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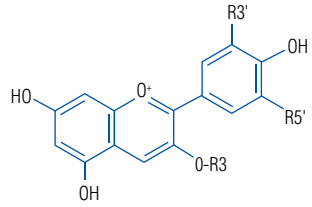
Rapid and Sensitive Determination of Anthocyanins in Bilberries Using UHPLC

INTRODUCTION

Bilberry (*Vaccinium myrtillus* L.) is a low-growing shrub that is native to temperate regions of North America and Europe. The shrubs are closely related to the native North American wild blueberries, but one characteristic difference is that bilberry plants produce single or paired berries on the bush, unlike blueberries that grow in clusters. In addition, bilberries are smaller, darker, hard, less juicy, easier to transport, and have a different flesh color than blueberries. The two berries also have different phytochemical profiles, with the anthocyanin content of fresh bilberry fruits being almost 4× higher than that of blueberries.[1] Bilberries—known to have a high anthocyanin content—cannot be cultivated, are hard to harvest and process, and therefore are one of the most expensive botanical ingredients in the health food industry. The high price of the extract makes it more susceptible to adulteration.

Bilberry extracts are widely used in nutritional supplements and pharmaceuticals for improving visual acuity and treating circulatory disorders. Chemical and pharmacological studies have identified anthocyanins as the main components responsible for the therapeutic effect of the extracts that are used in these supplements. Clinical trials on therapeutic products using bilberry extracts have shown that a 36% anthocyanin level is effective in the treatment of peripheral vascular disease and venous sensitivity.[2]



R3'	R5'	R3	Anthocyanin
H	H	Glucose/Arabinose/Galactose	Pelargonidin 3-Glucose/Arabinose/Galactose
OH	H	Glucose/Arabinose/Galactose	Cyanidin 3-Glucose/Arabinose/Galactose
OH	OH	Glucose/Arabinose/Galactose	Delphinidin 3-Glucose/Arabinose/Galactose
OCH_3	OH	Glucose/Arabinose/Galactose	Petunidin 3-Glucose/Arabinose/Galactose
OCH_3	H	Glucose/Arabinose/Galactose	Peonidin 3-Glucose/Arabinose/Galactose
OCH_3	OCH_3	Glucose/Arabinose/Galactose	Malvidin 3-Glucose/Arabinose/Galactose

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Figure 1. Basic structure of anthocyanins.

The anthocyanins present in bilberries are C-3 glucosides, galactosides, and arabinosides of the anthocyanidin: delphinidin, cyanidin, petunidin, peonidin, and malvidin (Figure 1).[3-5] Therefore, a reliable, simple, and reproducible analytical method is needed to determine anthocyanins in bilberry-based nutraceutical products to ensure their efficacy.

High-performance liquid chromatography (HPLC) is the method of choice for the determination of anthocyanins because this technique allows an efficient separation, identification, and quantification of anthocyanins in bilberries and related products. These characteristics are demonstrated in Dionex Application Note 264,[6] which describes the determination of anthocyanins in pomegranate-related juices. For bilberry-related supplements, the European,[7] Italian,[8] and United States Pharmacopeias[9] specify HPLC for the determination of anthocyanins with an analysis time of 50 min or more. These methods use cyanidin 3-glucoside as an external standard to calculate the concentrations of the individual anthocyanins using a molecular weight correction factor.[10]

This study describes a sensitive, fast, and accurate HPLC method to determine anthocyanins in bilberry products. The method uses a high-resolution silica-based 2.2 µm Thermo Scientific Acclaim® RSLC 120 C18 column and a wavelength of 520 nm to separate, detect, and quantify anthocyanins in several commercially available bilberry nutritional supplements. The method demonstrates good sensitivity, enabling the detection of a wide variety of anthocyanins with concentrations ranging from 0.25 µg/mL for peonidin 3-galactoside to 24.3 µg/mL for delphinidin 3-glucoside and a total run time of less than 30 min. The reported limits of detection (LOD) using the method ranged from 0.20 µg/mL for petunidin 3-glucoside to 1.56 µg/mL for delphinidin 3-glucoside, and limits of quantitation (LOQ) ranged from 0.78 µg/mL for petunidin 3-glucoside to 6.25 µg/mL for delphinidin 3-glucoside. The method described here is ideal for simple, sensitive, accurate, rapid, and routine analysis of anthocyanins in different bilberry containing nutritional products.

