A Variety of Agilent ZORBAX RRHD Phases Offers Selectivity Options for the Determination of Anthocyanins in Blueberries with UHPLC/MS

Application Note

Food and Beverage

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Abstract

Blueberries are extracted into acidified methanol and analyzed for anthocyanins with a simple methanol/formic acid gradient via UHPLC/MS; four different Agilent ZORBAX RRHD stationary phases are quickly evaluated: Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, StableBond SB-Aq, and StableBond SB-Phenyl. The Phenyl-Hexyl column separates the most anthocyanin peaks from the blueberry sample, while the SB-Phenyl is the most orthogonal of the columns. Glycosides and acylglycosides of five common anthocyanidins (cyanidin, delphinidin, peonidin, petunidin, and malvidin) are observed.

Introduction

Blueberries have among the highest antioxidant capabilities of all fruits and vegetables, while being low in calories, high in fiber, and full of flavor, making them often referred to as a super food [1]. Antioxidants get a lot of attention because of their commonly recognized health benefits, such as their anti-cancer abilities and cardiovascular benefits. The majority of blueberries' antioxidant capabilities can be attributed to their abundance of anthocyanins. Anthocyanins are natural red-blue water soluble pigments found not only in blueberries, but also in many other fruits, flowers and plants. Anthocyanins are glycosides and acylglycosides of anthocyanidins [2]. Five common anthocyanidins are observed in blueberry extract and addressed in this application note, cyanidin, delphinidin, peonidin, petunidin, and malvidin. See Figure 1 for the basic chemical structure of these anthocyanidins.

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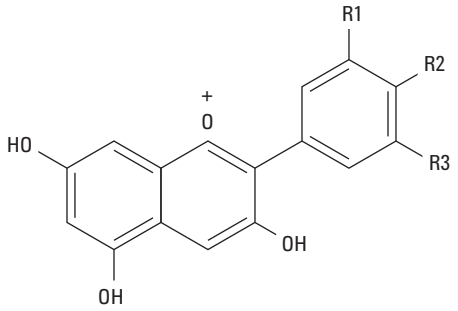

Anthocyanidin	R1	R2	R3	MW
Cyanidin	OH	OH	H	287
Delphinidin	OH	OH	OH	303
Peonidin	OCH_3	OH	H	301
Petunidin	OCH_3	OH	OH	317
Malvidin	OCH_3	OH	OCH_3	331

Figure 1. Chemical structures of five common anthocyanidins found in blueberry extract.

Advancements in liquid chromatography have lead to significantly improved sample throughput, which is advantageous to analytical method development. The Agilent 1290 Infinity UHPLC and Agilent ZORBAX Rapid Resolution High Definition (RRHD) columns are manufactured to withstand pressures up to 1200 bar. This allows the use of faster flow rates and rapid column screening to easily take advantage of stationary phase selectivity differences during method development.

Two different phenyl columns are currently available within the ZORBAX RRHD family, Eclipse Plus Phenyl-Hexyl and StableBond SB-Phenyl. Both phases excel with the analysis of anthocyanins due to the compounds' abundant conjugation. The π electrons in double bonds in these compounds interact with the π electrons in the phenyl stationary phase, providing a unique selectivity mechanism over traditional alkyl phases, such as C18s [3]. While the π-π interactions are not as strong as the hydrophobic interactions responsible for retention with alkyl phases, they may provide slight selectivity advantages for phenyl columns when analyzing closely related conjugated compounds.

Experimental

This application note is based on a recent publication by W. Long, where anthocyanin method parameters are scaled from a 250 mm, 5 µm StableBond SB-C18 column to a superficially porous 75 mm, 2.7 µm Poroshell 120 StableBond SB-C18 column [4]. The blueberry extract sample was prepared by W. Long and provided for this experiment.

