DIONEX

Application Note 264

Now sold under the Thermo Scientific brand

Thermo SCIENTIFIC

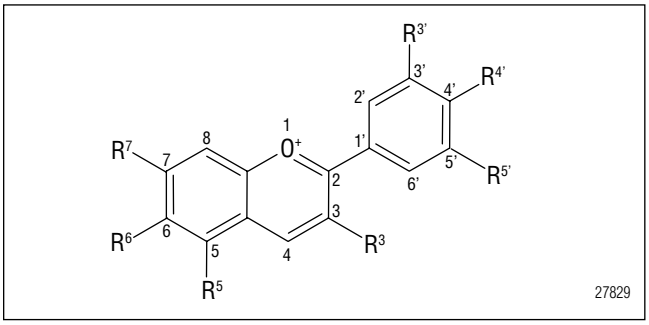
Fast Determination of Anthocyanins in Pomegranate Juice

INTRODUCTION

Anthocyanins are a subclass of molecules known as flavonoids that are responsible for the brilliant red, orange, and blue colors of most fruits and flowers. Anthocyanidins lack the sugar component of the parent anthocyanin. Six of the anthocyanidins that occur most commonly in nature are pelargonidin, cyanidin, peonidin, delphinidin, petunidin, and malvidin. Anthocyanins are the mono and diglycosylated forms of anthocyanidins with substitutions at the 3 and 5 positions (Figure 1).[1] The most common carbohydrates encountered on anthocyanins include glucose, galactose, rhamnose, and arabinose.

Due to their strong antioxidant properties, anthocyanins are of considerable interest to the scientific community and consumer market. The naturally electron-deficient chemical structure of anthocyanins makes them highly reactive toward free radicals and, consequently, makes them powerful natural antioxidants. Increased understanding of their health benefits has led to a growing interest in determining anthocyanins in foods, nutraceuticals, and natural products.[2, 3]

Major sources of anthocyanins include blueberries, cherries, raspberries, bilberries, strawberries, black currants, purple grapes, and pomegranates. Pomegranate juice (PJ) has been reported to contain 3× more antioxidant activity than green tea and higher total polyphenol concentrations, compared to common fruit juices (e.g., orange, grapefruit, grape, cranberry, pineapple, and apple). Due to the increased health consciousness of consumers, combined with the potential health benefits of PJ, the demand for PJ and pomegranate-related products has grown rapidly in recent years.



R3'	R5'	Anthocyanidin	R3	R5	Anthocyanin
H	H	Pelargonidin	Glucose		Pelargonidin 3-glucoside
H	H	Pelargonidin	Glucose	Glucose	Pelargonidin 3,5-diglucoside
OH	H	Cyanidin	Glucose		Cyanidin 3-glucoside
OH	H	Cyanidin	Glucose	Glucose	Cyanidin 3,5-diglucoside
OH	OH	Delphinidin	Glucose		Delphinidin 3-glucoside
OH	OH	Delphinidin	Glucose	Glucose	Delphinidin 3,5-diglucoside
OCH_3	OH	Petunidin	Glucose		Petunidin 3-glucoside
OCH_3	OH	Petunidin	Glucose	Glucose	Petunidin 3,5-diglucoside
OCH_3	H	Peonidin	Glucose		Peonidin 3-glucoside
OCH_3	H	Peonidin	Glucose	Glucose	Peonidin 3,5-diglucoside
OCH_3	OCH_3	Malvidin	Glucose		Malvidin 3-glucoside
OCH_3	OCH_3	Malvidin	Glucose	Glucose	Malvidin 3,5-diglucoside

Figure 1. Basic structure of anthocyanins.

Pomegranate is extensively cultivated worldwide and has become a high-value crop for juice production. The retail market now contains numerous pomegranate-related products such as juices, smoothies, flavored waters, and sports and energy drinks.[4] From 2006 to 2008, nearly 320 products containing pomegranate or pomegranate flavoring were launched and PJ currently remains one of the most popular drinks in the *super juice* category.[5]

Due to the high demand for pomegranates outstripping the supply, adulteration of PJ has become widespread. The United States Food and Drug Administration (U.S. FDA) has proposed a working definition of economic adulteration as "The fraudulent, intentional substitution or addition of a substance in a product for the purpose of increasing the apparent value of the product or reducing the cost of its production (i.e., for economic gain)." According to the U.S. FDA, the globalization of the food market has raised economic adulteration as a key concern because imports have increased annually by approximately 14% since 1997.[6] Manufacturers have attempted to extend the limited supply of PJ by blending with filler ingredients such as cane sugar, corn syrup sweeteners, and lower-quality juices containing sorbitol, malic acid, and sucrose (e.g., grape, apple, and blackberry).[7]

