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Comparison of HPLC Methods for Determination of Anthocyanins and Anthocyanidins in Bilberry Extracts

ZHE ZHANG,* XIAOLAN KOU, KEN FUGAL, AND JERRY MCLAUGHLIN

Department of Research and Development, Nature's Sunshine Products, Inc., 1655 North Main Street, Spanish Fork, Utah 84660

An HPLC method and an acid hydrolysis HPLC method for the analysis of anthocyanins and anthocyanidins in bilberry extracts have been developed. The HPLC method coupled with a mass detector has identified 11 anthocyanins in bilberry extracts. The method provides anthocyanin profiles that are very useful in verifying the identity of botanical raw materials, monitoring the consistency of the raw material source, and quantitating the total anthocyanins. The acid hydrolysis HPLC method greatly simplifies the anthocyanin profile in bilberry samples and converts anthocyanins to five major anthocyanidin aglycones: delphinidin, cyanidin, petunidin, peonidin, and malvidin. Each of these aglycones can be separated completely and quantitated accurately with external standards. Various extraction and hydrolysis conditions were investigated, and the advantages and disadvantages of the HPLC and acid hydrolysis methods are discussed.

KEYWORDS: HPLC; HPLC-MS; anthocyanin; anthocyanidin; bilberry extract

INTRODUCTION

Anthocyanins exist widely in fruits, flowers, and vegetables and are responsible for their bright colors such as orange, red, and blue (*1*). Recent and renewed interest in anthocyanins is due to not only their bright colors as natural colorants (*2*, *3*) but also their potential health benefits as antioxidants and antiinflammatory agents (*4*−*6*). Recent studies with human cells and rats demonstrated that dietary anthocyanins may have beneficial actions against the initiation and development of vascular diseases. They may also aid in the reduction of age-related deficiencies in neurological impairments (*7*−*9*).

In developed countries, public interest in the health benefits of phytoceuticals for reducing and inhibiting chronic diseases and aging has stimulated the nutritional supplement industry to develop "functional foods" and herbal supplements containing these ingredients. Product quality control, label claim verification, and raw material source identification require the development of simple, accurate, quantitative methods. There are many excellent papers on analysis of anthocyanins in juices, wines, berries, grapes, and vegetables (*10*, *11*). However, few papers deal with the analysis of anthocyanins in botanical extracts used in the supplement industry (*12*).

The most studied methods for separation and quantitation of anthocyanins are HPLC with UV−Vis or diode array detectors (DAD). HPLC coupled with a mass spectrometer (MS) detector is one of the most powerful approaches for identification of individual anthocyanins (*13*−*15*). HPLC analyses of anthocyanins in wines, juices, and fruits have been reviewed (*10*, *11*). The major challenge for HPLC quantitation of individual anthocyanins is often the difficulty in obtaining the anthocyanin reference compounds. There are more than 250 naturally existing anthocyanins which consist of one of six aglycones glycosylated with various sugar substitutes (*16*). Only a few anthocyanin reference compounds are commercially available.

Fortunately, the complex anthocyanidin glycoside pattern can be reduced to six major anthocyanidins by acid hydrolysis (*17*−*20*). These major aglycones are delphinidin, cyanidin, pelargonidin, petunidin, peonidin, and malvidin. Currently, all of these anthocyanidins except petunidin are commercially available. To our surprise, most people only used the hydrolysis to identify anthocyanin aglycones and associated sugars. Only a few papers reported the quantitation of anthocyanidin aglycones (*21*).

The antioxidant activity of anthocyanins varies with their aglycones (*5*−*7*, *22*). For example, among the anthocyanidins, delphinidin, but not malvidin or cyanidin, showed endothelium-dependent vasorelaxation (*7*). Cyanidin had a higher oxygen radical absorbing capacity than that of malvidin, peonidin, and pelargonidin (*6*). Recent studies in anthocyanin metabolism in rats have shown that cyanidin-3-*O*-β-D-glucoside is hydrolyzed in the intestines to aglycone cyanidin, then degraded to protocatechuic acid, which has a protective action in carcinogenesis (*23*, *24*). These results suggest the necessity for the development of simple and accurate methods for quantitation of individual anthocyanidins.

In this work, we developed an acid hydrolysis HPLC method for quantitation of individual anthocyanidins in bilberry extracts. We also developed a convenient HPLC method for checking the consistency of anthocyanin sources and quantitation of the total amount of anthocyanins. The direct HPLC method is most suitable for quality control, standardization of plant materials, and verification of raw material origins. The acid hydrolysis HPLC method is more suitable for quantitation of individual

* To whom correspondence should be addressed. Tel.: 801-798-4178. Fax. 801-798-4108. E-mail: zhez@natr.com.

