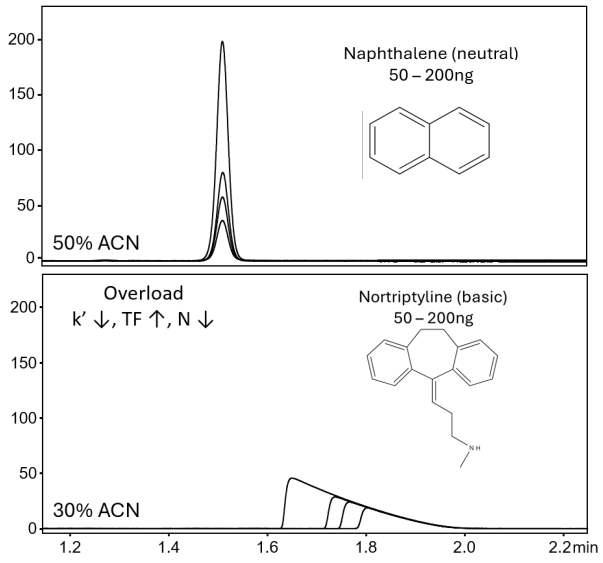
Reversed Phase Positive Charge Surface Phenyl-Hexyl Phase for Improved Separations of Basic Analytes

Introduction

Achieving symmetrical peak shapes for ionic analytes by reversed-phase liquid chromatography (RPLC) can be difficult. Basic analytes are particularly challenging when run using low pH mobile phases as they overload at significantly lower masses on-column, compared to neutral or non-ionized solutes. This is because basic compounds are protonated at low pH. The peak shape of these cationic solutes can be characterized as having high asymmetry and low efficiencies,which present as broad and tailed. When using low ionic strength mobile phases, like those favored in liquid chromatography mass spectrometry (LCMS) such as formic acid, these characteristic peak shapes are worsened.

The example in **Figure 1** emphasizes the differences between the performance of neutral and ionized basic solutes, when operating under typical RPLC mobile phase conditions of water and acetonitrile with 0.1% (v/v) formic acid (FA) as the modifier. Here a standard 2.1 x 100 mm analytical column is used, packed with 90 Å 2.7 µm superficially porous particles (SPPs) with a C18 bonded phase. A comparison of the neutral analyte, naphthalene, and the basic analyte, nortriptyline, visually shows the poor peak shape of the latter. Over the 50-200 ng of load on-column, nortriptyline already shows signs of overload at 50 ng. Overload is identified by increases in tailing factor (TF), and decreases in both the number of theoretical plates (N) and retention factor (k'). These same measurements stay consistent and do not show signs of overload from 50 - 1,000 ng for naphthalene, a neutral compound. This behavior of basic analytes under low ionic strength mobile phases in most cases makes performing high resolution separations difficult.

The overloading and poor peak shapes of basic solutes have been well investigated for many years now [1-2]. In 2003 McCalley proposed that mutual repulsion of the cationic solutes led to the poor chromatography of base [1]. This mutual repulsion effect was studied using mobile phases with 20mM formic acid as an additive, which has a resulting pH of 2.7 [1]. The 2014 publication by Gritti [2] studied and modeled the adsorption of basic solutes on C18 bonded phases. Their results found three items related to poor basic peak shapes: there is heterogenous adsorption of basic solutes onto the stationary phase, at low mass loads the overload is caused by adsorption onto a small number of high energy sites near the surface, and at higher mass loads mutual repulsion occurred on a larger number of low energy sites on the stationary phase [2]. This study was carried out with phosphate buffer at



Test Conditions:
Column: 90 Å SPP C18 2.7 µm, 2.1 x 100 mm
Mobile Phase A: Water/0.1% FA
Mobile Phase B: ACN/0.1% FA
Isocratic Separation at specified %B
Absorbance: PDA, 254 nm, mV
Injection: 1.0 µL
Temperature: 35 °C
Flow Rate: 0.50 mL/min
Instrument: Shimadzu Nexera

Figure 1. Comparison of neutral and basic analyteperformance under low ionic strength mobile phase conditions

pH =2.60 [2]. A simplistic summary of these theories would be that cationic solutes interact poorly with each other under acidic conditions and have negative interactions with the stationary phase.