To establish an authentication criterion, an International Multidimensional Authenticity Specifications algorithm was developed based on the analysis of commercial juice samples from 23 manufacturers in the United States, Iran, Turkey, Azerbaijan, Syria, India, and China.[8] There is universal agreement that the anthocyanin profile in PJ consists of a constant group of six anthocyanins, regardless of the origin. However, the anthocyanin concentrations can vary depending on the geographic source of the PJ. The anthocyanin profile is one of several chemical analyses that are required to determine the authenticity of PJ. Additional chemical profiling methods include measuring other polyphenols (i.e., ellagitannins), monosaccharides (e.g., fructose and glucose), organic acids, amino acids, and potassium in PJ samples. Determinations of monosaccharides, organic acids, and punicalagins in fruit juices have been previously described in AN 82, 143, and CAN 106, respectively.[9-11]

The method described here is a sensitive, fast, and accurate way to determine anthocyanins in commercially available fruit juices using a simple dilution. Anthocyanins were separated using a 2.2 µm, Acclaim® RSLC 120, C18 rapid separation liquid chromatography column and detected at a visible wavelength of 540 nm. The silica-based column used in this application is designed for rapid, high-resolution separations, which is compatible with ultrahigh pressure instrumentation. The six anthocyanins of interest were separated in <5 min in various beverages that included PJ, grape juice, simulated adulterated PJ, pomegranate cherry juice, and pomegranate wildberry juice.

EQUIPMENT

Dionex UltiMate® 3000 RSLC system including:

- SRD-3600 Solvent Rack with 6 degasser channels (P/N 5035.9230)
- Eluent Organizer, including pressure regulator and 2 L glass bottles for each pump, eluents maintained under helium or nitrogen head space (5–8 psi)
- HGP 3400RS Pump (P/N 5040.0046)
- WPS-3000TRS Well Plate Sampler (P/N 5840.0020)
- TCC-3000RS Thermostatted 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 column, 2.1 × 150 mm (P/N 071399)

Centrifuge equipped with a 10-place, aluminum fixed-angle rotor (Beckman Spinchron R, GS-6R Series, Beckman Coulter, P/N 358702 or equivalent)

Viper™ SST fingertight fitting including capillary for 10-32 fitting, i.d. × L 0.13 × 250 mm (P/N 6040.2325)

Viper SST fingertight fitting including capillary for 10-32 fitting, i.d. × L 0.13 × 350 mm (P/N 6040.2335)

Viper SST fingertight fitting including capillary for 10-32 fitting, 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)

REAGENTS AND STANDARDS

Reagent-grade water, Type I, 18 MΩ-cm resistance or better, filtered through a 0.2 µm filter immediately before use

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

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

Delphinidin 3-glucoside (Cerilliant P/N 89627)

Delphinidin 3,5-diglucoside (Cerilliant P/N 89626)

Cyanidin 3,5-diglucoside (Cerilliant P/N 89615)

Cyanidin 3-glucoside (Cerilliant P/N 89616)

Pelargonidin 3-glucoside (Cerilliant P/N 89753)

Pelargonidin 3,5-diglucoside (Cerilliant P/N 80334)

SAMPLES

100% Pomegranate juice

100% Grape juice

Simulated adulterated pomegranate juice

Pomegranate cherry juice

Pomegranate wildberry juice

CONDITIONS

Conditions for a 2.1 × 150 mm Column

Columns: Acclaim RSLC 120, C18, 2.2 µm Analytical, 2.1 × 150 mm (P/N 071399)

Flow Rate: 0.475 mL/min

Injection Volume: 0.5 µL

Tray Temp.: 4 ºC

Detection: Absorbance, visible, 540 nm

Column Temp.: 30 ºC

Eluents: A: 9% Acetonitrile, 10% formic acid
B: 36% Acetonitrile, 10% formic acid

System Backpressure: 6025–6200 psi over the gradient

Gradient Conditions:

Time (min)	Flow (mL/min)	% A	% B
0.0	0.475	100.0	0.0
0.9	0.475	100.0	0.0
8.0	0.475	71.5	28.5
10.0	0.475	71.5	28.5

Conditions for a 4.6 × 250 mm Column

Columns: Acclaim 120, C18, 5.0 µm Analytical, 4.6 × 250 mm (P/N 059149)

Flow Rate: 1.0 mL/min

Injection Volume: 5 µL

Gradient Conditions:

Time (min)	Flow (mL/min)	% A	% B
0.0	1.0	100.0	0.0
2.5	1.0	100.0	0.0
30.0	1.0	71.5	28.5
45.0	1.0	71.5	28.5

PREPARATION OF SOLUTIONS AND REAGENTS

9% Acetonitrile, 10% Formic Acid

Transfer 200 mL of acetonitrile into a glass 2 L volumetric flask containing approximately 700 mL of deionized water. Mix by inverting the volumetric flask, bring to volume with deionized water, and mix again. Remove 200 mL of the mix and dispose in organic waste, then add 200 mL of formic acid to the volumetric flask and invert to mix.

