HALO® PCS: New Column Chemistry Options for Basic Compounds

INTRODUCTION

The goal for chromatographic separations is to achieve well resolved peaks with adequate retention and demonstrate Gaussian peak shapes, so the compounds being separated can be assessed accurately both qualitatively and quantitatively. Peak shape is one of the most common observations chromatographers see in a chromatogram.

Peak fronting occurs when an asymmetric peak is broader in the first half and narrower in the second half while peak tailing behaves in the opposite trend. Whether the peaks in a chromatogram are fronting (asymmetry <1), symmetric, or tailing (asymmetry >1) can tell you a lot about the different types of interactions occurring during an HPLC separation.[1] Examples of this can be seen in Figure 1. Achieving a good peak shape in a separation is always a goal during method development, however, this can be more of a challenge than one might think.

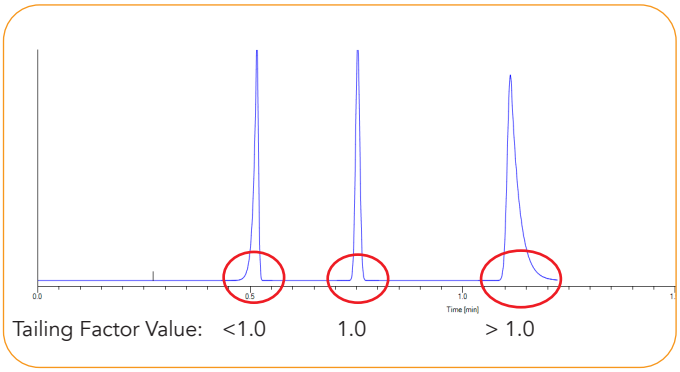


Figure 1: DryLab® software modeling demonstrating fronted peak, symmetric, and tailing

Poor peak shape developing over time can be a sign that the performance of an HPLC column has deteriorated. After all, HPLC columns are consumables and will need to be replaced on a regular basis. This is one reason that a validated method will have certain system suitability requirements including a tailing factor (TF) specification. Not only is this a sign that the column will need to be replaced, but the results of the chromatographic test can be compromised due to a decrease in resolution between two closely eluted peaks. An increase in peak tailing will also make it more difficult to accurately measure peak areas, especially for low abundance impurity peaks within the chromatogram. It is usually a good idea to track the peak tailing over time in order to make sure that the column is still good or that something has not changed within the HPLC instrument. A bad connection or partially blocked flow path can also lead to poor peak shape within the chromatogram.

It is important to note that most peaks within a chromatogram are going to tail or front a bit. Perfectly symmetrical peaks are actually quite rare. Column manufacturers will provide a QC report with a tailing factor (TF) specification range that is acceptable. (for example: 0.95 < TF < 1.3) For many applications a peak tailing factor under 1.5 is acceptable for good quantitation and resolution purposes and tailing above a 2.0 usually requires attention.

There are several factors that can cause poor peak shape other than the column deterioration. This can include the pH of the mobile phase being too close to the pKa of the solute, column oven temperature too hot and causing degradation of solutes, or even wrong/no buffer concentration. It is important to precisely measure these variables and, if your peak shape changes over time, to verify that these measurements are accurate.

TAILING CAUSED FROM BASIC COMPOUNDS

Exchanges between the analytes of interest and the column's stationary phase can lead to unwanted interactions leading to a poor peak shape as well. Many chromatographers are familiar with the difficulties of separating basic compounds on silica-based columns due to the increased peak broadening and tailing that may occur, however, it is important to note that silica-based columns are preferred due to faster equilibration times and mobile phase changes, along with much higher-pressure stability (compared to polymer-based columns). One theory that peak tailing occurs is due to the basic analyte interacting with the stationary phase and free silanols, which allow peak broadening as a result. This becomes significantly worse at higher sample loads, as seen in Figure 2. Another theory for peak tailing (especially when the compound is anionic) is mutual repulsion wherein the adsorption of a small amount of charge to the stationary phase surface results in repulsion of similarly charged analyte molecules that enter the zone of previously adsorbed analytes, thus leading to broadening of the zone.[2]