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anthocyanidins. The advantages and disadvantages of these two methods are discussed, and the optimizations of extraction and hydrolysis conditions were determined.

MATERIALS AND METHODS

Chemicals. All solvents were HPLC grade from Fisher (Pittsburgh, PA). Water was purified by a reverse osmosis system (Culligan, Miami, FL) and prior to use passed through a 0.45-μm filter (Whatman, Maidstone, England). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO). All anthocyanins and anthocyanidins were purchased from ChromaDex (Santa Ana, CA) and used as received. Their purities were cyanidin-3-glucoside chloride 99.4%, pelargonidin-3-glucoside chloride 95.8%, peonidin-3-glucoside chloride >95.0%, malvidin-3-glucoside chloride 93.1%, delphinidin chloride 99.7%, cyanidin chloride 97.7%, peonidin chloride >99.0%, pelargonidin chloride 99.8%, and malvidin chloride 98.9%.

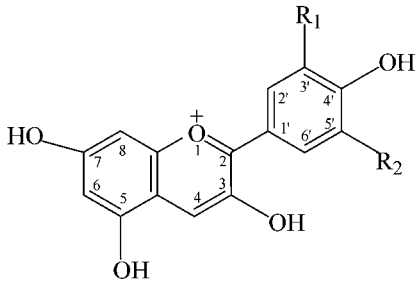
Standards and Calibration Curves. Commercially available reference anthocyanins were dissolved together in 1:1 water/methanol solution containing 2% HCl (50 mL of methanol + 48 mL of water + 2 mL of 37% concentrated HCl) to form a standard mixture of cyanidin-glucoside chloride (0.115 mg/mL), pelargonidin-3-glucoside chloride (0.066 mg/mL), peonidin-3-glucoside chloride (0.040 mg/mL), and malvidin-3-glucoside chloride (0.130 mg/mL). The anthocyanidins were dissolved together in a methanol solution containing 2 N HCl (17 mL of 37% concentrated HCl + 83 mL of methanol) to form a standard mixture of delphinidin chloride (0.161 mg/mL), cyanidin chloride (0.192 mg/mL), pelargonidin chloride (0.066 mg/mL), peonidin chloride (0.056 mg/mL), and malvidin chloride (0.125 mg/mL). The anthocyanin standard mixture was diluted to 1/5 and 1/10 of the initial concentrations with 1:1 water/methanol containing 2% HCl. The anthocyanidin standard mixture was diluted to 3/5, 2/5, 1/5, and 1/10 of the initial concentrations with the methanol solution containing 2 N HCl. Standard solutions were injected separately under direct HPLC conditions and acid hydrolysis HPLC conditions to generate calibration curves for all reference compounds. All calibration curves were linear, with $R^2 \geq 0.999$.

Sample Preparation. Bilberry extract powder was obtained from Van Drunen Farms (Momence, IL) and Indena (Seattle, WA). In the direct analysis, samples were dispersed in a 1:1 water/methanol solution containing 2% HCl and sonicated for 20 min. The concentrations were ~1 mg/mL for bilberry extract. A portion of each sample solution was passed through a 0.45-μm PTFE filter (Fisher, Pittsburgh, PA) prior to HPLC injection.

In the hydrolysis analysis, samples were dispersed in 1:1 water/methanol solution containing 2 N HCl (50 mL of methanol + 33 mL of water + 17 mL of 37% HCl) and sonicated for 20 min. Sample concentrations were similar to those in HPLC analyses. A portion of each sample solution (~3 mL) was filtered through a 0.45-μm PTFE filter and transferred to a vial with a Teflon-lined screw cap. The vial was placed in a preheated dry-bath (Pierce, Model Reacti-therm III, Rockford, IL) and hydrolyzed at 100 ± 2 °C for 60 min. Hydrolyzed samples were immediately cooled to room temperature for analysis.

HPLC-DAD Analysis and HPLC-MS Identification. HPLC analyses were conducted on a Hewlett-Packard 1090 HPLC with a DAD. The separation of anthocyanins and anthocyanidins was accomplished on a Beckman Ultrasphere ODS column (5-μm, 4.6 × 250 mm). In direct analyses, mobile phases were A, 0.4% TFA in water and B, 0.4% TFA in acetonitrile. The gradient condition was 0−6 min, 15% B; 6−20 min, 15−22% B; and 20−35 min, 22−30% B. Other chromatographic conditions were as follows: flow rate, 1 mL/min; column temperature, 35 °C; detection, 525 nm; 20 μL injection; and postrun time, 5 min. In hydrolysis analyses, the anthocyanidins were separated isocraticly with 18% B and the detection wavelength was 530 nm. Other conditions were the same as in direct analyses.

