Analysis of Anthocyanins in Common Foods Using an Agilent Poroshell 120 SB-C18

Application Note

Food/Pharmaceutical

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Abstract

Methods separating anthocyanins compounds originally developed on an Agilent StableBond SB-C18 4.6 × 250 mm 5 µm column or a 4.6 mm × 150 mm, 3.5 µm column are transferred to an Agilent Poroshell 120 SB-C18, 4.6 mm × 75 mm, 2.7 µm column using an Agilent 1260 Rapid Resolution Liquid Chromatograph. The gradient, injection volume, and flow rate are scaled, maintaining retention index for each column evaluated. One Method reduces the time per analysis from 100 minutes to 20 minutes and reduces solvent consumption by 70%. The second transfer reduces time from 67 minutes to 40 minutes and reduces solvent consumption by 40%.

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Introduction

Anthocyanins are water soluble plant pigments responsible for red, blue and purple colors found in many fruits, flowers and plants. The analysis of anthocyanins by HPLC and HPLC MS has been shown to be useful in identifying the fingerprint of different varieties of fruits and or assist in determination of authenticy of fruit juices.

Interest in anthocyanin pigments has intensified because of their possible health benefits as dietary antioxidants. Over 300 structurally distinct anthocyanins have been identified in nature. Anthocyanins are one class of flavonoid compounds, which are widely distributed plant polyphenols. Flavonols, flavan-3-ols, flavones, flavanones, and flavanonols are additional classes of flavonoids that differ in their oxidation state from the anthocyanins.

Qualitative and quantitative analysis of anthocyanins can be used to distinguish between different cultivars of blueberry plants and determine their quality. Therefore, the chromatographic separation of anthocyanins is of increasing importance to the agricultural and wine industries. Recent interest in medicinal use of anthocyanins, as antioxidants/anticancer agents, has also stimulated interest in their chromatographic separation [1].

Traditionally, a low-pH mobile phase (containing formic acid) in these types of separations has caused degradation of the column and change in the separation [2]. Agilent ZORBAX StableBond SB-C18 columns provide the chromatographer with long-term stability for reverse-phase separations requiring very low pH. Many published methods use 50 mL/L (5%) formic acid or 30 mL/L (3%) phosphoric acid. In this work, the methods using phosphoric acid and formic acid are scaled for use with an Agilent Poroshell 120 SB-C18. Several fruit or juice samples are assayed with this new method including blueberries, blackberries, cranberries, strawberries and pomegranate juice.

Experimental

- G1312B Binary Pump SL with mobile phase A: 3% phosphoric acid or 5% Formic Acid in Water and B: Methanol.
- G1367E Automatic Liquid Sampler (ALS) SL.
- G1316B Thermostatted Column Compartment (TCC) SL with temperature set to 30 °C.
- G4212C Diode Array Detector (DAD) SL with the signal set to 525, 16 nm and reference not used, using a G4212-60008 micro flow cell (10-mm path, 1-µL variance).
- ChemStation version B.04.02 was used to control the HPLC and process the data.
- Agilent Poroshell 120 SB-C18, 4.6 mm × 75 mm, 2.7 µm, (p/n 689775-902)
- Agilent ZORBAX SB-C18, 4.6 mm × 250 mm, 5 µm (p/n 880975-902)
- Agilent ZORBAX SB-C18, 4.6 mm × 150 mm, 3.5 µm, (p/n 863953-902)

The formic acid, and phosphoric acid were purchased from Sigma Aldrich (Bellefonte, PA). Methanol was purchased from Honeywell, Burdick and Jackson High Purity, (Muskegon, MI). Water used was 18 M-Ω Milli-Q water (Millipore, Bedford, MA). Fresh blackberries, blueberries, strawberries, cranberries as well as pomegranate juice were purchased from a local grocery store.