EQUIPMENT

Thermo Scientific Dionex UltiMate® 3000 RSLC System

SRD-3600 Solvent Rack with 6 degasser channels (P/N 5035.9230) and Eluent Organizer, including pressure regulator, and 2 L glass bottles for each pump. Eluents were maintained under helium or nitrogen headspace (5–8 psi)

HGP 3400RS Pump (P/N 5040.0046)

WPS-3000TRS Well Plate Sampler (P/N 5840.0020)

Sample Loop, 25 µL (P/N 6820.2415)

TCC-3000RS UltiMate 3000RS Column Compartment (P/N 5730.0000)

DAD-3000RS Photodiode Array Detector (P/N 5082.9920)

Semi-Micro Flow Cell for DAD-3000 and MWD-3000 Series, SST, 2.5 µL Volume, 7 mm Path Length (P/N 6080.0300)

CONSUMABLES

Acclaim RSLC 120 C18, 2.2 µm, Analytical, 2.1 × 150 mm (P/N 059130)

Centrifuge equipped with a ten-place, aluminum fixed-angle rotor (Beckman Spinchron R, GS-6R Series, Beckman Coulter P/N 358702 or equivalent)

Thermo Scientific Dionex Viper™ SST Flex. –Cap., i.d. × L: 0.13 × 250 mm (P/N 6040.2325)

Dionex Viper SST Flex. –Cap., i.d. × L: 0.13 × 350 mm (P/N 6040.2335)

Dionex Viper SST Flex. –Cap., i.d. × L: 0.18 × 450 mm (P/N 6040.2365)

Static mixer, mixing volume: 350 µL (P/N 6040.0040)

Glass injection vials with caps and septa, 1.5 mL (P/N 055427)

Borosilicate glass scintillation vials with closures attached, 20 mL (VWR P/N 66022-129)

REAGENTS AND STANDARDS

Reagent-grade water Type I, 18 MΩ-cm resistance or better, filtered through a 0.2 µm filter immediately before use. Referred to here as deionized (DI) water.

Acetonitrile, HPLC Grade (Honeywell P/N AH015-4)

Formic Acid, 98% Pure (Fluka P/N 06440)

Delphinidin 3-Glucoside (Cerilliant P/N 89627)

Cyanidin 3-Galactoside (Cerilliant P/N C-070)

Cyanidin 3-Glucoside (Cerilliant P/N 89616)

Petunidin 3-Glucoside (Cerilliant P/N P-057)

Peonidin 3-Galactoside (Cerilliant P/N P-058)

Malvidin 3-Galactoside (Cerilliant P/N 80600)

Peonidin 3-Arabinoside (Cerilliant P/N 82247)

Cyanidin Chloride (Cerilliant P/N 80022)

Malvidin Chloride (Cerilliant P/N 80083)

Petunidin Chloride (Cerilliant P/N 80225)

Peonidin Chloride (Cerilliant P/N 80085)

Delphinidin Chloride (Cerilliant P/N 89625)

Note: The anthocyanidin standards were only used to confirm retention times.

Mixture of 15 monoglycosides from bilberry (Polyphenols P/N 1826), see Table 1.

Table 1. Mixture of 15 Monoglycosides from Bilberry

Analyte	Abbreviation
Delphinidin 3-galactoside	Dp3Gal
Delphinidin 3-glucoside	Dp3Glu
Cyanidin 3-galactoside	Cy3Gal
Delphinidin 3-arabinoside	Dp3Ara
Cyanidin 3-glucoside	Cy3Glu
Petunidin 3-galactoside	Pet3Gal
Cyanidin 3-arabinoside	Cy3Ara
Petunidin 3-glucoside	Pet3Glu
Peonidin 3-galactoside	Peo3Gal
Petunidin 3-arabinoside	Pet3Ara
Peonidin 3-glucoside	Peo3Glu
Malvidin 3-glactoside	Mal3Gal
Peonidin 3-arabinoside	Peo3Ara
Malvidin 3-glucoside	Mal3Glu
Malvidin 3-arabinoside	Mal3Ara

SAMPLES

National Institute of Standards and Technology (NIST) Standard Reference Materials (SRM) 3291 – Bilberry Extract

Powdered Bilberry Extract (USP Reference Standard, 1071268)

