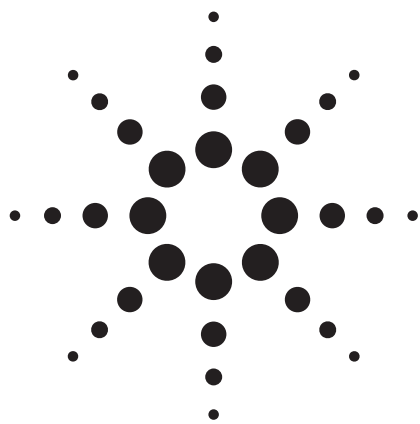




Selective Analysis of Non-Nutritive Food Additives Using Agilent ZORBAX Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl Columns

Application Note

Food

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Abstract

Nine common food additives are examined using 3.5-micron ZORBAX Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl Rapid Resolution columns with an isocratic solvent system. The mobile phases used include methanol or acetonitrile with 20 mM phosphate buffer at pH 2.5 or 0.2 percent formic acid. While excellent peak shape is found on all columns examined, selectivity and resolution differences are found when comparing C18 and various phenyl columns, especially the Eclipse Plus Phenyl-Hexyl and StableBond SB-Phenyl columns. In addition, selectivity differences and peak shape are preserved even when using a formic acid mobile phase modifier. The use of formic acid would allow verification of unknown materials using mass spectrometry.



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Introduction

Non-nutritive food additives are often found in food and non-food consumer products; they serve to maintain freshness and enhance flavor. Some of these compounds include preservatives, such as parabens and benzoic acid, which have antibacterial properties, and also acids, such as ascorbic acid and sorbic acid, to act as sacrificial oxidants. Non-nutritive sweeteners, like aspartame and saccharin, are commonly used in products where natural sweeteners would not be welcome, such as diet beverages or dental products. Finally, materials such as caffeine are frequently added to beverages, gums, and candies as mild stimulants. When present in appropriate amounts, each of these additives is nontoxic and serves an important function; however, at higher levels some can evoke allergic reactions or, after prolonged exposure, they can cause sensitization in certain individuals. To prevent these potentially negative side effects, it is important that the levels of additives be regulated and monitored. One common structure found in these additive compounds is their conjugation, which may interact with phenyl stationary phases, resulting in varied selectivity when compared to alkyl and other phenyl phases.

Alkyl columns, such as the Agilent ZORBAX Eclipse Plus C18, are often regarded as an all-purpose column, as they are sufficient for a variety of analyses. Many chromatographers center HPLC method development on C18 columns; however, other columns can offer advantages in selectivity. While the C18 separation is based primarily on hydrophobic interactions, other columns, such as phenyl columns, can also separate based on a degree of aromatic selectivity using π - π interactions. The degree of π - π interactions is based on many factors, including the type of phenyl phase and the type of silica. This has been shown in references 1 through 3. Other examples of applied selectivity with phenyl columns can be found [4–8]; they are shown with 1.8-, 3.5-, and 5.0- μ m particles. Agilent offers three phenyl phases in 3.5-micron size, which include the new Eclipse Plus Phenyl-Hexyl, the Eclipse XDB-Phenyl (an ethyl phenyl phase), and the StableBond SB-Phenyl (an ethyl phenyl phase attached to nonendcapped type B silica). The 3.5-micron particles have been previously shown to offer increased efficiency over 5-micron materials. They offer the efficiency of a 25-cm 5-micron column in a 15-cm column length. In many conservative laboratories, changing to these proven columns provides 40 percent or better time and solvent savings. These columns are ideal for use in food laboratories, as they provide advantages of efficiency and robustness.

In this work, a group of non-nutritive food additives will be used to advocate the use of phenyl columns in HPLC method development with conjugated samples. Select consumer product samples will also be analyzed to demonstrate how the level of complexity of a sample might also dictate column choice with respect to resolution and speed of analysis.