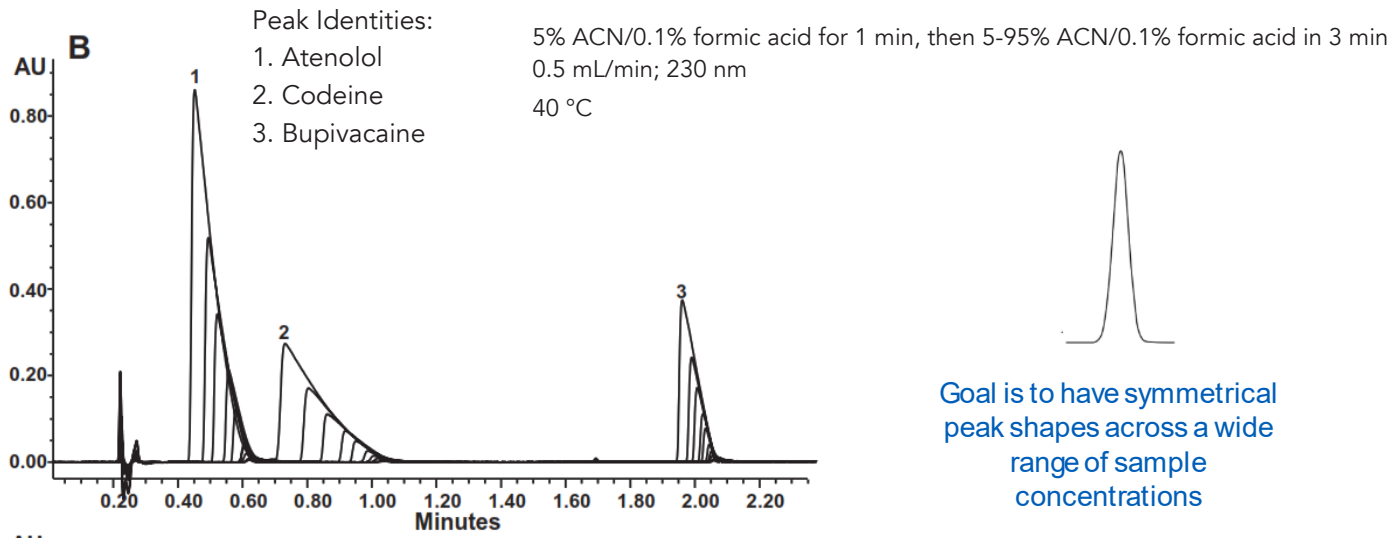
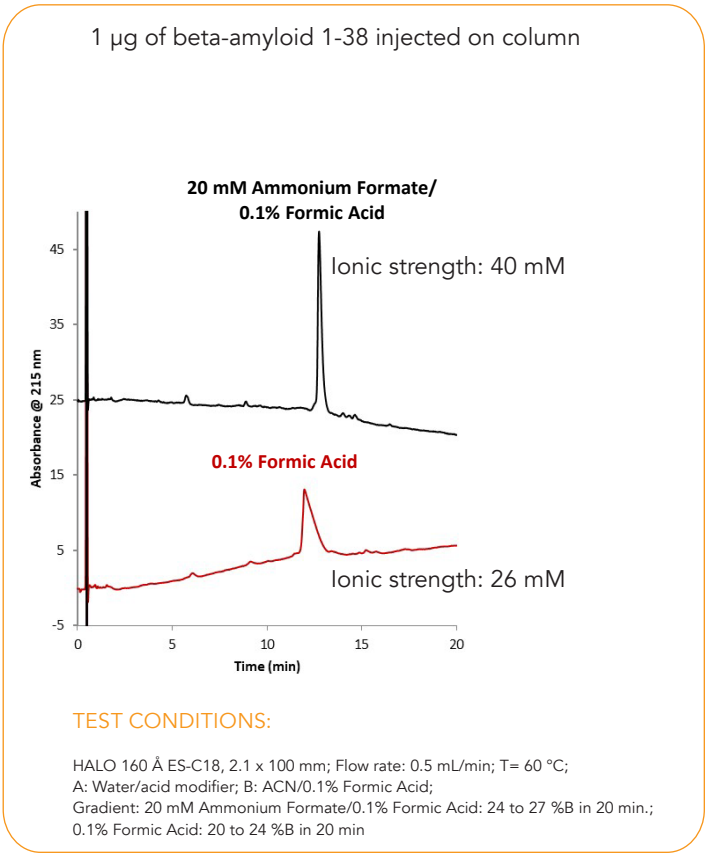