An Agilent 1290 Infinity UHPLC with an Agilent 6410 Triple Quadrupole Mass Spectrometer was used in this experiment:

Mobile phase	A - 5% formic acid in water B - methanol
Flow rate	0.65 mL/min
Gradient	10–50% B in 15 minutes
Sample	5 µL injection of blueberry extract
TCC	30 °C
DAD	Sig = 525, 8 nm; Ref = Off
MS	MS2 Scan: 200-1000, ESI positive mode
Scan time	100 ms, 0.2 amu step
Fragmentor	180 V
Drying gas	10 L/min, 350 °C
Nebulizer pressure	50 psig
Capillary voltage	3500
EICs for anthocyanidins	Cyanidin *m/z* 286 Delphinidin *m/z* 302 Peonidin *m/z* 300 Petunidin *m/z* 316 Malvidin *m/z* 330

MassHunter versions B.03.01, B.02.00, and B.03.01 were used for data acquisition, qualitative and quantitative analyses respectively

Four Agilent ZORBAX RRHD 2.1 × 100 mm, 1.8 µm columns were used:

- Eclipse Plus C18 (p/n 959758-902)
- Eclipse Plus Phenyl-Hexyl (p/n 959758-912)
- StableBond SB-Aq (p/n 858700-914)
- StableBond SB-Phenyl (p/n 858700-912)

Anthocyanins were extracted from fresh, locally purchased blueberries (Wilmington, DE, USA) as follows:

- Blend 10 g blueberries + 10 mL 70:28:2 CH_3OH:H_2O:HCOOH for 10 min on dry ice, allow to sublime
- Filter through glass wool in a 10 mL syringe, allow filtrate to sit for 1 h
- Filter through a 0.2 µm filter

Results and Discussion

Ten grams of blueberries are extracted into acidified methanol and analyzed by LC/UV in Figure 2. Four different Agilent ZORBAX RRHD stationary phases are used, Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, StableBond SB-Aq, and StableBond SB-Phenyl. All columns are of the same 2.1 × 100 mm dimension, packed with 1.8 µm particles, and stable to 1200 bar. A rapid screening of these four columns is possible within this 1200 bar system and column pressure limit. With this methanol/formic acid gradient at 0.65 mL/min, the maximum pressure generated is 1020 bar, and each analysis is accomplished in only 15 minutes. Because the UV detector is not optimal for identifying unknown peaks or locating coeluting peaks, it is difficult to determine a best stationary phase for this analysis based solely on the UV data shown in Figure 2.

To determine more information regarding the compound for each peak, an MS detector can be used. Figure 3 shows the total ion chromatograms (TIC) from a scan from 200–1000 with the blueberry extract on each column. MS detection is more sensitive than UV, revealing many more peaks, including several small late eluting peaks that are not present on the UV trace. While the TIC itself does not reveal significantly more information than the UV data, extracted ion chromatograms (EIC) reveal the glycosides and acylglycosides of five common anthocyanidins (cyanidin, delphinidin, peonidin, petunidin, and malvidin) in this blueberry extract.