Experimental

An Agilent 1200 Rapid Resolution LC (RRLC) system was used for this work:

- G1312B Binary Pump SL with mobile phase A: 20 mM monobasic potassium phosphate in water, pH 2.5 or 0.2% formic acid; B: methanol or acetonitrile. Flow rate was 1.5 mL/min. For methanol mobile phase, the analysis was isocratic with A/B (70:30); when equivalent solvent strength acetonitrile was substituted for methanol, the analysis was isocratic with A/B (79:21).
- G1376C Automatic Liquid Sampler (ALS) SL. Injection volume was 2.0 μ L.
- G1316B Thermostated Column Compartment (TCC) SL. Temperature was 25 °C.
- G1315C Diode Array Detector (DAD) SL. Wavelength was 220, 16 nm Ref = 420, 20 nm, with a G1314-60083 semi-micro flow cell (6-mm path, 5- μ L volume).
- ChemStation version B.02.01 was used to control the HPLC and process the data.

Four Agilent ZORBAX columns were used in this work:

- ZORBAX Rapid Resolution Eclipse Plus C18, 4.6 mm $\times$ 150 mm, 3.5 μ m, Agilent p/n 959963-902
- ZORBAX Rapid Resolution Eclipse Plus Phenyl-Hexyl, 4.6 mm $\times$ 150 mm, 3.5 μ m, Agilent p/n 959963-912
- ZORBAX Rapid Resolution Eclipse XDB-Phenyl, 4.6 mm $\times$ 150 mm, 3.5 μ m, Agilent p/n 963967-912
- ZORBAX Rapid Resolution StableBond SB-Phenyl, 4.6 mm $\times$ 150 mm, 3.5 μ m, Agilent p/n 863953-912

Figure 1 shows the compounds that were examined in this work with their respective structures, pKa values, and additive functions. All compounds were dissolved in water to a concentration of about 2 mg/mL. A composite sample was then made by combining equal aliquots of each solution.

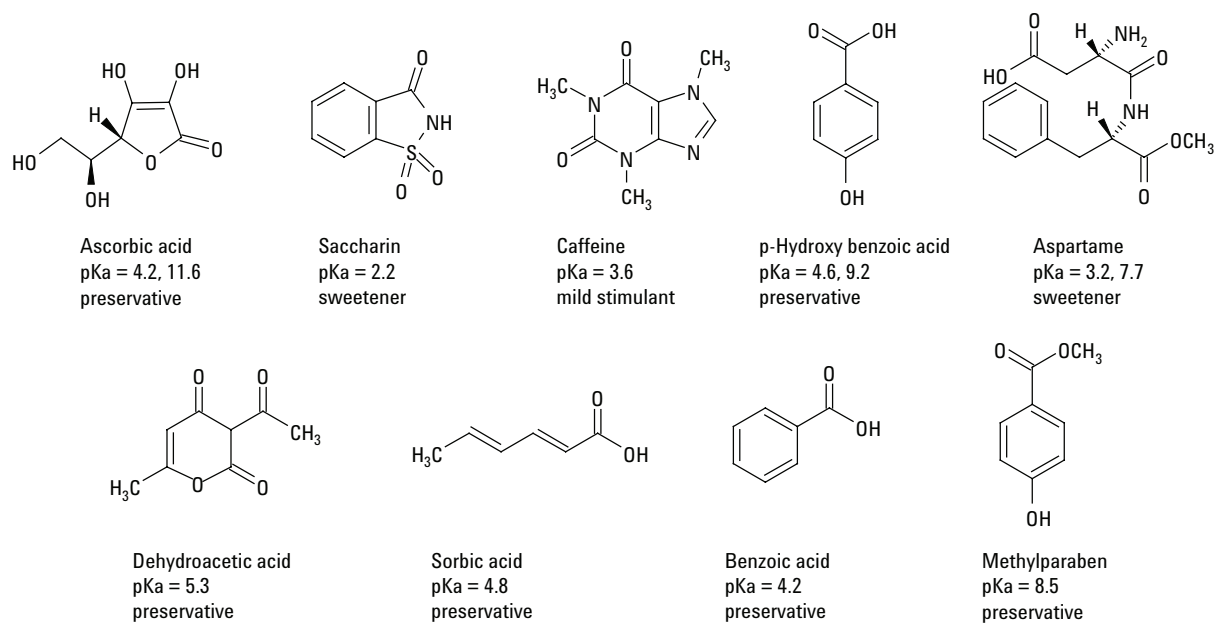


Figure 1. Compounds of interest (non-nutritive additives).

