Analysis of Polar Compounds Using 100% Aqueous Mobile Phases with Agilent ZORBAX Eclipse Plus Phenyl-Hexyl and Other ZORBAX Phenyl Columns

Application Note

Pharmaceuticals and Food

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Abstract

The analysis of polar compounds, such as acids and bases, typically requires ion pairing reagents or Hydrophobic Interaction Chromatography (HILIC) columns in HPLC. In this work, the utility of phenyl columns is demonstrated using 100% aqueous mobile phase, without the inconvenience of phase collapse. Selectivity of different Agilent ZORBAX phenyl phases (StableBond SB-Phenyl, Eclipse XDB-Phenyl and Eclipse Plus Phenyl-Hexyl) is shown with both aliphatic acids and catecholamines (bases). The selectivity of these compounds is further shown to be a function of mobile phase additive.

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Introduction

Polar compounds, like acids and bases, are generally difficult to separate using reversed phase liquid chromatography. Bases can be more easily retained at higher pH where the compounds are not charged. However, in some cases, other compounds may be in the sample that will not be separable at high pH. Another possible solution is to use an ion pair reagent to increase retention. In general, ion pair reagents are not compatible with mass spectrometry. Acidic compounds are also noted as difficult samples to separate or even retain. In general, it is necessary to work below the pKa of the compound where it will be fully protonated (not charged) and decrease the organic content of the mobile phase [1]. A problem that can occur with alkyl columns such as C8 or C18 phases is poor retention or reproducibility of retention in low organic mobile phase. One of the unique properties of phenyl columns is their resistance to "dewetting", or what is sometimes referred to as phase collapse [2]. Phenyl columns may be a good choice when 100% aqueous mobile phase will be utilized. This work will show separation of both basic and acidic compounds using 100% aqueous conditions.

Catecholamines are basic compounds that act as neurotransmitters. Control of many body functions can be altered by a lack or overabundance of these materials. Changes in catecholamine levels have been shown to be useful in the diagnosis of several pathological states [3].

Acids and their salts serve a variety of functions in foods, including the following: flavoring to provide a desired taste, controlling the pH to retard the growth of microorganisms, chelating of metal ions that can cause lipid oxidation (Cu, Fe), reducing color and texture changes in some fruits and vegetables, and changing the texture of foods by modifying gels made from pectin or proteins [4].

Experimental

HPLC analysis was performed with the Agilent 1200 Series Rapid Resolution LC (RRLC) system:

- G1312B binary pump SL, mobile phase Channel A only various mobile phase additives in water, 1 mL/min
- 1376C automatic liquid sampler (ALS) SL, injection volume was 5 µL
- G1316B Thermally Controlled Column (TCC) Compartment SL, temperature was 25 °C
- G1316C diode array detector (DAD), wavelength settings were 268,4 and 360,50 nm for the catecholamines and 220,4 and 360,50 nm for the acidic compounds, with a G1315-60024 micro flow cell (5 mm path, 6 µL volume)

ZORBAX Columns

- Eclipse Plus Phenyl-Hexyl 4.6 mm × 100 mm, 5 µm (p/n 959996-912)
- Eclipse Phenyl 4.6 mm × 100 mm, 5 µm (custom)
- StableBond Phenyl 4.6 mm × 100 mm, 5 µm (custom)
- Eclipse Plus Phenyl-Hexyl 4.6 mm × 150 mm, 3.5 µm (p/n 959961-912)
- Eclipse Phenyl 4.6 mm × 150 mm, 3.5 µm (p/n 963967-912)
- StableBond Phenyl 4.6 mm × 150 mm, 3.5 µm (p/n 863953-912)

Chemicals

The 18 MΩ Milli-Q water was produced on site. Formic acid, trifluoroacetic acid (TFA) and acetic acids were purchased from Sigma Aldrich (Bellfonte, PA). Catecholamines ephedrine, norephedrine, dopamine, levadopa, and tyrosine were also purchased from Sigma Aldrich. They were prepared in water at 1 mg/mL and mixed together to produce a final concentration of 0.2 mg/mL. L-(+)-tartaric acid, DL-malic acid, DL-lactic acid, acetic acid, citric acid, and propionic acid were dissolved in water at 1 mg/mL, and mixed to a final concentration of 0.2 mg/mL. All compounds were also injected separately in order to identify the peak and any impurities. Structures and pKa values for these chemicals are shown in Figure 1.