Method of Preparing Fruit Extracts

Begin by mixing: 10 g blueberries (or other fruit), 10 mL solvent (70:28:2, MeOH:H_2O, Formic acid), Blend for 10 minutes on dry ice allow ice to sublime. Filter through glass wool in a 10 mL syringe. Allow filtrate to sit for 1 hour. Filter through a 0.2 µm filter. Inject 50 µL immediately for HPLC analysis. (4.6 mm × 250 mm columns) [3,4]. The resulting clear solution is then transferred to an Agilent MS Analyzed write on vial (p/n 5190-2278).

Equation 1: $$k^* = (t_G F)/(d/2)^2 L(\Delta\%B)$$

Where:

t_G is the gradient time,
F is the flow rate
L is the column length
d is the column diameter
Δ%B is the change in organic content across the gradient segment

Figure 1 shows the separation of blueberry extract using totally porous 5 and 3.5 µm SB-C18 columns as well as with a shorter, superficially porous Agilent Poroshell 120 SB-C18 2.7 µm column. Injection volumes are scaled to column volume. The selectivity on both columns should be very similar as they both have the same bonded phase [5,6,7]. Agilent ZORBAX StableBond SB-C18 is particularly well suited to very low pH methods. Previous work has shown that Poroshell 120 achieves approximately 90% of the peak capacity of 1.8 µm totally porous columns at roughly half the pressure. In addition, the efficiency of Poroshell 120 was shown to be 2 × of a 3.5 µm column[6,7]. In this work, the logical progression from long 5 µm columns to shorter 3.5 µm columns to shorter still Poroshell 120 columns is demonstrated. By scaling the gradients to the column length (diameter is kept constant), retention index (k') is kept constant using Equation 1.