Compounds were purchased from Sigma Aldrich (Bellefonte, PA). In addition, formic acid, phosphoric acid, and monobasic potassium phosphate were also purchased from Sigma Aldrich. Methanol was purchased from Honeywell, Burdick and Jackson (Muskegon, MI). Water used was 18 M-Ω Milli-Q water (Bedford, MA).

Results and Discussion

The compounds shown in Figure 1 are found as ingredients in many food and non-food consumer products. These include, but are not limited to, beverages and dental care products.

In this work we investigated the selectivity between ZORBAX Eclipse Plus C18, Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl. In the chromatograms shown in Figure 2 we compare the separation of these compounds using Rapid Resolution 4.6 mm × 150 mm, 3.5-micron columns.

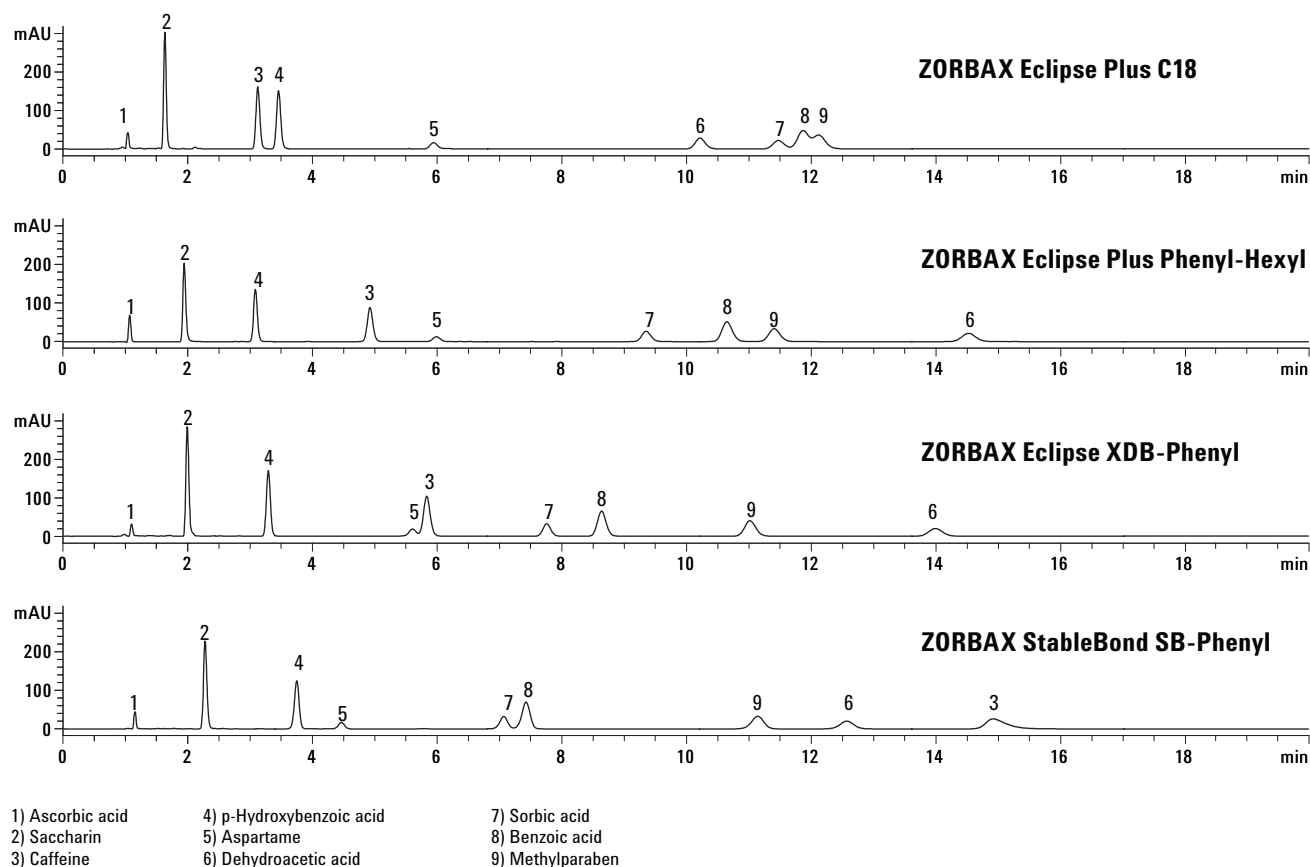


Figure 2. Selectivity of additives on C18 and different phenyl columns, using 30% methanol 70% 20 mM phosphate buffer, pH 2.5; flow rate was 1.5 mL/min.