The mobile phase used in RPLC methods for the separations of basic analytes also plays a crucial role in minimizing these negative interactions. Increases in mobile phase pH, the use of ion pairing reagents like trifluoroacetic acid (TFA), and increases in the ionic strength all help to improve peak shape of basic analytes [1, 3, 4]. These mobile phase modifications, though beneficial, are not always preferred. In LCMS applications poor ionization and signal intensities can result from the use of TFA [1, 4]. LCMS is routinely used for the analysis of small molecule pharmaceuticals which often contain basic functional groups. The preferred additive of 0.1% formic acid is volatile, low in ionic strength, and produces efficient ionization.

Positively Charged Surface Bonded Phase Technology

The addition of a positively charged ligand to the silica stationary phase has been shown to improve basic peak shape, using low ionic strength mobile phases. These bonded ligands will carry a positive charge under acidic conditions, such as 0.1% formic acid. The positively charged ligand will electrostatically repulse cationic solutes away from the particle surface, effectively screening out many of the negative interactions previously discussed. Many versions of this technology have been previously described in literature and are commonly found with C18 bonded phases on fully porous particles (FPPs) and SPPs. The mechanism of retention is still reversed-phase based, not ion-pairing.

The HALO® positively charged surface (PCS) technology is a positively charged ligand bonded to a 90 Å 2.7 µm Fused-Core® particle. The first offering of this was the 90 Å HALO® PCS C18 2.7 µm packing. As with other positively charged ligands, acidic mobile phases are required to charge the surface. This charge will repel cationic solutes away from the surface and screen analytes from negative interactions.

Chromatographically this leads to improvements in TF, N, and resolution (Rs) of basic analytes. This technology is designed for RPLC and LCMS separations of basic small molecules, when using acidic low ionic strength mobile phases like 0.1% formic acid. **Figure 2** 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.

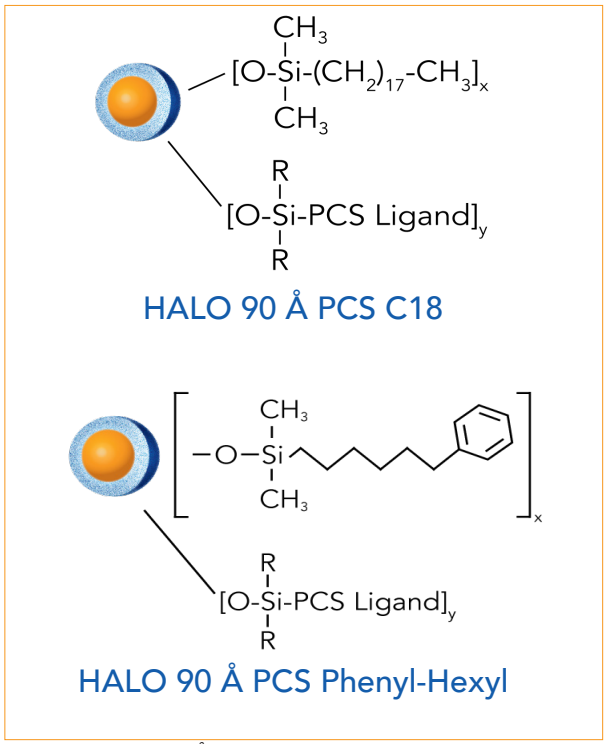
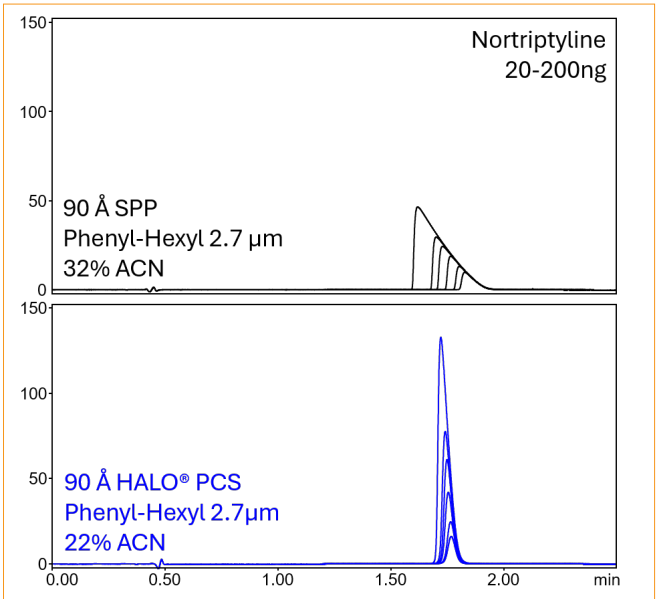


