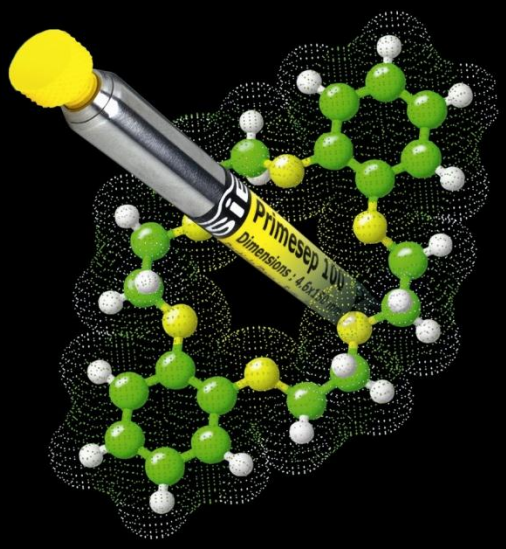
SIELC

Primesep® 100

Columns
Methods
Applications



“Creating New Dimensions in the World of Chromatography”

Primesep 100 Columns

Introduction

For decades, liquid chromatography (LC) stationary phase design has been dominated by the elimination of the multiple or “unwanted” interactions that occur in separations. For instance, in single-mode ion-exchange (IE) and ion-exclusion chromatography, nonionic interactions are generally viewed as complications to separation and, thus, are undesirable. However, there is a way to benefit from multiple interactions in the stationary phase: mixed-mode columns.

SIELC’s Primesep 100 HPLC columns are designed for mixed-mode separations and ~~is~~ are capable of separating a tremendous range of compounds by different separation modes. These modes can be controlled and varied via the mobile phase. With an embedded ion-pairing group, the columns require no ion-pairing reagents in the mobile phase to retain and separate ionizable polar compounds.

These columns have been shown to efficiently separate organic and inorganic ions in ion-exchange (bases) and ion-exclusion (acids) modes. The columns can be used to provide efficient separation in single-mode reverse phase (RP), as well, and in several combinations of all these 3 modes. Different modes of separation offer different column selectivity.

In most cases, separations require a simple mobile phase containing acetonitrile, water, and an acid or salt buffer. The key is to create a strong enough mobile phase in order to elute ionic and non-ionic compounds. The presence of ions in the mobile phase is required to facilitate the elution of ionizable compounds. The nature of the buffer can be changed during analytical method development and allows switching from one detection technique to another without changing a separation method. All common detection techniques such as MS, ELSD, UV, and RI are compatible with this volatile mobile phase. The mobile phase allows simple scale-up from analytical to preparative separations with no changes in the separation conditions.

This column is resistant to dewetting in 100% aqueous mobile phase and is stable in pure organic and highly acidic conditions down to pH 1.0. Any silanol or metal chelating interactions are completely eliminated and do not affect the efficiency of the separation.

A ligand on the surface shields residual silanols from interacting with the analytes. The ligand contains all the functionalities (reversed-phase, ion-exchange, polar interactions) for the column and is attached to the surface of the silica gel. Primesep silica gels are not physical mixtures of silica with different functionalities. This approach guarantees very good reproducibility from lot to lot, and absolute and relative retentions of neutral and charged compounds, which are maintained within close tolerances.

The two functional groups utilized in Primesep 100 columns provide for varying interactions with analytes. One functional group is a very hydrophobic alkyl chain, and the other is a very hydrophilic acid residue. With two functional groups with such a large difference in polarity, multiple separation modes can be performed on a single column. Neutral compounds can be resolved in reverse, normal, or polar organic mode. Charged molecules can be resolved in reverse, normal, polar organic, ion-exchange, or ion-exclusion modes. Also, the combination of more than one mode is typical for ionizable molecules. The selection of the separation mode is governed by the composition of the mobile phase used in each particular separation.

Benefits of Mixed-Mode Columns

1. The presence of ionizable groups along with a hydrophobic chain provides additional mechanisms to adjust retention selectivity (reverse-phase, normal-phase, ion-exchange, ion-exclusion).
2. Retention time of neutral hydrophobic and ionizable polar compounds can be adjusted separately.
3. Increased capacity of the column towards ionizable analytes provides longer retention and higher loadability. This allows for the use of smaller and less expensive columns for preparative separations..
4. Longer retention for polar ionizable analytes compare to traditional reversed-phase approaches.
5. Ability to simultaneously analyze polar neutral, polar ionizable and hydrophobic ionizable compounds in reversed-phase ion-exchange modes..
6. Ability to do multiple gradients to achieve high efficiency high throughput separation using traditional HPLC systems and not UPLC.
7. Overall charge of the column can be changed based on the pH of the mobile phase.
8. Ability to operate in several single modes or in several mixed modes.
9. Compatibility with all detection techniques and preparative separation.
10. Ligand-shielding from silanols.

Good column care requires little additional time and will extend the lifetime of your SIELC HPLC columns. The following are general recommendations. More detailed information is available at www.sielc.com.

Try to avoid using alcohols with Primesep 100. Alcohols can esterify carboxylic acids attached to the surface of the silica, significantly affecting reproducibility and the retention time of some analytes.

Do not use or store SIELC HPLC columns in pH outside of recommended ranges. SIELC columns require buffered or acidified mobile phases to be present in the column at all times (Table 1).

Do not store SIELC HPLC columns in mobile phases containing modifiers that degrade easily, such as triethylamine (TEA), tetrahydrofuran (THF), and trifluoroacetic acid (TFA). TEA and TFA are more likely to become contaminated when stored at ambient temperatures. Use formic acid as an acidic modifier for long-term storage. Contamination may change the column and may change chromatography results. Use only HPLC-grade solvents and additives for your analysis.

To achieve reproducible results, please use a column thermostat. Recommended operating temperatures for all SIELC columns are between 0 and 50 ^{o}C.

To achieve reproducible results, always equilibrate your SIELC column prior to the injection. In some cases, the replacement of the mobile phase inside the column does not make the column sufficiently equilibrated. SIELC columns' stationary phase retain components of the mobile phase as counter ions. Equilibrating the column, in this case requires a replacement of all the ions adsorbed in the column stationary phase. Typically, a 50 mM buffer will equilibrate a column in 1 hour. If only the concentration of the buffer changed, then 2-4 volumes of the column are sufficient to reach an equilibration state.

It is important to operate columns below the specified maximum pressure limits. The pressure limit is 5000 psi for most columns and 9000 psi for columns packed with solid-core particles. If you exceed maximum pressure, the column can be destroyed. A leaking column is an indication of excessive pressure usage.

Limit the use of tetrahydrofuran (THF) to 10% with all SIELC columns.

An arrow on the column label is used to specify the direction of flow through the column. Typically you should operate the column in the direction marked. If the column needs to be washed in the opposite direction, use a reduced flow rate in order to not generate pressure that can disturb the packing.

Use only your fingers to tighten PEEK fittings to the column connection.

Test the column performance using conditions described in the Certificate of Analysis and compare your results with those in the report. Due to the variations in particular LC systems, you may observe the slightly different results.

Table 1

Column packing	pH Limits/ Recommended Operating Range	Short Term Storage	Long Term Storage	Alcohol in mobile phase
Primesep 100	1.5-7.0/ 2.0-6.5	Buffered or acidified mobile phases within recommended pH range	MeCN/H_2O/formic or acetic acid – 75/25/0.1%	Not allowed

Retention and Selectivity

Mixed-mode chromatography provides multiple interactions for many analytes. The presence of these interactions allows you to use mobile phase modifications with greater efficiency than in traditional reversed-phase chromatography. Ionizable compounds can interact with the stationary phase by reverse-phase, ion-exchange, or ion-exclusion mechanisms. The amount of the acid in the mobile phase (i.e. buffer strength) influences the retention attributed to the ion-exchange interaction just as the organic component affects the retention in reverse-phase separation (i.e. hydrophobic interactions). Thus, the amounts of organic and acidic modifiers are both important for controlling the retention of ionizable analytes.

The stationary phase of the Primesep 100 mixed-mode column can interact with multiple types of analytes in a variety of ways. These properties offer multiple ways to adjust the selectivity of the Primesep 100 column. If two or more peaks are not resolved with traditional reverse-phase or ion-exchange technology, the mixed-mode column can be a powerful tool to find optimum separation conditions.

Mixed-Mode vs. Single -Mode

Every compound has two parameters that are important for LC: hydrophobicity (characterized by the log P parameter) and ionization properties (characterized by the log D parameter). Placing a mixture of compounds on a two-dimensional plane with Log P and Log D coordinates creates a two-dimensional distribution of the compounds on the plane.