The largest selectivity differences are seen in caffeine (3), dehydroacetic acid (6), sorbic acid (7), and benzoic acid (8). For all columns, there is no significant difference in the elution of ascorbic acid (1), saccharin (2), p-hydroxybenzoic acid (4), aspartame (5), and methylparaben (9). The Eclipse Plus Phenyl-Hexyl column provides the best separation for this application under the given conditions, with a minimum resolution of $R = 2.32$. The Eclipse Plus C18 column also delivers excellent separation for the first six compounds; however, the separation of sorbic acid (7), benzoic acid (8), and methylparaben (9) is not as good as that shown on the Eclipse Plus Phenyl-Hexyl column. Also, the caffeine (3) and p-hydroxybenzoic acid (4) peaks are in reverse order on the phenyl-hexyl column compared to the C18 column. The StableBond SB-Phenyl column provides the second best separation (minimum resolution, $R = 1.51$), with substantially different selectivity;

most significant is the movement of the caffeine to the end of the chromatogram, preceded by dehydroacetic acid, which elutes in the sixth position on the C18 column. Figure 3 demonstrates the same separation with an equal strength acetonitrile mobile phase compared to the methanol separation shown in Figure 2. What is most notable with acetonitrile is the significant compression of the phenyl chromatograms and the loss of selectivity differences among the C18 and phenyl columns as compared to the methanol separation. It has been theorized that the π - π interactions between the analyte and bonded phase are overwhelmed by the π bonds in acetonitrile [9]. It is evident that the methanol separation is superior to the acetonitrile separation in these cases.