Eye Antioxidant

Nutritional Supplement Brand A

Nutritional Supplement Brand B

CONDITIONS

Column: Acclaim RSLC 120 C18, 2.2 µm (2.1 × 150 mm)

Eluents: A: 10% Formic Acid
B: 10% Formic Acid, 22.5% Methanol, 22.5% Acetonitrile

Flow Rate: 0.475 mL/min

Inj. Volume: 2.0 µL

Tray Temp.: 4 ºC

Column Temp.: 35 ºC

Detection: Absorbance, vis, 520 nm

System Backpressure: ~6700–7400 psi during the gradient

Gradient Conditions:	*Time (min)*	*A %*	*B %*
	0	91	9
	12	91	9
Step Change	25	65	35
	25	50	50
Step Change	30	50	50
	30	91	9
	35	91	9

PREPARATION OF SOLUTIONS AND REAGENTS

Formic Acid (10%)

Transfer 200 mL of formic acid into a glass 2 L volumetric flask containing approximately 1700 mL of DI water. Bring to volume using DI water and invert flask several times to mix.

Formic Acid (10%), Methanol (22.5%), Acetonitrile (22.5%)

Transfer 200 mL of DI water into a 1 L glass volumetric flask, then add 100 mL of formic acid. Transfer 225 mL of methanol and 225 mL of acetonitrile to the flask. Invert the flask several times to mix the contents and bring to volume using DI water.

Acidified Methanol (2% Hydrochloric Acid in Methanol)

Transfer 20 mL of hydrochloric acid to 800 mL of methanol in a glass 1 L volumetric flask. Allow the mixture to cool, invert to mix several times, and bring to volume using methanol.

Phosphoric Acid (10%)

Transfer 100 mL of phosphoric acid to 800 mL of DI water in a glass 1 L volumetric flask. Invert to mix several times and bring to volume using DI water.

Standards (100 µg/mL)

Prepare anthocyanin standards of delphinidin 3-glucoside, cyanidin 3-galactoside, cyanidin 3-glucoside, petunidin 3-glucoside, peonidin 3-galactoside, malvidin 3-galactoside, and peonidin 3-arabinoside by accurately weighing 10 mg of solid into a 100 mL glass volumetric flask and adding 2 mL of acidified methanol, followed by approximately 90 mL of 10% phosphoric acid. Invert several times to dissolve all the contents and bring to volume using 10% phosphoric acid. The stock solids can be stored at -40 ºC in glass vials until needed. All standard concentrate solutions can be stored for up to six months at -40 ºC and protected from light.

Mixed 15 Anthocyanin Standard (1.25 mg/mL)

Accurately weigh 125 mg of solid (the mixture of 15 monoglycosides from bilberry, obtained from Polyphenols) into a 25 mL glass volumetric flask, then add 15 mL of acidified methanol. Mix by inversion to dissolve, then bring the volume to 25 mL using acidified methanol. Pipet 5.0 mL of this solution into a 20 mL glass volumetric flask and bring to volume using 10% phosphoric acid. The standard can be stored at -40 ºC in glass vials protected from light for up to six months until needed.

Working Standards and Standards for Method Linearity

To prepare working standards, use a calibrated pipette to deliver the appropriate volume of the 100 µg/mL stock standard into a glass vial containing the appropriate volume of 10% formic acid in water. To prepare mixed anthocyanin working standards, combine appropriate volumes of the stock mixed anthocyanin standard into a glass vial containing the appropriate volume of mobile phase A. Diluted intermediate standards are stable for three months at -40 °C and working and mixed standards are stable for four weeks at 2–4 °C.

SAMPLE PREPARATION

The SRM 3291 used in this study was provided by NIST as part of a separate collaborative study. All commercial nutritional and eye antioxidants were purchased locally.

Commercial Samples, NIST SRM 3291, and USP Standard

To prepare samples, empty the entire contents of one capsule into a glass vial. The amount of solid per capsule can vary from 220 mg to 330 mg, depending on the brand of bilberry-based nutritional supplement. Accurately weigh 12.5 mg of the solid stored in the glass vial into a 25 mL glass volumetric flask and add 15 mL of acidified methanol. Mix by inversion to dissolve, then bring the volume to 25 mL using acidified methanol. Pipet 5.0 mL of this solution into a 20 mL glass volumetric flask and bring to volume using 10% phosphoric acid. Prior to analysis, filter the sample using a 0.2 µm cellulose acetate sterile syringe filter. Once the sample is prepared, discard the remaining solid obtained from the capsule and use a new capsule for every sample preparation.