In Reverse-Phase Chromatography, the elution order corresponds to the position of the projection of spots on the log P coordinate (Fig. 2a). Compounds with similar hydrophobicity will co-elute or overlap in a single-mode RP column (Fig. 2d). In an Ion-Exchange Chromatography, the elution order corresponds to the position of the projection of spots on the log D coordinate (Fig. 2c). Compounds with similar ionization constants also co-elute or overlap in the single-mode IE column (Fig. 2f).

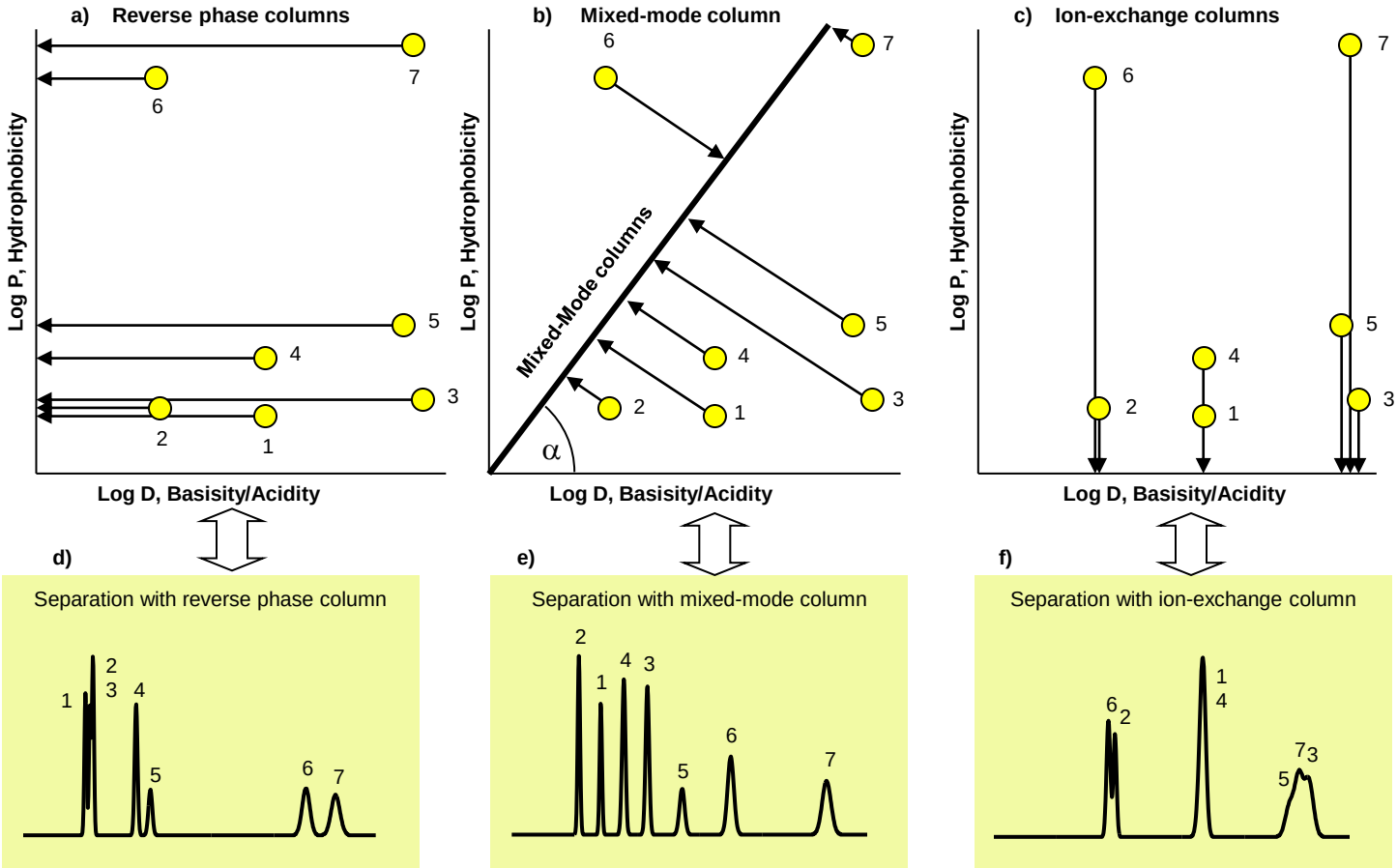
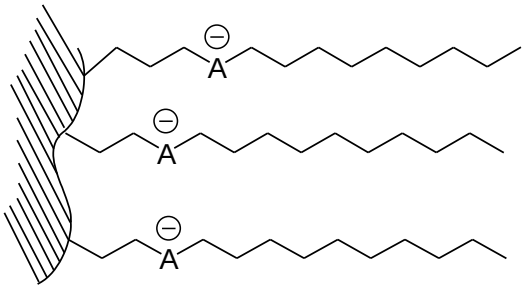


Figure 1

Mixed-mode chromatography is characterized by the diagonal in Fig. 2b, where the slope, α, is a function of the ratio of ion-exchange vs. reversed-phase governed by the mobile phase composition. The selectivity of the mixed-mode column is governed by the composition of the mobile phase – specifically, by the buffer and organic component concentrations. If the mobile phase has a high organic concentration or the analytes have very little hydrophobicity, the slope angle will be close to 0º and the separation will be due to IE interactions. If the organic concentration is low with a high buffer, then the angle of the slop will be close to 90º and the the separation will be due to typical RP interactions.

Surface Chemical Composition

Figure 2



With an embedded acidic ion-pairing group, a Primesep 100 column requires no ion-pairing reagent in the mobile phase to retain and separate ionizable polar compounds. The ligand itself is a chain 12 carbons long (C12), and its acidic functional group has a pKa of ~1. Reversed-phase and cation-exchange functionality are assembled on one molecule to provide good reproducibility and stability.

Column Properties

Primesep 100 columns are cation-exchange columns with an embedded negatively-charged compound on the silica surface. The foundational Primesep stationary phase is a silica-based gel of nano-porous microparticles. Polar compounds are separated on Primesep columns by a degree of polar interaction. Polar interaction includes electrostatic interaction which can be utilized by ion-exchange mechanism or hydrogen bonding which are adjustable by the amount of water in the mobile phase.

Primesep 100 columns are available in lengths of 10, 25, 50, 100, 150, and 250 mm and inner diameters of 0.5, 1.0, 2.1, 3.2, 4.6, 10, 22, 30 and 50 mm. Particles are spherical and available from 2.7 to 10 µm. Guard columns are integrated with a male connector and do not require holders. The 90 Å pore size is only available on solid-core columns.

Typical mobile phases used with Primesep 100 columns are based on acetonitrile, water, and an acid buffer such as $HClO_4$, H_2SO_4 or H_3PO_4, and the like. Mass spectrometry-compatible buffers ammonium formate (pH 3) and ammonium acetate (pH 5) can also be used. If it is necessary to detect in low UV (<220 nm) then a phosphate buffer is recommended.

