

THE RAPID ANALYSIS OF ANTHOCYANIDINS IN BERRIES

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INTRODUCTION

Anthocyanins are plant pigments that provide vibrant blues and reds in fruits, fruit juices, wine, and vegetables. Konczak et al. has reported that anthocyanins are rich antioxidant compounds thus providing great health benefits to the consumer.¹ There are reports of over 600 anthocyanins identified in food samples including berries thus making direct analysis of these compounds very challenging. However, the analysis of these compounds can be simplified by converting them into their aglycon form (anthocyanidin) by performing an acid hydrolysis on the sample. Acid hydrolysis will convert any of the over 600 anthocyanins into 1 of the 6 common anthocyanidins (delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin). Thus, estimation of the relative abundance of anthocyanins through the analysis of anthocyanidins becomes a simpler task.

Previously, methods for analyzing anthocyanidins have taken anywhere from 34 minutes on a SuperPac Pep-S (4 x 250 mm, 5 μm) RP C18 column to 55 minutes on a C18 Inertsil ODS-3 (4.0 x 150 mm, 3 μm) column.^{2,3} The method, developed with Waters® ACQUITY UPLC™ System performs the separation in 2.1 minutes on an ACQUITY UPLC™ BEH C18 (2.1 x 50 mm, 1.7 μm) column, which is 16 and 26 times FASTER, respectively, than previous HPLC methods while still maintaining baseline resolution of all 6 anthocyanidins (Figure 1).

MATERIALS AND METHODS

Materials

A variety of fresh and frozen berry samples were purchased from a local store. A sample of dried blueberries was also purchased from the same store. All berries were frozen and stored in a freezer. Anthocyanin standards were purchased from ChromaDex (Santa Ana, CA). Hydrochloric acid Gold, phosphoric acid, and acetonitrile (Optima) were purchased from Fisher Scientific (Agawam, MA). Water was purified with a Milli-Q system (Millipore, Billerica, MA).

UPLC Conditions

The Waters® ACQUITY UPLC™ System consisted of the ACQUITY UPLC Binary Solvent Manager (BSM), the ACQUITY UPLC Sample Manager (SM) fitted with a 10 μL loop, and the ACQUITY UPLC Tunable UV (TUV) Detector. All instruments were controlled and data collected and analyzed using Waters Empower™ 2 Software.

UPLC Conditions Continued

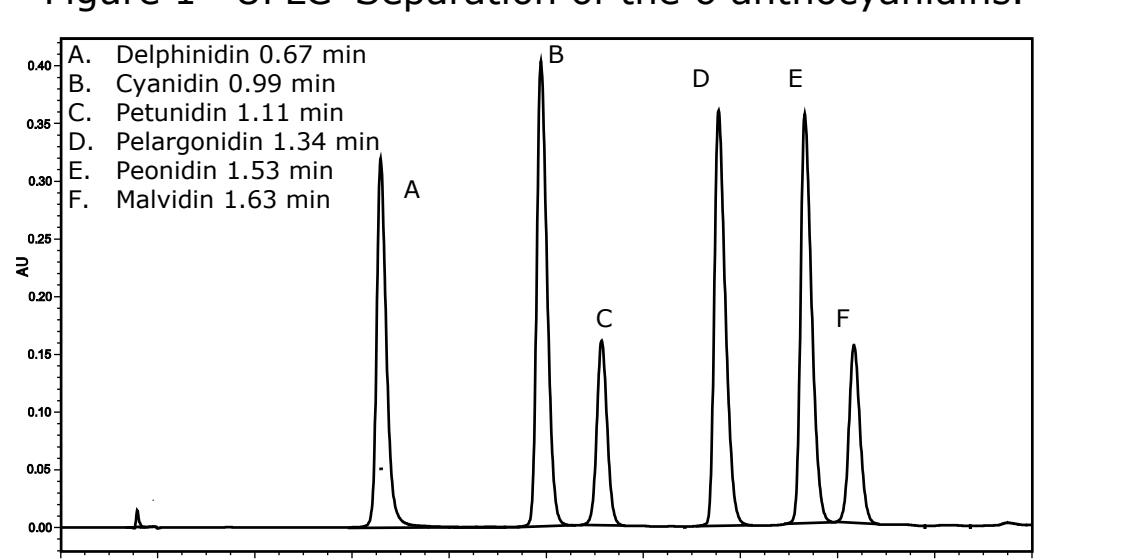
Separations were performed on a 2.1 x 50 mm ACQUITY UPLC BEH C18 column, 1.7 μm at a flow rate of 1.00 mL/minute. Column temperature was set at 40°C and injection volumes for all samples and standards were set to 2 μL .