RESULTS AND DISCUSSION

Separation of the Mixture of 15 Monoglycoside Bilberry Standard

The initial investigation for the separation of anthocyanins used a 2.2 µm Acclaim RSLC 120 C18 column in the 2.1 × 150 mm format. This column format was chosen to increase sample throughput and reduce sample and eluent consumption. Shorter column formats were also evaluated, but the 2.1 × 150 mm format was chosen because this column provided the best resolution of the target compounds. Figure 2 shows the separation of a standard mixture containing 15 monoglycosides derived from bilberries in less than 30 min.

The five anthocyanidins inherent to bilberries—delphinidin, petunidin, cyanidin, peonidin, and malvidin—were also separated in the standard mixture using this method. The identity of each of the peaks was confirmed by LC-mass spectrometry (MS) using a Thermo Scientific MSQ Plus™ mass spectrometer, which is described in Dionex AB 134.[11]

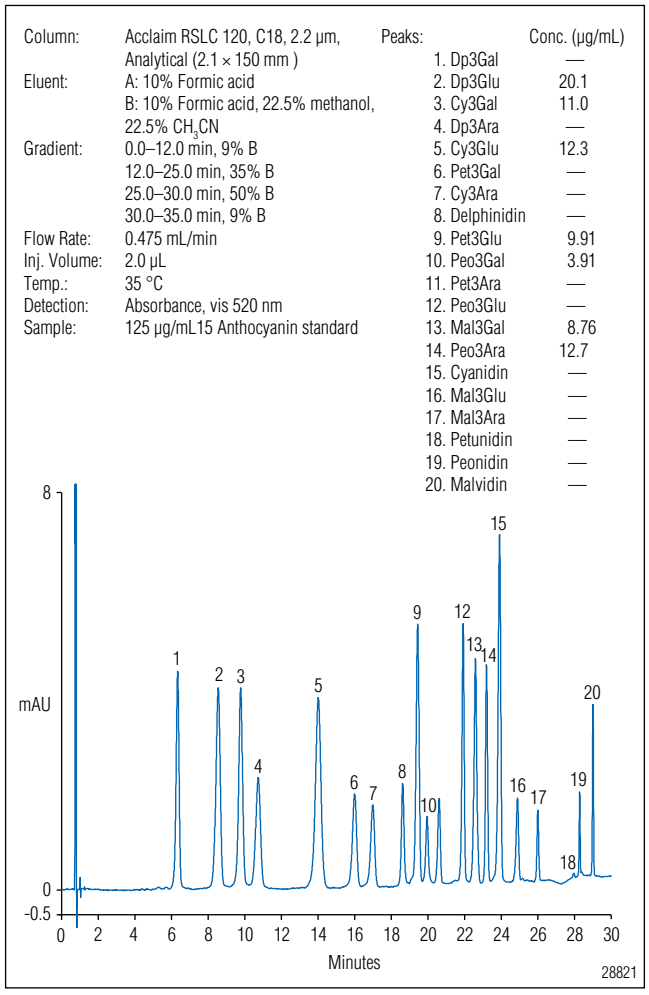

Figure 2. Separation of 15 anthocyanins and five anthocyanidins on the Acclaim RSLC 120 C18 column.

Table 2. Data for Linearity, LOD, and LOQ of Anthocyanins							
Analyte	Range (µg/mL)	Coefficient of Determination (r^2)	LOD[a] (µg/mL)	LOQ[b] (µg/mL)	RSD		
					Ret. Time (n=30)	Peak Area (n=30)	Peak Height (n=30)
Dp3Glu	0.39–50	0.9997	1.56	6.25	0.15	0.60	0.88
Cy3Gal	0.39–25	0.9996	0.78	3.13	0.12	0.97	0.89
Cy3Glu	0.39–25	0.9999	0.78	3.13	0.14	1.29	0.76
Pet3Glu	0.39–25	0.9999	0.20	0.78	0.08	1.51	1.32
Peo3Gal	0.39–10	0.9988	0.56	2.34	0.03	1.05	1.12
Peo3Ara	0.39–25	0.9997	0.78	3.13	0.03	0.50	0.52
Mal3Gal	0.39–25	0.9993	0.20	0.78	0.01	0.72	0.97

[a]Estimated from 3 × S/N
[b]Estimated from 10 × S/N

Preliminary Sample Analysis

Prior to analyzing commercial nutraceutical samples, a NIST reference standard was evaluated for its anthocyanin profile using the method described here. The reference standard was prepared as described in the Sample Preparation section. The chromatography demonstrated that all peaks were resolved, suggesting that the method could be used for further system suitability studies (data not shown).

