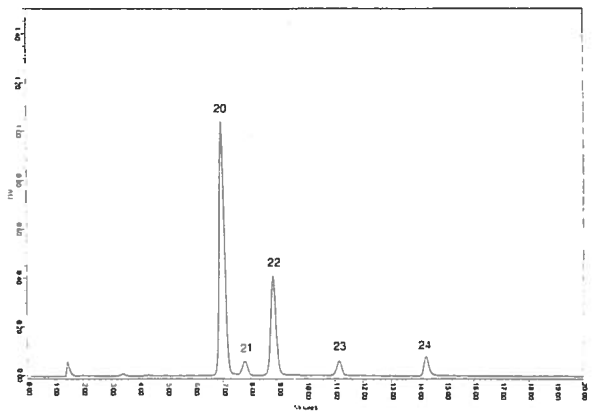


Figure 6. Typical HPLC profile of anthocyanins in chokeberry powder. Compounds: **20**, cyd-3-galactoside, $M^+ = 449$; **21**, cyd-3-glucoside, $M^+ = 449$; **22**, cyd-3-arabinoside, $M^+ = 419$; **23**, cyd-3-xyloside, $M^+ = 419$; **24**, cyd, $M^+ = 287$; (cyd = cyanidin).

3-rutinoside or cyanidin-3-glucoside chloride) from the calibration curve; *DF* = dilution factor (*DF* for fresh fruits was calculated as initial sample weight (g) + 20 g water/weight of 5 mL aliquot (g) × 10 mL; *DF* for powder samples was 10 mL); *S Wt* = initial sample weight (g); and *CF* = molecular weight correction factor to convert individual anthocyanins (mg/g) calculated as the external standard equivalents to their respective forms.

Spike Studies. Two aliquots of HPLC sample solutions (1 mL each) of Balaton tart cherry powder containing 0.0755 mg/mL of cyanidin-3-rutinoside chloride (original amount) were spiked separately with 100 μL (containing 0.0441 mg) and 50 μL (containing 0.02205 mg) of freshly prepared cyanidin-3-rutinoside chloride standard solution (0.441 mg/mL). This theoretically afforded 0.1087 and 0.09290 mg/mL cyanidin-3-rutinoside chloride in resultant spiked solutions. Analysis of these solutions against the calibration curve of cyanidin-3-rutinoside chloride resulted in 0.110 and 0.0950 mg/mL amounts of the target analyte. This indicated a spiked recovery of 101.95% and 102.26%, respectively, at spike levels of 158% and 129% of the originally present cyanidin-3-rutinoside chloride, respectively (Figure 7).

Direct Sampling versus Solid-Phase Extraction (SPE). Two separate aliquots of juiced Balaton tart cherry samples (1 mL each) were collected. The first aliquot (1 mL) was diluted by 2% HCl−MeOH soln (1 mL) for direct HPLC analysis (total volume 2 mL), while the second aliquot (1 mL) was subjected to SPE. The second aliquot of Balaton tart cherry juice (1 mL) was quantitatively loaded on a preconditioned (4 mL of 2% HCl−MeOH) Sep-pak plus C-18 cartridge (1 mL) (Waters Corporation, Milford, MA). Juice (1 mL) was gently adsorbed in the cartridge and the colorless liquid was eluted out and discarded. The (red colored) anthocyanin band was washed with 1 mL × 2 0.5% phosphoric acid solution. The anthocyanin band was eluted quantitatively by 1 mL × 2 2% HCl−MeOH solution for HPLC analysis (total volume 2 mL). Both the samples (with and without SPE) were qualitatively analyzed by HPLC, and their HPLC profiles at similar volumes of sample preparation were compared (Figure 8).

RESULTS AND DISCUSSION

This method is validated using Balaton tart cherry (*Prunus cerasus* L.) and elderberry (*Sambucus nigra* L.) raw materials as templates and can be adapted to several anthocyanin containing raw materials.