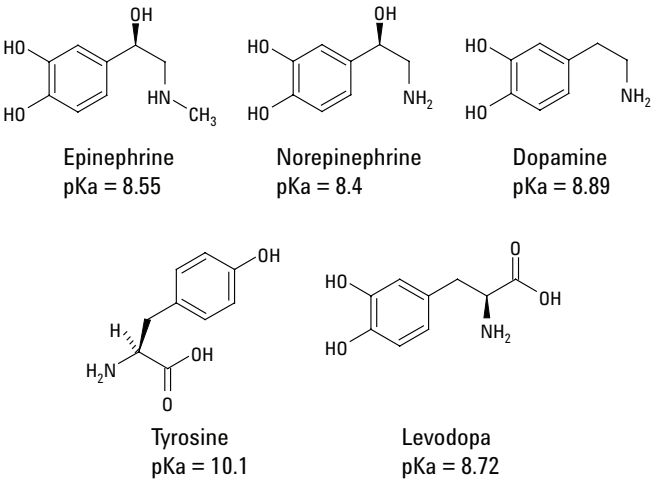


Figure 1a. Structures and pKa of catecholamines.

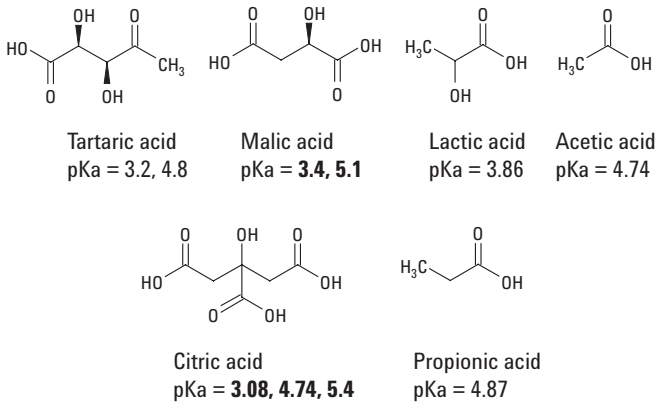


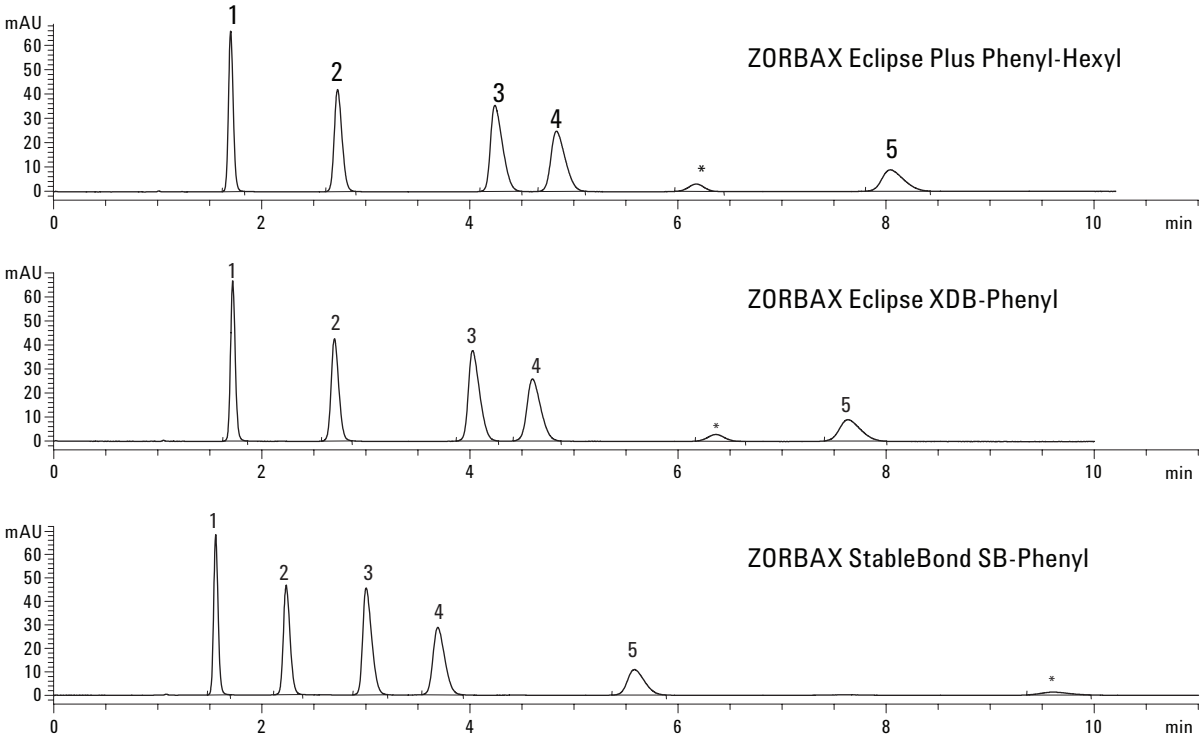
Figure 1b. Structures and pKa of aliphatic acids.

Results and Discussion

The ZORBAX Eclipse Plus Phenyl-Hexyl and Eclipse XDB-Phenyl (an ethyl phenyl phase) columns provide very similar separations (see Figure 2). For most of the components in the mixture, the separation appears more similar than different. The most notable exception appears to be tyrosine, which is only slightly more retained on the phenyl-hexyl column, perhaps due to its longer attaching group or higher carbon load (9.5 vs. 7%). StableBond SB-Phenyl, with its exposed silica silanol groups but larger protecting groups, leads to an alternative selectivity in which most of the peaks elute in the same order, with the exception of the impurity peak and the tyrosine, which have exchanged positions. The more highly retained and later-eluting impurity peak is in an excellent position for isolation. However, in general, the peaks in the StableBond SB-Phenyl separation are not as highly retained as those in the endcapped ethyl phenyl, Eclipse XDB-Phenyl separation.

Figure 3 shows the Eclipse Plus Phenyl-Hexyl column run with different acid mobile phases. The pKa's of these compounds are listed in Figure 1. As these compounds are all amines it is interesting to see how alternative mobile phases will affect this separation. Even though all of these compounds are weak bases, it can be noted that the strongest retention is found with the higher concentration of trifluoroacetic acid (TFA) mobile phase.

The varied retention shown in Figure 3 can be explained in many ways. If we are working close to the pKa of a group of compounds, we will typically see more retention if the charge