HPLC-MS analyses were performed on a Hewlett-Packard 1100 coupled with a DAD and a Finnigan LCQDUO mass spectrometer. The mass spectrometer was operated at positive ion mode with an atmospheric pressure chemical ionization (APCI) probe at a temperature of 450 °C and a voltage of 4.5 kV. The mass scan range was 200−800 amu. HPLC conditions were the same as in the direct HPLC analysis.



Anthocyanidin	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidin	OH	OH
Petunidin	OCH_3	OH
Malvidin	OCH_3	OCH_3

Figure 1. The structure of anthocyanidins.

RESULTS AND DISCUSSION

Structure and Visible Spectra of Anthocyanidins. Anthocyanidins belong to flavonoid families that are based on the $C_6-C_3-C_6$ configuration in the flavan nucleus (*1*). There are six common anthocyanidins naturally occurring in berry fruits. **Figure 1** shows the structure of these anthocyanidins. Anthocyanins are anthocyanidins linked with one or more sugar moieties. The most common sugars are glucose, galactose, and arabinose. These sugars can also be acylated by acetic acid and coumaric acid.

Anthocyanidins vary in the pattern of hydroxylation and methoxylation, and this variation greatly influences the spectra of individual anthocyanidins. The solution acidity also dramatically affects spectra of anthocyanidins. Under our hydrolysis HPLC separation condition, the spectra obtained from HPLC-DAD showed pelargonidin had a maximum absorption at 512 nm. Cyanidin and peonidin had almost identical spectra and gave a maximum absorption at 524 nm. Delphinidin, petunidin, and malvidin had similar spectra with maximum absorption at wavelength of 530 nm, 532 nm, and 534 nm, respectively.

Extraction. Usual solvents for extraction of anthocyanins are an acidic methanol solution or an acidic aqueous/methanol solution (*12*, *25*). We investigated the extraction yields with various ratios of aqueous/methanol, water, and methanol, and found the acidic aqueous/methanol solution gave the best extraction yield

HPLC-MS Identification. In direct HPLC chromatograms (**Figure 2**), more than 15 peaks in bilberry extracts were detected by the UV−vis detector. Nine major peaks have been identified by HPLC-MS as delphinidin-3-galactoside, $M^+ = 465$ (**1**); delphinidin-3-glucoside, $M^+ = 465$ (**2**); delphinidin-3-Arabinoside, $M^+ = 435$ + cyanidin-3-galactoside, $M^+ = 449$ (**3**); cyanidin-3-glucoside, $M^+ = 449$ (**4**); petunidin-3-glucoside, $M^+ = 479$ (**5**); cyanidin-3-arabinoside, $M^+ = 419$ (**6**); unknown, $M^+ = 369$ (**7**); peonidin-3-glucoside, $M^+ = 463$ (**8**); malvidin-3-galactoside, $M^+ = 493$ (**9**); malvidin-3-glucoside, $M^+ = 493$ + unknown, $M^+ = 519$ (**10**).

Hydrolysis Method. After acid hydrolysis, the anthocyanin profile had fewer peaks. More than 15 peaks in bilberry samples became 5 major anthocyanidin peaks (**Figure 3**). They were delphinidin, $M^+ = 303$ (**11**); cyanidin, $M^+ = 287$ (**12**); petunidin, $M^+ = 317$ (**13**); peonidin, $M^+ = 301$ (**14**); and malvidin, $M^+ = 331$ (**15**).

At various times, the hydrolysis of anthocyanins was conducted with 2 N HCl in 1:1 water/methanol solution. A 30