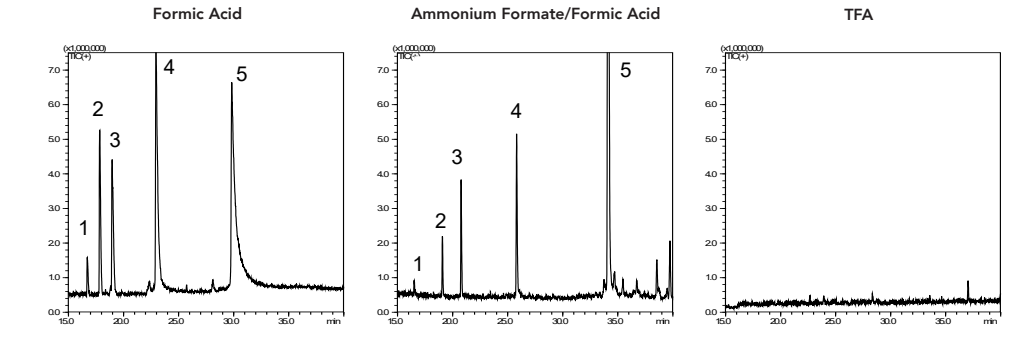
Figure 2: A separation of basic analytes using a standard C18 column. Tailing factors increase as sample load increases. (J. Chromatogr. A 1228 (2012) 221-231)

There are several ways to improve the peak shape of basic compounds such as increasing the ionic strength of the mobile phase (adding a salt/buffer), use of an ion pair agent, elevating the pH of the mobile phase, using a non-silica-based column, or even using an alternative stationary phase such as a charged surface material. For example, Figure 3 shows how the peak shape of beta-amyloid improved after the addition of ammonium formate (AF) to the mobile phase. The use of AF along with formic acid (FA) as a mobile-phase modifier was found to be compatible with LC-MS/MS analysis of peptides. Peak widths were reduced by ~15%, leading to an increase of ~30% in peak capacity when compared with FA-modified mobile phases. Significant improvements in peak shape were displayed by FA/AF when peak asymmetry factors were compared.[3]



Addition of ammonium formate will improve peak shape and increase retention, however, a reduction in ionization efficiency with MS detection will be observed. The same will be true for other acidic modifiers such as difluoroacetic acid or even trifluoroacetic acid. Furthermore, use of trifluoroacetic acid will require routine cleaning within the mass spectrometer and is not recommended. Figure 4 (next page) shows the reduction in ionization efficiency with MS detection compared to formic acid. For these reasons it is desirable to use low ionic strength modifiers, such as formic acid when developing MS compatible methods.

