Rapid Determination of Anthocyanins in Pomegranate Juice and Bilberries

Pranathi Perati, Brian De Borba, and Jeffrey Rohrer
Dionex Corporation, Sunnyvale, CA, USA

INTRODUCTION

Anthocyanins are a subclass of naturally electron deficient, powerful antioxidants called flavonoids that are responsible for the red, orange, and blue coloration of most fruits, vegetables, and flowers. Major sources of anthocyanins include pomegranates, bilberries, strawberries, black currants, and purple grapes. Pomegranate juice (PJ) and bilberries have been reported to contain higher antioxidant activity than most commercially available fruit juices and extracts. Due to the increased health consciousness of consumers combined with the potential health benefits of PJ and bilberries, the demand for these products has grown rapidly. The high demand and short supply of both products has resulted in an increased rate of adulteration. Analytical methods are therefore required to quantify anthocyanins in both sources.

This work describes two sensitive, fast, and accurate UHPLC methods to determine anthocyanins in pomegranate beverages and bilberry-based nutritional supplements. The methods demonstrate good sensitivity, enabling detection and quantification over a wide concentration range of anthocyanins. Six anthocyanins of interest are separated in less than 10 min in various fruit juices that included PJ (Method A). Fifteen anthocyanins of interest and five anthocyanidins are separated in several bilberry-based supplements, ranging in concentration from 0.25–24.3 µg/mL with a total analysis time of less than 30 min (Method B). The linearity, LODs, LOQs, and accuracy of each method for determining the target compounds in these products is reported.

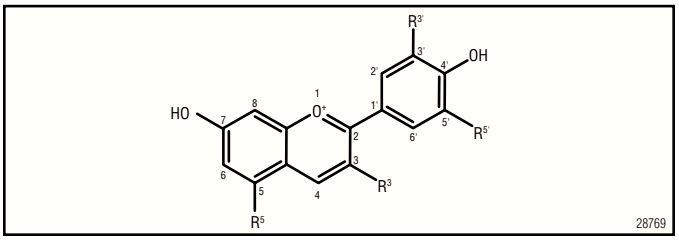

Figure 1. Basic structure of anthocyanins.

Table 1. Anthocyanins and Anthocyanidins					
R3'	R5'	R3	R5	Analyte	Abbreviation
OH	H	Arabinose	OH	Cyanidin 3-arabinoside	Cy3Ara
OH	H	Galactose	OH	Cyanidin 3-galactoside	Cy3Gal
OH	H	Glucose	OH	Cyanidin 3-glucoside	Cy3Glu
OH	H	Glucose	Glucose	Cyanidin 3,5-diglucoside	Cy3,5Glu
OH	OH	Arabinose	OH	Delphinidin 3-arabinoside	Dp3Ara
OH	OH	Galactose	OH	Delphinidin 3-galactoside	Dp3Gal
OH	OH	Glucose	OH	Delphinidin 3-glucoside	Dp3Glu
OH	OH	Glucose	Glucose	Delphinidin 3,5-diglucoside	Dp3,5Glu
OCH_3	OCH_3	Arabinose	OH	Malvidin 3-arabinoside	Mal3Ara
OCH_3	OCH_3	Galactose	OH	Malvidin 3-galactoside	Mal3Gal
OCH_3	OCH_3	Glucose	OH	Malvidin 3-glucoside	Mal3Glu
H	H	Glucose	OH	Pelargonidin 3-glucoside	Pg3Glu
H	H	Glucose	Glucose	Pelargonidin 3,5-diglucoside	Pg3,5Glu
OCH_3	H	Arabinose	OH	Peonidin 3-arabinoside	Peo3Ara
OCH_3	H	Galactose	OH	Peonidin 3-galactoside	Peo3Gal
OCH_3	H	Glucose	OH	Peonidin 3-glucoside	Peo3Glu
OCH_3	OH	Arabinose	OH	Petunidin 3-arabinoside	Pet3Ara
OCH_3	OH	Galactose	OH	Petunidin 3-galactoside	Pet3Gal
OCH_3	OH	Glucose	OH	Petunidin 3-glucoside	Pet3Glu
OH	H	OH	OH	Cyanidin	Cy
OH	OH	OH	OH	Delphinidin	Dp
OCH_3	OCH_3	OH	OH	Malvidin	Mal
OCH_3	H	OH	OH	Peonidin	Peo
OCH_3	OH	OH	OH	Petunidin	Pet