System Suitability

The linearity, limits of detection, and limits of quantification were evaluated to determine the suitability of the method for this analysis. To determine the appropriate calibration ranges for the target compounds, each sample was analyzed and compared to a mixed anthocyanin standard containing known amounts of Dp3Glu, Cy3Gal, Cy3Glu, Pet3Glu, Peo3Gal, Mal3Gal, and Peo3Ara.

As shown in Table 2, the anthocyanins exhibited linear peak area responses in their respective target ranges. The LODs for the anthocyanins were determined based on the concentration of the analyte that provides a peak height of 3× the measured baseline noise (S/N = 3), whereas the LOQs were determined as the concentration of the analyte that provides a peak height of 10× the measured baseline noise (S/N = 10). The LODs ranged from 0.20 µg/mL for Mal3Gal to 1.56 µg/mL for Dp3Glu and the LOQs from 0.78 µg/mL for Mal3Gal to 6.25 µg/mL for Dp3Glu.

Retention time precisions of the standards were excellent with relative standard deviations (RSD) ranging from 0.01% for Mal3Gal to 0.15% for Dp3Glu. This demonstrates good precision of the gradient delivered by the HPG-3400RS. Peak area precision ranged from 0.50% for Peo3Ara to 1.51% for Pet3Glu, whereas peak height precision ranged from 0.52% for Peo3Ara to 1.32% for Pet3Glu (n=30).

Sample Analysis

A NIST reference sample was provided by the National Institute of Health Office of Dietary Supplements (NIH-ODS) for a collaborative study to determine organic acids, but did not have any certified anthocyanin values to compare with the values obtained in this study. This reference sample and a USP bilberry extract were used to determine anthocyanins prior to the analysis of commercial nutritional supplements. Both samples were prepared as described in the Sample Preparation section.

Table 3 summarizes the anthocyanin concentrations of seven anthocyanins in the USP and NIST reference samples. The individual anthocyanin concentrations for the NIST sample ranged from 1.53 µg/mL for Peo3Gal to 14.91 µg/mL for Dp3Glu. The anthocyanin concentrations for the USP sample ranged from 1.45 µg/mL for Peo3Gal to 12.22 µg/mL for Dp3Glu.

Three different nutraceutical products were evaluated for their anthocyanin content. The samples investigated in this study included two different brands of bilberry extract-based nutritional supplements and a bilberry extract-based eye antioxidant. Figure 3 shows the separation of anthocyanins in the bilberry-based eye antioxidant. This commercially available product contains an eye support blend with bilberry fruit extract, lutein derived from marigold flower extract, and zexanthin from marigold and paprika extract. Lutein and zexanthin levels of this product were not quantified, but selected anthocyanins were quantified and ranged from 0.28 µg/mL for Peo3Gal to 5.15 µg/mL for Dp3Glu. Fourteen different anthocyanins were identified in this sample, but only seven were quantified due to availability issues with standards for the rest of the anthocyanins. No interferences were observed and all the anthocyanins were well separated.

Table 3. Determination of Anthocyanins in NIST and USP Reference Samples

Analyte	NIST (µg/mL)	USP (µg/mL)
Dp3Glu	14.9	12.2
Cy3Gal	8.01	7.00
Cy3Glu	7.59	6.61
Pet3Glu	6.08	5.14
Peo3Ara	5.76	5.22
Peo3Gal	1.53	1.45
Mal3Gal	2.58	2.24