Figure 3: Peak shape improvement of beta-amyloid after the addition of ammonium formate



10FA	LeuEnk.	Angio. I	Subst. P	β-Endor.	Mellitin
Ret.Time	16.7	17.9	19.0	23.0	29.8
MIC(+)	9.3E+06	4.4E+07	3.0E+07	7.3E+07	1.0E+08
Relative	100%	100%	100%	100%	100%

10AFFA	LeuEnk.	Angio. I	Subst. P	β-Endor.	Mellitin
	16.5	19.1	20.8	25.8	34.1
	1.1E+06	6.1E+06	1.2E+07	2.0E+07	1.6E+08
	12%	14%	41%	28%	150%

10TFA	LeuEnk.	Angio. I	Subst. P	β-Endor.	Mellitin
	19.0	22.5	23.7	28.2	36.9
	1.4E+05	8.8E+05	8.0E+05	1.0E+06	2.9E+06
	1%	2%	3%	1%	3%

10 mM acid in mobile phases; 2.1 x 150 mm HALO 160 Å C18, 0.3 mL/min, 2-47% ACN in 40 min, 60°C, 300-1800 m/z, 4 kV, 0.33 s

Figure 4: Reduction in ionization efficiency with the use of TFA and MS detection compared to formic acid and ammonium formate/formic acid

THE POSITIVELY CHARGED SURFACE COLUMN SOLUTION:

One of the easiest ways to improve the peak shape of basic analytes while using low ionic strength mobile phases such as formic acid is to alter the stationary phase of the HPLC packing material. This would include a positively charged surface material that Advanced Materials Technology recently introduced.

The new HALO® positively charged surface (PCS) column offers improved performance of basic compounds under acidic conditions. With the new line of HALO® PCS columns, built on Fused-Core® technology, chromatographers can rely on fast and efficient separations of basic compounds in both small molecule and biological separations. Figure 5 shows the new HALO® PCS available phases. The HALO® PCS small molecule chemistries are offered in C18 and Phenyl-Hexyl for USP L1 and L11 categories respectively, allowing selectivity options for method developers.

The HALO® PCS C18 Peptide is engineered with a 160 Å pore size, ideal for peptide analysis. Peptides have acidic and basic sites (amphiprotic) and can be difficult to analyze especially when trying to run a separation using MS detection. Formic acid conditions for peptides may lead to high tailing factors while using a more traditional C18 stationary phase.

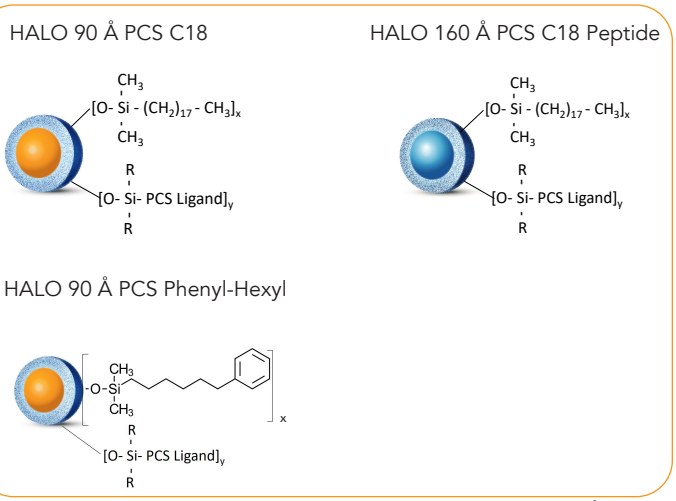
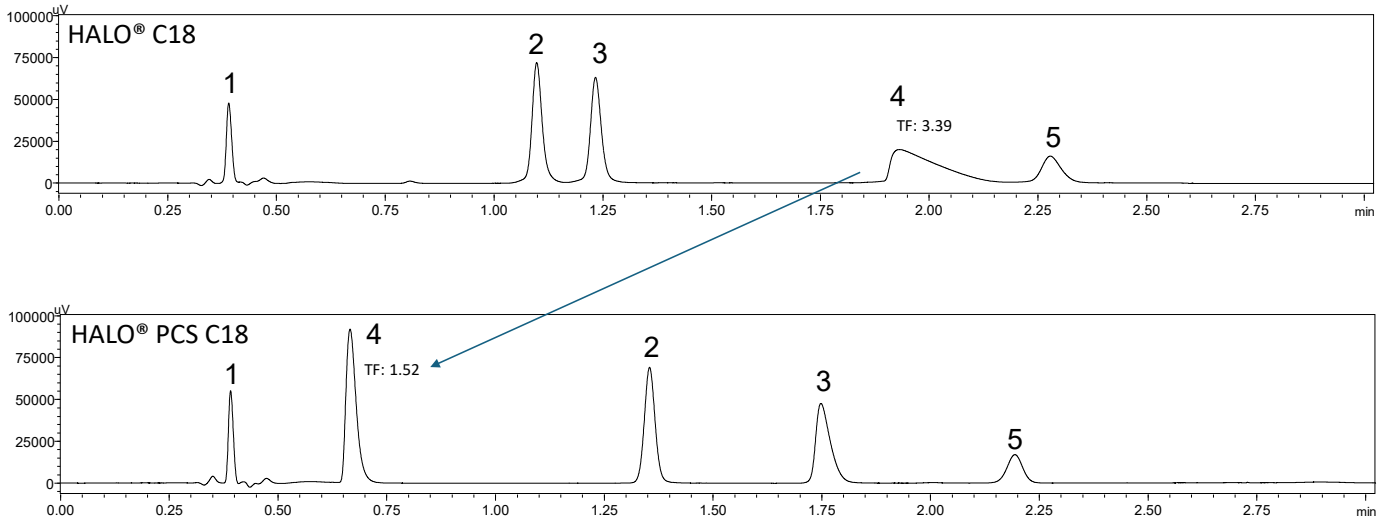