EQUIPMENT

A Dionex UltiMate® 3000 Rapid Separation LC System was used for this study. For individual components of the system and preparation of solutions, standards, and samples refer to Dionex Application Notes 264 and 281.[1,2]

Method Conditions

	Method A Determination of Anthocyanins in PJ	Method B Determination of Anthocyanins in Bilberries
Column	Dionex Acclaim® RSLC 120, C18, 2.2 µm Analytical, 2.1 × 150 mm	Dionex Acclaim RSLC 120 C18, 2.2 µm Analytical, 2.1 × 150 mm
Mobile Phases	A: 9% Acetonitrile, 10% formic acid B: 36% Acetonitrile, 10% formic acid	A: 10% Formic acid B: 10% Formic acid, 22.5% methanol, 22.5% acetonitrile
Total Run Time	10 min	35 min
Flow Rate	0.475 mL/min	0.475 mL/min
Column Temperature	30 °C	35 °C
Detection	Vis at 540 nm	Vis at 520 nm
Injection Volume	0.5 µL	2 µL
System Backpressure	6025–6200 psi during the gradient	6700–7400 psi during the gradient

Gradient Conditions Method A:

Time (min)	% A	% B
0.0	100.0	0.0
0.9	100.0	0.0
8.0	71.5	28.5
10.0	71.5	28.5

Gradient Conditions Method B:

Time (min)	% A	% B	Comments
0	91	9	
12	91	9	
25	65	35	
25	50	50	Step Change
30	50	50	
30	91	9	Step Change
35	91	9	

RESULTS AND DISCUSSION

Chromatography

System suitability was determined by separating standards of the fingerprint anthocyanins present in pomegranates and bilberries, listed in Table 1. Figure 2A shows a standard chromatogram containing six anthocyanins that are characteristic of pomegranate using a 2.1 × 150 mm column. As shown, all anthocyanins are well separated with a total run time of less than 10 min. Figure 2B shows fifteen anthocyanins and five anthocyanidins separated in a total analysis time of less than 30 min.

Method Performance

The linearity, limits of detection (LODs), and limits of quantification (LOQs) were evaluated to determine the suitability of the method for this analysis. All the anthocyanins showed a linear peak response in the ranges chosen and produced coefficient of determinations between 0.9992–0.9999 (Table 2). The LODs ranged from 0.12 µg/mL for Dp3Glu to 0.37 µg/mL for Pg3,5Glu, and the LOQs ranged from 0.63 µg/mL for Dp3Glu to 1.25 µg/mL for Pg3,5Glu. Retention time precisions of the standards were excellent with RSDs ranging from 0.06% for Dp3,5Glu to 0.12% for Cy3,5Glu. This demonstrates good precision of the gradient delivered by the HPG-3400RS pump.