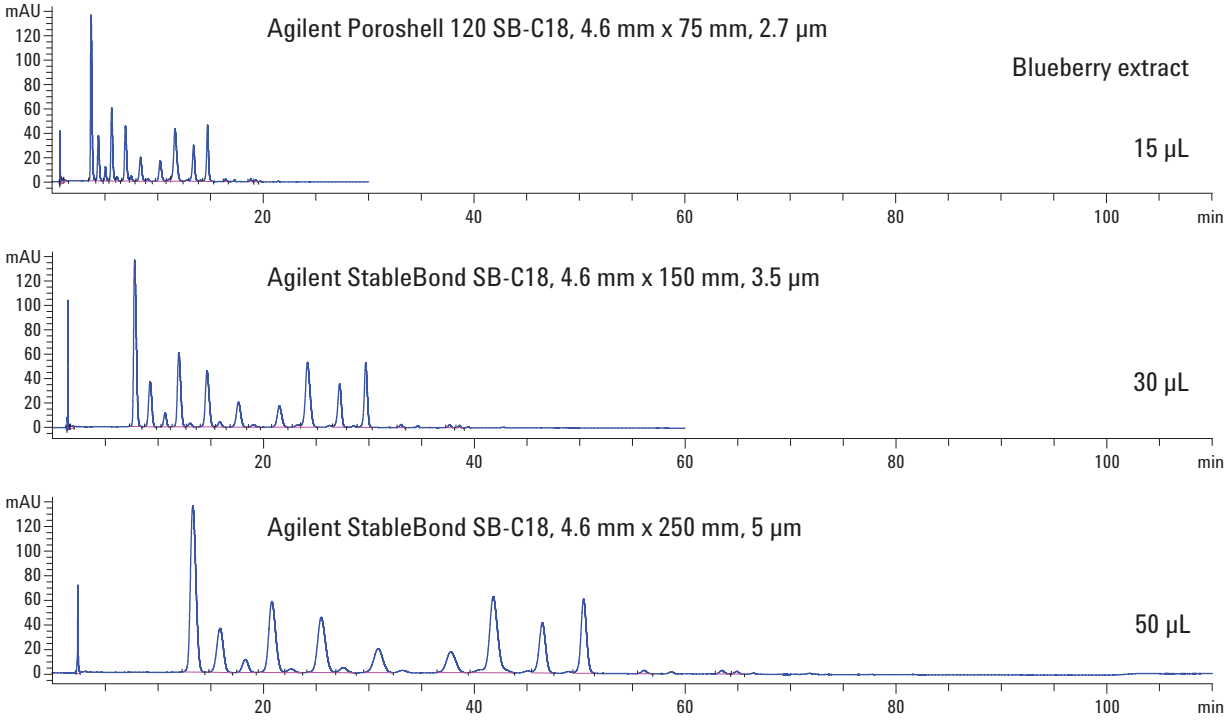

Figure 1. Blueberry anthocyanin analysis on totally porous and superficially porous stableBond C18 columns. Overlay of anthocyanin method with 250 mm 5 µm, 150 mm 3.5 µm, and 75 mm 2.7 µm at 1 mL/min.

Even in the complex example of blueberry extract, all peaks are separated with identical (albeit faster) separations saving both time and solvent. Table 1 shows the gradients used, and the reduction of time from 97 minutes to 29 minutes. No additional sample preparation was performed as all columns evaluated use the same 2 µm column frits. These frits have been shown to be more resistant to clogging than those used on totally porous 3 µm columns [8].

Table 1. Phosphoric Acid Gradients Used in Figures 1,2, and 3a Scaled from Reference 3

Length	**4.6 mm × 250 mm**	**4.6 mm × 150 mm**	**4.6 mm × 75 mm**	**4.6 mm × 75 mm**	**4.6 mm × 75 mm**
Particle	5	3.5	2.7	2.7	2.7
Part number	880975-902	863953-902	689775-902	689775-902	689775-902
Flow rate	1	1	1	1.5	2
Max pressure	183 bar	236 bar	236 bar	349 bar	448 bar
Injection volume	50	30	15	15	15
% B	**Time**	**Time**	**Time**	**Time**	**Time**
23	0	0	0	0	0
26	35	21	10.5	7.5	5.25
60	97	58	29.1	20	10

In Figure 2, flow rate or linear velocity is increased. Previous work has shown that higher peak capacity for Agilent Poroshell 120 EC-C18 can be obtained at higher linear velocity between 1.5 and 2.5 mL/min on a 4.6 mm column [9]. By employing equation 1 and keeping k' constant the separation is maintained. In this case pressure maxima of 448 bar is reached at 2 mL/min at 50% Methanol content as show in the chromatograms. However at 1.5 mL/min the maximum pressure is under 400 bar. Gradients used in this work are listed in Table 1.