Figure 2. HALO 90 Å PCS 2.7 µm available bonded phases

For alternative selectivity compared to C18, the 90 Å HALO® PCS Phenyl-Hexyl 2.7 µm bonded phase is now available. The pi-pi interactions between the Phenyl-Hexyl bonded phase and aromatic analytes can change the separation selectivity compared to the alkyl C18 phase. The PCS Phenyl-Hexyl phase should be considered for separations of aromatic compounds, like small molecule pharmaceuticals. Many of these drugs contain both basic and aromatic functional groups.

The improvements in basic analyte performance can be seen for the PCS technology in **Figure 3**. Here nortriptyline is injected over a load range of 20-200 ng, using water and acetonitrile (ACN) with 0.1% formic acid as the mobile phase. A non-charged 90 Å SPP Phenyl-Hexyl on a 2.7 µm SPP is compared to the 90 Å HALO® PCS Phenyl-Hexyl 2.7 µm. The addition of the positively charged ligand greatly decreases peak tailing and increases column efficiency. The PCS Phenyl-Hexyl column shows less signs of overload compared to the non-charged Phenyl-Hexyl. The amount of ACN in the mobile phase has been decreased for the PCS Phenyl-Hexyl in order to have comparable retention between the PCS and non-charged Phenyl-Hexyl columns. The addition of the PCS ligand to particle's decreases retention of cationic analytes compared to a non-charged column. These same chromatographic improvements have also been demonstrated using HALO® PCS C18.



Test Conditions:
Column: 2.1 x 100 mm
Mobile Phase A: Water/0.1% FA
Mobile Phase B: ACN/0.1% FA
Isocratic Separation at specified %B
Absorbance: PDA, 254 nm, mV
Injection: 1.0 µL
Temperature: 35 °C
Flow Rate: 0.50 mL/min
Instrument: Shimadzu Nexera

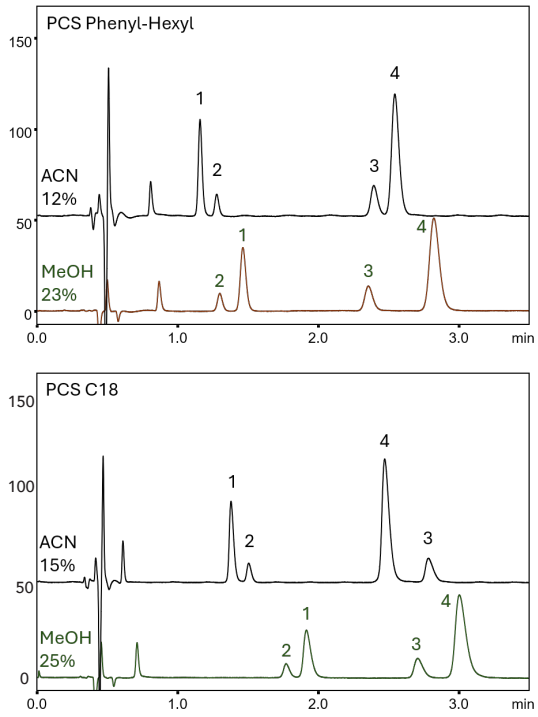
Figure 3. Improvements in basic (nortriptyline) peak shape with HALO® PCS Phenyl-Hexyl (bottom) compared to non-charged SPP Phenyl-Hexyl (top)