Water/Acetonitrile (95:5) was used as the weak wash solvent and a mixture of acetonitrile/isopropyl alcohol/water (7:2:1) was used as the strong wash solvent. Mobile phase components and gradient conditions are outlined in Table 1. Detection was set at 525 nm, which is common to all anthocyanidins, using a sampling rate of 20 points per second and a filtering constant of 0.1 seconds.

Calibration

A standard solution of cyanidin, petunidin, pelargonidin, and peonidin (0.100 mg/mL) was prepared from crystalline material in methanol. A seven point standard curve was created from 0.100 mg/mL to 0.001 mg/mL. A second standard solution was created for delphinidin from a pre-dissolved purchased sample because crystalline material was not available. The concentrations of delphinidin were the same as those of the previously mentioned standard solution. A third standard solution for malvidin (0.300 mg/mL) was prepared from crystalline material in methanol. A five point standard curve was created covering a range of 0.300 mg/mL to 0.001 mg/mL. Calibration curves were created for each standard and fit to linear equations with R^2 values all greater than 0.998.

Figure 1—UPLC® Separation of the 6 anthocyanidins.



Time (min)	%A	%B	Curve
Initial	90	10	-
2.0	80	20	6
2.1	90	10	6

Table 1 – Gradient separation conditions, where:
Solvent A = 0.3% Phosphoric Acid in water
Solvent B = 100% Acetonitrile

Sample Preparation, Extraction and Hydrolysis

Anthocyanidins were extracted from the samples by weighing out 25 \pm 0.3 g of berries into a weighing boat. The berries were then transferred into a 100 mL graduated cylinder and 30 mL of extraction solution (80:20 Acetonitrile: 0.3% Phosphoric Acid in water) was added. The berries were then homogenized using a Janke & Kunkel homogenizer for 1.5 minutes. The 1.5 minute duration ensured that all 25 grams of sample would be completely homogenized. The homogenized liquid was then transferred into a graduated centrifuge tube. The graduated centrifuge tube was centrifuged for 10 minutes at 2500 rpm. A 2 mL aliquot of the supernatant was pipetted into a 4 mL vial along with 200 μL of concentrated hydrochloric acid. The vial was then capped with a cap that contained a self sealing septum to minimize loss of liquid to evaporation during hydrolysis. The vial was then placed on a vortex mixer for 5 seconds. The vial was then placed into a chemical oven at 150 \pm 2°C for 30 minutes. After 30 minutes, the vial was removed and placed into a freezer for 10 minutes to stop the hydrolysis. Samples were removed from the freezer and allowed to come to room temperature. Once the vial reached room temperature, the berry sample was filtered through a 0.45 μm filter into a 2 mL vial. The vial was capped and then analyzed by Waters ACQUITY UPLC™ SYSTEM.

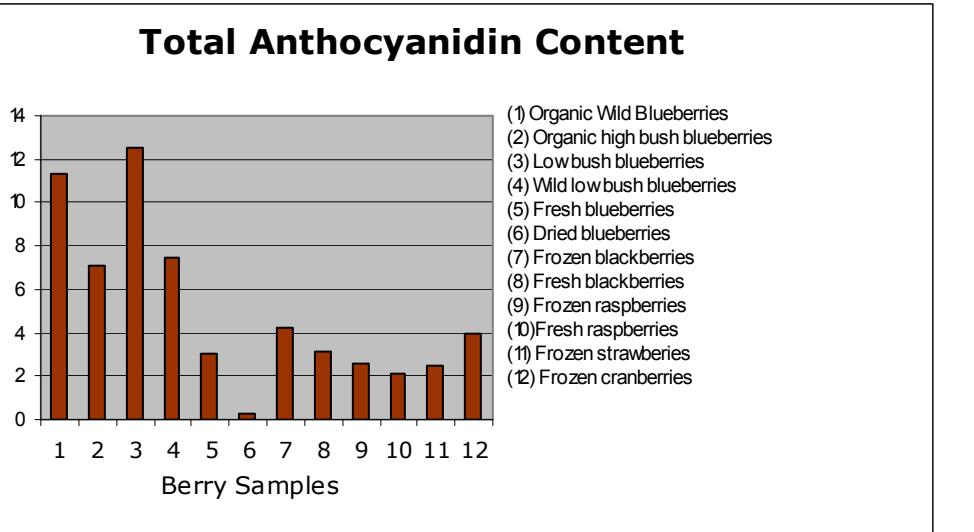


Figure 2—Comparison of total anthocyanidin content in berry samples.