A majority of the HPLC methods used today involve the use of high volumes of acids (1.0 to 15% v/v) as mobile phases to maintain a low pH as a requirement for maintaining the stability of anthocyanins in solution in the form of flavylium cation (*19*). Our method uses relatively low volumes of phosphoric acid and acetic acid (0.5 to 1.0%). It was observed that the mobile phase used in our method was not only enough to maintain the optimum pH (below 1.5) for anthocyanin stability and separation, but was also more gentle on the instrumentation for long term use. Separated anthocyanins were subjected to identification by monitoring the molecular weights of their respective cations by LC−ES/MS (*17*). The anthocyanins identified in Balaton tart cherry and elderberry samples are listed in Table 1. The major anthocyanins identified in these products were in agreement with those previously reported in the literature (*2, 3*).

Solid-phase extraction is a routinely practiced procedure for cleaning up a sample prior to HPLC analysis (*19*). This was mostly performed to clean the HPLC sample of phenolic and related impurities in order to identify anthocyanins from their UV/PDA spectra against reference substances (*21*). Identification of anthocyanins by LC/ES−MS enabled us to eliminate this cleanup procedure completely leading to a simplified single-step sample preparation for quantitative HPLC analysis, and it prevented any pigment loss during the experimental procedures. The elimination of this step was substantiated by comparing HPLC profiles of Balaton tart cherry juice sample prepared with and without SPE (Figure 8). Results indicated that there was no significant difference between the anthocyanin profiles of the two samples. We also avoided extraction of samples at elevated temperatures so as to protect anthocyanins from thermolability. 2% HCl−MeOH was used as the extraction solvent for all samples and standards as to afford parity between samples and standard solutions for accurate results (*16*).

UV/PDA spectra of the separated anthocyanins in Balaton tart cherry and elderberry samples were very similar to that of cyanidin-3-glucoside, the commercially available external standard used for their quantification. Spectral and structural similarities of target anthocyanins with external standard led to the use of molecular-weight-correction factors for their specific calculations. The molecular-weight-correction factors for specific calculation of individual anthocyanins were determined by dividing the molecular weight of the anthocyanin to be quantified by that of the standard anthocyanin (Table 1). Use of the molecular-weight-correction factors was justified by calculating recoveries of known amounts of cyanidin-3-glucoside and cyanidin-3-rutinoside from their standard solutions against each other at three different concentrations at 520 nm. Mutual recoveries of these two compounds were calculated by using molecular-weight-correction factors for specific quantification ranged between 97 and 101%. Use of molecular-weight-correction factors was further substantiated by quantifying cyanidin-3-rutinoside in Balaton tart cherry powder against cyanidin-3-rutinoside standard and cyanidin-3-glucoside standards, separately. Multiplication with the molecular-weight-correction factor was used to convert cyd-3-rutinoside calculated as cyd-3-glucoside to cyd-3-rutinoside in the latter case. Results from two approaches yielded 3.51 and 3.45 mg/g cyd-3-rutinoside in the sample, respectively (within ± 2%). These results thus established a direct correlation between molecular weights and responses (absorbance/concentration) between anthocyanins containing similar aglycons (anthocyanidins).

On the basis of the parameters discussed above, this method was applied and validated for individual as well