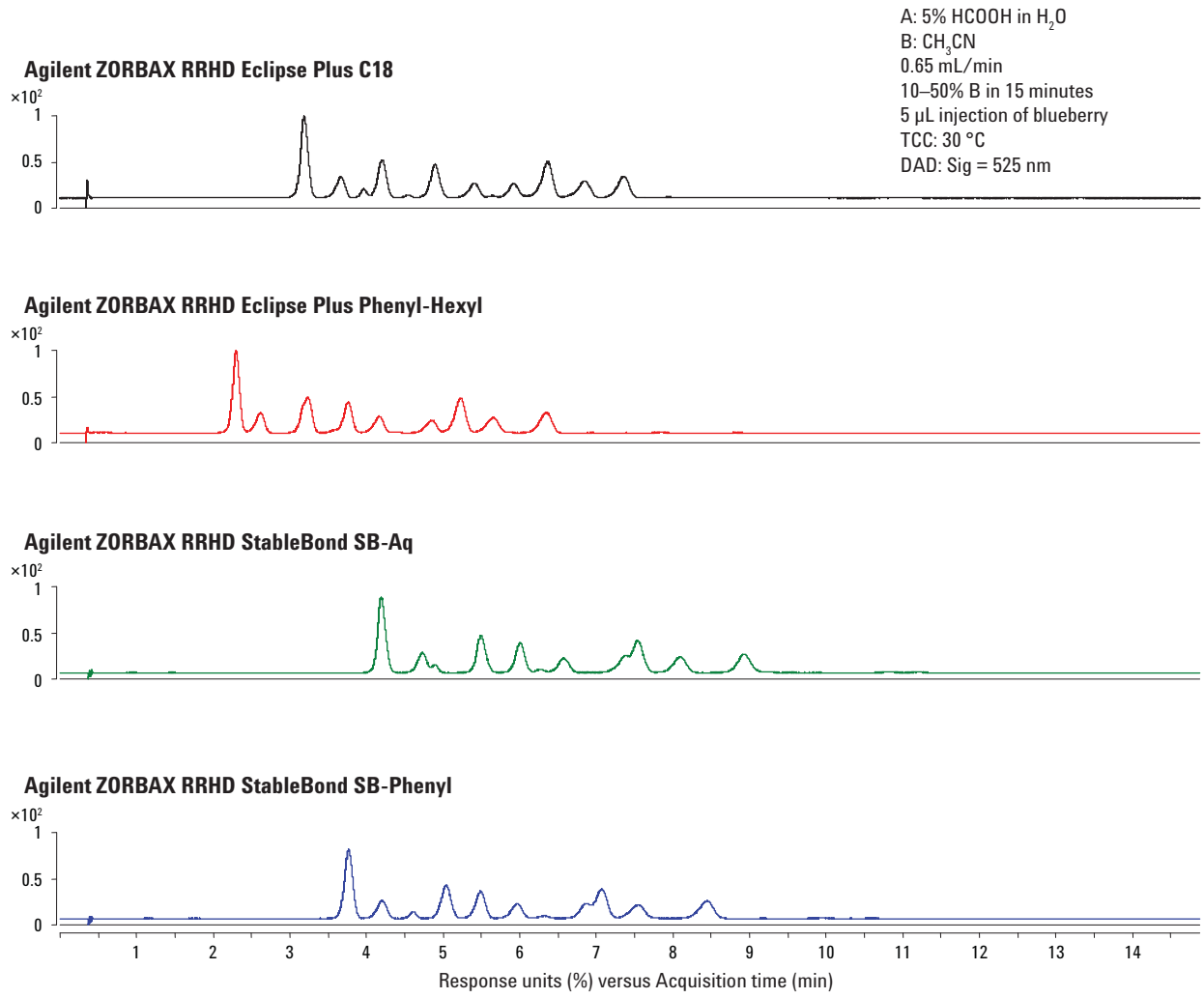

Figure 2. Analysis of anthocyanins extracted from blueberries on four Agilent ZORBAX RRHD columns, with detection by UV. See Experimental section for detailed method parameters.