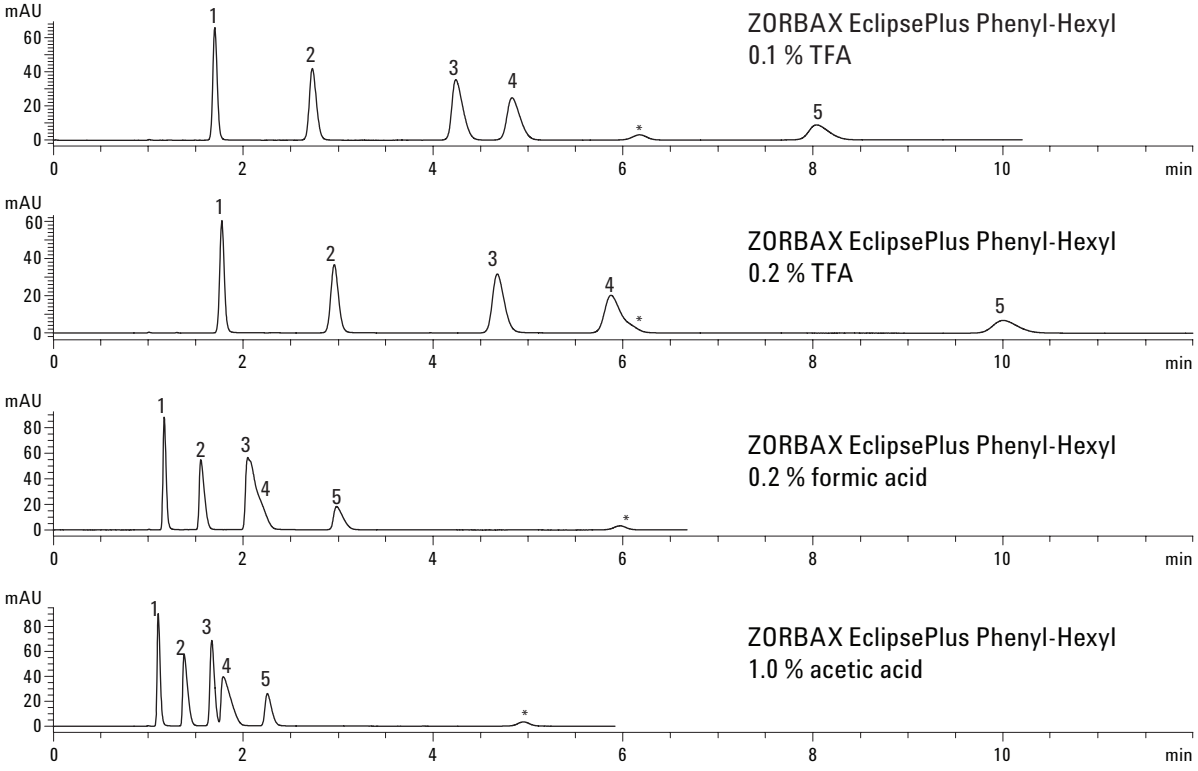


Norepinephrine, epinephrine, dopamine, levodopa, impurity*, tyrosine 0.2 mg/mL each 5 µL 4.6 mm × 100 mm, 5 µm columns.
Mobile phase = 0.1% TFA in water, 1 mL /min, 265 nm.

Figure 2. Comparison of ZORBAX phenyl column selectivity using 0.1% TFA mobile phase.

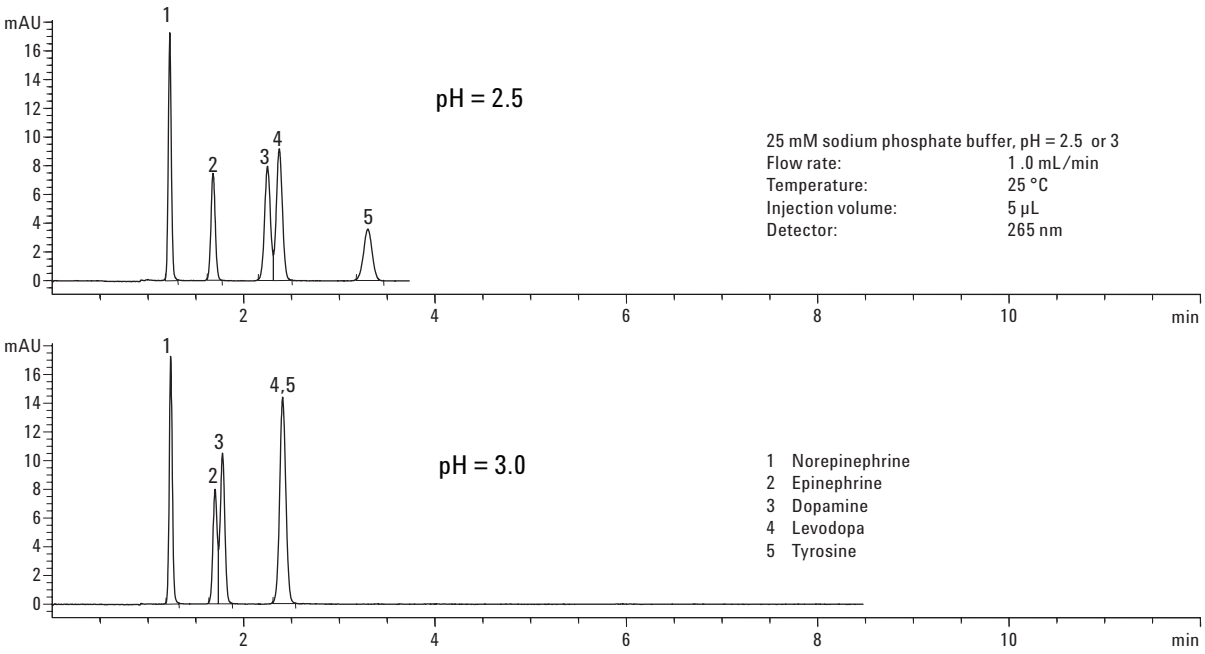
on the compounds is neutralized. Uncharged compounds are more highly retained than charged compounds [5,6]. An alternative explanation is ion pairing. TFA and formic acid are commonly referred to as ion pairing reagents, while acetic acid is not. As can be seen, retention is increased with additional TFA. A further experiment with two phosphate buffers in Figure 4 shows better retention at lower pH, but not as good as that using the TFA mobile phase [7,8].