Solid-Core

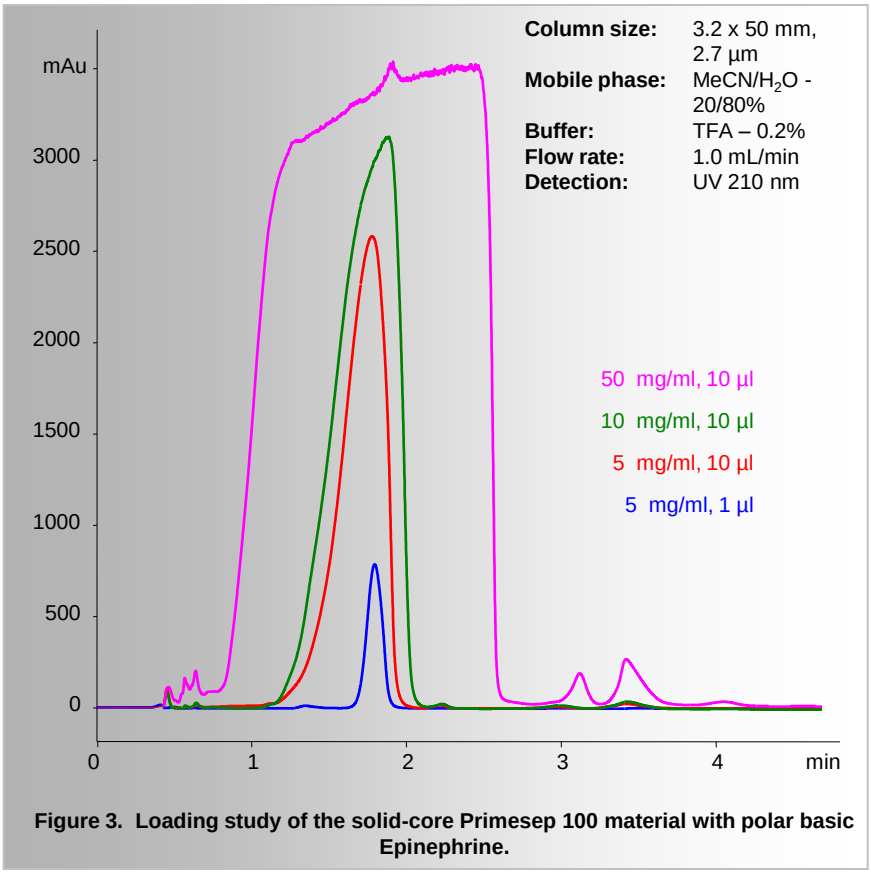
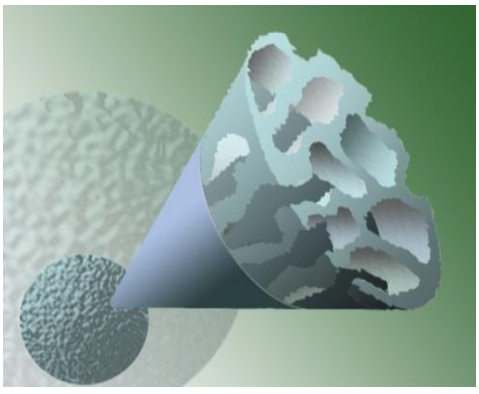


Figure 3. Loading study of the solid-core Primesep 100 material with polar basic Epinephrine.

Primesep 100 columns are also available in a solid-core (or core-shell) model. Made from a gel of solid core (as opposed to fully porous) silica particles, this version of the Primesep 100 column provides a shorter diffusion distance and allows for faster separation speeds with a high mobile phase velocity. The silica particles are only-partially porous near the surface (i.e. the shell), and this reduced pore length means less time analyte spends on the silica surface, and therefore faster elution times with high column efficiency.



While the solid core columns have a lower loading capacity, the mixed-mode properties help recover some of that loading capacity. Fig. 3 shows a study of the loading capacity of the core-shell Primesep 100 column with Epinephrine.

Core-shell technology offers the unique ability to achieve high efficiency at higher flow rates while generating relatively mild back pressures which is compatible with traditional HPLC systems. This offers the end-user the opportunity to improve separation speed and quality to UHPLC-like performance without needing to purchase expensive UHPLC equipment. Figs. 4-8 show this high efficiency and speed. These retentions last at most under 5 minutes and can be as fast as just a few seconds. The speed, however, does not affect the quality of the separations. The column can still retain and separate a wide variety of different, complex compounds.

Column size: 3.2 × 50 mm, 2.7 µm
Mobile phase: MeCN/H_2O – 5/95%
Buffer: TFA – 0.05%
Flow rate: 2.0 mL/min
Detection: UV 270 nm

1. Uracil
2. Xantine
3. Hypoxantine
4. Cytosine
5. Guanine
6. Adenine

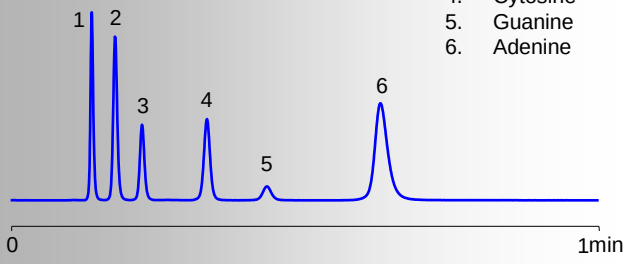


Figure 4. Separation of nucleobases in reversed-phase cation-exchange modes on a core-shell column

Column size: 2.1 x 50 mm, 2.7 µm
Mobile phase: MeCN/H_2O – 10/90%
Buffer: H_3PO_4 – 0.5%
Flow rate: 0.2 mL/min
Detection: UV 200 nm

1. Triazole Acetic Acid
2. 1,2,3-Triazole

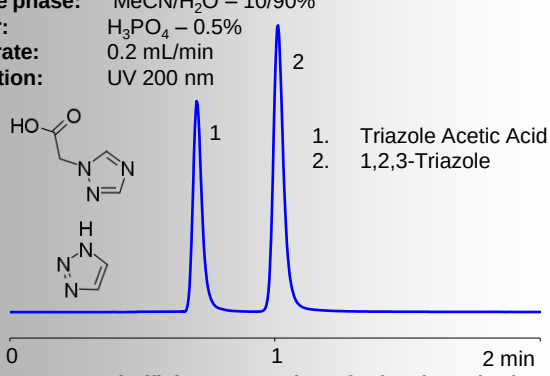


Figure 5. Fast and efficient separation of Triazole and Triazole Acetic Acid on mixed-mode core-shell column

1. Serine
2. Glycine
3. Alanine
4. Valine
5. Dopa

Column Size: 3.2 x 50 mm, 2.7 µm
Mobile phase: MeCN/H_2O – 3/97%
Buffer: TFA – 0.03%
Flow rate: 1 mL/min
Detection: UV 210 nm

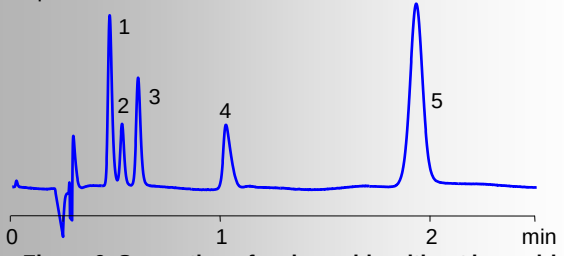


Figure 6. Separation of amino acids without ion-pairing reagent in reversed-phase and cation-exchange mode on a core-shell column

Column: 3.2 x 50 mm, 2.7um
Mobile phase: MeCN/H_2O 10/90% to 65/35% in 5 min
Buffer: AmFm pH 3.5, 30 mM to 70 mM in 5 min
Flow rate: 1.2 mL/min
Detection: 270 nm

1. Adenosine
2. 3,4-Difluroaniline
3. 4-Amino-2-chloropyridine
4. 4-Amino-3-chloropyridine
5. 5-Aminoindole
6. 2-Amino 5-methylthiadiazole
7. 4-Ethylaniline

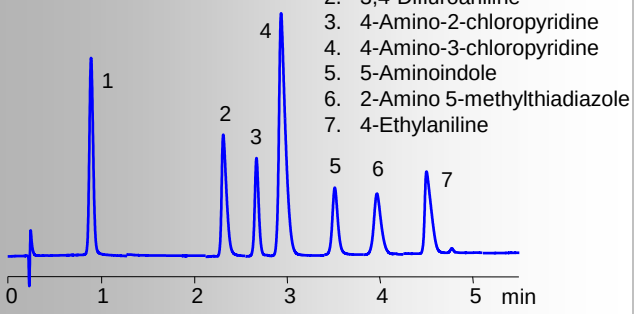


Figure 7. Separation of model compounds in reversed-phase cation-exchange modes on two core-shell Columns

Column Size: 2.1 x 50 mm, 2.7 µm
Mobile phase: MeCN/H_2O – 5/95%
Buffer: H_2SO_4 – 0.05%
Flow rate: 1.0 mL/min
Detection: UV 200 nm

1. DL-Methionine
2. Tyrosine
3. L-Phenylalanine
4. L-Tryptophan

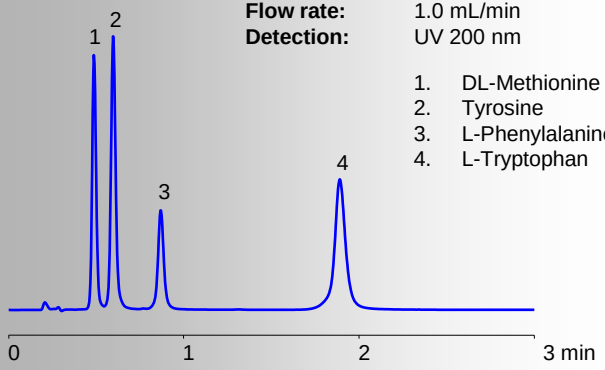


Figure 8. Fast separation of nucleobases on a mixed-mode core-shell column

Positively-Charged Polar Molecules

As mentioned on Page 4, the Primesep 100 column can interact with a wide variety of analytes in a number of ways, depending on the properties of the mobile phase, such as the buffer pH, organic and buffer concentrations, and the gradient type (if applicable).