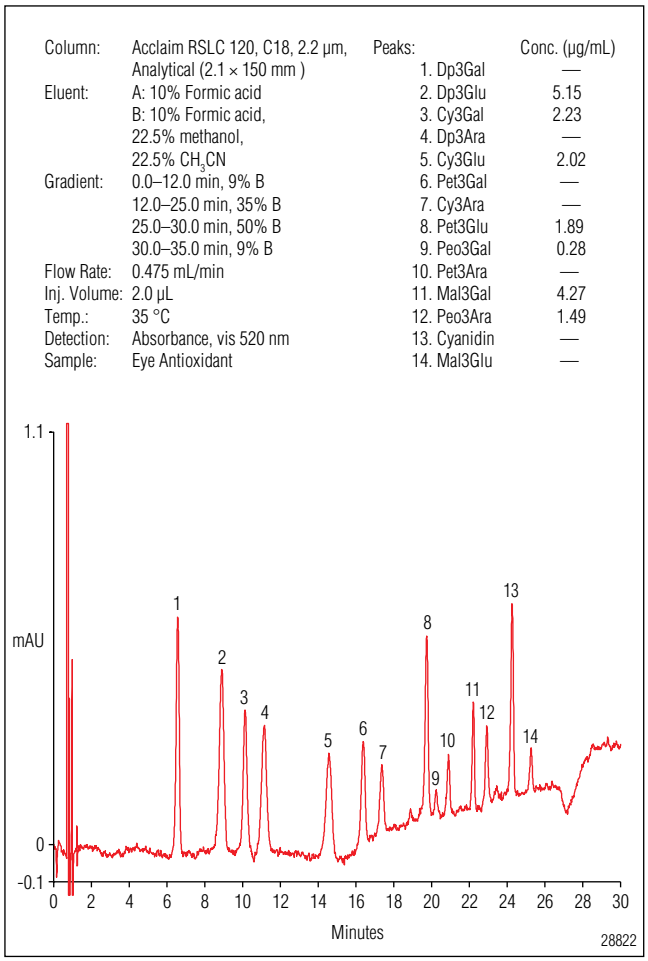

Figure 3. Separation of anthocyanins in a sample of bilberry-based eye antioxidant.

Figure 4 shows the separation of anthocyanins in bilberry-based Nutritional Supplement A. The label of this product indicates that it contains a blend of bilberry leaf and bilberry extract. Other ingredients include modified vegetable cellulose and magnesium stearate. Cellulose and magnesium stearate levels of this product were not quantified, but selected anthocyanins were quantified and ranged from 3.31 µg/mL for Peo3Gal to 21.69 µg/mL for Dp3Glu. All 15 anthocyanins that are present in bilberries were found in this product. Additionally, five anthocyanidins were also separated, which included delphinidin, cyanidin, peonidin, petunidin, and malvidin.

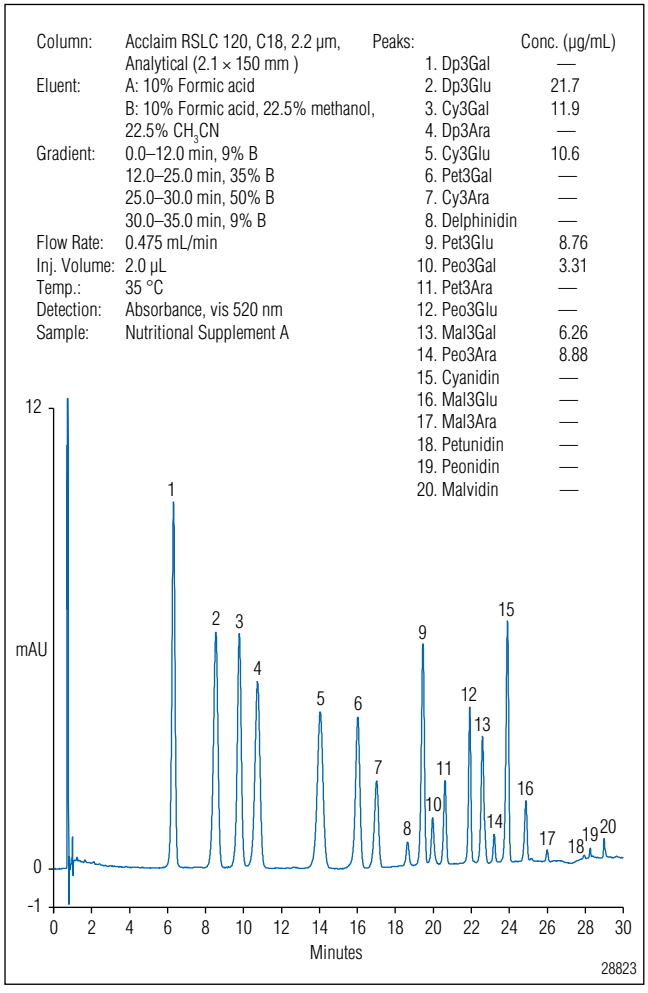

Figure 4. Separation of anthocyanins in a sample of bilberry-based Nutritional Supplement A.