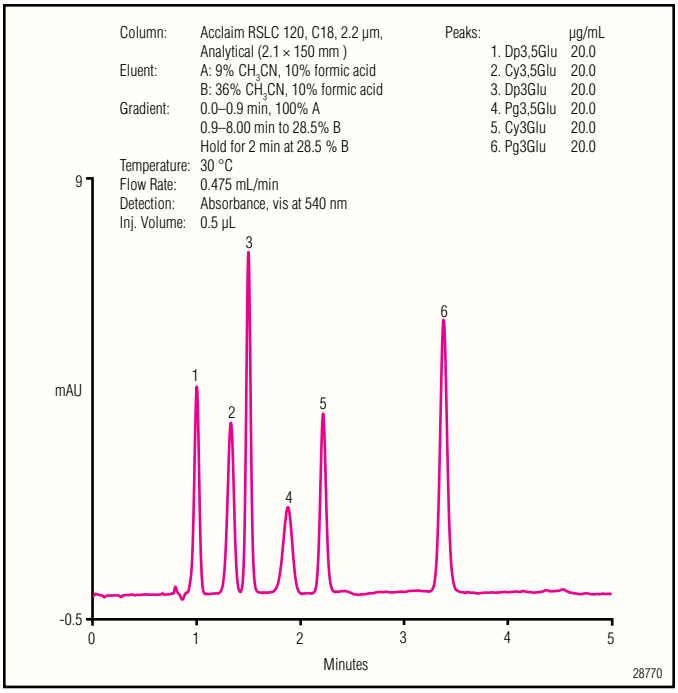

Figure 2A. Separation of a standard of mixed anthocyanins inherent to pomegranates.

Table 2. Data for Linearity, LOD and LOQ of Anthocyanins for Methods A and B

Analyte	Range (µg/mL)	Coeff. of Deter. r^2	LOD (µg/mL)	LOQ (µg/mL)	RSD	
					Ret. Time (n=30)	Peak Area (n=30)
Method A						
Dp3,5Glu	0.31–160	0.9992	0.21	0.66	0.06	1.82
Cy3,5Glu	0.31–160	0.9995	0.19	1.25	0.12	1.60
Dp3Glu	0.31–160	0.9996	0.12	0.63	0.06	1.45
Pg3,5Glu	0.31–160	0.9984	0.37	1.25	0.07	1.80
Cy3Glu	0.31–160	0.9994	0.15	1.25	0.06	1.46
Pg3Glu	0.31–160	0.9996	0.20	0.63	0.09	1.70
Method B						
Dp3Glu	0.39–50	0.9997	1.56	6.25	0.15	0.60
Cy3Gal	0.39–25	0.9996	0.78	3.13	0.12	0.97
Cy3Glu	0.39–25	0.9999	0.78	3.13	0.14	1.29
Pet3Glu	0.39–25	0.9999	0.20	0.78	0.08	1.51
Peo3Gal	0.39–10	0.9988	0.56	2.34	0.03	1.05
Peo3Ara	0.39–25	0.9997	0.78	3.13	0.03	0.50
Mal3Gal	0.39–25	0.9993	0.20	0.78	0.01	0.72

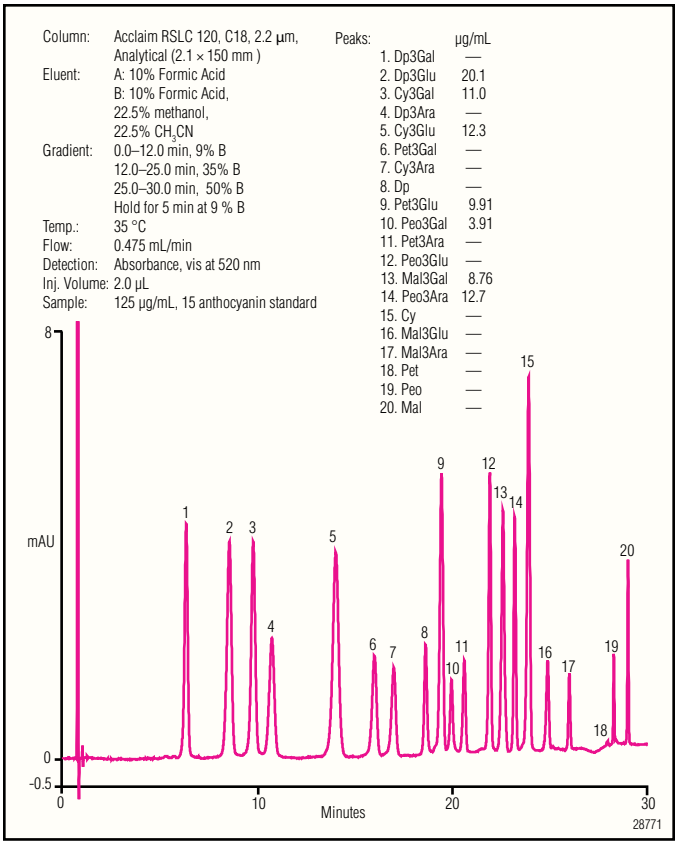

Figure 2B. Separation of a standard of mixed anthocyanins inherent to bilberries.