Below in Figs. 9 and 10, the column was 'tuned' to an ion-exchange mode in order to retain and separate positively charged ions. Notice that the relative concentration of MeCN is fairly high in Fig. 9 and the gradient increases in MeCN concentration in Fig. 10; since the ions are not as soluble in MeCN as they are when in water, they favor interacting with the stationary phase and it's embedded charged functional group and thus are able to be retained and separated.

When a complex mixture is analyzed using Primesep columns, two or more interaction mechanisms help to tune the separation. Elution order and retention time can be adjusted in accordance with your analytical needs. The typical combinations of the mechanisms are reverse-phase – ion-exchange; reverse phase – ion exclusion; hydrophilic interaction – ion-exchange; chelating - reverse phase.

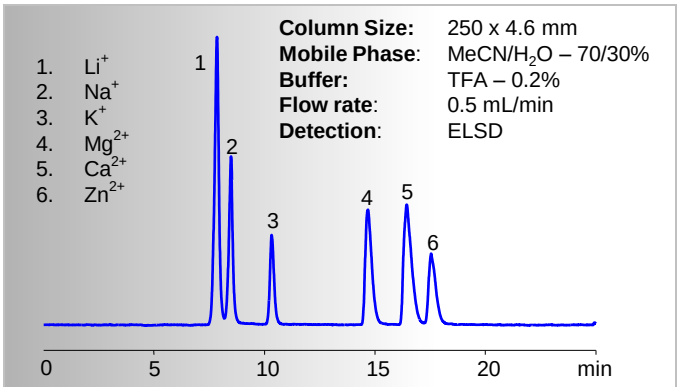


Figure 9. Cation separation via ion-exchange mode

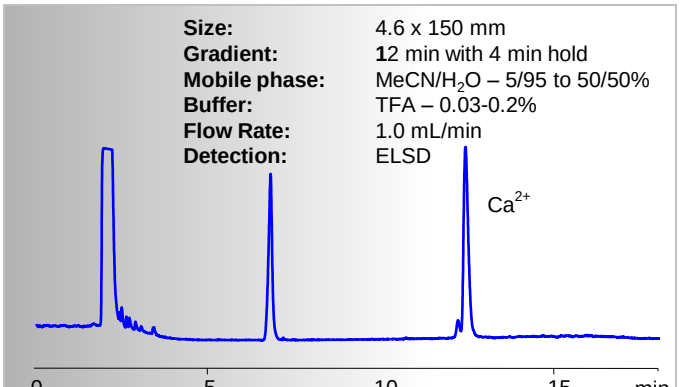


Figure 10. Retention of Calcium from maple syrup

Negatively-Charged Hydrophobic Molecules

In this section, the column has been tuned to retain negatively-charged, hydrophobic acids, such as Octanoic Acid (Fig. 11), Naproxen (part of the 2-arylpropionic acid family of NSAIDS; Fig. 12), and Tranexamic Acid (Fig. 20), among others. These retentions are possible due to the column's 2D, mixed-mode features. In these chromatograms, the column takes advantage of both ion-exchange interactions and reverse-phase interactions with the analytes.

In each experiment, however, no two mobile phases are identical, using various organic component concentrations and buffer strengths to accurately tune the influences of the IE and RP modes on each different analyte.

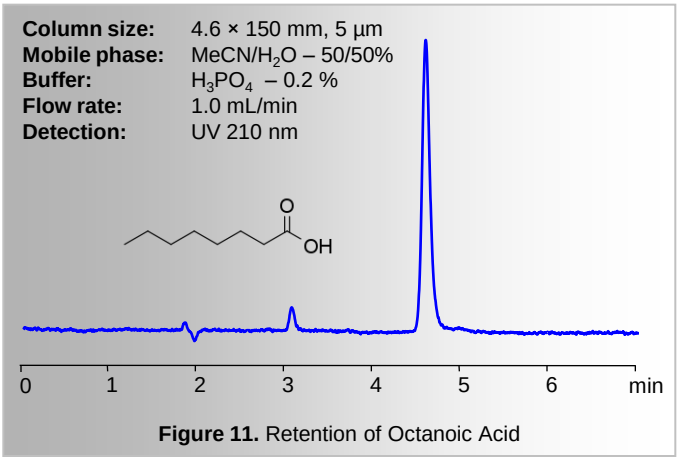


Figure 11. Retention of Octanoic Acid

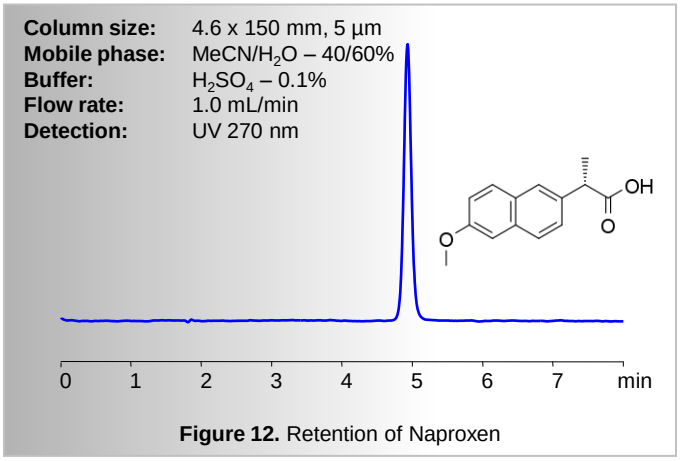


Figure 12. Retention of Naproxen

More retentions and separations of negatively-charged hydrophobic molecules are shown below, like Cephalosporin-class antibiotics (Fig. 13), Glutathione (Fig. 14), Fenofibric acid (Fig. 15), Daptomycin (Fig. 17), tetracycline antibiotics (Fig. 18), Glyphosate (Fig. 19), and Triazole Acetic Acid (Fig. 21).

In single-mode ion-exclusion chromatography, acids (lower pKa) are usually eluted in order of strength (i.e. in rising order of pKa) because they are repulsed by the surface's negative charge. Fig. 16, if done in a single-mode column, would have had Fumaric acid elute after Maleic acid, but because of the RP interactions, it actually elutes last. These chromatograms show the power and flexibility of mixed-mode columns.