36% Acetonitrile, 10% Formic Acid

Transfer 400 mL of acetonitrile into a glass 1 L volumetric flask containing approximately 400 mL of deionized water. Mix by inverting the volumetric flask, bring to volume with deionized water, and mix again. Remove 100 mL of the mix and dispose in organic waste, then add 100 mL of formic acid to the volumetric flask and invert to mix.

Standards

All standard concentrates can be stored for up to 6 months at -40 °C protected from light. Diluted intermediate standards are stable for 3 months at -40 °C and working and mixed standards are stable for 4 weeks at 2 to 4 °C.

1 mg/mL Standard Concentrates

Prepare anthocyanin standards of delphinidin 3,5-diglucoside (Dp3,5), cyanindin 3,5-diglucoside (Cy3,5), delphinidin 3-glucoside (Dp3), pelargonidin 3,5-diglucoside (Pg3,5), cyanidin 3-glucoside (Cy3), and pelargonidin 3-glucoside (Pg3) by weighing 1 to 2 mg of solid and adding 1 to 2 mL of mobile phase A to make a stock solution of 1.0 mg/mL for each individual anthocyanin. Prepare the stocks in 10 mL glass vials, vortex to mix, and store at -40 °C until needed.

Working Standards and Standards for Method Linearity

To prepare working standards, use a calibrated pipette to deliver the appropriate volume of the 1 mg/mL stock standard into a glass vial containing the appropriate volume of mobile phase A. For method linearity studies, the following standards were used: 160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.62, and 0.31 µg/mL.

Mixed Standards

To prepare mixed anthocyanin standards, combine appropriate volumes of the individual stock anthocyanin standards into a glass vial containing the appropriate volume of mobile phase A.

SAMPLE PREPARATION

Centrifuge all samples at 5000 rpm for 10 min. Aspirate the supernatant and store in a glass vial at -40 °C until needed. Prepare a 1:5 dilution of the supernatant of all the juices (with the exception of pomegranate cherry) in mobile phase A prior to analysis. The anthocyanin content of pomegranate cherry is low; therefore, sample dilution is not required.

RESULTS AND DISCUSSION

Separation of Anthocyanin Standards

The initial investigation for the separation of anthocyanins was evaluated using a 5 µm Acclaim 120 C18 column in the 4.6 × 250 mm format (gradient specified in the Conditions section). To increase sample throughput and reduce sample and eluent consumption, this application was transferred to an UltiMate 3000 RSLC system. The *Dionex Method Transfer Calculator* was used to accelerate the method by using an RSLC column format (2.2 µm, 2.1 × 150 mm).

Figure 2 shows a chromatogram of a mixed anthocyanin standard with all six anthocyanins using a 2.1 × 150 mm column. The retention times of Dp3,5, Cy3,5, Dp3, Pg3,5, Cy3, and Pg3 are 1.02, 1.34, 1.51, 1.90, 2.23, and 3.42 min, respectively. All anthocyanin compounds are well separated and the analysis time is <8 min, compared to approximately 30 min when using the larger column format and larger particle diameter. The accelerated method saves 40 mL of solvent per injection.

System Suitability

The linearity, limits of detection (LOD), and limits of quantification (LOQ) were evaluated to determine suitability of the method for this analysis. Dp3,5, Cy3,5, Dp3, Pg3,5, Cy3, and Pg3 exhibited a linear peak area response in the range of 0.31 to 160 µg/mL, which produced correlation coefficients between 0.9984 and 0.9996 (Table 1). The LOD for the anthocyanins were determined based on the concentration of the analyte that provides a peak height of 3× the measured noise (S/N = 3), whereas the LOQ was determined as the concentration of the analyte that provides a peak height of 10× the measured noise (S/N = 10). The LODs ranged from 0.12 µg/mL for Dp3 to 0.37 µg/mL for Pg3,5, whereas the LOQs ranged from 0.63 µg/mL for Dp3 to 1.25 µg/mL for Pg3,5. Retention time precisions of the standards were excellent, with RSDs ranging from 0.06% for Dp3,5 to 0.12% for Cy3,5. This demonstrates good precision of the gradient delivered by the HPG-3400RS. Peak area precision ranged from 1.45% for Dp3 to 1.82% for Dp3,5, whereas peak height precision ranged from 1.19% for Cy3 to 1.85% for Pg3,5 over 30 runs at a 10 µg/mL concentration.