Sample Analysis

Figure 3 shows separation of the six signature anthocyanins present in 100% PJ. This confirms previous reports that claim the presence of six anthocyanins isolated and identified in pomegranates from different cultivars.[3]

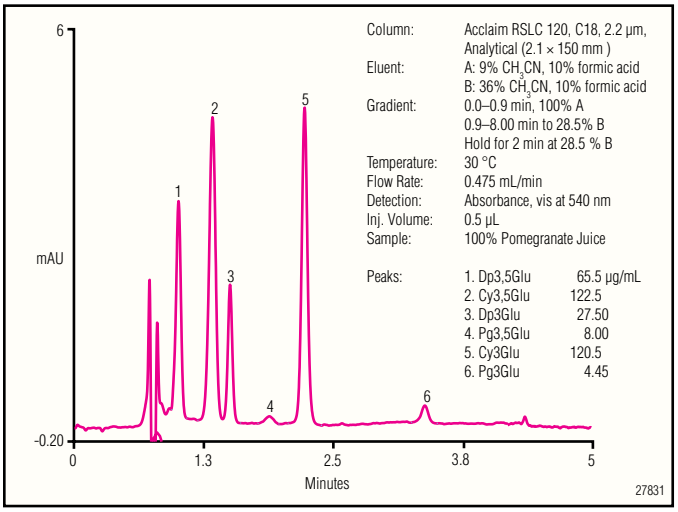

Figure 3. Separation of anthocyanins in a pomegranate juice sample.

Figure 4a shows the separation of anthocyanins present in pomegranate cherry juice. A low concentration of Dp3,5Glu (1.503 µg/mL) was observed in the undiluted juice. No other anthocyanins were observed in pomegranate cherry juice, which implies that very little PJ was added to this juice blend. Figure 4b shows a good separation of the anthocyanins in pomegranate wildberry juice. Dp3,5Glu (39.0 µg/mL), Cy3,5Glu (54.0 µg/mL), Dp3Glu (10.0 µg/mL), and Cy3Glu (50.5 µg/mL) are the four detected anthocyanins in pomegranate wildberry juice, which indicates a good proportion of PJ was added to the product.

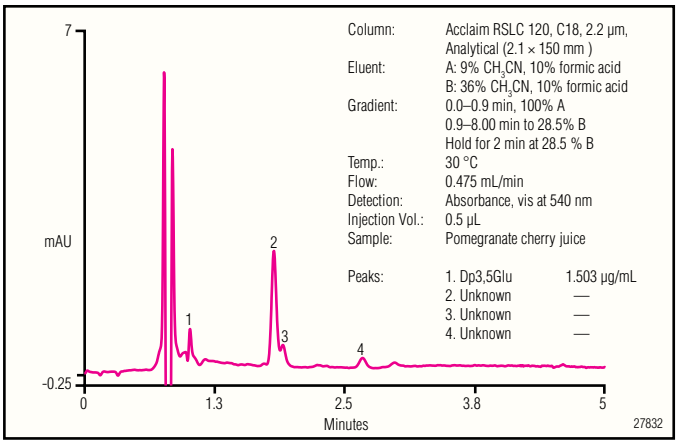

Figure 4a. Determination of anthocyanins in pomegranate cherry juice.