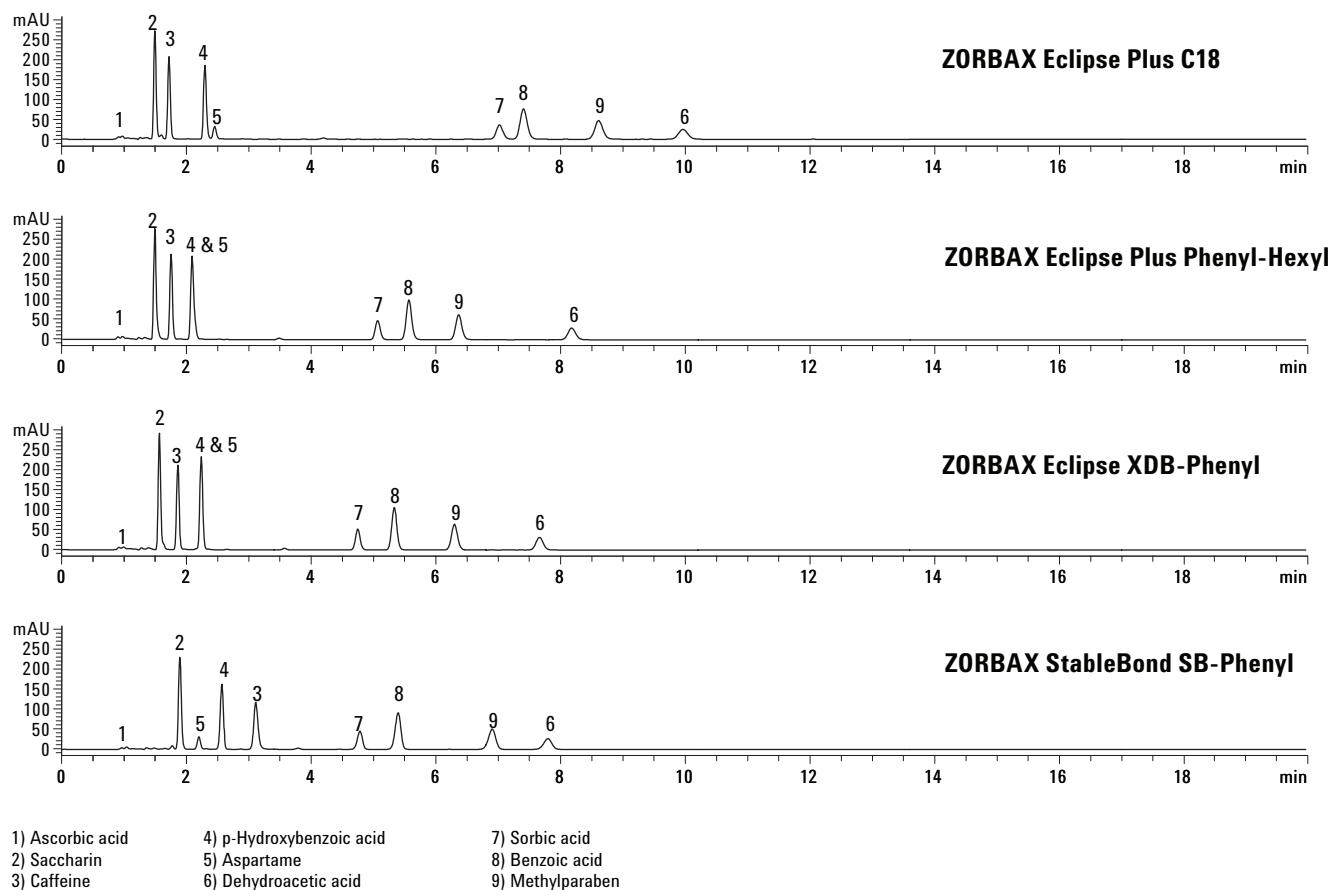


Figure 3. Selectivity of additives on C18 and different phenyl columns, using 21% acetonitrile 79% 20 mM phosphate buffer, pH 2.5; flow rate was 1.5 mL/min.

In many QC laboratories, phosphate buffers are used to maintain pH control of mobile phases. However, due to their lack of volatility, phosphate buffers are not compatible with mass spectrometers. In Figure 4, comparison chromatograms of C18 and phenyl columns are shown using methanol and either phosphate buffer or 0.2 percent formic acid. What is noteworthy is the lack of change in selectivity and elution order when shifting from one solvent to another. This indicates that the formic acid mobile phase can act as a supplementary mobile phase and would be ideal if MS detection were required.

As mentioned earlier, non-nutritive sweeteners and preservatives are found in many beverages and dental products. A simple survey of most prepared beverages will show at least

one or two of the compounds in this study. We examined several products including TaB cola (The Coca-Cola Co., Atlanta, GA) and Monster Energy drink (Monster Beverages Co., Corona, CA), as well as Listerine (McNeil-PPC, Inc., Skillman, NJ) and Scope (The Procter & Gamble Co., Cincinnati, OH) mouthwashes. A list of the ingredients of each product is shown in Table 2. In at least one case an ingredient is listed twice (sodium benzoate and benzoic acid, which are combined by analyzing at low pH, as the acidity of the mobile phase ensures full protonation of the benzoic acid). As can be seen, a great number of other ingredients are not analyzed during this work. In many cases, a second HPLC test or other test might be used, for example LCMS.