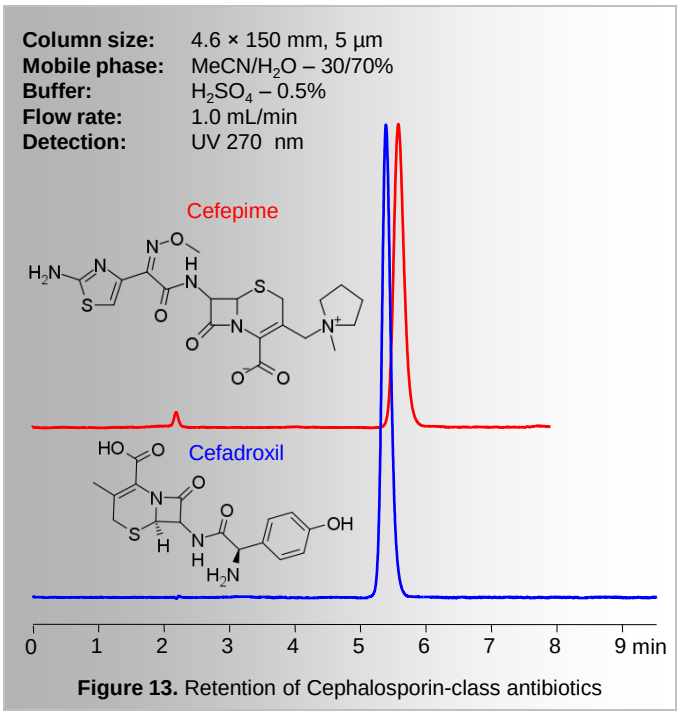


Figure 13. Retention of Cephalosporin-class antibiotics

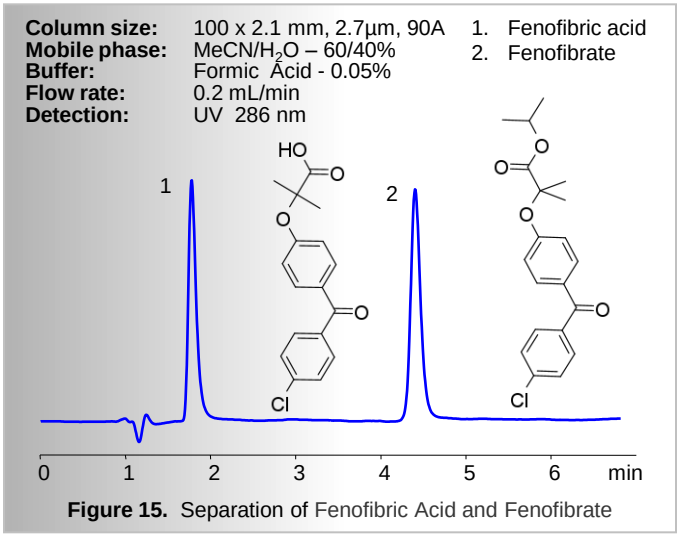


Figure 15. Separation of Fenofibric Acid and Fenofibrate

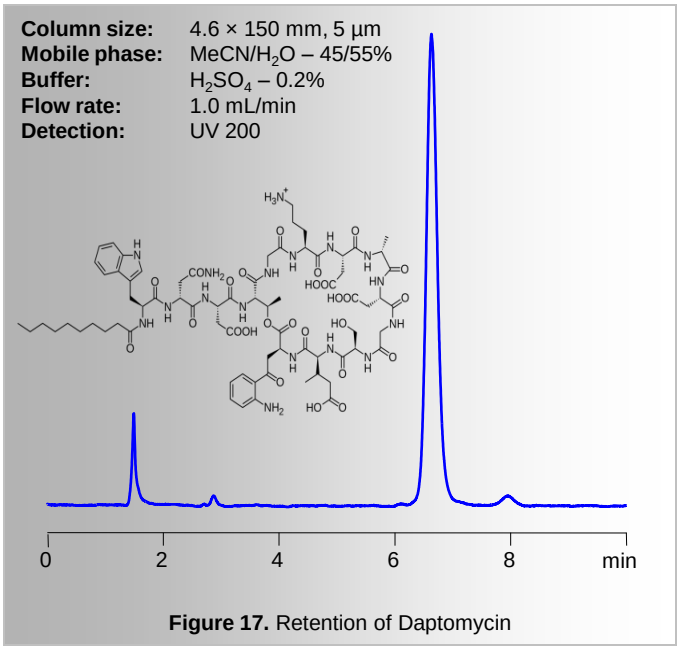


Figure 17. Retention of Daptomycin

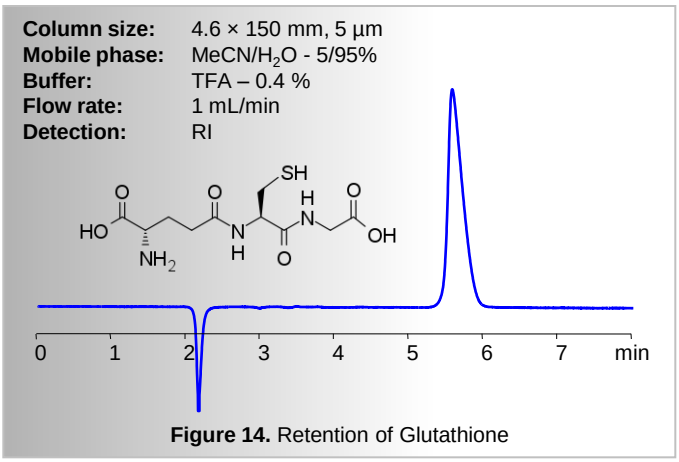


Figure 14. Retention of Glutathione

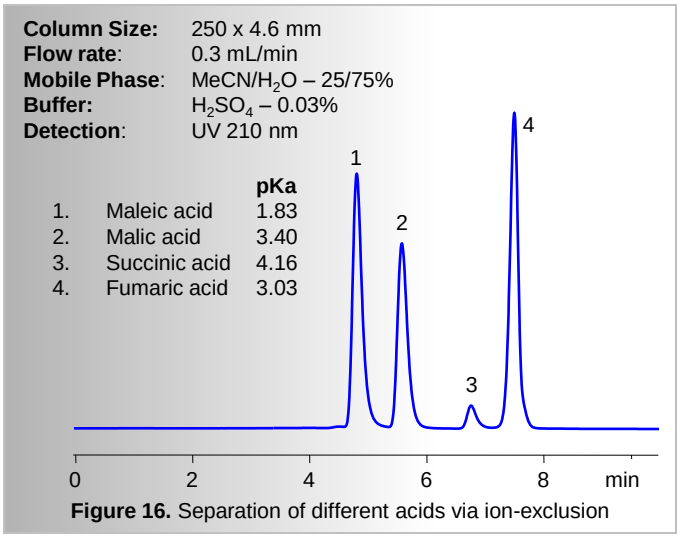


Figure 16. Separation of different acids via ion-exclusion

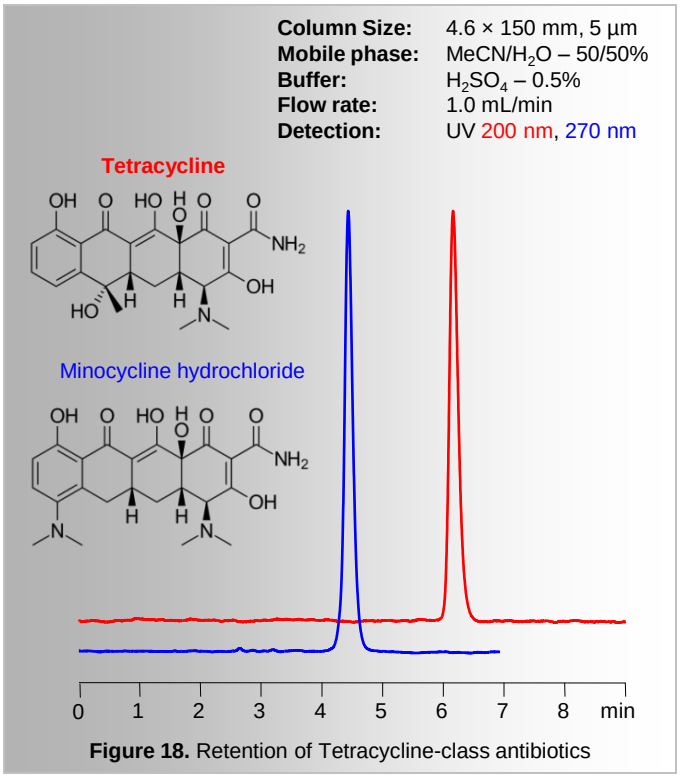


Figure 18. Retention of Tetracycline-class antibiotics

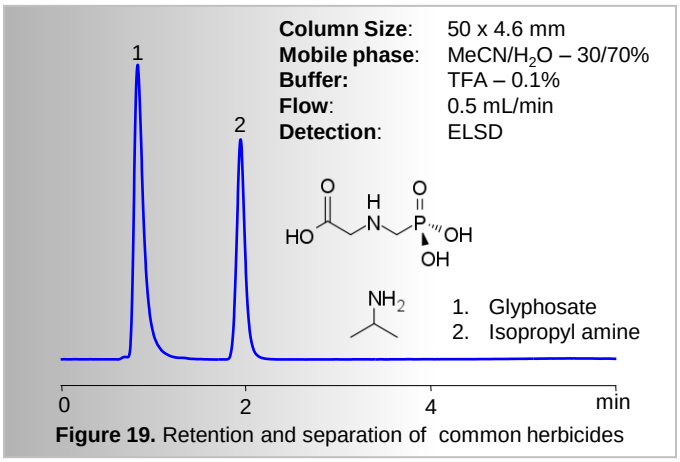


Figure 19. Retention and separation of common herbicides

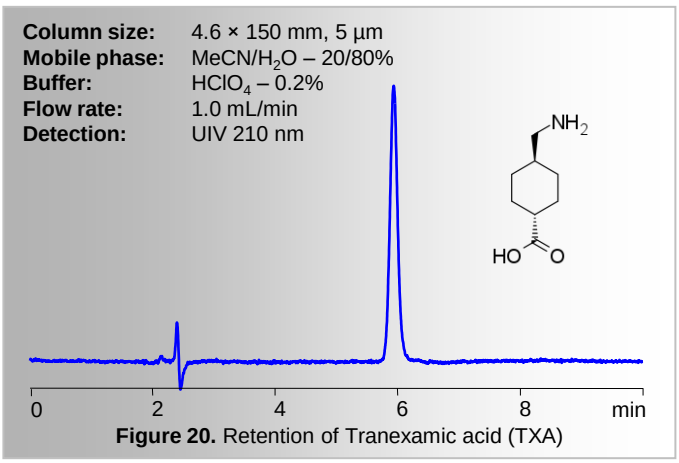


Figure 20. Retention of Tranexamic acid (TXA)