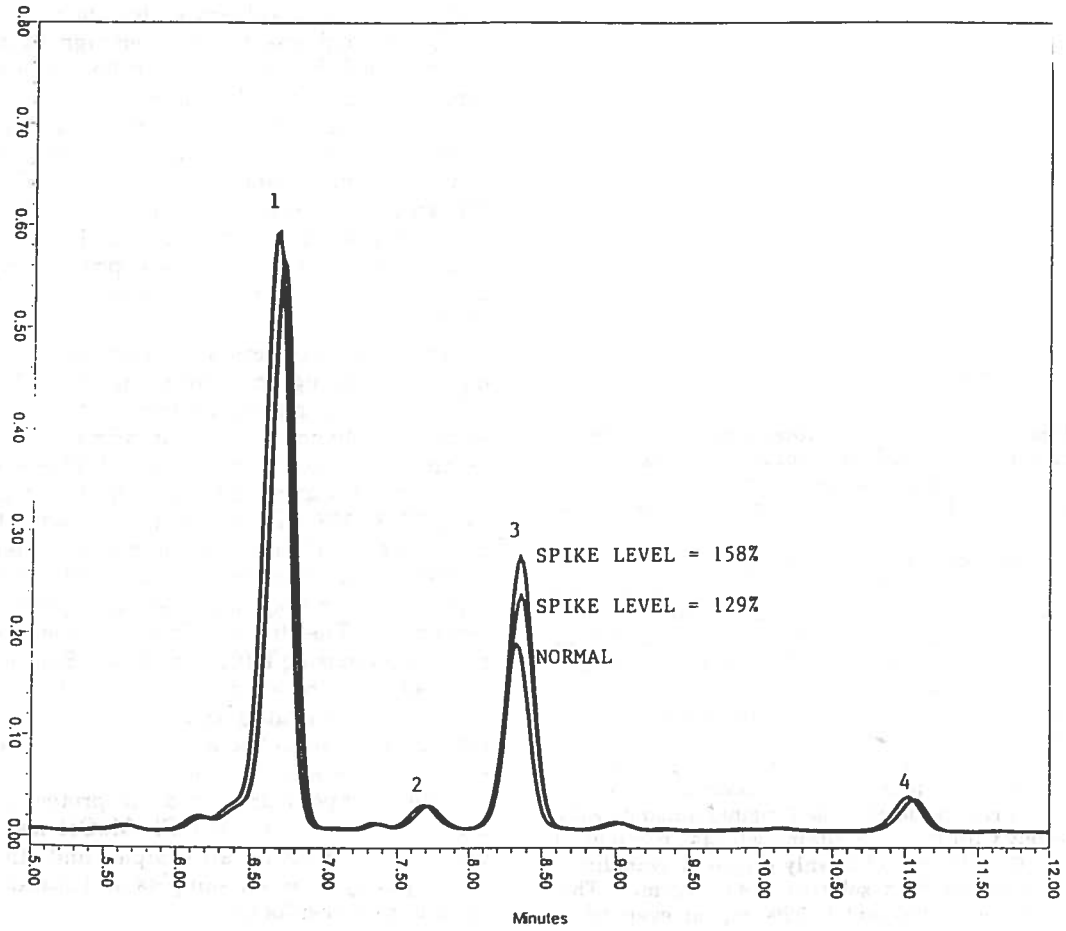


Figure 7. Overlayed HPLC chromatogram of Balaton tart cherry powder spiked with two concentrations of cyd-3-rutinoside standard.

as total anthocyanins in Balaton tart cherry powder, Balaton tart cherry fresh fruit, and elderberry powder samples. Criteria addressed for validation were precision, accuracy, linearity, and specificity. Experiments were carried out by two analysts with five replicates per sample. Results for precision are expressed in Tables 2, 3, and 4.

Alternatively, and depending on manufacturer's requirements, total anthocyanins in these and related samples and blends also can be expressed as "cyanidin-3-glucoside chloride equivalents". This is possible by summing up the peak areas of separated anthocyanins according to this procedure and calculating it together against the cyanidin-3-glucoside chloride standard at 520 nm. Total anthocyanins calculated by this approach will vary from that of the previous approach (calculated using molecular-weight-correction factors). This is due to the variability in responses (absorbance/concentration) of individual anthocyanins present in the sample.

Accuracy of the method was determined by spiking known amounts of cyd-3-rutinoside standard in Balaton tart cherry powder sample. Spike recoveries at levels of 129% and 158% were found to be 102% and 101%, respectively (Figure 6). Calibration curve ranges for cyd-3-rutinoside chloride and cyd-3-glucoside chloride were 4−400 ppm and 3.5−350 ppm, respectively, and afforded R^2 values of more than 0.999. Specificity of the method was determined by positively identifying the target anthocyanins on the basis of their molecular weights for each respective anthocyanin cation by LC/ES−MS prior to quantification. Accuracy of total anthocyanins using the molecular-weight-correction factor relies on identification of individual anthocyanins with aglycons having similar UV absorbance at detection wavelength.