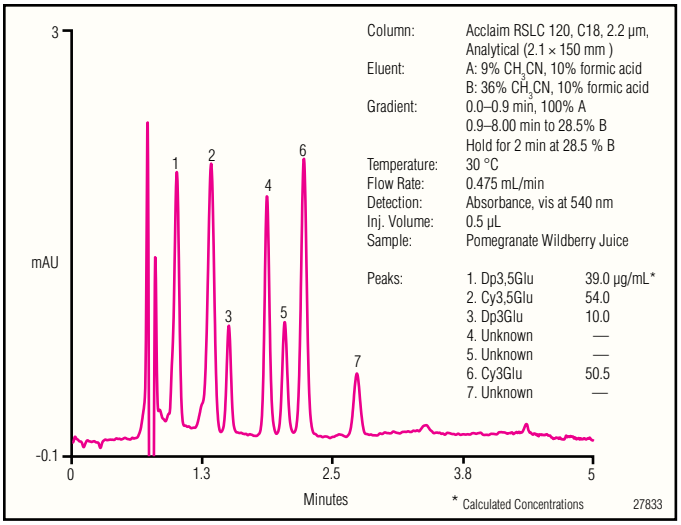

Figure 4b. Determination of anthocyanins in pomegranate wildberry juice.

Figure 5 shows an overlay of 100% grape juice and simulated adulterated PJ. The grape juice separation shows the presence of Cy3,5Glu, Dp3Glu, and Cy3Glu. Several later eluting unknown peaks are also present. Grape juice contains three of the six anthocyanins present in PJ, but at much lower concentrations. Simulated adulterated PJ was prepared by combining PJ and grape juice, and then diluting 1:5 in mobile phase A prior to analysis. The adulterated juice shows all six signature anthocyanins with several other late eluting peaks, which are not characteristic of PJ. The anthocyanin content of the adulterated juice is also lower than that of PJ, as expected.

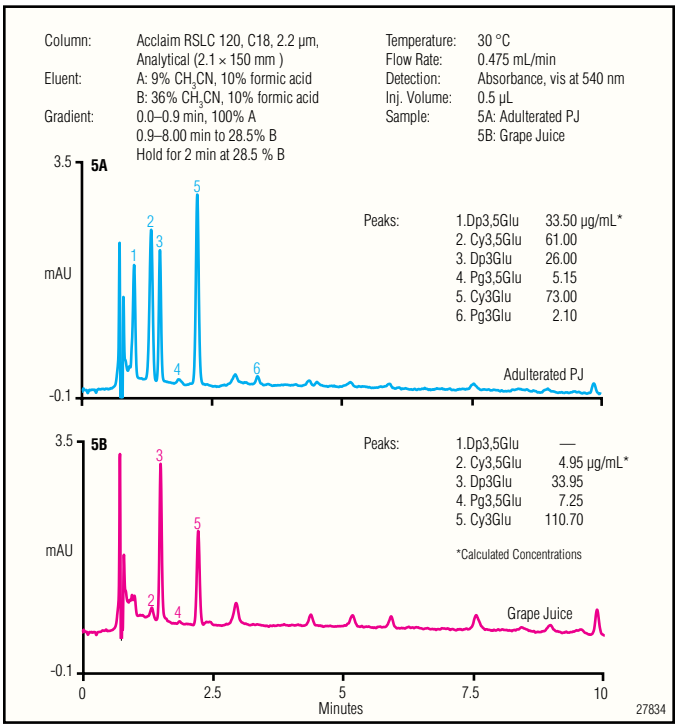

Figure 5. Separation of anthocyanins in A) simulated adulterated pomegranate juice and B) grape juice.

Two different bilberry based nutraceutical products were evaluated for their anthocyanin content. The first, marketed as an eye antioxidant product, contains an eye support blend with bilberry fruit extract, lutein derived from marigold flower extract, and zeaxanthin 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. Figure 6 shows the separation of the fourteen different anthocyanins and antothocyanidins identified in the eye antioxidant sample. No interferences were observed and all the anthocyanins were well separated.

Figure 7 shows the separation of anthocyanins in a bilberry-based nutritional supplement. 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 present in this product. Additionally, five anthocyanidins were also separated, namely delphinidin, cyanidin, peonidin, petunidin, and malvidin.