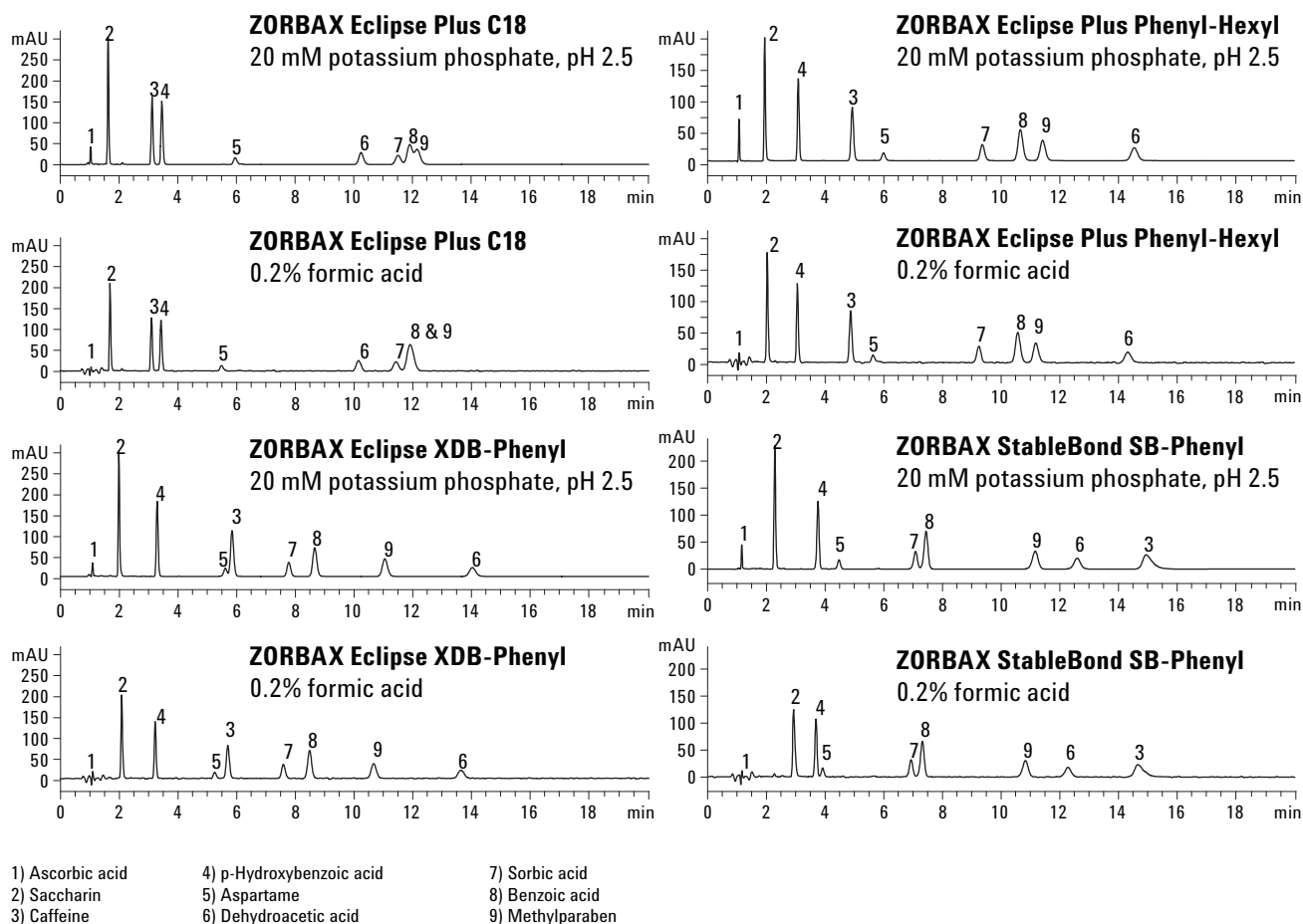


Figure 4. Comparison of 70%, 20 mM phosphate buffer, pH 2.5, and 70%, 0.2% formic acid with 30% methanol on food additives separation with C18 and phenyl columns; flow rate was 1.5 mL/min.