This method affords choice of selection of an external anthocyanin standard that is commercially available for the quantification of related anthocyanins irrespective of their commercial availability in pure form. The basis of such selection should be its similarity with the anthocyanins present in the test sample on the basis of their UV spectrum. This can be easily achieved by selecting a commercially available anthocyanin standard that has an aglycon moiety (anthocyanidin) similar to those present in the anthocyanins in test sample. This would enable, as well as justify, the use of molecular-weight-correction factors for specific quantifications of anthocyanins in test samples. On the basis of this discussion, this method can be applied/adapted for source verification and quantification of individual, as well as total, anthocyanins in several other botanical raw materials that are standardized for specific amounts of anthocyanins: e.g., bilberry (*Vaccinium myrtillus* L.) and chokeberry (*Aronia melanocarpa* Elliott). Our preliminary experiments afforded a characteristic HPLC profile of anthocyanins present in bilberry and chokeberry raw materials (Figures 4 and 5). The peaks were positively identified according to the molecular weights obtained for various anthocyanin cations by LC−ES/MS. The major anthocyanins are in agreement with those reported earlier for bilberry (*11*) and chokeberry (*22*). Selecting the right external standards (e.g., cyanidin-

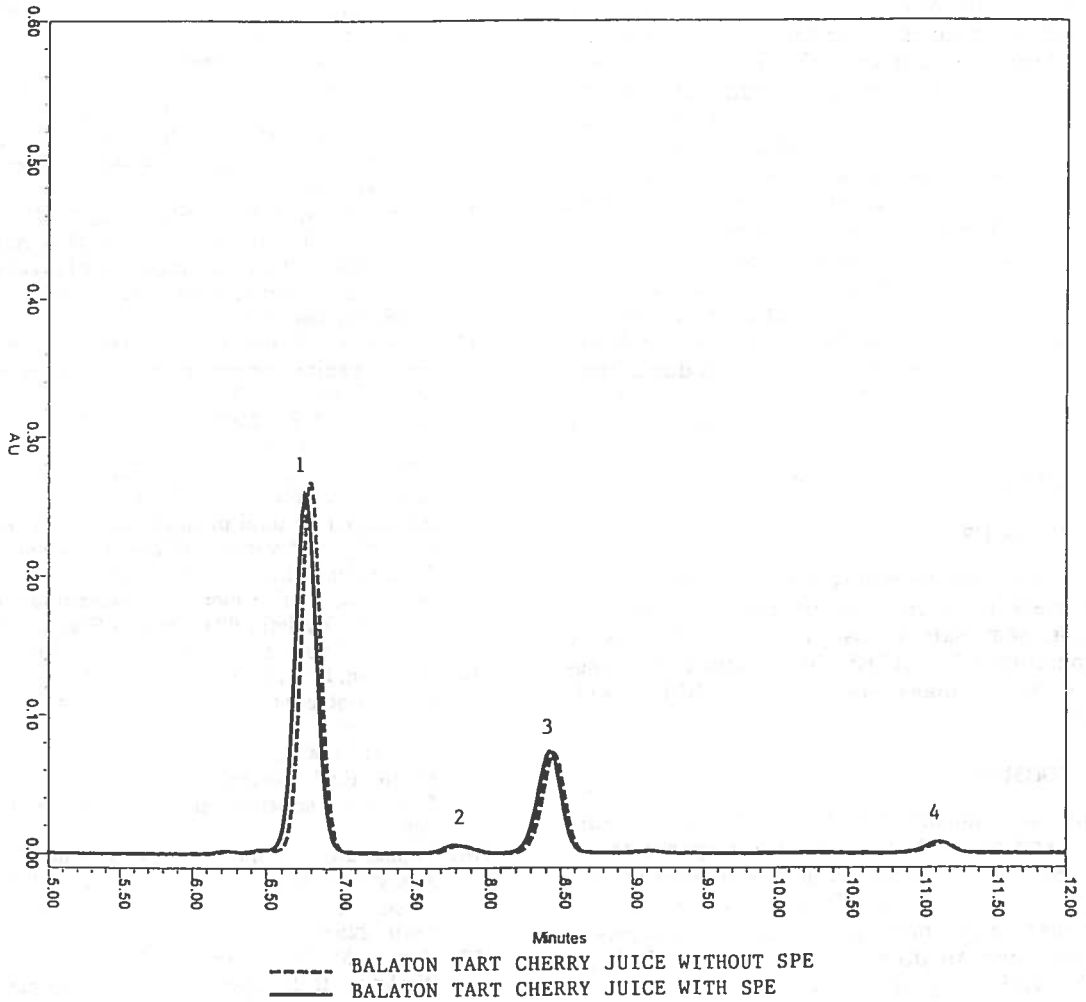


Figure 8. Overlayed HPLC chromatogram of Balaton tart cherry juice sample with and without SPE.