Selectivity

Changes in separation selectivity, and resolution, drive the need for various bonded phase chemistries. The standard C18 RPLC bonded phase is a great first choice for most separations and provides a common frame of reference for changes in selectivity. Therefore, the following examples will examine why a chromatographer would want to switch from C18 to a Phenyl-Hexyl bonded phase. The examples shown below will compare the 90 Å HALO® PCS C18 to the 90 Å HALO® PCS Phenyl-Hexyl bonded phase for the separation of basic drugs under acidic mobile phase conditions.

Acetonitrile Mobile Phase				
Bonded Phase	Peaks 1 and 2		Peaks 3 and 4	
	Selectivity	Rs	Selectivity	Rs
PCS Phenyl-Hexyl	1.17	2.22	1.08	1.56
PCS C18	1.13	2.08	1.15	3.07
Methanol Mobile Phase				
Bonded Phase	Peaks 1 and 2		Peaks 3 and 4	
	Selectivity	Rs	Selectivity	Rs
PCS Phenyl-Hexyl	1.20	2.29	1.25	3.95
PCS C18	1.11	1.61	1.13	2.30

Table 1. Selectivity and resolution measurements from Figure 4



Test Conditions:
Column: 90 Å SPP C18 2.7 µm, 2.1 x 100 mm
Mobile Phase A: Water/0.1% FA
Mobile Phase B: Specified solvent/0.1% FA
Isocratic Separation at specified %B
Absorbance: PDA, 230 nm, mV
Injection: 1.0 µL
Temperature: 30 °C
Flow Rate: 0.50 mL/min
Instrument: Shimadzu Nexera

Peak Identities:
1. Bisoprolol (beta-blocker)
2. Bupivacaine (local anesthetic)
3. Tetracaine (local anesthetic)
4. R-propranolol (beta-blocker)

Figure 4. Selectivity changes between 90 Å HALO® PCS Phenyl-Hexyl and PCS C18 when run using acetonitrile and methanol mobile phases with 0.1% formic acid

Measurements of selectivity and resolution will be examined to illustrate the utility of bonded phase changes.

The four-component separation in **Figure 4** of two beta-blockers and two local anesthetics highlights typical selectivity differences between PCS C18 and PCS Phenyl-Hexyl columns. The amount of ACN delivered by the pump has been adjusted to more closely match retention of R-propranolol between both phases. The measured values for selectivity and resolution are in **Table 1**.

Both pairs of peaks in this separation are baselined resolved (Rs > 1.5) for both columns. The first peak pair of bisoprolol and bupivacaine show a small change in selectivity between PCS C18 and PCS Phenyl-Hexyl. The slight increase in PCS Phenyl-Hexyl selectivity (α= 1.17) provides an increase in resolution (Rs= 2.22). The second peak pair of tetracaine and propranolol demonstrates an example of retention order switch. The retention order of propranolol/tetracaine is reversed when changing to a PCS Phenyl-Hexyl column. The resolution of the PCS C18 (Rs = 3.07) is larger than the PCS Phenyl-Hexyl (Rs = 1.56) for this peak pair. It is reasonable to assume that in other sample mixtures a retention order switch could be beneficial in resolving a co-elution.

The use of different organic mobile phase modifiers can also provide changes in chromatographic selectivity. **Figure 4** also shows the same four-component mixture run with methanol instead of acetonitrile. Methanol is a weaker organic solvent than acetonitrile, and the percent methanol in the mobile phase has been increased to match retention for propranolol. As can be seen the switch to methanol has changed the retention order for both columns. Bupivacaine is now less retained than bisoprolol, and tetracaine is less retained than propranolol for the PCS C18 column. With regards to selectivity, once again small increases help to improve resolution. The PCS Phenyl-Hexyl column has larger selectivity values for this mixture than the PCS C18, which results in increased resolution values for both pairs.