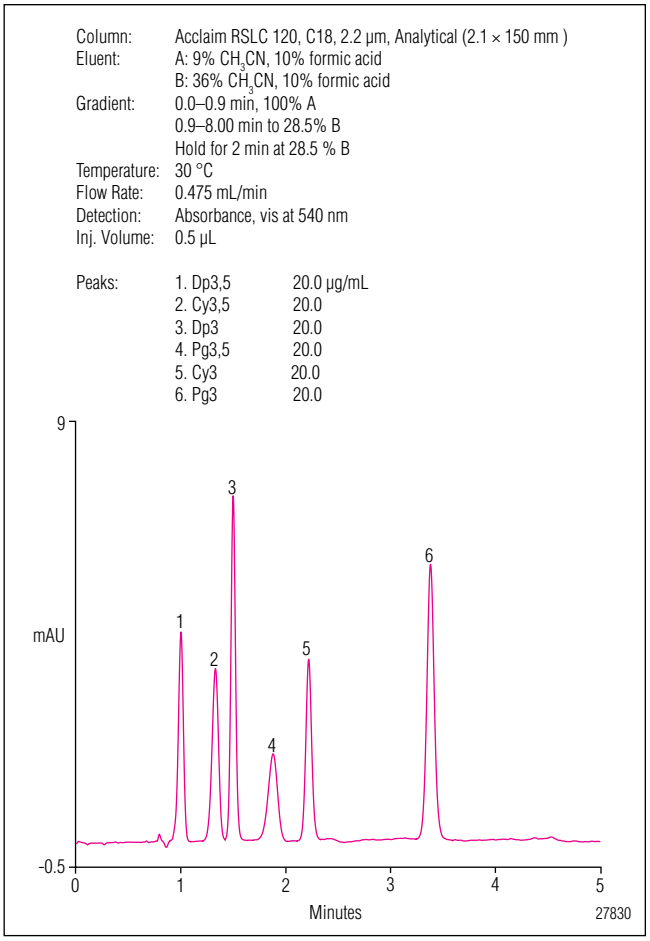


Figure 2. Separation of a mixed anthocyanin standard using the Acclaim RSLC 120 C18 column.

Table 1. Data for Linearity, LOD, and LOQ of Anthocyanins

Analyte	Range (µg/mL)	Correlation Coefficient r^2	LOD (µg/mL)	LOQ (µg/mL)	RSD		
					Ret. Time* (n=30)	Peak Area* (n=30)	Peak Height (n=30)
Dp3,5	0.31-160	0.9992	0.21	0.66	0.06	1.82	1.40
Cy3,5	0.31-160	0.9995	0.19	1.25	0.12	1.60	1.45
Dp3	0.31-160	0.9996	0.12	0.63	0.06	1.45	1.35
Pg3,5	0.31-160	0.9984	0.37	1.25	0.07	1.80	1.85
Cy3	0.31-160	0.9994	0.15	1.25	0.06	1.46	1.19
Pg3	0.31-160	0.9996	0.20	0.63	0.09	1.70	1.50

*Analyte concentrations for precision = 10 µg/mL

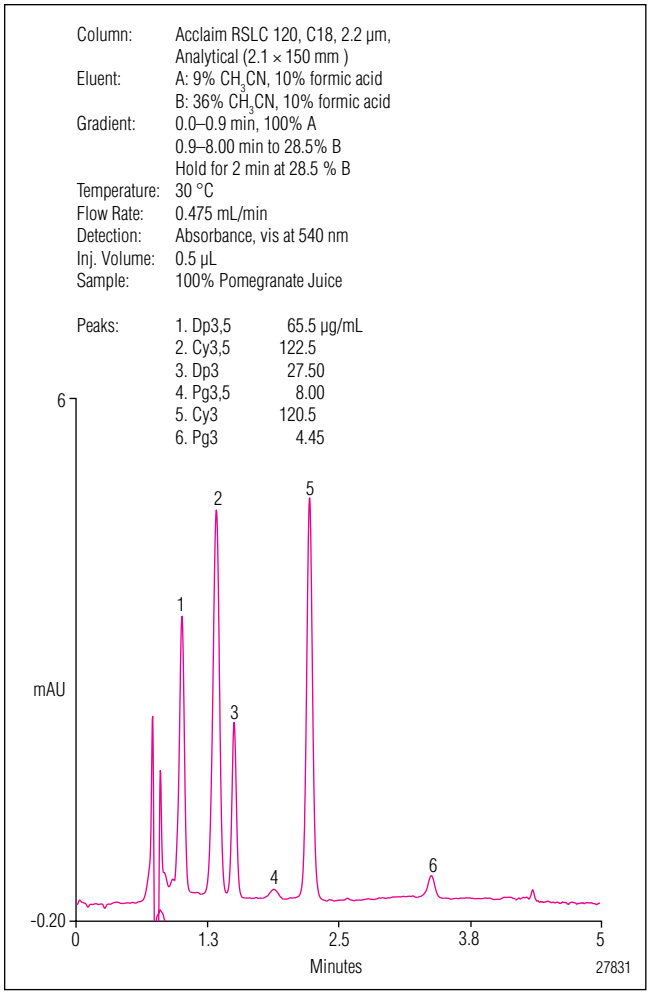


Figure 3. Separation of anthocyanins in a pomegranate juice sample.