Figure 5: HALO® PCS 2.7 µm particle available with 90 and 160 Å pore size.

HALO® PCS can be run with most standard mobile phases, but does rely on acidic conditions to make use of the charged surface interaction. By running under low pH conditions, the basic compounds gain a proton becoming positively charged and repel from the positively charged surface of PCS. With the reduced retention of the basic compounds, tailing is reduced, but the strength of the organic mobile phase may need to be reduced to have adequate retention. It should be noted that this is true for any positively charged surface stationary phase. Operation at neutral to high pH conditions will negate the benefits contributed by a positive charge from the stationary phase.

For example, Figure 6 (next page) shows a separation of acidic, neutral, and basic analytes on a standard C18 column compared to a HALO® PCS C18 column. The peak shape of imipramine significantly improves when the positive charge surface stationary phase is used.



TEST CONDITIONS:

Column: HALO 90 Å, 2.7µm, 2.1 x 100 mm
Mobile Phase: A: Water/ 0.1% FA
B: Acetonitrile/ 0.1% FA
Isocratic Separation: 30 %B
Wavelength: PDA, 254 nm

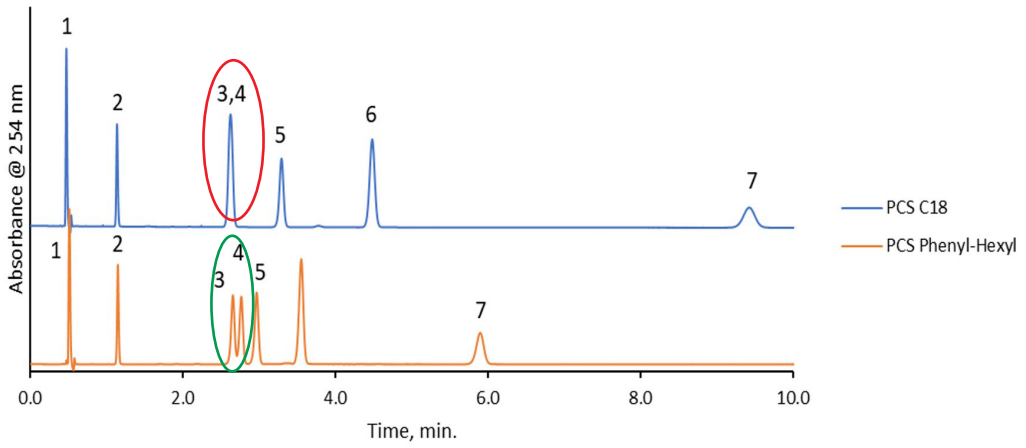
Injection: 0.5 µl
Temperature: 30 ºC
Flow Rate: 0.5 mL/min.
Instrument: Shimadzu Nexera X2