Column Performance and Overloading Comparison

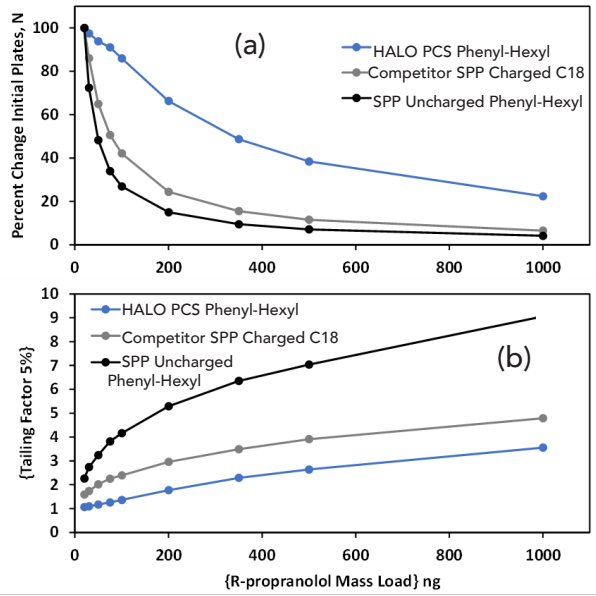
Protonated basic analytes overload at significantly lower on-column mass loads than neutral analytes when using standard RPLC columns. The use of a positively charged surface ligand, like HALO® PCS technology, can greatly improve the performance of protonated basic analytes when using mobile phases with a 0.1% formic acid modifier. Improvements in column efficiencies (plates) and decreases in peak tailing factors are observed when switching to a column with a positively charged surface modification.

Improvements in the number of theoretical plates and tailing factor for a given analyte can be altered by changes in column bonded-phase chemistry. As with selectivity, the standard C18 RPLC bonded phase is normally a good choice for efficient separations and provides a common frame of reference for chromatographers. The next examples will compare the 90 Å HALO® PCS Phenyl-Hexyl

to a commercially available competitor surface charged C18 column. Both columns are packed with 90 Å pore size, 2.7 µm superficially porous particles that are modified with different positively charged surface ligands. **Figure 5** also includes the same curve for a non-charged, 90 Å 2.7µm SPP Phenyl-Hexyl column. Measurements of percent change from initial plate count at 20 ng and tailing factor at 5% peak height will be examined for the basic analyte, R-propranolol, as the mass load is increased on column from 20 to 1,000 ng. The plate counts, retention factor, and tailing factor measurements for 20 and 200 ng are in **Table 2**.

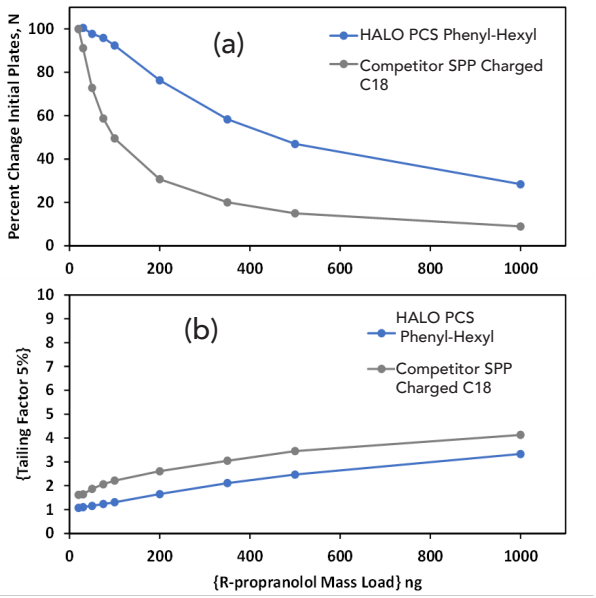
Acetonitrile Mobile Phase from Figure 5			
Column	20 ng R-propranolol		
	k'	TF 5%	Plates, N
HALO PCS Phenyl-Hexyl	3.18	1.06	12357
Competitor SPP Charged C18	2.99	1.59	10713
SPP Uncharged Phenyl-Hexyl	3.27	2.26	6317
Methanol Mobile Phase from Figure 6			
Column	20 ng R-propranolol		
	k'	TF 5%	Plates, N
HALO PCS Phenyl-Hexyl	3.46	1.07	8809
Competitor SPP Charged C18	3.41	1.62	8640