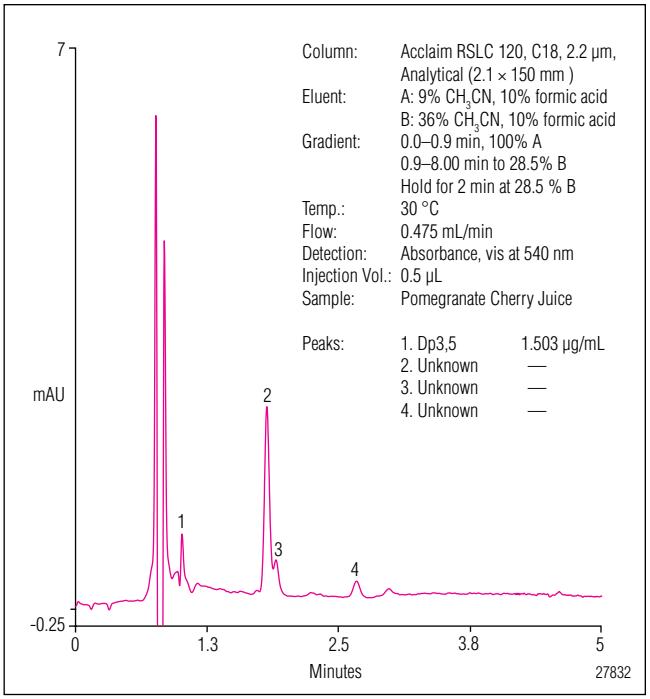


Figure 4a. Determination of anthocyanins in pomegranate cherry juice.

Sample Analysis

This method was applied to determination of the six common anthocyanins that are expected in PJ. The samples investigated in this study included 100% PJ, 100% grape juice, pomegranate cherry juice, pomegranate wildberry, and simulated adulterated PJ. The 100% PJ was used as a reference sample to compare its anthocyanin profile and concentrations to other juices on the market that feature pomegranate on the label. Figure 3 shows the separation of the six signature anthocyanins present in 100% PJ. This confirms previous reports that claim the presence in pomegranates of six anthocyanins that can be isolated and identified from different cultivars.[8]

The pomegranate cherry and pomegranate wildberry juices do not have a claim that states 100% PJ. Therefore, all six anthocyanins were not expected to be detected in these samples. Figure 4a shows the separation of anthocyanins present in pomegranate cherry juice. When this fruit juice was diluted, the anthocyanin concentrations were below the LODs; therefore, the juice was not diluted prior to analysis. A low concentration of Dp3,5 (1.50 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.

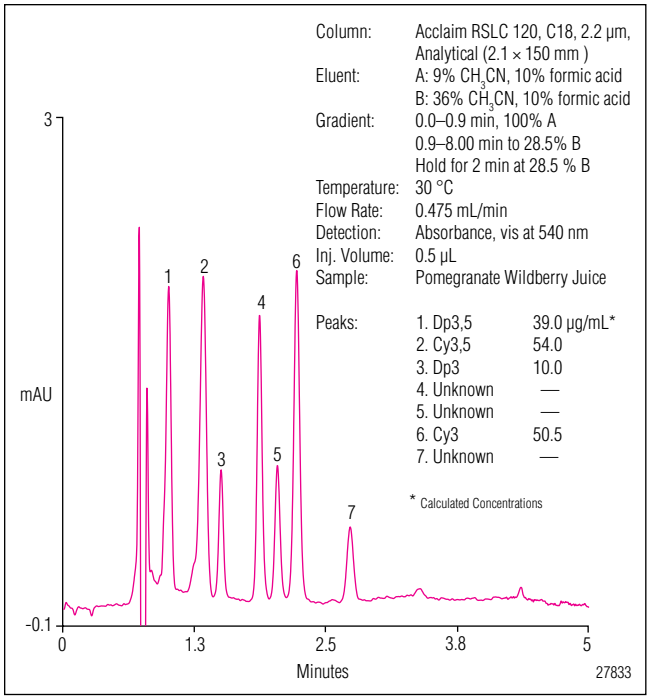


Figure 4b. Determination of anthocyanins in pomegranate wildberry juice.

Figure 4b shows a good separation of the anthocyanins in pomegranate wildberry juice. The four detected anthocyanins in pomegranate wildberry juice are Dp3,5 (39.0 µg/mL), Cy3,5 (54.0 µg/mL), Dp3 (10.0 µg/mL), and Cy3 (50.5 µg/mL), which indicates a significant proportion of PJ was added to the product. However, Pg3,5 and Pg3 were not detected in this sample, although these anthocyanins typically are present at significantly lower concentrations in 100% PJ. Therefore, it is possible that these anthocyanins were present but at concentrations that were < LODs.

Grape juice is one of several juices used to adulterate PJ. Therefore, a 50:50 mixture of grape and 100% PJ was used in this study to simulate an adulterated sample.[7] Figure 5B shows a separation of grape juice with the presence of Cy3,5, Dp3, Cy3, and Pg3,5. Several other later-eluting unknown peaks are also present.