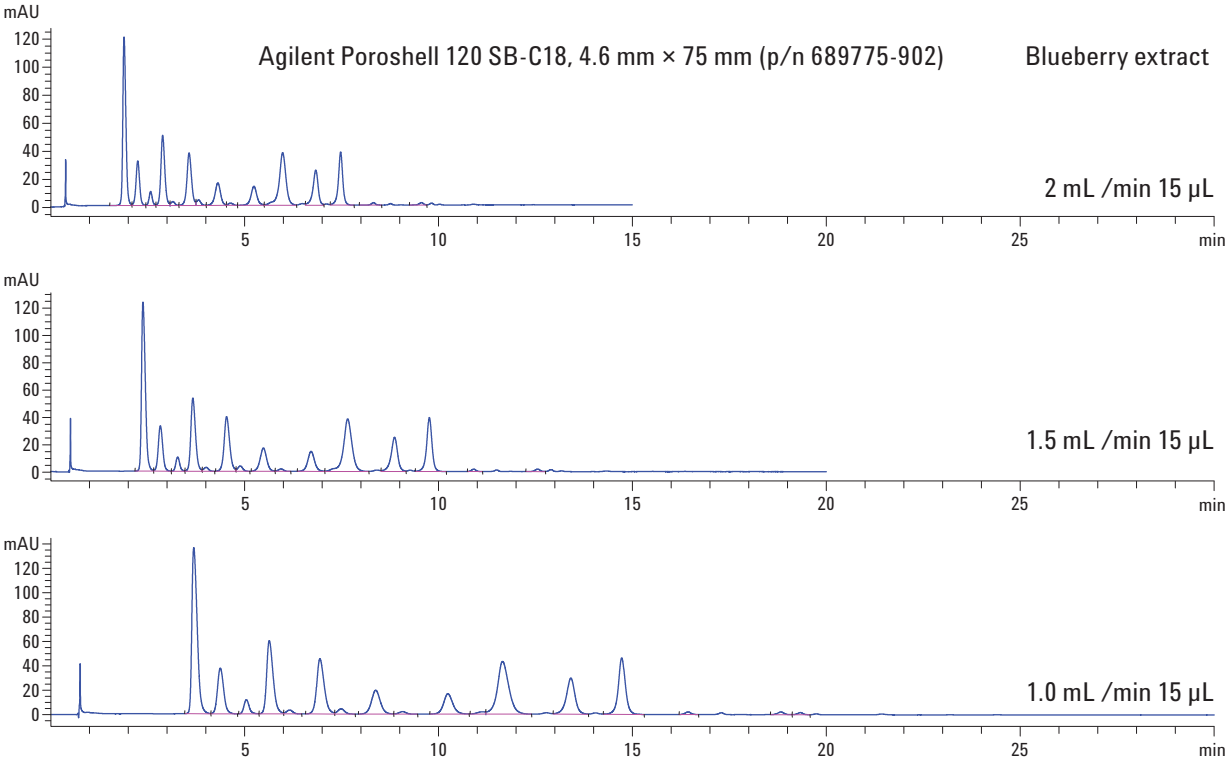

Figure 2. Overlay of blueberry extract on Anthocyanin Method using an Agilent Poroshell 120 SB-C18 4.6 mm × 75 mm 2.7 µm at varied flow rates.

Figure 3a shows real samples as analyzed using the 1.5 mL/min method as described in Table 1. Samples of blueberry, blackberry, cranberry and strawberry were prepared using acidified methanol [3,4]. Pomegranate juice was injected without further preparation. As shown in Figure 1, the complex blueberry chromatogram shows approximately 20 peaks (16 major, 4 minor). Work by Kalt has shown that the more wild and stressed blueberry varieties have more Anthocyanin peaks [4]. Blackberry, a cultivated variety shows few peaks, but is consistent with previously reported data [10]. Cranberry shows the distinctive (cyd-3-gal, cyd-3-glu, cyd-3-arab, pnd-3-gal, pnd-3-glu, pnd-3-arab) depicted in reference [11], and also shown in references [10, 12, 13]. Strawberry also appears consistent with previously reported data. Figure 3b shows similar results using 5% formic acid on a slightly different gradient. The original gradient run was 1.5 mL/min and no attempt was made to increase the linear velocity of the mobile phase. The original and resulting scaled gradient used is listed in Table 2. The use of formic acid instead of phosphoric acid allows the use of mass spectrometry for identification, but the chromatography using either mobile phase modifier leads to similar fingerprinting results.