In Figure 5, these aliphatic acids are well resolved using phenyl-hexyl and phenyl-ethyl columns. This suggests that phenyl columns may be good choices when separating highly polar compounds. The ZORBAX Eclipse Plus Phenyl-Hexyl column, with its longer alkyl linking group, is more lipophilic in nature than the ethyl phenyl Eclipse XDB-Phenyl or StableBond SB-Phenyl columns. Furthermore, by working well below the pKa's of these aliphatic acids, we can fully separate these aliphatic compounds. The acidic compounds are best retained on the more lipophilic Eclipse Plus Phenyl-Hexyl column; however, the column with the best separation, the separation of all compounds, is actually the Eclipse XDB-Phenyl (see Figure 5). This can be seen in the lack of overlap with any compound as shown in Figure 5.



Norepinephrine, epinephrine, dopamine, levodopa, impurity*, tyrosine 0.2 mg/mL each 5 µL 4.6 mm × 100 mm, 5 µm columns.
Mobile phase = 0.1% TFA in water, 1 mL /min, 265 nm.

Figure 3. Comparison of mobile phase selectivity using ZORBAX Eclipse Plus Phenyl-Hexyl column.



Norepinephrine, epinephrine, dopamine, levodopa, tyrosine 0.2 mg/mL each 5 µL 4.6 mm × 100 mm, 5 µm columns.
Mobile phase = Sodium phosphate buffer 25 mM, 1 mL /min, 265 nm.

Figure 4. Comparison of mobile phase selectivity using ZORBAX Eclipse Plus Phenyl-Hexyl (non-ion pair).