Table 2. Chromatographic measurements from Figure 5 and 6



Test Conditions:
Columns: 2.1 x 100 mm
90 Å HALO PCS Phenyl-Hexyl, 2.7 µm (14 %B)
90 Å Competitor SPP Charged C18, 2.7µm (19 %B)
90 Å SPP Uncharged Phenyl-Hexyl, 2.7 µm (25 %B)
Mobile Phase A: Water/0.1% FA
Mobile Phase B: ACN/0.1% FA
Flow Rate: 0.50 mL/min
Injection: 1.0 µL
Absorbance: PDA, 280 nm
Temperature: 35 °C
Flow Rate: 0.50 mL/min
Instrument: Shimadzu Nexera

Figure 5. Load tolerance comparisons in acetonitrile mobile phase for R-propranolol



Test Conditions:
Columns: 2.1 x100 mm
90 Å HALO PCS Phenyl-Hexyl, 2.7 µm (25 %B)
90 Å Competitor SPP Charged C18, 2.7µm (31 %B)
Mobile Phase A: Water/0.1% FA
Mobile Phase B: MeOH/0.1% FA
Injection: 1.0 µL
Absorbance: PDA, 280 nm
Flow Rate: 0.50 mL/min
Temperature: 35 °C
Instrument: Shimadzu Nexera

Figure 6. Load tolerance comparisons in methanol mobile phase for R-propranolol

The plot of percent change from initial plate count versus mass load of propranolol shown in **Figure 5a** clearly shows improvements in column efficiencies across all loads for the PCS Phenyl-Hexyl. The PCS Phenyl-Hexyl provides improved tolerance to higher loads as measured by percent change from initial. This improvement in plates is observed whether using acetonitrile (**Figure 5**) or methanol (**Figure 6**) as the strong organic solvent. The two positively charged surface ligand materials demonstrate the usefulness of this technology, as both provide more plates across the entire load range when compared to the non-charged Phenyl-Hexyl column.

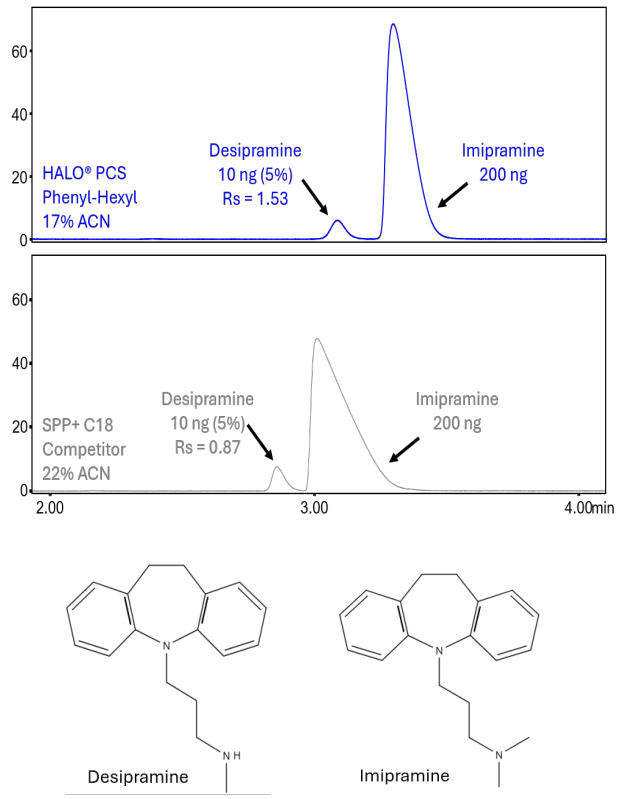
For this comparison acetonitrile is the preferred organic mobile phase for higher efficiencies. For the competitor C18 column the two mobile phases have equal plates at loads greater than 200 ng. For the PCS Phenyl-Hexyl acetonitrile provides higher plates at all loads. The advantage in plates for the PCS Phenyl-Hexyl is observed over a range of analyte loads with two common RPLC organic mobile phases.