Table 1. Molecular-Weight-Correction Factors (MWCF) for Quantification of Individual Anthocyanins Identified in Balaton Tart Cherry and Elderberry against Cyd-3-glucoside (cyd + hex) as External Standard

HPLC peak no.	name[a]	analyte M^+ (cation)	standard M^+ (cation)	MWCF
1	cyd + hex + rut	757.22	449.1	1.69
2	cyd + pent + rut	727.21	449.1	1.62
3	cyd + rut	595.16	449.1	1.33
4	peo + rut	609.18	449.1	1.36
5	cyd + sam + hex	743.20	449.1	1.65
6	cyd + sam	581.20	449.1	1.29
7	cyd + hex	449.10	449.1	1.00
8	cyd	287.00	449.1	0.64

[a] Cyd, cyanidin; peo, peonidin; hex, hexose; pent, pentose; rut, rutinose; sam, sambubiose.

Table 2. Precision Results on Quantification of Individual as Well as Total Anthocyanins in Balaton Tart Cherry Fruits

analyst	replicates (n)	individual anthocyanins (mg/g) 1	2	3	4	total anthocyanins (mg/g)
1	5	0.373	0.017	0.094	0.017	0.501
2	5	0.390	0.018	0.099	0.018	0.526
	Std. Dev	0.012	0.001	0.004	0.001	0.018
	% RSD	3.14%	5.71%	4.15%	5.71%	3.51%

3-glucoside for chokeberry and delphinidin-, petunidin-, and malvidin-based anthocyanins for bilberry) according to the criteria mentioned in this article, quantification

Table 3. Precision Results on Quantification of Individual as Well as Total Anthocyanins in Balaton Tart Cherry Powder

analyst	replicates (n)	individual anthocyanins (mg/g) 1	2	3	4	total anthocyanins (mg/g)
1	5	13.54	0.67	3.41	0.75	18.37
2	5	13.61	0.68	3.49	0.75	18.45
	Std. Dev	0.05	0.007	0.056	0.00	0.06
	% RSD	0.37%	1.04%	1.62%	0.00%	0.31%

Table 4. Precision Results on Quantification of Individual as Well as Total Anthocyanins in Elderberry Powder

analyst	replicates (n)	individual anthocyanins (mg/g) 1	2	3	4	total anthocyanins (mg/g)
1	5	14.71	80.81	67.37	0.68	163.56
2	5	14.63	80.60	66.39	0.71	162.34
	Std. Dev	0.057	0.148	0.693	0.021	0.86
	% RSD	0.39%	0.18%	1.04%	0.01%	0.53%

of individual, as well as total, anthocyanins can be carried out despite the commercial unavailability of the anthocyanins present in these raw materials.

This method is very suitable for the generation and verification of characteristic fingerprints of anthocyanins present in several herbal raw materials used in the nutritional supplement industry as sources for anthocyanins in natural form. It involves direct sample preparation of raw materials for HPLC analysis and

avoids any thermal and acid hydrolysis steps, thus maintaining the integrity of anthocyanins in the test sample. It also affords a cost-effective, yet accurate, approach for quantification of anthocyanins that are not yet available as commercial standards. Experimental procedures have been substantiated using Balaton tart cherry and elderberry as type examples with validation parameters that are acceptable by most laboratories conducting nutritional supplement analysis. Good repeatability makes it a viable quality assurance method for quantification of individual, as well as total, anthocyanins in botanical supplements. It also can be used for optimization of a product that has to be preferentially standardized for specific anthocyanins due to their respective/preferential biological activities. As with any other analytical method, minor modifications might be necessary to adapt it to the requirements of product specifications and changes in sample matrix.

ABBREVIATIONS USED

Cyd, cyanidin; peo, peonidin; dpd, delphinidin; pet, petunidin; malv, malvidin; hex, hexose; pent, pentose; rut, rutinose; sam, sambubiose; HPLC, high-pressure liquid chromatography; LC/ES−MS, liquid chromatography/electrospray−mass spectrometry; SPE, solid-phase extraction.

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