Table 2. Products and Ingredients

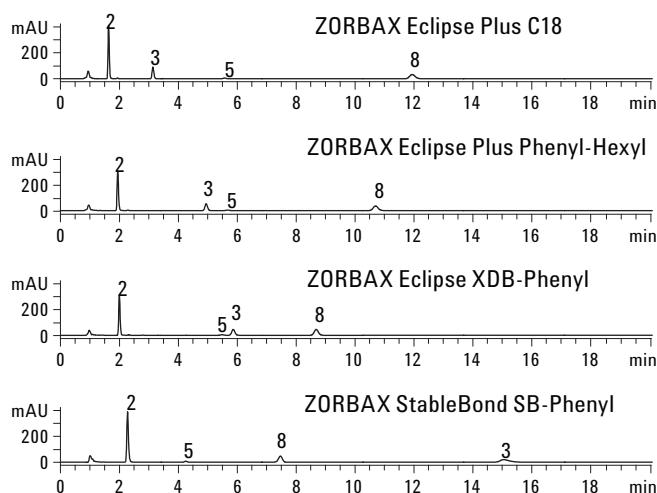
Product	Analyzed ingredients	Other ingredients
TaB	Calcium saccharin, potassium benzoate, caffeine, aspartame	Carbonated water, caramel color, natural flavors, phosphoric acid
Monster Energy	Caffeine, sorbic acid, benzoic acid	Carbonated water, sucrose, glucose, citric acid, natural flavors, panax ginseng root extract, L-carnitine, niacinamide, sodium chloride, glucuronolactone, inositol, guarana seed extract, pyridoxine, hydrochloride, sucralose, riboflavin, maltodextrin, cyanobalamin
Listerine	Benzoic acid, sodium saccharin, sodium benzoate	Eucalyptol, menthol, methyl salicylate, thymol Inactive ingredients: water, alcohol (21.6%), sorbitol solution, flavoring, poloxamer 407, FD&C Green no. 3
Scope	Sodium saccharin, sodium benzoate, benzoic acid	Water, alcohol, glycerin, flavor, polysorbate 80, Blue 1, Yellow 5, cetylpyridinium chloride

Equation 1. Resolution Equation

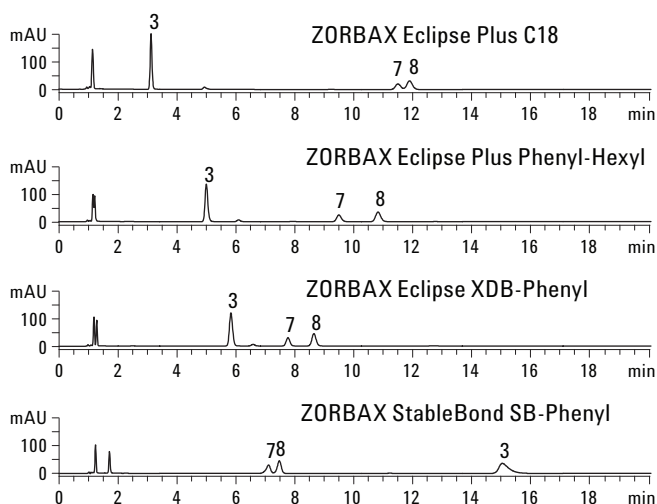
$$R_s = \frac{\sqrt{N}}{4} \cdot \frac{(\alpha - 1)}{\alpha} \cdot \frac{k'}{k' + 1}$$