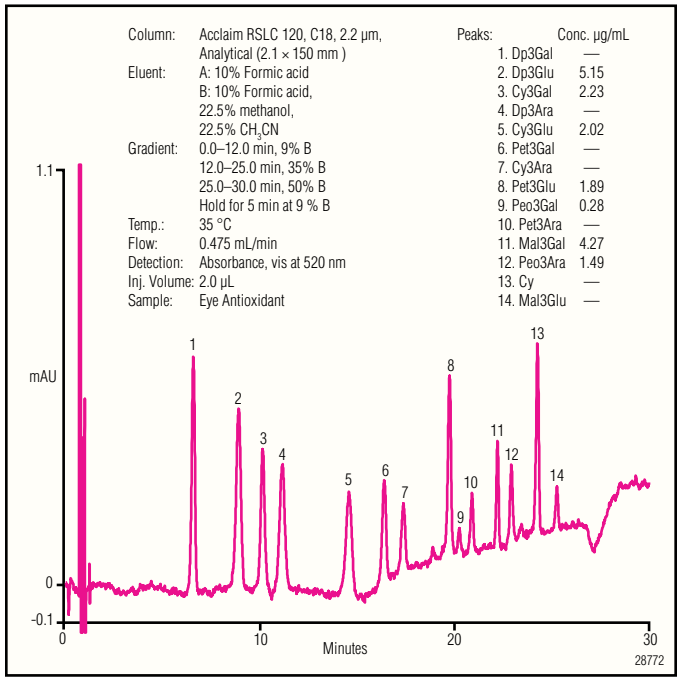

Figure 6. Separation of anthocyanins in a bilberry-based eye antioxidant.

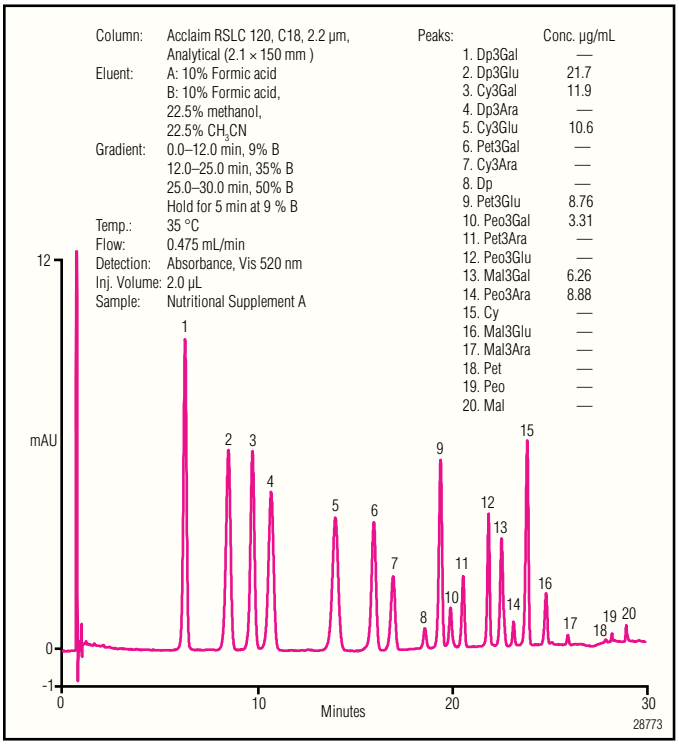

Figure 7. Separation of anthocyanins in a bilberry-based nutritional supplement.

Recovery

Recovery studies were performed on several fruit juices and nutritional supplements by spiking in known amounts of anthocyanins. Table 3 summarizes the amounts spiked and the calculated recoveries. Recoveries ranged from 64.2% for Dp3,5Glu in the pomegranate cherry to 110.0% for Cy3,5Glu in pomegranate cherry juice. Recoveries are low for Dp3,5Glu in pomegranate cherry because increased background noise and the low concentration of Dp3,5Glu.