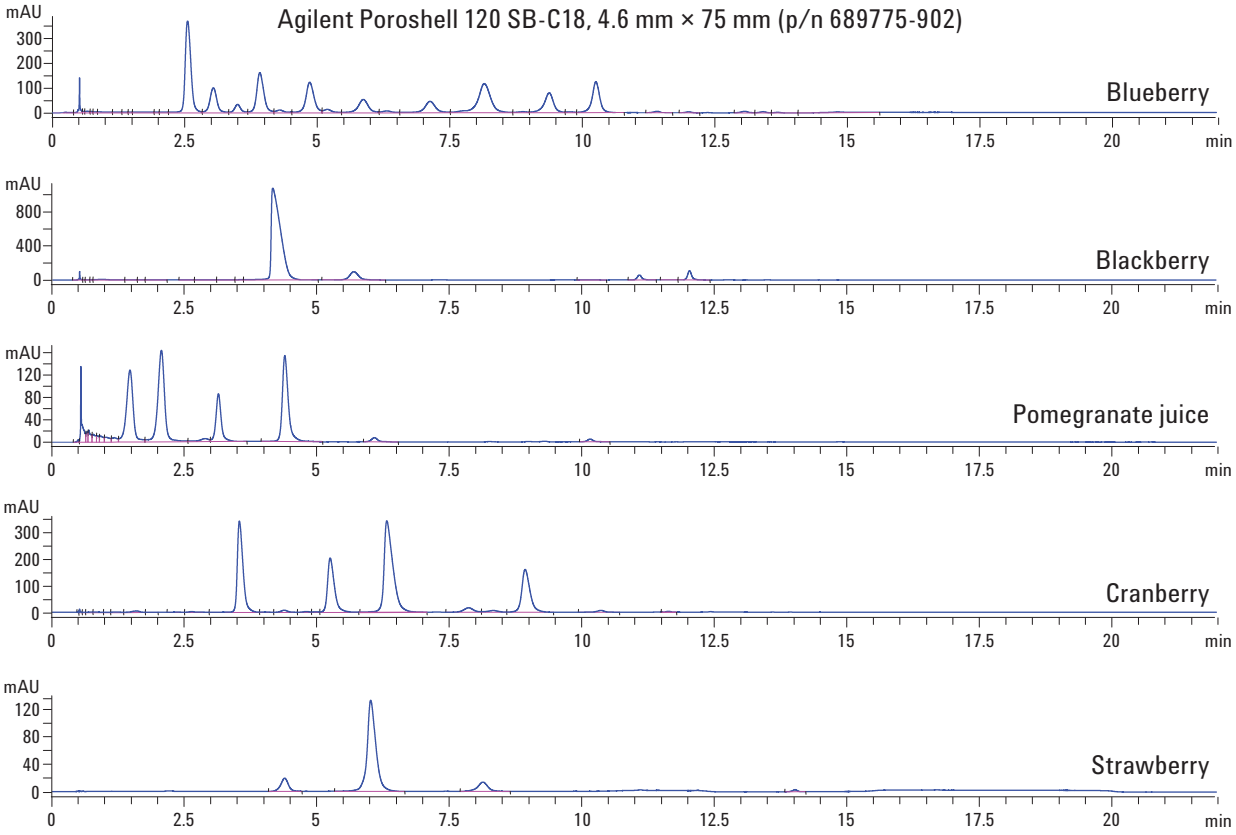

Figure 3a. Overlay of Anthocyanin Method using an Agilent Poroshell 120 SB-C18 of varied samples using a H_3PO_4 gradient.