Theoretical plates Selectivity Retention

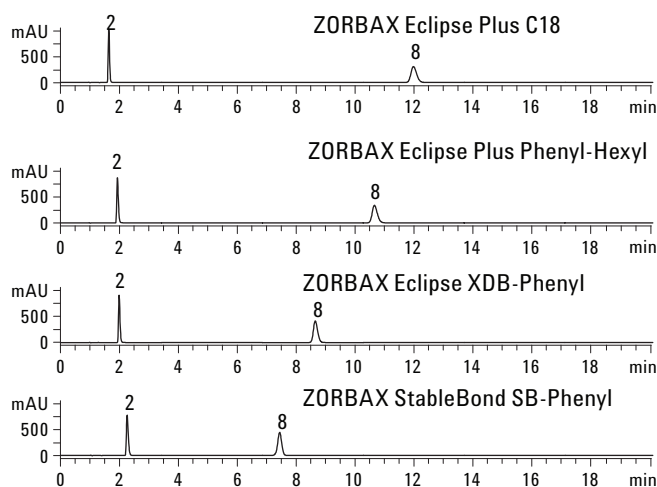
TaB



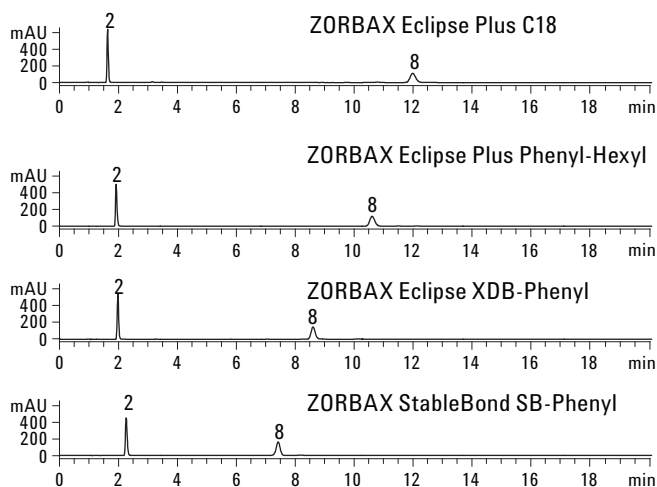
Monster Energy



Cool Mint Listerine



Original Mint Scope



- | | | |
|------------------|--------------------------|------------------|
| 1) Ascorbic acid | 4) p-Hydroxybenzoic acid | 7) Sorbic acid |
| 2) Saccharin | 5) Aspartame | 8) Benzoic acid |
| 3) Caffeine | 6) Dehydroacetic acid | 9) Methylparaben |

Figure 5. Soft drink, energy drink, and mouthwash analysis with varied columns.

As can be seen in Figure 3 the best separation of the nine-compound mixture with this mobile phase is achieved with the Eclipse Plus Phenyl-Hexyl column. However, it is obvious that not all samples contain the same challenging mixture. For the TaB sample shown in Figure 5, it can be seen that the Eclipse Plus C18, Phenyl-Hexyl, and StableBond SB-Phenyl columns all provide, good resolution for all of the analytes in the sample. In the case of the Monster Energy drink, the Eclipse Plus Phenyl-Hexyl column provides excellent resolution of all compounds in this mixture, but the Eclipse XDB-Phenyl column provides similar resolution in less time. Finally, in the analysis of mouthwash samples, the StableBond SB-Phenyl column provides the fastest separation, while maintaining sufficient resolution.

Conclusions

The importance of selectivity in analysis cannot be underestimated. As depicted in the resolution equation (Equation 1), selectivity is the most important factor in this equation. This work has examined selectivity derived from choice of solvent (methanol vs. acetonitrile) and selectivity derived from different columns. The importance of selectivity is further underscored in the examination of real samples, containing only selected components of the mixture, leading to altered analysis requirements, especially when speed of analysis is considered.

References

1. K. Croes et al., "Relevance of π - π and Dipole-Dipole Interactions for Retention on Cyano and Phenyl Columns in Reversed Phase Liquid Chromatography," *J. Chromatogr.*, 1098, 123–130, 2005.
2. M. R. Euerby, et al., "Chromatographic Classification and Comparison of Commercially Available Reversed Phase Columns Containing Phenyl Moieties Using Principal Component Analysis," *J. Chromatogr. A*, 1154, 138–151, 2007.

3. V. R. Meyer, *Practical High Performance Liquid Chromatography*, Fourth Ed., p 34, Wiley, 2004.
4. J. Henderson and W. Long, "Resolving Potentially Harmful Azo-Colorant Amines Using the Distinct Selectivities of the Agilent ZORBAX Eclipse Plus Phenyl-Hexyl and StableBond Phenyl Columns," Agilent Technologies publication 5989-8542EN, 2007.
5. W. Long and J. Henderson, "High Resolution Analysis of Taxanes Using Rapid Resolution HT (1.8 μ m) Agilent Eclipse Plus Phenyl-Hexyl Columns," Agilent Technologies publication 5989-9340EN, 2008.
6. William Long, John Henderson, and Maureen Joseph, "Comparing Selectivity of Phenylhexyl and Other Types of Phenyl Bonded Phases," *LC GC Magazine* June 2008.
7. J. Henderson and W. Long, "Exceptional Selectivities of Agilent ZORBAX Eclipse Plus Phenyl-Hexyl Phenyl Columns to Separate Estrogens," Agilent Technologies publication 5989-9130EN, 2008.
8. W. Long and J. Henderson, "Unique Selectivity and High Throughput Applications of SB-Phenyl RRHT," Agilent Technologies publication 5989-6067EN, 2007.
9. M. Yang, et al., "Impact of Methanol and Acetonitrile on Separations Based on π - π Interactions with Reversed Phase Phenyl Columns," *J. Chromatogr.*, 1097, 124–129, 2005.

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