PEAK IDENTITIES:

1. Uracil
2. 4-Methoxybenzoic acid
3. 2-Chlorobenzoic acid
4. Imipramine (base)
5. Dimethyl phthalate

Figure 6. Basic analyte peak shape (imipramine) improvement with HALO® PCS C18

For an alternative selectivity compared to C18, a Phenyl-Hexyl option is available. The Phenyl-Hexyl ligand will take advantage of pi-pi interactions between the analyte and the stationary phase. For aromatic compounds the PCS Phenyl-Hexyl option should be considered. For example, Figure 7 shows a separation of benzodiazepines and neutrals showing a separation advantage on Phenyl-Hexyl.



PEAK IDENTITIES:

1. Uracil
2. Phenol
3. Oxazepam
4. Flunitrazepam
5. Diazepam
6. 1-Cl-4-Nitrobenzene
7. Naphthalene

TEST CONDITIONS:

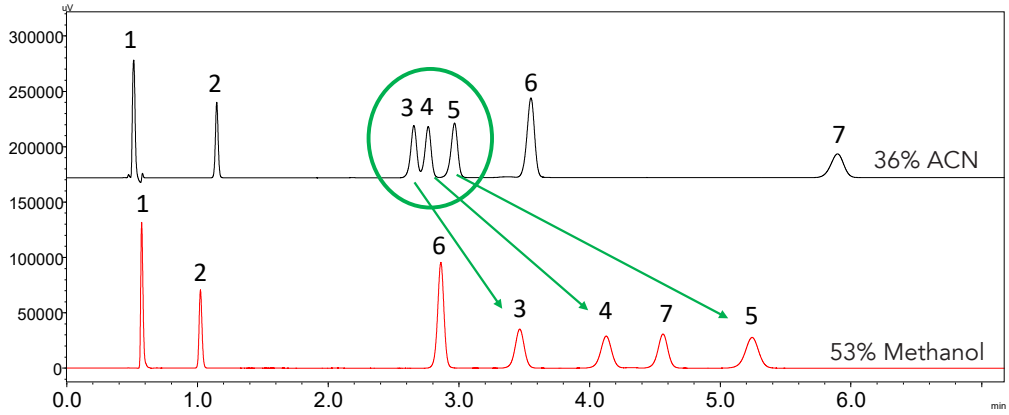
Column: HALO 90 Å PCS C18, 2.7µm, 2.1 x 100 mm
Part Number: 92812-617
HALO 90 Å PCS Phenyl-Hexyl, 2.7µm, 2.1 x 100 mm
Part Number: 92812-618
Mobile Phase:
A: Water, 0.1% Formic Acid
B: Acetonitrile, 0.1% Formic Acid

Isocratic: 36 %B
Flow Rate: 0.4 mL/min
Back Pressure: 254 bar
Temperature: 30 ºC
Injection: 0.5 µL
Sample Solvent: 70/30 Water/ACN
Wavelength: PDA, 280 nm

Flow Cell: 1 µL
Data Rate: 100 Hz
Response Time: 0.05 sec.
LC System: Shimadzu Nexera X2

Figure 7: Separation improvements of benzodiazepines using HALO® PCS Phenyl-Hexyl

This separation can further be improved with the incorporation of methanol as the organic modifier. Methanol is better for enhancing pi-pi interactions between the analytes and the phenyl stationary phase. It should be noted that using methanol over acetonitrile may provide selectivity advantages; however, it will significantly increase the system back pressure.