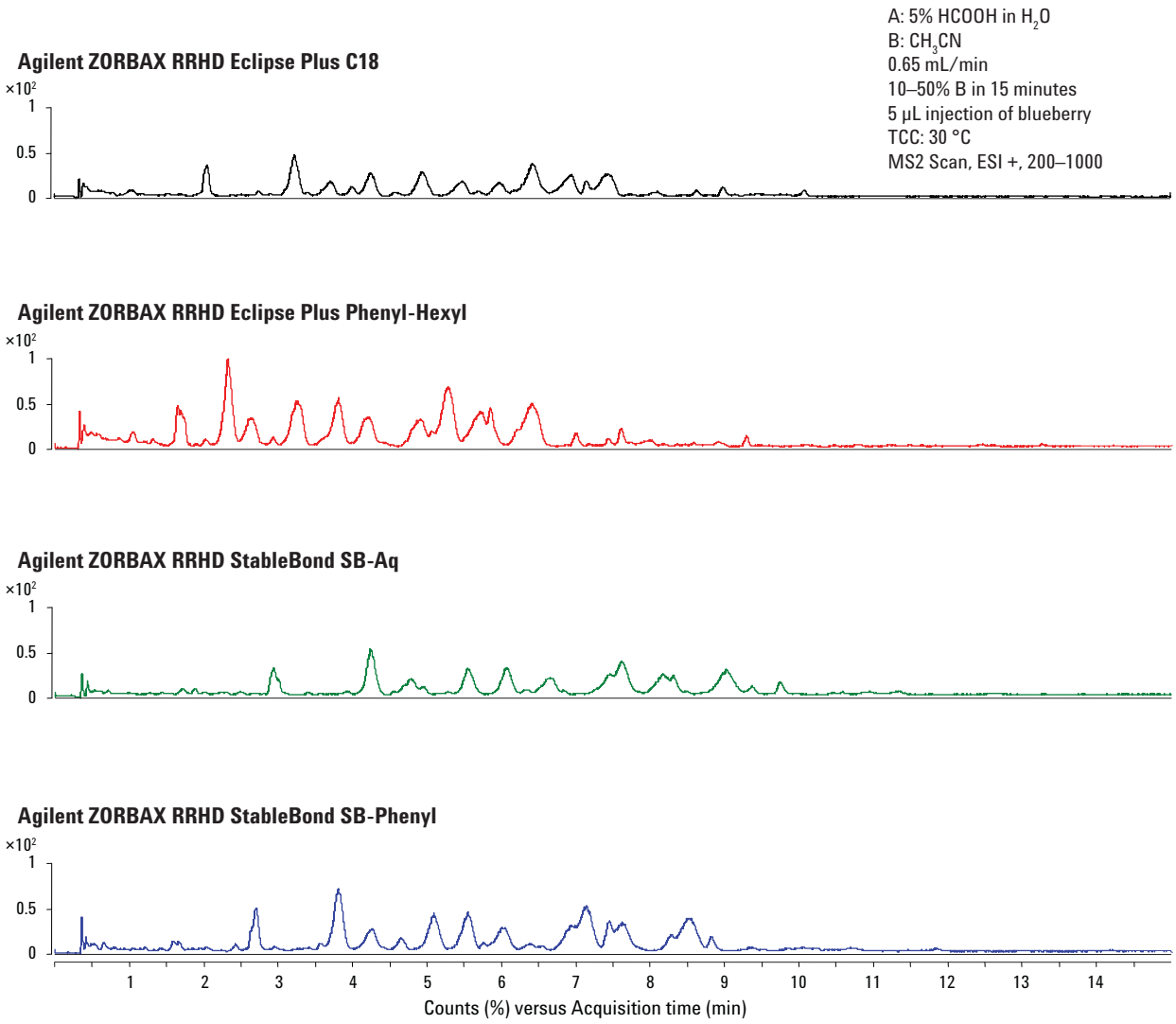

Figure 3. Analysis of anthocyanins extracted from blueberries on four Agilent ZORBAX RRHD columns, with detection by MS scan. See Experimental section for detailed method parameters.

The EIC's displayed in Figure 4 clearly show the distinct glycosides and acylglycosides of the five different anthocyanidins, each marked with a unique color. By looking at the EICs it is easier to see many of the smaller peaks in the chromatogram, several of which coelute with larger peaks and were not noticeable with the UV or TIC chromatograms in Figures 2 and 3 respectively. From these EICs, the total number of resolved anthocyanin peaks is determined and summarized in Table 1. This data shows that the Eclipse Plus Phenyl-Hexyl column resolves a few more anthocyanin peaks with this methanol/formic acid gradient than the other three phases.

Additionally, a scatter plot to determine column orthogonality is shown in Figure 5. The retention factor, k', is plotted for all anthocyanin peaks on each column versus the best column, the Eclipse Plus Phenyl-Hexyl. The StableBond SB-Phenyl exhibits the most varied retention, as shown by its low slope, m = 0.9175, and low correlation coefficient, R^2 = 0.8711. The StableBond SB-Aq also shows very different retention, as compared to the Eclipse Plus Phenyl-Hexyl. This different retention is most likely due to the StableBond phases having more exposed silanols than the more end-capped Eclipse Plus phases.