Table 3. Recovery of Anthocyanins in Various Matrices

Sample	Analyte	Amount (µg/mL)	Amount Spiked (µg/mL)	% Recovery
100% Pomegranate Juice, 1:5 Dilution	Dp3,5Glu	13.2	15.0	101.8
	Cy3Glu	23.8	25.0	98.0
	Dp3Glu	6.50	5.0	106.9
	Pg3,5Glu	1.04	1.0	104.1
	Cy3Glu	23.6	25.0	102.2
	Pg3Glu	0.88	1.0	108.3
100% Grape Juice, 1:5 Dilution	Dp3,5Glu	< LOD	2.5	104.6
	Cy3,5Glu	1.05	1	105.1
	Dp3Glu	5.12	5	89.5
	Pg3,5Glu	< LOD	1	87.3
	Cy3Glu	4.24	5	85.5
	Pg3Glu	0.86	1	102.2
Simulated Adulterated Pomegranate Juice, 1:5 dilution	Dp3,5Glu	6.5	10.0	102.6
	Cy3,5Glu	12.8	10.0	80.1
	Dp3Glu	5.48	5.0	103.4
	Pg3,5Glu	0.56	0.75	102.0
	Cy3Glu	14.9	10.0	97.2
	Pg3Glu	0.43	0.75	87.6
Pomegranate Cherry Juice	Dp3,5Glu	1.55	1.0	64.2
	Cy3,5Glu	< LOD	10.0	110.0
	Dp3Glu	< LOD	5.0	81.1
	Pg3,5Glu	< LOD	1.0	97.0
	Cy3Glu	< LOD	5.0	106.0
	Pg3Glu	< LOD	1.0	93.7
Pomegranate Wildberry Juice, 1:5 Dilution	Dp3,5Glu	9.15	7.5	75.7
	Cy3,5Glu	8.39	10.0	84.4
	Dp3Glu	1.70	2.0	74.9
	Pg3,5Glu	< LOD	5.0	89.7
	Cy3Glu	8.37	5.0	94.1
	Pg3Glu	< LOD	2.0	70.3

Table 3. Recovery of Anthocyanins in Various Matrices

Sample	Analyte	Amount (µg/mL)	Amount Spiked (µg/mL)	% Recovery
Eye Antioxidant	Dp3Glu	6.24	10	89.3
	Cy3Gal	2.53	3.0	94.8
	Cy3Glu	2.45	3.0	81.1
	Peo3Gal	0.28	0.5	96.2
	Pet3Glu	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	90.2
	Cy3Gal	13.5	10	82.8
	Cy3Glu	12.01	10	78.7
	Peo3Gal	3.26	4.0	98.1
	Pet3Glu	9.94	10	78.4
	Mal3Gal	6.47	10	107
	Peo3Ara	10.3	10	104

CONCLUSION

- Two sensitive, accurate and rapid methods to separate and quantify anthocyanins in different fruit juices and bilberry-based supplements were demonstrated.
- Several fruit juices and nutritional supplements with varying concentrations of anthocyanins ranging from 122.5–1.5 µg/mL were determined by the two methods.
- Good recoveries were observed ranging from 75%–110%, with the exception of Dp3,5Glu in pomegranate cherry juice and Pg3Glu in pomegranate wildberry juice. In both cases some matrix interferences were observed that made quantification difficult.

REFERENCES

1. Dionex Corporation. *Fast Determination of Anthocyanins in Pomegranate Juice;* Application Note 264, LPN 2647: Sunnyvale, CA, 2010.
2. Dionex Corporation. *Fast Determination of Anthocyanins in Bilberries;* Application Note 281, LPN 2848: Sunnyvale, CA, 2011.
3. Zhang, Y.; Krueger, D.; Durst, R.; Lee, R.; Wang, D.; Seeram, N.; Herber, D. International Multidimensional Authenticity Specification (IMAS) Algorithm for Detection of Commercial Pomegranate Juice Adulteration. *J. Agric. Food Chem.* **2009**, *57* (6), 2550–2557.

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