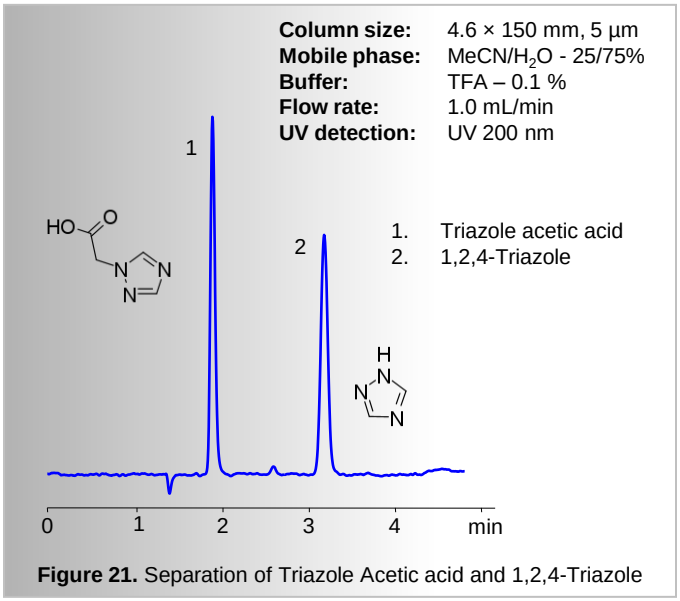


Figure 21. Separation of Triazole Acetic acid and 1,2,4-Triazole

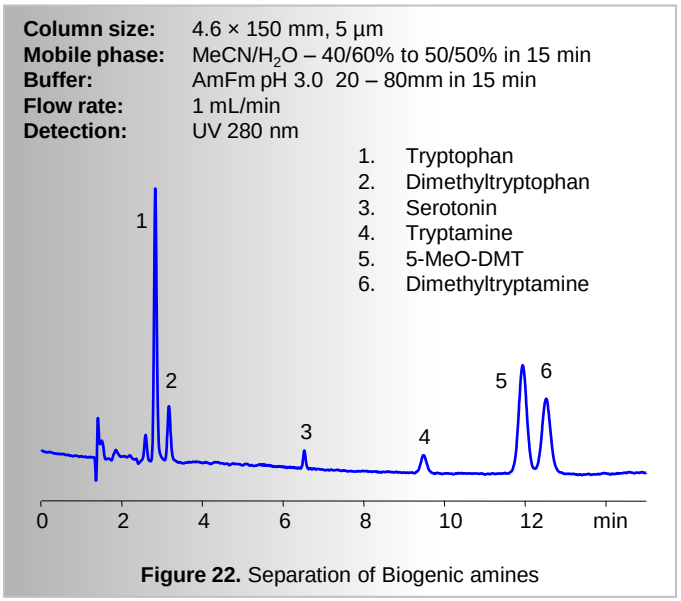


Figure 22. Separation of Biogenic amines

Neutral Molecules

By relying more heavily on the reverse-phase mode, the Primesep 100 column can also retain and separate neutral molecules such as Benzoquinone (Fig. 23), Divinyl Sulfone (Fig. 24), amines (Fig. 25), sulfonamides (Fig. 26), and Fentin (Fig. 28).

Some complex mixtures with compounds varying in polarity can be resolved with a gradient method. The gradual change in the organic composition of the MP allows even greater tuning of the column's selectivity, more than a gradient flow on a single-mode, reverse phase column would allow. The power of the gradient method can be seen in Fig. 22, where a mixture of biogenic amines with wide variations in polarity (very polar to very hydrophobic) is shown very clearly in Fig. 22.