RESULTS AND DISCUSSION

Area counts from injections of hydrolyzed berry extracts, were compared to the standard calibration curves and the amounts (mg/mL) of each component were calculated using Empower™ 2 software. These amounts were then used to back calculate to the original concentration of anthocyanidins in the berries as dry weight, Tables 3 and 4. Dry weight was determined by taking a representative sample of each berry (\sim 15 g), accurately weighing it, and then placing it into a chemical oven at 40°C overnight to remove the water content. The change in weight was considered to be the water content of the berry. The results for this experiment were consistent with those of Wu et al.⁴

As shown in Figure 2, low bush blueberries contained the highest total concentration (12.84 ± 0.59 mg/g) of anthocyanidins per gram of dry berry while purchased dried blueberries contained the lowest (0.26 ± 0.06 mg/g). Table 3 and 4 outline the concentration of each anthocyanidin and the total anthocyanidin concentration for each berry sample tested. Each berry was tested in triplicate and the results reported in Table 3 and 4 are the mean of the three trials with its standard deviation. One reason that blueberries contain the highest concentration can be seen in Figures 3–8. Blueberries contain either 5 or all 6 anthocyanidins while other berries contain anywhere from 1 to 4 anthocyanidins. Notably each berry contained one of the six anthocyanidins in a much higher concentration than the rest of the berries. In the case of blueberries, malvidin is present at almost twice the concentration of any other anthocyanidin. In addition, blueberries were the only berry to contain malvidin. Fresh blackberries contained primarily cyanidin while frozen blackberries contained both cyanidin and a slight trace of pelargonidin. Cyanidin in blackberries was 2 to 3 times higher than in any other berry. Strawberries gave a distinct pelargonidin peak while other berries only contained trace amounts of it. The concentration of peonidin in cranberries was 3 to 4 times higher than in blueberries while most other berries contained no peonidin at all. Cyanidin was the major anthocyanidin in raspberries, but was contained at concentrations approximately half the level found in blackberries. Even though these differences give each berry a unique chromatogram, there was one common link throughout the samples. Cyanidin was the only common anthocyanidin present in each berry sample.