Similar trends are found when examining measured tailing factors at 5% peak height. This measurement also supports how positively charged surface technologies improve basic peak shape under low ionic strength mobile phases. Both charged surface C18 and Phenyl-Hexyl columns show more resistance to overloading than a non-charged Phenyl-Hexyl column. In both **Figure 5b and 6b**, the tailing factors are lower for PCS Phenyl-Hexyl compared to the competitor

C18. For the competitor C18, tailing factors were decreased by roughly 8-15% in the methanol mobile phase at loads greater than 30ng. The PCS Phenyl-Hexyl tailings factors are nearly equivalent in acetonitrile and methanol, with methanol having a 6% decrease at loads higher than 200 ng.
The HALO® PCS Phenyl-Hexyl offers enhanced loading capabilities compared to a competitor positively charged surface C18 column. These improvements in column performance are observed as sharper and more symmetrical peak shapes over a range of mass loads.

Imipramine Spiked Impurity Analysis

An isocratic separation of imipramine and its N-demethylation product, desipramine, highlights how the combination of improvements in load tolerance and changes in selectivity for the HALO®PCS Phenyl-Hexyl can resolve two closely retained solutes. The separation in **Figure 7** is of imipramine at 200 ng load on-column, with a 5% (10 ng) spiked impurity of desipramine. A resolution value of 1.53 is obtained for this separation. Baseline resolution is maintained as this impurity is increased from 5 to 15% (not shown). The SPP competitor charged surface C18 column was unable to baseline resolve this critical pair at these load levels.
The HALO®PCS Phenyl-Hexyl was able to resolve this spiked impurity from the main peak due to its improvements in peak shape and changes in selectivity. The PCS Phenyl-Hexyl has a selectivity value of 1.08 for this peak pair, which is a small yet useful increase over the measured competitor C18 value of 1.06. This selectivity increase is complemented by the sharper peaks at both 20 and 200 ng, to achieve baseline resolution.



Test Conditions:
Columns: 2.1 x100 mm
90 Å HALO® PCS Phenyl-Hexyl 2.7 µm (17 %B)
90 Å SPP Comp. Charged Surface C18 2.7µm (22 %B)
Mobile Phase A: Water/0.1% FA
Mobile Phase B: ACN/0.1% FA
Injection: 1.0 µL
Absorbance: PDA, 254 nm, mV
Flow Rate: 0.50 mL/min
Temperature: 35 °C
Instrument: Shimadzu Nexera

Figure 7. Baseline resolved separation of spiked desipramine impurity from imipramine.

Conclusions

The HALO® PCS Phenyl-Hexyl provides an alternative selectivity compared to HALO® PCS C18 and demonstrates similar high performance for the separation of basic small molecules. The HALO®PCS Phenyl-Hexyl is another versatile option for separations of these analytes with LCMS preferred 0.1% formic acid mobile phases. Alternative selectivity can be found by changing both the bonded phase (PCS C18 vs PCS Phenyl-Hexyl) and the organic mobile phase modifier (ACN vs MeOH). The new HALO®PCS Phenyl-Hexyl material also demonstrated improvements in plates and tailing over a large mass range for basic analyte, when compared to a SPP competitor positively charged C18 column and shows favorable performance for the separations of aromatic, basic pharmaceuticals. The advantages in load tolerance were shown to resolve the closely retained impurity of desipramine from the larger imipramine peak. When using low ionic strength mobile phases, the addition of a positively charged ligand to the silica surface can greatly improve basic solute peak shape when compared to non-charged bonded phases.

References

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