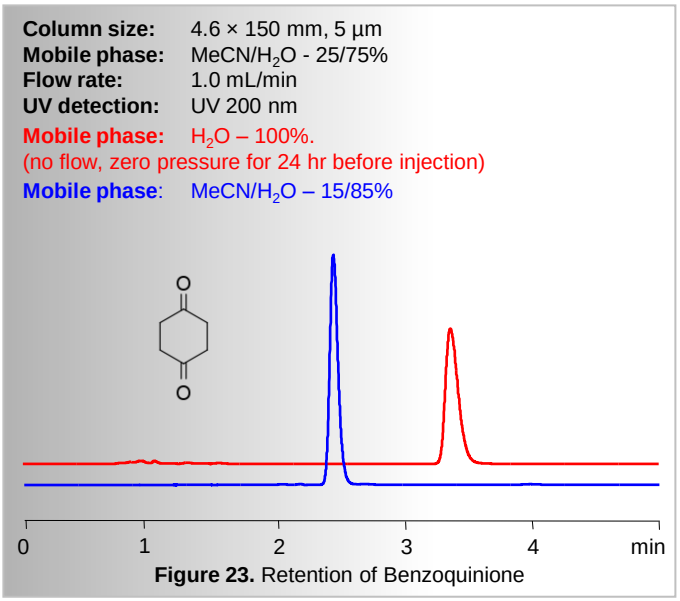


Figure 23. Retention of Benzoquinione

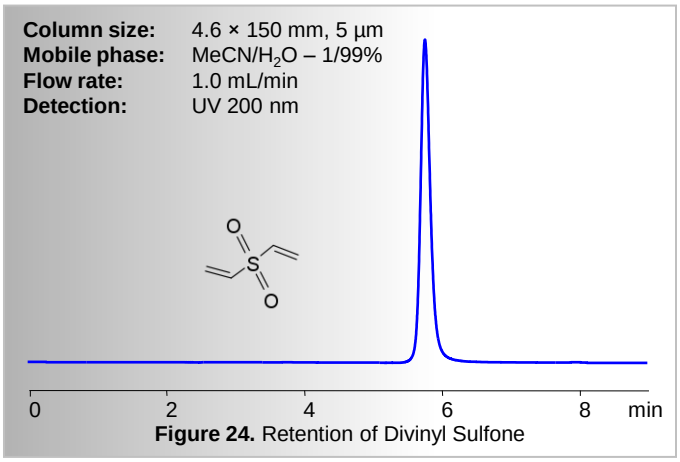


Figure 24. Retention of Divinyl Sulfone

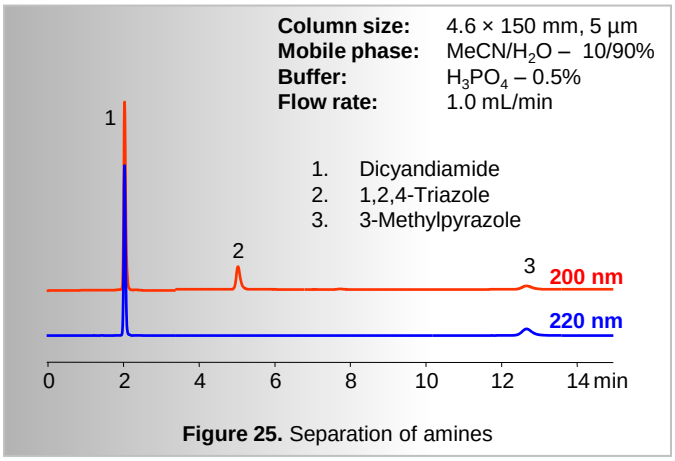


Figure 25. Separation of amines

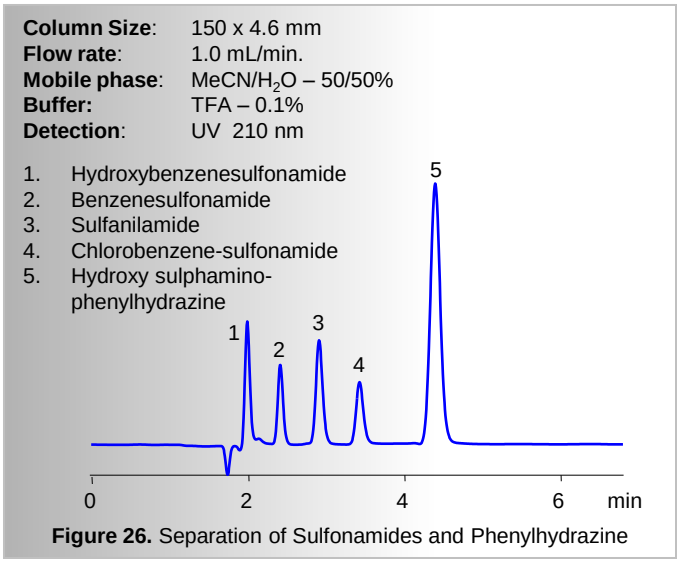


Figure 26. Separation of Sulfonamides and Phenylhydrazine

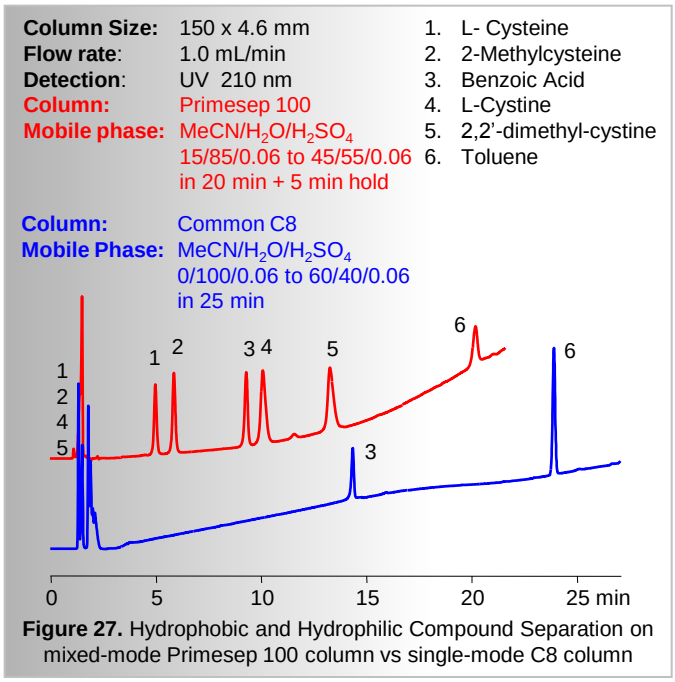


Figure 27. Hydrophobic and Hydrophilic Compound Separation on mixed-mode Primesep 100 column vs single-mode C8 column

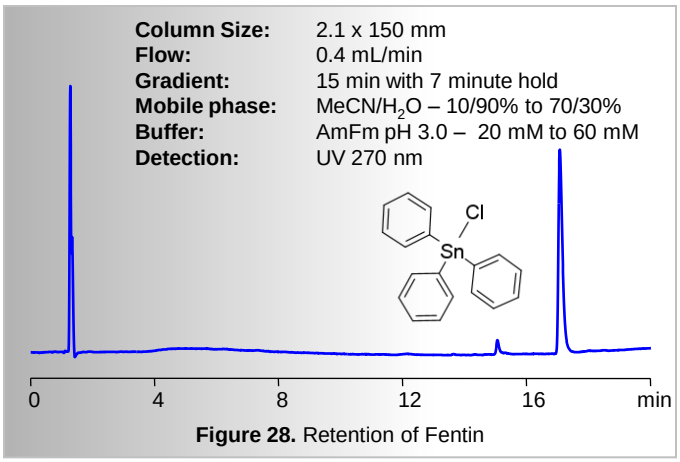


Figure 28. Retention of Fentin

The power of using a gradient on a mixed-mode column compared to a single-mode C8 column is shown in Fig. 27. The single-mode column was only properly able to separate the two polar compounds (#3 and #6). The non-polar compounds eluted quickly without any noticeable separation. In order to achieve the same results on a single-mode column, an ion-pairing reagent would need to be added to the MP. With a mixed-mode column, this extra component is not necessary for the separation of this type of mixture.

Column Tuning via Mobile Phase - pH

Previously, we have only discussed tuning the mobile phase and column with respect to different compounds. Here, we see just how influential altering the mobile phase can be on the retention time and elution order of the same analytes.

In simpler terms, adjusting the strength of the buffer (i.e. the pH) can cause significant changes in the retention of your analytes. This is most apparent in Fig. 29, where the amount of H_2SO_4 alters the retention time of the anti-viral compounds. Fig. 30 also shows how the amount of H_2SO_4 can alter the retention times of analytes, such as Doxycycline, while others, like Ivermectin, are unaffected.