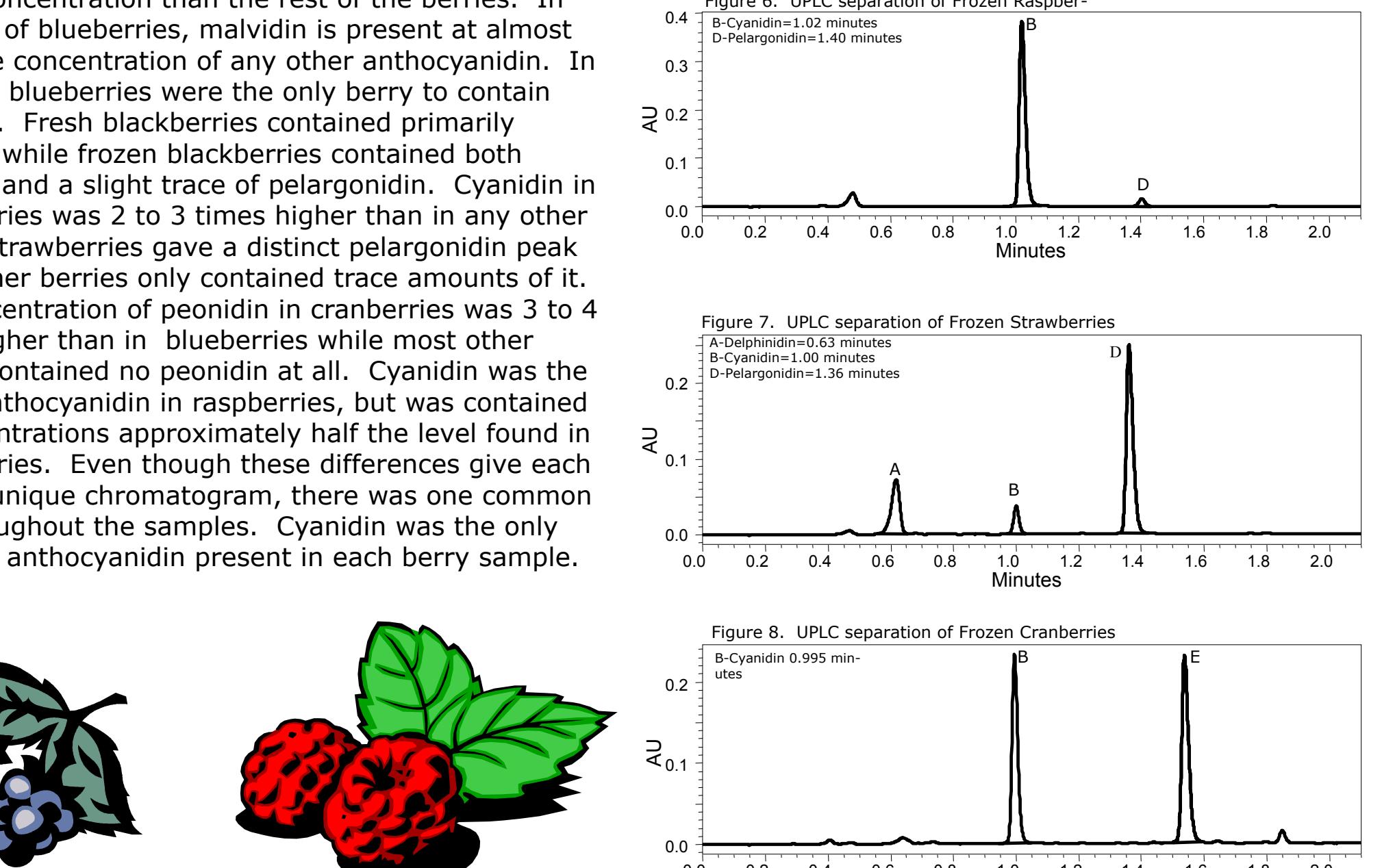


Table 3. Quantitative comparison of blueberry samples

Anthocyanidin	Organic Wild Blueberries	Organic High Bush Blueberries	Low Bush Blueberries	Wild Low Bush Blueberries	Fresh Blueberries	Purchased Dried Blueberries
Delphinidin	0.59 \pm 0.09	0.22 \pm 0.07	0.89 \pm 0.13	0.34 \pm 0.02	0.09 \pm 0.01	-
Cyanidin	1.59 \pm 0.09	0.25 \pm 0.02	1.75 \pm 0.06	0.82 \pm 0.08	0.14 \pm 0.00	0.01 \pm 0.00
Petunidin	3.14 \pm 0.33	1.52 \pm 0.20	3.91 \pm 0.25	2.03 \pm 0.21	0.79 \pm 0.05	0.04 \pm 0.01
Pelargonidin	0.03 \pm 0.00	-	0.03 \pm 0.00	0.03 \pm 0.00	-	-
Peonidin	0.67 \pm 0.02	0.14 \pm 0.02	0.61 \pm 0.02	0.47 \pm 0.09	0.09 \pm 0.00	0.01 \pm 0.00
Malvidin	5.63 \pm 0.57	5.07 \pm 0.11	5.66 \pm 0.22	4.01 \pm 0.68	2.00 \pm 0.08	0.21 \pm 0.05
Total (mg/g)	11.66 \pm 1.09	7.21 \pm 0.33	12.84 \pm 0.59	7.70 \pm 1.05	3.11 \pm 0.09	0.26 \pm 0.06

Table 4. Quantitative comparison of other berry samples

Anthocyanidin	Frozen Blackberry	Fresh Blackberry	Frozen Raspberry	Fresh Raspberry	Frozen Strawberry	Frozen Cranberry
Delphinidin	-	-	-	-	0.27 \pm 0.04	-
Cyanidin	4.18 \pm 0.26	3.14 \pm 0.43	2.40 \pm 0.14	2.03 \pm 0.12	0.25 \pm 0.05	1.73 \pm 0.30
Petunidin	-	-	-	-	0.03 \pm 0.04	0.04 \pm 0.00
Pelargonidin	0.02 \pm 0.02	-	0.12 \pm 0.02	0.10 \pm 0.02	1.96 \pm 0.62	0.04 \pm 0.00
Peonidin	-	-	0.03 \pm 0.00	-	-	2.13 \pm 0.43
Malvidin	-	-	-	-	-	-
Total (mg/g)	4.20 \pm 0.23	3.14 \pm 0.43	2.54 \pm 0.15	2.13 \pm 0.13	2.51 \pm 0.68	3.94 \pm 0.73

CONCLUSIONS

Each berry tested contained one of the six anthocyanidins at a higher concentration than the rest of the berries. However, blueberries had the highest total anthocyanidin content compared to the other berries. Among the blueberries tested low bush blueberries contained the highest concentration of total anthocyanidins per gram of dry berry. After blueberries, blackberries contain the highest concentration followed by cranberries, raspberries, and strawberries respectively.

The ACQUITY UPLC™ System combined with ACQUITY UPLC™ Column technology provides rapid analysis of anthocyanidins and baseline resolution of each of the six standard anthocyanidin components. The ability to decrease analysis time while maintaining resolution creates more efficient and productive laboratories. UPLC™ technology, when applied to the burgeoning field of natural products research, opens possibilities for more rapid and extensive analysis of natural compounds than ever before.

References

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