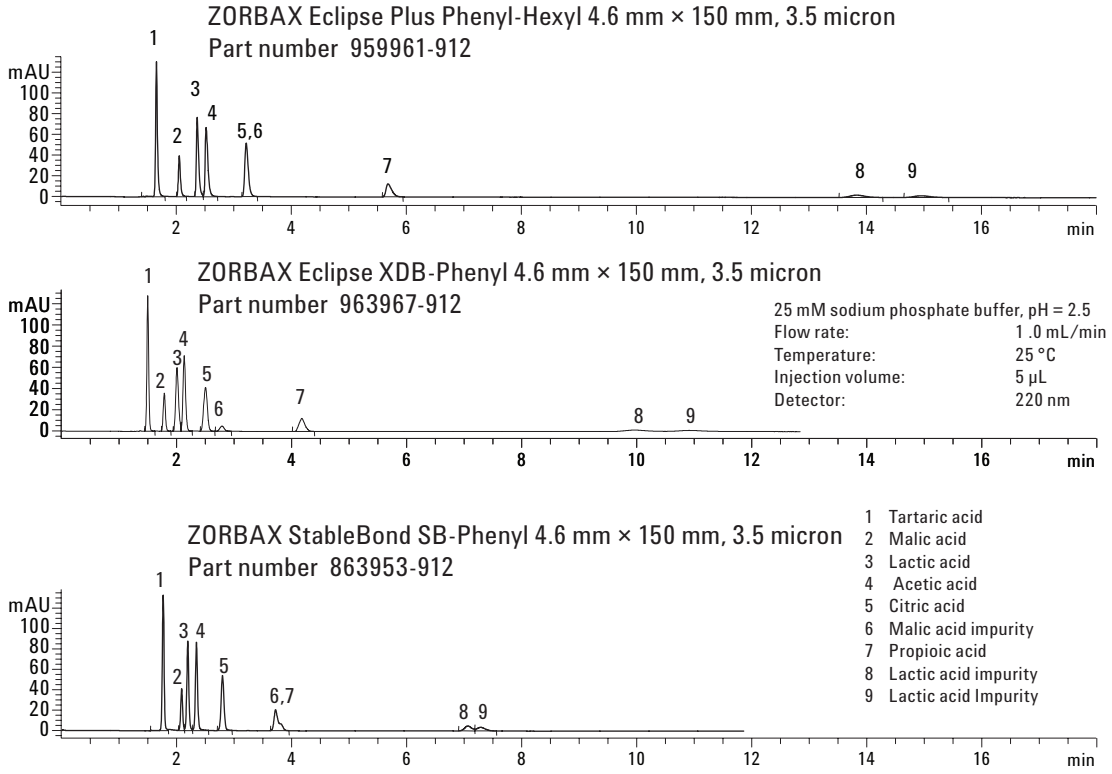