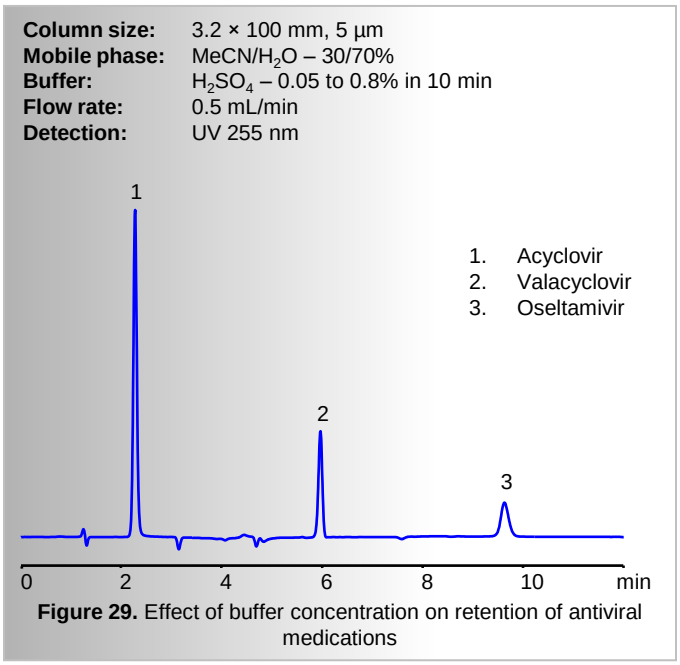


Figure 29. Effect of buffer concentration on retention of antiviral medications

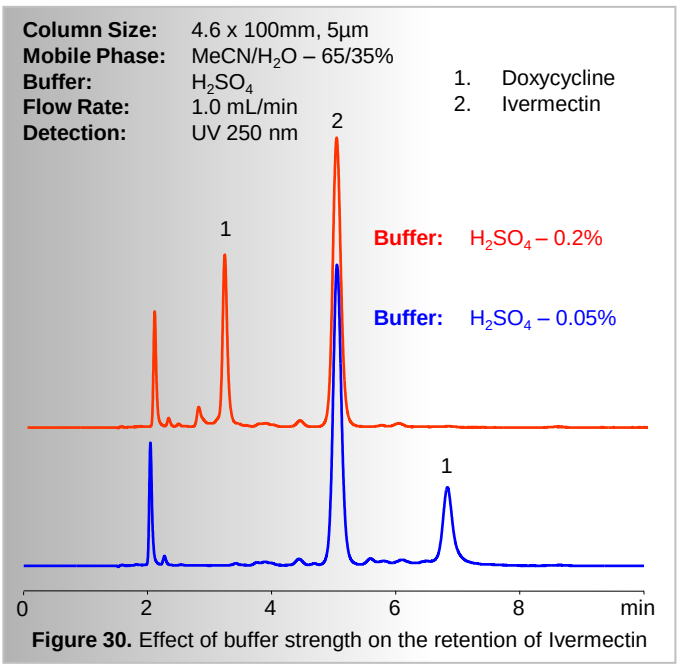


Figure 30. Effect of buffer strength on the retention of Ivermectin

Column Tuning via Mobile Phase – Concentration

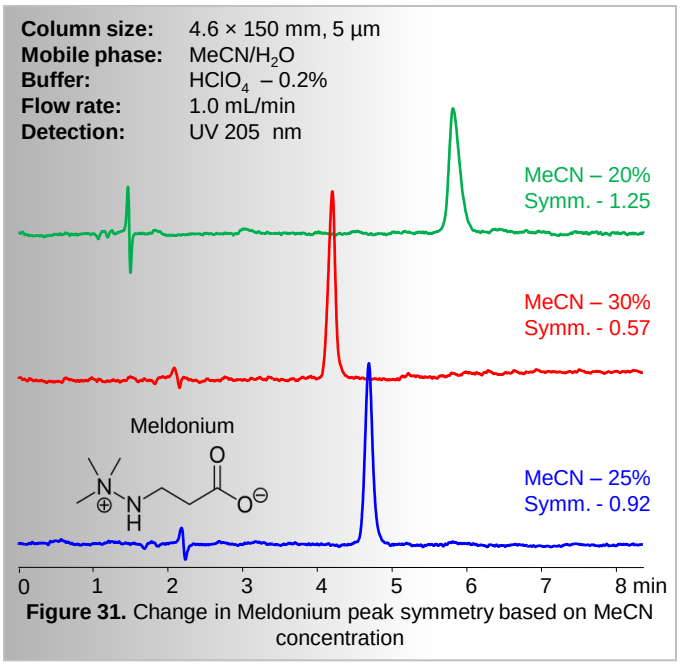


Figure 31. Change in Meldonium peak symmetry based on MeCN concentration

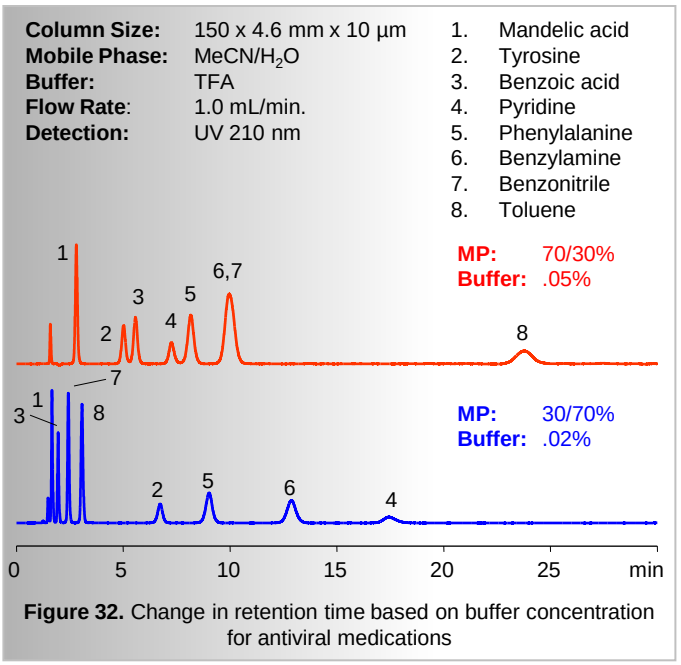


Figure 32. Change in retention time based on buffer concentration for antiviral medications

Modifying the buffer concentration is not the only way to significantly modify the retention times of your analytes. Adjusting the organic component of your mobile phase, in this case Acetonitrile (ACN or MeCN) can also have significant effects on the retention times.

Fig. 31 shows how increasing the MeCN content in the MP changes the symmetry of the Meldonium peaks. We normally would not expect changes in the (nonpolar) organic modifier's concentration to have any effect on polar compounds, but here we see how hydrophobic interactions play a small role in the retention of polar molecules.

Fig. 32 shows how the elution order can be drastically changed by inverting the MP composition ratios. For example, toluene goes from a retention in about 25 minutes to a retention in under 10 minutes with the reduction in MeCN content in the MP.

Column Tuning via Mobile Phase – Gradient

Column Size: 150 x 4.6 mm
Flow rate: 1.0 mL/min
Detection: UV 250 nm

Mobile Phase: $MeCN/H_2O/TFA$ 60/40/0.2% to 80/20/0.2% in 12 min
Detection: UV 250
Mobile phase: $Hexane/Me_3COH$ 99.5/0.5% to 95/5 in 10 min
Detection: UV 220 nm

1. Benzoin
2. Deoxybenzoin
3. Benzil
4. 4-Methyl-deoxybenzoin
5. Benzylbenzoate
6. Bibenzyl

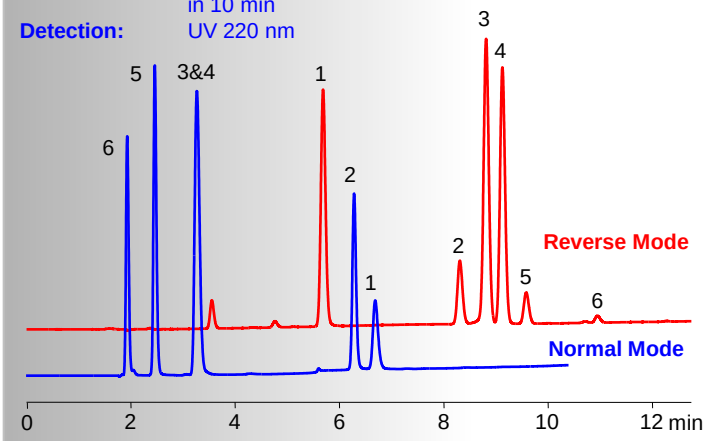


Figure 34. Change in retention order of aromatic compounds by switching modes

Column Size: 250 x 4.6 mm
Flow rate: 1.0 mL/min.
Detection: UV - 240 nm
Mobile Phase: Hexane/EtOH 98/2% to 80/20% in 30 min

Mobile Phase: $MeCN/H_2O$ 80/20% to 0/100% in 25 min

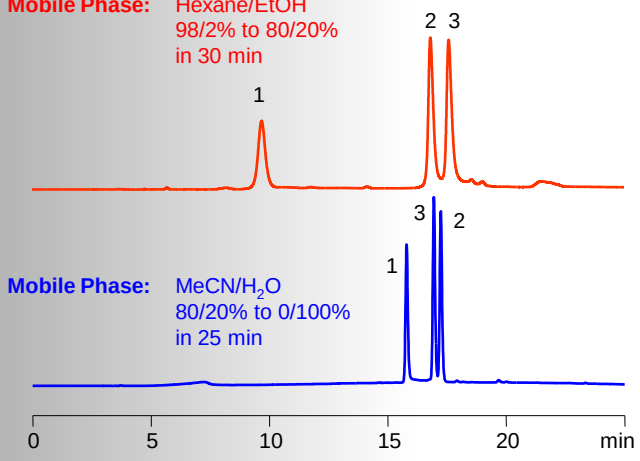


Figure 35. Change in retention order of steroids by switching modes

Altering the type of gradient, in addition to the mobile phase composition, can cause drastic changes with how the analytes interact with the column.

Fig. 34 shows a complete change from Reverse Phase mode to Normal mode chromatography on the same Primesep 100 column. The same exact aromatic compounds interact in the exact opposite mode and elute in the opposite order.

Fig. 35 shows a similar change in elution order based on two different gradient separations and mobile phase compositions.

Fig. 36 shows the separation of a mixture of amino acids with an acid (buffer) gradient. Where a mobile phase gradient can help separate compounds with significantly different polarities, an acid gradient can help separate compounds with significantly different pKa values within a single run through the column.

Column Size: 250 x 4.6 mm
Mobile phase: $MeCN/H_2O$ – 30/70%
Buffer: TFA gradient 0.05 to 0.3% in 25 min
Flow rate: 1.0 mL/min
Detector: ELSD

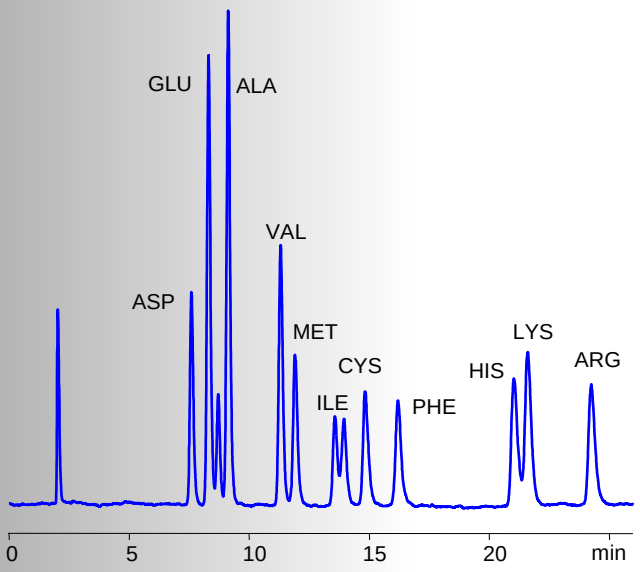


Figure 36. Separation of Amino Acids with Acid Gradient

All data were obtained in SIELC Technologies labs.

SIELC

Primesep 100 part number generator **100 – 32 . 100 . 0210**

Type of packing		Column id		Column length		Particle size		Pore size	
Primesep 100	**100**	50 mm	500	250 mm	250	2.7 µm	**02**	90 Å	09
		30 mm	300	150 mm	150	3 µm	03	100 Å	**10**
		22 mm	220	100 mm	**100**	5 µm	05		
		10 mm	100	50 mm	50	10 µm	10		
		4.6 mm	46	25 mm	25				
		3.2 mm	**32**	10 mm	10				
		2.1 mm	21	Guard	G				
		1.0 mm	10						
		0.5 mm	05						

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