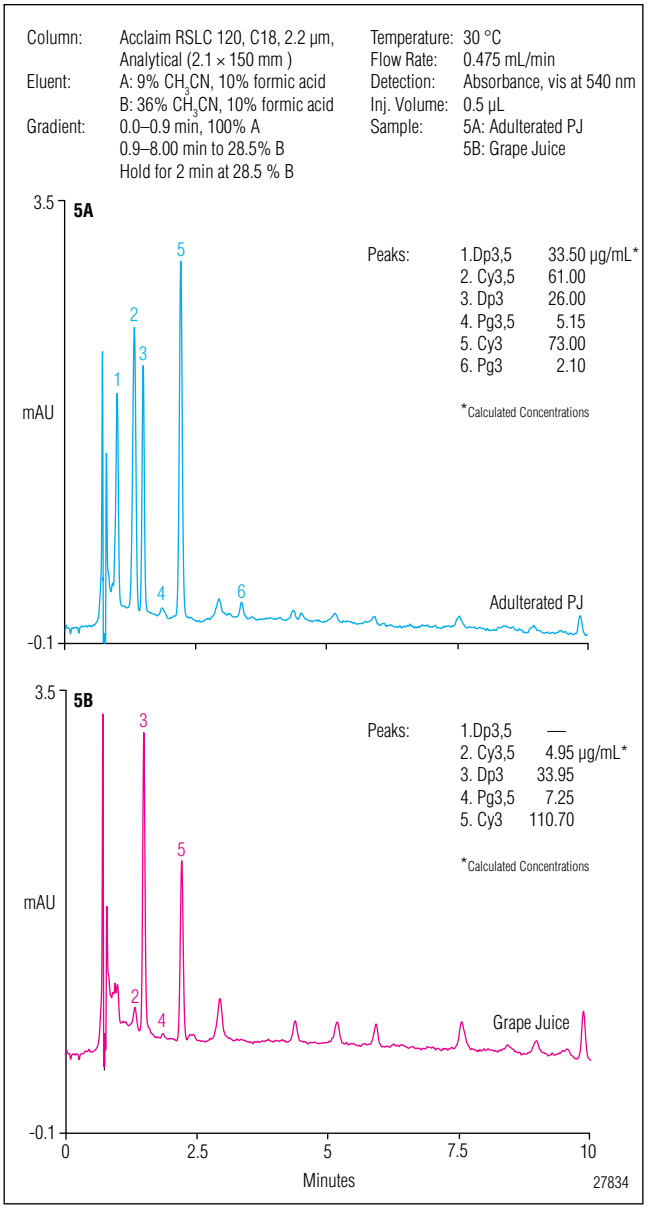


Figure 5. Separation of anthocyanins in simulated adulterated pomegranate juice (5A) overlayed with a separation of anthocyanins in grape juice (5B).

Grape juice contains four of the six anthocyanins present in PJ but at much lower concentrations. Simulated adulterated PJ was prepared by combining PJ and grape juice, then diluting 1:5 in mobile phase A prior to analysis. The chromatogram in Figure 5A shows a separation of Dp3,5, Cy3,5, Dp3, Pg3,5, Cy3, and Pg3 at concentrations of 33.5 µg/mL, 61.0 µg/mL, 26.0 µg/mL, 5.15 µg/mL, 73.0 µg/mL, and 2.10 µg/mL, respectively. The adulterated juice shows all of the signature anthocyanins and several other late-eluting peaks not characteristic of PJ. The anthocyanin content of the adulterated juice is also lower than that of PJ, as expected.

Table 2. 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)**
1:5 Dilute 100% Pomegranate Juice	Dp3,5	13.0	0.010	1.61	0.81	2.10
	Cy3,5	23.2	0.010	0.18	0.16	1.18
	Dp3	5.35	0.006	1.09	0.69	1.65
	Cy3	22.8	0.178	1.41	1.28	2.16
	Pg3,5	1.03	0.085	0.86	0.81	1.48
	Pg3	0.85	0.056	1.16	1.08	1.67
1:5 Dilute 100% Grape Juice	Cy3,5	0.86	0.148	1.17	1.04	1.87
	Dp3	6.24	0.125	1.07	1.19	1.83
	Cy3	6.72	0.004	0.89	0.84	1.42
1:5 Dilute Simulated Adulterated Pomegranate Juice	Dp3,5	6.99	0.010	0.70	0.70	1.05
	Cy3,5	12.1	0.007	0.65	1.11	1.38
	Dp3	5.29	0.006	1.04	1.12	1.86
	Cy3	14.5	0.082	1.43	1.92	2.51
	Pg3,5	0.58	0.371	1.07	0.83	1.39
	Pg3	0.43	0.096	0.62	1.16	1.87
1:5 Dilute Pomegranate Wildberry Juice	Dp3,5	8.24	0.010	1.34	1.57	1.74
	Cy3,5	11.2	0.007	1.98	1.31	2.28
	Dp3	2.10	0.006	1.71	1.20	2.52
	Cy3	10.5	0.088	2.71	2.72	2.68
Pomegranate Cherry Juice	Dp3,5	1.55	0.012	2.65	2.30	4.16