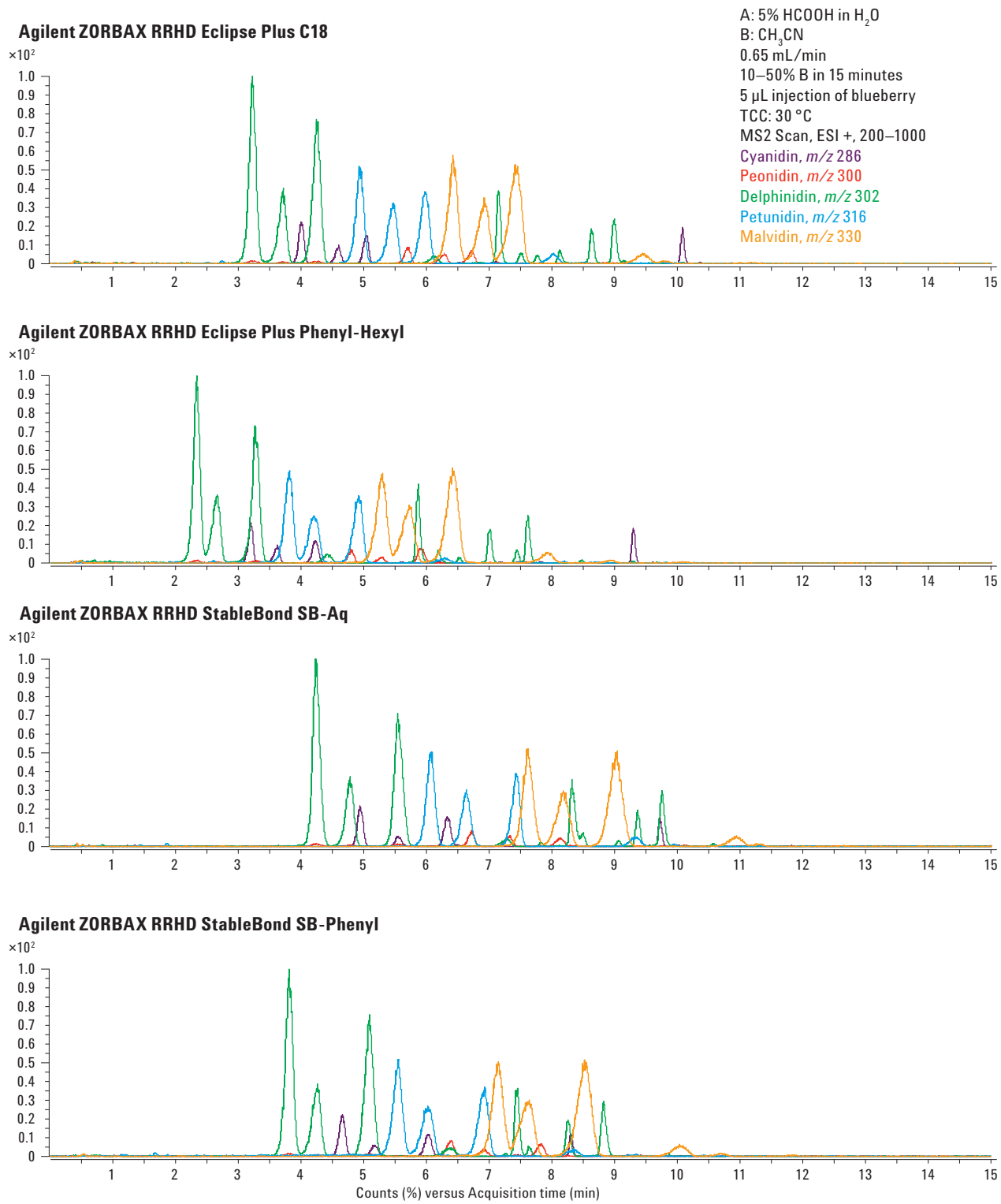

Figure 4. Extracted ion chromatograms from LC/MS scan data of blueberry anthocyanins shown in Figure 3. See Experimental section for detailed method parameters.