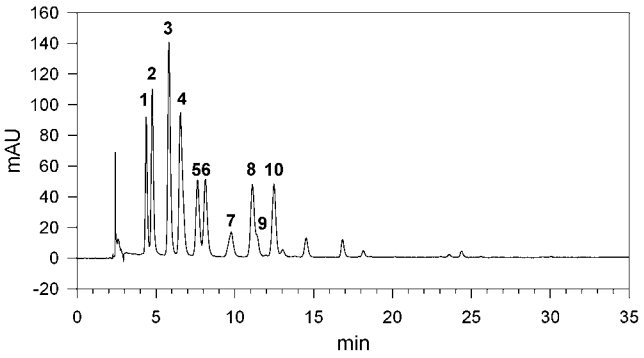


Figure 2. HPLC chromatograms for bilberry extracts. Samples were extracted with 2% HCl in 1:1 water/methanol. Peaks: delphinidin-3-galactoside, $M^+ = 465$ (1); delphinidin-3-glucoside, $M^+ = 465$ (2); delphinidin-3-arabinose, $M^+ = 435$ + cyanidin-3-galactoside, $M^+ = 449$ (3); cyanidin-3-glucoside, $M^+ = 449$ (4); petunidin-3-glucoside, $M^+ = 479$ (5); cyanidin-3-arabinoside, $M^+ = 419$ (6); unknown, $M^+ = 369$ (7); peonidin-3-glucoside, $M^+ = 463$ (8); malvidin-3-galactoside, $M^+ = 493$ (9); malvidin-3-glucoside, $M^+ = 493$ + unknown, $M^+ = 519$ (10).

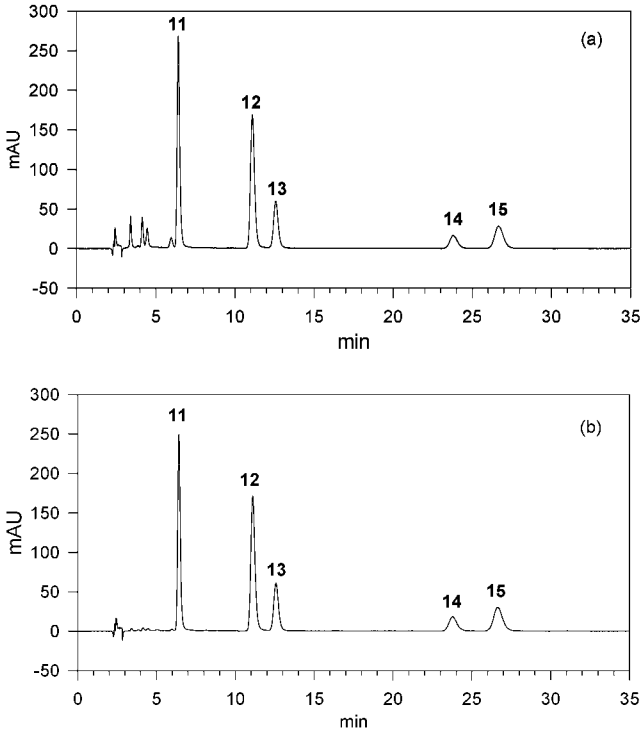


Figure 3. HPLC chromatograms of bilberry extracts after 30 min acid hydrolysis (a) and 60 min acid hydrolysis (b). Peaks: delphinidin, $M^+ = 303$ (11); cyanidin, $M^+ = 287$ (12); petunidin, $M^+ = 317$ (13); peonidin, $M^+ = 301$ (14); malvidin, $M^+ = 331$ (**15**).

min hydrolysis reaction converted most anthocyanins to anthocyanidins, with approximately 10% anthocyanins (peaks with retention time 3−6 min) still detected (**Figure 3a**). After 60 min of hydrolysis, anthocyanin peaks almost completely disappeared (**Figure 3b**). **Table 1** gives individual anthocyanidin percentages at various hydrolysis times. Hydrolyses at 1 and 2 hours gave similar amounts of total anthocyanidins and similar amounts of individual anthocyanidins. Delphinidin was an exception, the delphinidin amount decreased slightly (~3%) with increased hydrolysis time. This suggests delphinidin is not stable under hydrolysis conditions.

Recovery and Repeatability. The recovery test was done in triplicate by spiking the bilberry extract with cyanindin-3-glucoside before hydrolysis. The amount of cyanidin aglycone was calculated using the cyanidin chloride standard curve after complete hydrolysis of glycosides in the sample. The average recovery and standard deviation of three spike samples was 102 ± 2 (%). The repeatability was tested by analyzing a bilberry sample three times over 24 days. The relative standard deviations (RSD%) were 1−3% for individual anthocyanidin and 2% for total anthocyanidins.

Table 1. Effect of Hydrolysis Time on Anthocyanidin Yields[a]

hydrolysis (hour)	delphinidin (%)	cyanidin (%)	petunidin (%)	peonidin (%)	malvidin (%)	total (%)
0.5	7.85	9.05	2.97	3.39	2.95	26.2
1	7.71	9.85	3.31	3.99	3.18	28.0
2	7.44	9.79	3.25	4.00	3.20	27.7

[a] Results were obtained with triplicate samples.