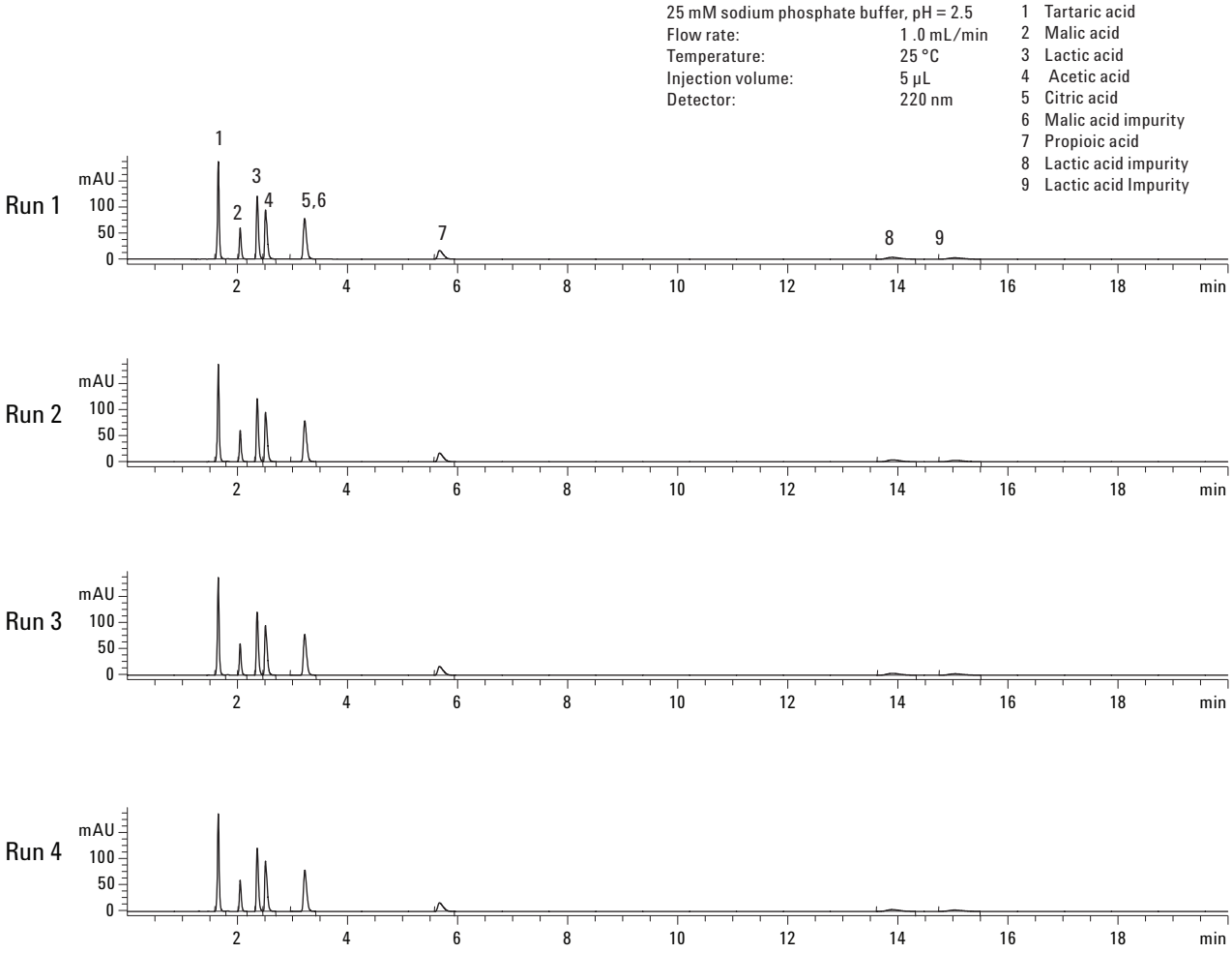