PEAK IDENTITIES:

1. Uracil
2. Phenol
3. Oxazepam
4. Flunitrazepam
5. Diazepam
6. 1-chloro-4-nitrobenzene
7. Naphthalene

TEST CONDITIONS:

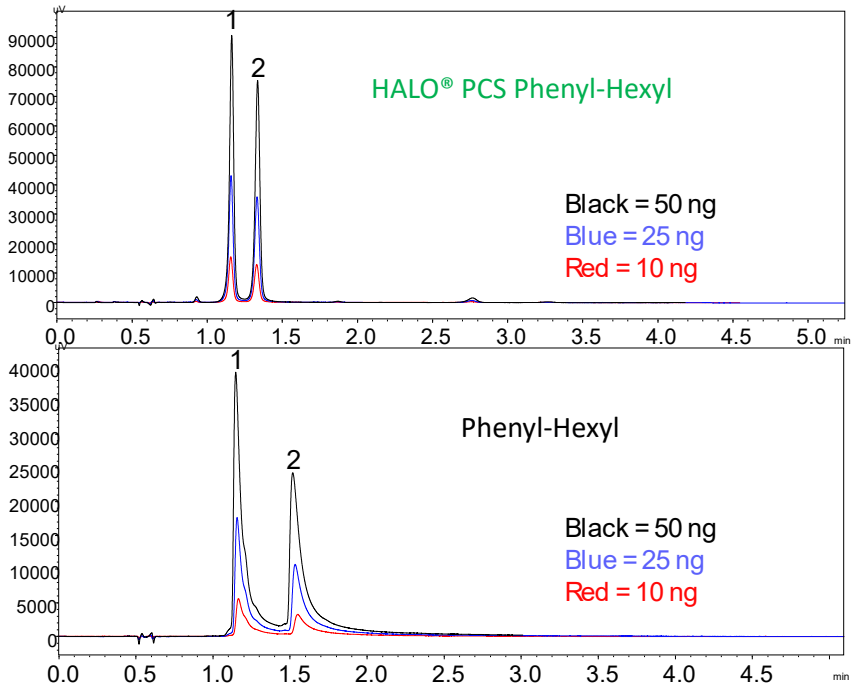
Column: HALO 90 Å PCS Phenyl-Hexyl, 2.7 µm, 2.1 x 100mm
Mobile Phase A: Water/ 0.1% Formic Acid
B: Acetonitrile or Methanol
Isocratic: 36% ACN or 53% Methanol

Injection: 0.5 µL
Temperature: 30 °C
Flow Rate: 0.4 mL/min
Instrument: Nexera

Figure 8: Use of methanol instead of acetonitrile as the organic modifier enhances pi-pi interactions

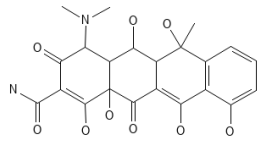
SAMPLE LOADING:

It is widely recognized that ionizable analytes such as basic drugs have much lower sample loading capacities compared to low polarity neutral compounds.[4] The HALO® PCS stationary phase allows for higher sample loads on column due to the increased performance (maintaining peak shape) for basic analytes. This is demonstrated in Figure 9, observing a mixture of tetracyclines at different sample loads. (10-50 ng on the column) Sharp, symmetrical peaks with ~50% smaller peak peaks are observed on the HALO® PCS Phenyl-Hexyl column. (ex. Tetracycline: 0.036 vs. 0.073 PW@50% at 25 ng)

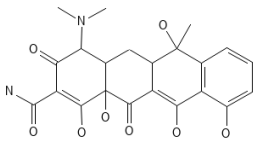


TEST CONDITIONS:

Column: 2.7 µm, 2.1 x 100 mm phase as labeled
Mobile Phase A: Water/ 0.1% Formic Acid
B: Acetonitrile/ 0.1% Formic Acid
Isocratic: 12% B HALO® PCS Phenyl-Hexyl
18% B HALO® Phenyl-Hexyl
Flow Rate: 0.4 mL/min
Injection: 0.2, 0.5, 1.0 µL (10,25,50 ng)
Temperature: 35 °C
Instrument: Nexera



1. Oxytetracycline



2. Tetracycline

Figure 9: Separation of tetracyclines on HALO® PCS Phenyl-Hexyl vs. Phenyl-Hexyl. Improvement in peak shape over increasing sample load is observed with the positive charge surface material.

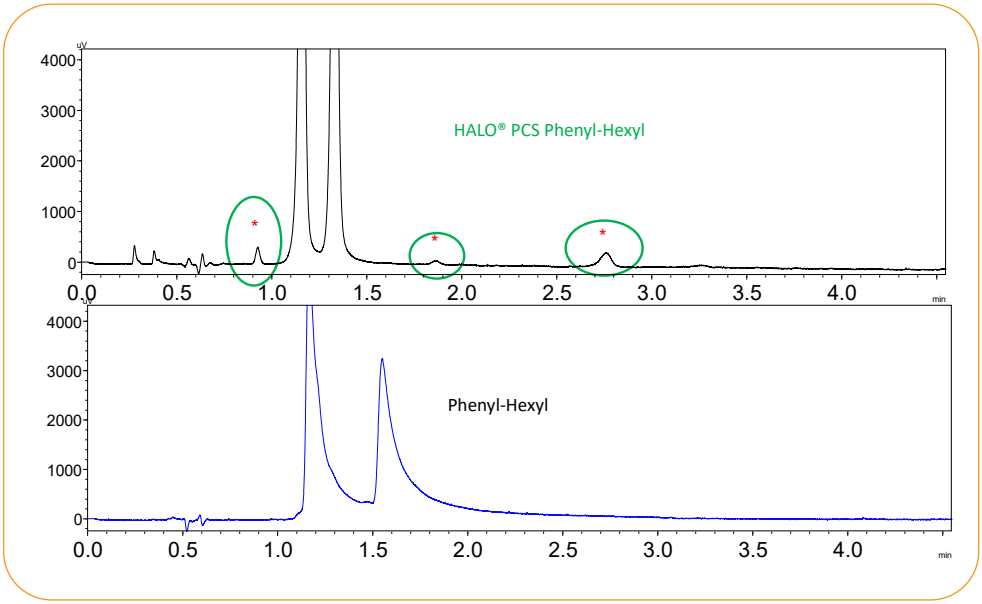


Figure 10: PCS allows for higher sample loading of basic compounds, allowing easier detection of impurities

Higher sample loads can also allow easier detection and quantification of impurities within a sample which is crucial for quality assurance purposes. This can be seen on the same chromatogram in Figure 10 (left).

CONCLUSIONS:

In summary, the HALO® PCS stationary phase provides chromatographers with a surface chemistry that can improve separations of basic analytes for small molecules and peptides. Having multiple bonded phase choices will enhance selectivity and increase method development success. HALO® PCS should be used under acidic conditions to benefit from the positively charged surface and is a helpful addition to MS method development, allowing for the use of formic acid, meaning higher ionization efficiencies, without sacrificing peak shape and/or inviting instrument contamination from ion pair reagents, such as TFA. The positive charge surface allows for higher sample loading, enabling easier detection of impurities within a sample. With all of the benefits to basic compound analyses, chromatographers should consider HALO® PCS as a screening option for method development.

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4. Jun Dai, Peter W. Carr, David V. McCalley, A new approach to the determination of column overload characteristics in reversed-phase liquid chromatography, Journal of Chromatography A, Volume 1216, Issue 12, 2009, Pages 2474-2482,

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