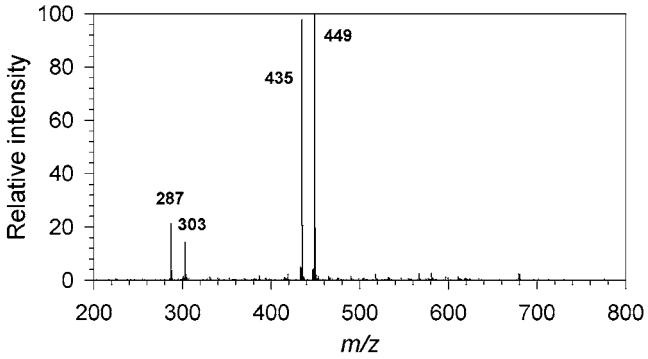


Figure 4. Mass spectrum of peak (3) in **Figure 2**, HPLC chromatograms of bilberry extracts. *m/z* 287, cyanidin; *m/z* 303, delphinidin; *m/z* 435 cyanidin-3-arabinoside; *m/z* 449, cyanidin-3-galactoside.

Comparison of HPLC Analysis and Hydrolysis HPLC Analysis. The HPLC analysis gives information of the anthocyanin profile and is very useful for the control of product quality, identification of sample origins, and examination of consistency in raw materials. The greatest disadvantage of anthocyanin HPLC quantitation is the unavailability of reference compounds. Cyanidin-3-glucoside is usually used as a reference compound to calculate total anthocyanins (*12*). The molar absorbance difference of individual anthocyanidins at selective wavelength and the incomplete separation of anthocyanins will influence the accuracy of quantitative results. For example, peak 3 and peak 10 in bilberry HPLC chromatograms (**Figure 2**) apparently are singlets. When mass spectrometry was used for detection, the spectra clearly indicated that peaks 3 and 10 were mixtures of two different compounds. The complete separation of all these anthocyanins in single HPLC run is very difficult. The mass spectrum of peak 3 is given in **Figure 4**. The *m/z* values of 435 and 449 corresponded to the M^+ of delphinidin-3-arabinoside and cyanidin-3-galactoside, respectively, and the *m/z* 287 and 303 corresponded to cyanidin and delphinidin, respectively. Peak 10 was a mixture of malvidin-3-glucoside (*m/z* 493) and an unknown compound (*m/z* 519). The mass spectrum of this unknown compound contained two characteristic fragments at *m/z* 303 (delphinidin) and 465 (delphinidin-3-glucoside or delphinidin-3-galactoside). This incomplete separation makes the quantitation of individual anthocyanins very difficult and also influences the accuracy of total anthocyanin calculations.

Acid hydrolysis greatly simplifies the anthocyanin profile, and the five anthocyanidin aglycones can be completely separated (**Figure 3**). Moreover, all six aglycone reference

Table 2. Comparison of Anthocyanidin Results Obtained by the HPLC Method and the Acid Hydrolysis Method

HPLC[a]	delphinidin (%)	cyanidin (%)	petunidin (%)	peonidin (%)	malvidin (%)	total (%)
bilberry	6.93	7.65	2.30	1.81	2.89	21.6
RSD	1.4	1.6	2.7	1.8	2.7	1.8
Hydrolysis[b]						
bilberry	6.86	8.48	3.29	1.47	3.08	23.2
RSD	0.22	0.25	1.18	1.22	0.99	0.26

[a] Results were obtained with triplicate samples and converted to the correspondent anthocyanidins. [b] Hydrolysis was done with 2 N HCl in 1:1 water/methanol for 1 h. Results were obtained with six samples.

standards except petunidin are commercially available. This makes accurate quantitation possible.

Anthocyanidin results (from the conversion of anthocyanins to anthocyanidins) of HPLC analysis and acid hydrolysis HPLC analysis are given in **Table 2**. Both methods gave good precision for bilberry extracts. The relative standard deviation (RSD) for total anthocyanidins was 1.8% for the HPLC method and 0.3% for the hydrolysis method.

The HPLC acid hydrolysis method gave results of greater quantity than the HPLC method. The difference may be due to incomplete separation and some small peaks not being integrated in the HPLC analysis. For instance, the peaks in the range of 14−25 min retention time were not integrated (**Figure 2**). The visible spectra of these peaks were similar to those of anthocyanidins, but we could not confirm this by LC-MS due to weak MS signals.

The HPLC analysis before hydrolysis provides an anthocyanin profile that is very useful for the control of product quality and the identification and consistency of raw materials. However, the acid hydrolysis HPLC analysis is more suitable for quantitation of individual anthocyanidins. Both methods could be applicable to other botanical extracts.

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