Figure 5. Comparison of mobile phase selectivity using ZORBAX Eclipse Plus Phenyl-Hexyl aliphatic acids 25 mM Na phosphate buffer.

This work has shown separations using 100% aqueous mobile phase. One of the less known features of ZORBAX phenyl columns is their ability to work while using high aqueous mobile phases. In some applications it is necessary to retain and separate polar analytes using a nonpolar C18 stationary phase. This often requires a high aqueous or a buffered-water mobile phase to achieve the desired separation. Many polar analytes are not well retained on a reversed-phase column except when the organic content of the eluent is very low (< 5%).

The bonding on a C18 reversed-phase column gives it a highly hydrophobic surface. Decreasing the organic content of the mobile phase to the levels previously described may lead to a loss in analyte retention over time (or instantaneously, with flow stoppage). This phenomenon is best described as dewetting, retention loss or, more commonly, phase collapse [2]. The onset of dewetting is unpredictable, but stopping the flow of eluent through the column is known to initiate this effect. Figure 6 shows the effect of repeatedly stopping the flow for 30 minutes between injections. The Eclipse Plus Phenyl-Hexyl column surpasses this test with ease. As can be seen in the four stop-flow test cycles, no significant changes are observed in the retention time or shape of peaks. Work reported in reference 2 showed very good performance for StableBond SB-Phenyl using water-soluble vitamins (niacin) as a probe.



1 and 2) Run successively with no pause. 3) Pump stopped 30 minutes and restarted. 4) Pump stopped 30 minutes and restarted ZORBAX Eclipse Plus Phenyl-Hexyl 4.6 mm × 150 mm 3.5 micron, p/n 959961–912.

Figure 6. Resistance to dewetting.

Conclusions

The separation provided on ZORBAX Eclipse Plus Phenyl-Hexyl, Eclipse XDB-Phenyl, and StableBond SB-Phenyl columns can be performed on most commercial HPLC systems. The methods shown here can be easily modified to fit most researchers' needs. They are easily implemented and do not require complicated mobile phase compositions. These phenyl columns show varied selectivity and retention with acids and bases without the added complication of phase collapse.

References

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