Sample Precision and Accuracy

Five different kinds of juice were analyzed over three days to evaluate the precision of the method. Representative data from each of the juices are presented in Table 2. Intraday retention time RSDs ranged from 0.004% for Cy3 in grape juice to 0.317% for Pg3,5 (n=3) in simulated adulterated PJ. Intraday peak area RSDs ranged from 0.62% for Pg3 in simulated adulterated PJ to 2.71% for Cy3 in pomegranate wildberry juice (n=3). The between-day peak area RSDs ranged from 1.05% for Dp3,5 in adulterated pomegranate juice to 4.16% for Dp3,5 in pomegranate cherry juice (n=3). The imprecision observed in pomegranate cherry juice was attributed to the increased background noise and low concentration of Dp3,5, which made quantification challenging. Recovery studies were performed on all five fruit juices by spiking in known amounts of the six anthocyanins.

Table 3. Recovery of Anthocyanins in Various Matrices

Sample	Analyte	Amount (µg/mL)	Amount Spiked (µg/mL)	Recovery %
1:5 Dilute 100% Pomegranate Juice	Dp3,5	13.2	15.0	101.8
	Cy3,5	23.8	25.0	98.0
	Dp3	6.50	5.0	106.9
	Pg3,5	1.04	1.0	104.1
	Cy3	23.6	25.0	102.2
	Pg3	0.88	1.0	108.3
1:5 Dilute 100% Grape Juice	Dp3,5	< LOD	2.5	104.6
	Cy3,5	1.05	1	105.1
	Dp3	5.12	5	89.5
	Pg3,5	< LOD	1	87.3
	Cy3	4.24	5	85.5
1:5 Dilute Simulated Adulterated Pomegranate Juice	Dp3,5	6.5	10.0	102.6
	Cy3,5	12.8	10.0	80.1
	Dp3	5.48	5.0	103.4
	Cy3	14.9	10.0	97.2
	Pg3,5	0.56	0.75	102.0
	Pg3	0.43	0.75	87.6
Pomegranate Cherry Juice	Dp3,5	1.55	1.0	64.2
	Cy3,5	< LOD	10.0	110.0
	Dp3	< LOD	5.0	81.1
	Pg3,5	< LOD	1.0	97.0
	Cy3	< LOD	5.0	106.0
	Pg3	< LOD	1.0	93.7
1:5 Dilute Pomegranate Wildberry Juice	Dp3,5	9.15	7.5	75.7
	Cy3,5	8.39	10.0	84.4
	Dp3	1.70	2.0	73.9
	Pg3,5	< LOD	5.0	89.7
	Cy3	8.37	5.0	94.1
	Pg3	< LOD	2.0	70.3

Table 3 summarizes the amounts spiked and the calculated recoveries. Recoveries ranged from 64.2% for Dp3,5 in pomegranate cherry juice to 108.3% for Pg3 in PJ. Recoveries were low for Dp3,5 in the pomegranate cherry because of increased background noise and low concentration of Dp3,5.

Table 4. Peak Purity Results for PJ and Other PJ Blends

Sample	Analyte	Match	PPI (nm)	RSD PPI %
1:5 Dilute 100% Pomegranate Juice	Dp3,5	924	505	0.94
	Cy3,5	995	503	0.33
	Dp3	993	509	0.27
	Cy3	931	442	0.99
	Pg3,5	793	493	3.91
	Pg3	931	483	0.31
1:5 Dilute Simulated Adulterated Pomegranate Juice	Dp3,5	988	400	0.88
	Cy3,5	989	358	0.87
	Dp3	949	505	0.91
	Cy3	914	495	0.89
	Pg3,5	995	318	0.20
	Pg3	829	468	2.86
1:5 Dilute Pomegranate Wildberry Juice	Dp3,5	951	395	0.89
	Cy3,5	873	465	1.69
	Dp3	960	334	0.98
	Cy3	994	387	0.69
Pomegranate Cherry Juice	Dp3,5	992	319	0.17

Application of UV Spectral Information to Determine Purity

Spectral scanning was used for the analysis of the standard mix of anthocyanins. High match values of the standards suggested that the peaks were pure and the peak spectra were loaded to the spectral library to identify anthocyanins in different fruit juices. Table 4 displays the match factor and the peak purity index (PPI) values of different anthocyanins in four different fruit juices. The match factor expresses the similarity of two spectra (one from the standard and one from the sample). The match factor also refers to the correlation between the spectrum at its peak maximum and the leading and tailing edges. A 100% peak match indicates that the peak start and end do not deviate from the spectrum at the peak maximum, therefore resulting in a perfect match score of 1000. The match values for all anthocyanins were more than 900, with the exceptions of Pg3,5 in PJ, Pg3 in simulated adulterated PJ, and Cy3,5 in pomegranate wildberry juice. Therefore, the anthocyanins separated in all four fruit juices showed high spectral matches with the exception of three anthocyanins, each in only one sample. This suggests that matrix-related interfering peaks may have

co-eluted with the peaks for Pg3,5 in PJ, Pg3 in simulated adulterated PJ, and Cy3,5 in pomegranate wildberry juice, thereby causing the match score to be low.