Table 1. Total Number of Blueberry Anthocyanin Peaks Resolved by Four Agilent ZORBAX RRHD Columns with a Methanol/Formic Acid Gradient

	Agilent ZORBAX RRHD Eclipse Plus C18	Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl	Agilent ZORBAX RRHD StableBond SB-Aq	Agilent ZORBAX RRHD StableBond SB-Phenyl
Cyanidin, *m/z* 286	11	13	10	10
Peonidin, *m/z* 300	9	8	9	10
Delphinidin, *m/z* 302	12	13	12	11
Petunidin, *m/z* 316	9	12	9	9
Malvidin, *m/z* 330	6	6	6	6
Total number of resolved peaks	47	52	46	46

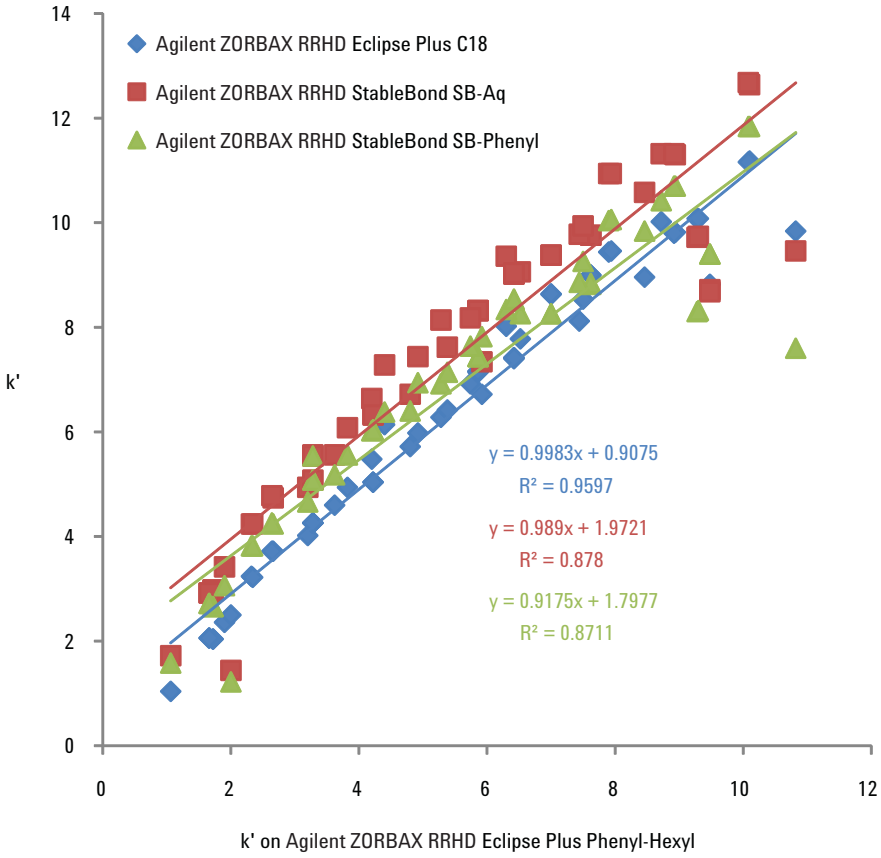

Figure 5. Scatter plot of column orthogonality, as compared to the Agilent ZORBAX RRHD Eclipse Plus Phenyl-Hexyl; retention index, k', is plotted for each anthocyanin. See Experimental section for detailed method parameters.

Conclusions

The LC/MS scan reveals that the Eclipse Plus Phenyl-Hexyl column separates more anthocyanins in this blueberry extract than the other three phases shown, among which five common anthocyanidins are observed, cyanidin, delphinidin, peonidin, petunidin, and malvidin. Anthocyanin compounds have many double bonds that interact with phenyl columns using π-π interactions. Additionally, the SB-Phenyl exhibits the most varied elution order, due to its exposed silanols, in addition to the phenyl properties.

References

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