Figure 5 shows the separation of anthocyanins in bilberry-based Nutritional Supplement B. The product label indicates that this product contains bilberry extract and other ingredients include vegetable cellulose, cellulose, silica, and magnesium stearate. Selected anthocyanins were quantified and ranged from 1.12 µg/mL for Peo3Gal to 20.8 µg/mL for Dp3Glu. All 15 anthocyanins and five anthocynidins were also separated in this sample. Both brands of bilberry-based supplements showed similar anthocyanin and anthocyanidin profiles.

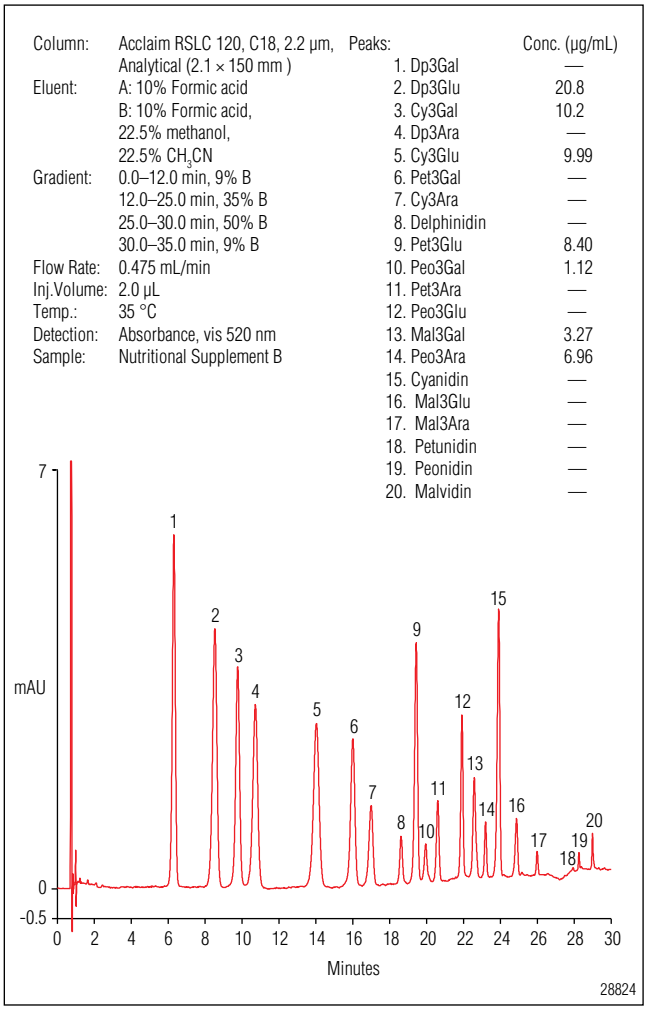

Figure 5. Separation of anthocyanins in a sample of bilberry-based Nutritional Supplement B.

Table 4. Sample Analysis for Intraday and Between-Day Precision						
Sample	Analyte	Amount (µg/mL)	Intraday Precision RSD			Between-Day Precision
			Ret. Time (n=3)	Peak Area (n=3)	Peak Height (n=3)	Peak Area (n=3, over 3 days)
Eye Antioxidant	Dp3Glu	6.24	0.14	2.79	1.79	3.56
	Cy3Gal	2.53	0.08	2.83	2.76	3.17
	Cy3Glu	2.45	0.02	2.10	2.38	3.61
	Peo3Gal	0.28	0.01	1.77	5.40	6.93
	Pet3Glu	2.25	0.06	2.13	0.89	2.63
	Mal3Gal	0.53	0.01	4.37	2.71	3.56
	Peo3Ara	1.78	0.17	5.99	1.24	3.78
Brand A Bilberry-Based Nutritional Supplement	Dp3Glu	24.3	0.06	1.12	0.83	2.21
	Cy3Gal	13.5	0.03	1.19	0.82	2.24
	Cy3Glu	12.0	0.05	0.75	0.55	2.19
	Peo3Gal	3.26	0.01	1.78	2.66	2.78
	Pet3 Glu	9.94	0.02	0.65	0.69	2.31
	Mal3Gal	6.47	0.02	1.59	0.97	2.35
	Peo3Ara	10.3	0.02	1.20	2.14	2.82
Brand B Bilberry-Based Nutritional Supplement	Dp3Glu	20.1	0.06	1.25	1.14	2.00
	Cy3Gal	9.86	0.05	0.79	0.42	2.04
	Cy3Glu	9.47	0.03	1.23	0.89	2.22
	Peo3Gal	1.64	0.02	1.66	0.63	2.66
	Pet3Glu	8.01	0.02	1.24	0.70	1.50
	Mal3Gal	2.76	0.03	0.88	0.95	1.90
	Peo3Ara	6.07	0.02	1.94	1.35	1.91