PPI is another measure for evaluating spectral purity. It represents the wavelength where the areas of the spectrum to the left and right are identical and, therefore, independent of the concentration. In the case of a pure peak, the individual PPI values result in a rectangular curve. The height of each single rectangle corresponds to the value of the central wavelength. The deviation from the rectangle shape can be mathematically expressed by the relative standard deviation of the PPI value. Low RSDs represent good spectral purity, which were observed for all the anthocyanins in all four fruit juices with the exception of Pg3,5 in PJ, Pg3 in simulated adulterated PJ, and Cy3,5 in pomegranate wildberry juice. The PPI values further confirm that some matrix interferences caused the PPI and match scores to be low for Pg3,5, Pg3, and Cy3,5 in PJ, simulated PJ, and pomegranate wildberry juice, respectively. A closer visual inspection of Pg3,5 peak in PJ chromatogram, Pg3 peak in simulated PJ chromatogram, and the Cy3,5 peak in pomegranate wildberry juice chromatogram reveals that all three peaks show a good amount of tailing or fronting, which correlates to the high PPI RSDs.

CONCLUSION

This work describes a sensitive and accurate method to separate and quantify anthocyanins in different fruit juices with a simple dilution of the sample. The method uses a high-resolution, silica-based, Acclaim RSLC C18 column and absorbance detection at a visible wavelength of 540 nm to separate and detect anthocyanins in < 5 min. Several fruit juices with varying concentrations of anthocyanins ranging from 122.5 µg/mL of Cy3,5 in PJ to 1.5 µg/mL of Dp3,5 in pomegranate cherry juice were determined by this method.

PRECAUTIONS

Supplier PhytoLab recommends dissolution of the anthocyanin standards in methanol acidified with 0.01% HCl; however, this experiment showed that using mobile phase A for standard dilution resulted in better peak shapes (tailing was observed with acidified methanol), retention time, peak area, and peak height precisions.

REFERENCES

1. Einbond, L. S.; Reynertson, K. A.; Luo, X.; Basile, M. J.; Kennelly, E. J. Anthocyanin Antioxidants From Edible Fruits. *Food Chem*. **2004**, 23–28.
2. Lee, HS. Characterization of Major Anthocyanins and the Color of Red-Fleshed Budd Blood Orange (Citrus sinensis). *J. Agric. Food Chem*. **2002**, *50*, 1243–6.
3. Lee, J.; et al. Determination of Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorant, and Wines by pH Differential Method: Collaborative Study. *J. of AOAC International* **2005**, *88 (5)*, 1269–1278.
4. Fuhrman, E. A Wonderful World: POM Wonderful Takes Pomegranate to Places It Has Never Gone Before, *Beverage Industry Magazine*, July 15, 2008. Available at http://www.bevindustry.com/Articles/Cover_Story/BNP_GUID_9-5-2006_A_1000000000000383065
5. Zegler, J. Ingredient Spotlight: Pomegranate Packs a Punch, *Beverage Industry Magazine*, January 11, 2010. Available at http://www.bevindustry.com/Articles/Departments/BNP_GUID_9-5-2006_A_1000000000000727599
6. Lutter, R. *Addressing Challenges of Economically-Motivated Adulteration*, Presented at the Public Meeting on Economically Motivated Adulteration, College Park, MD, 2009.
7. Murray, N. Pomegranate: New Target for Crooks. *Food News*, Oct 27, 2006.
8. 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.
9. Dionex Corporation, *Analysis of Fruit Juice Adulterated with Medium Invert Sugar From Beets*. Application Note 82, LPN 034751, 1992, Sunnyvale, CA.
10. Dionex Corporation, *Determination of Organic Acids in Fruit Juices*. Application Note 143, LPN 1415, 2003, Sunnyvale, CA.
11. Dionex Corporation, *Determination of Punicalagins found in Pomegranate by High Performance Liquid Chromatography*. Customer Application Note 106, LPN 2281, 2009, Sunnyvale, CA.

SUPPLIERS

Sigma-Aldrich, 3050 Spruce Street, St. Louis,
MO 63103, U.S.A. Tel: 800-521-8956.
www.sigmaaldrich.com

Sarstedt Inc., 1025 St. James Church Road,
P.O. Box 468, Newton NC 28658-0468, U.S.A.
Tel: +1 828 465 4000.
www.sarstedt.com

Praxair Specialty Gases and Equipment,
39 Old Ridgebury Road, Danbury, CT 06810-5113
U.S.A. Tel: 877-772-9247.
www.praxair.com

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

LPN 2647-01 PDF 08/16