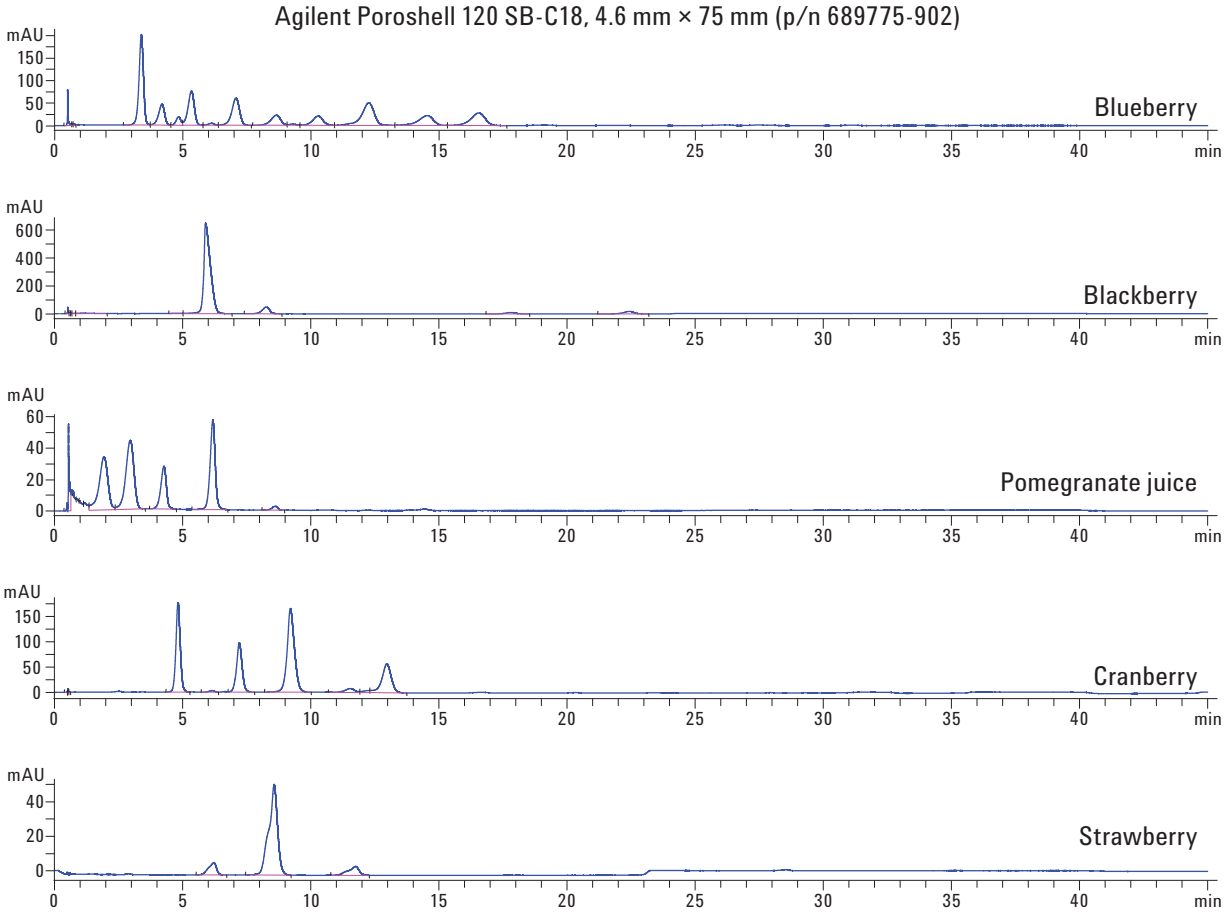

Figure 3b. Overlay of Anthocyanin Method using an Agilent Poroshell 120 SB-C18 of varied samples using a HCO_2H gradient.

Table 2. Formic Acid Gradients Used in Figure 3b Scaled From Reference 4

Length	4.6 x 250	4.6 x 75
Particle	5	
Part number	880975-902	68775-902
Flow rate	1.5	1.5
Max pressure	274 bar	349 bar
Injection volume	50	15
%B	**Time**	**Time**
14	0	0
17	10	6
23	35	21
47	65	39
14	67	40.2

Conclusion

HPLC columns packed with superficially porous particles offer many advantages over columns packed with conventional, fully porous particles. The superficially porous Agilent Poroshell 120 SB-C18, 2.7-µm offers similar selectivity to Agilent ZORBAX StableBond SB-C18 columns. The use of Poroshell 120 SB-C18 for the analysis of Anthocyanins has been shown to allow faster analysis with lower solvent use per sample. The importance of this faster and less solvent expensive change can be easily seen when applying the analysis to differentiate plant species [4] or assay varieties of foods [10, 12] where hundreds of samples have been analyzed requiring more than an hour to separate each sample. Substantial amounts of time could have been saved or more samples assayed allowing further differentiation of plant species. Using formic acid as a mobile phase additive would allow further identification using mass spectrometry.

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Printed in the USA
August 26, 2011
5990-8249EN

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