Sample Precision and Accuracy

All three samples were analyzed over three days to evaluate method precision. Representative data from each of the samples are summarized in Table 4. For the samples analyzed in this study, the intraday retention time RSDs ranged from 0.01% for Peo3Gal to 0.17% for Peo3Ara. Intraday peak area RSDs ranged from 0.75 for Cy3Glu to 5.99% for Peo3Ara. The between-day peak area RSDs ranged from 1.50% for Pet3Glu to 6.93% for Peo3Gal. Recovery studies were performed on all three samples by spiking known amounts of the seven anthocyanins to determine method accuracy.

Table 5. Recovery of Anthocyanins in Different Commercial Nutritional Supplements				
Sample	**Analyte**	**Amount (µg/mL)**	**Amount Spiked (µg/mL)**	**% Recovery**
Eye Antioxidant	Dp3Glu	6.24	10.0	89.3
	Cy3Gal	2.53	3.0	94.8
	Cy3Glu	2.45	3.0	81.1
	Peo3Gal	0.28	0.5	96.2
	Pet3 Glu	2.25	3.0	92.6
	Mal3Gal	0.53	0.5	94.2
	Peo3Ara	1.78	3.0	91.2
Brand A Bilberry-Based Nutritional Supplement	Dp3Glu	24.3	25.0	90.2
	Cy3Gal	13.5	10.0	82.8
	Cy3Glu	12.0	10.0	78.7
	Peo3Gal	3.26	4.0	98.1
	Pet3 Glu	9.94	10.0	78.4
	Mal3Gal	6.47	10.0	107
	Peo3Ara	10.3	10.0	104
Brand B Bilberry-Based Nutritional Supplement	Dp3Glu	20.1	20.0	97.6
	Cy3Gal	9.86	10.0	84.0
	Cy3Glu	9.47	10.0	93.6
	Peo3Gal	1.64	2.0	105
	Pet3 Glu	8.01	10.0	83.7
	Mal3Gal	2.76	3.0	101
	Peo3Ara	6.07	5.0	108

Table 5 summarizes the amounts spiked and the calculated recoveries. Recoveries ranged from 78.4% for Pet3Glu to 107% for Mal3Gal and suggest method accuracy.

CONCLUSION

This study describes a simple, sensitive, rapid, and accurate method to separate and quantify anthocyanins in different commercially available nutraceutical products with minimal sample preparation. The method uses a high-resolution silica-based Acclaim RSLC 120 C18 column and absorbance at 520 nm to separate and detect 15 anthocyanins and five anthocyanidins in 30 min, compared to competing methods with run times of 50 min or greater.[7-9]

PRECAUTIONS

Acetonitrile is a flammable liquid and may cause eye irritation. It may be harmful if swallowed, inhaled, or absorbed through the skin, and may cause skin and respiratory tract irritation. Acetonitrile is metabolized to cyanide in the body, which may cause headache, dizziness, weakness, unconsciousness, convulsions, coma, and possible death. Avoid contact with eyes, skin, and clothing. Keep container tightly closed. Keep away from heat, sparks, and flame. Use only with adequate ventilation. Keep away from sources of ignition and store in a tightly closed container. Keep away from contact with oxidizing materials. Store in a cool, dry, well-ventilated area away from incompatible substances and flammables-areas, and protected from moisture.

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SUPPLIERS

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PhytoLab GmbH & Co. KG, Dutendorfer Str. 5-7, 91487 Vestenbergsgreuth, Germany, Tel: +49 9163 88-330. www.phytolab.com

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