# Evaluation of Positively Charged Surface Stationary Phases for Improved Chromatographic Separations of Basic Analytes in Small Molecules and Peptides

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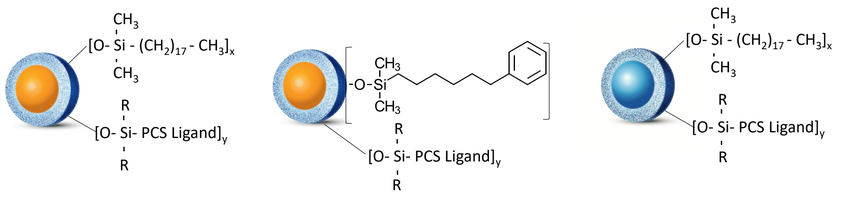
## Introduction

**Problem:** basic compounds become charged at low pH leading to tailed peak shape as sample load is increased under typical low ionic strength reversed-phase LC and LCMS conditions

**Solutions:** To improve peak shape, there are a few options such as adding an ion pair reagent or adding a buffer, but these options are not always 100% compatible with MS detection. Specifically, trifluoroacetic acid (TFA) reduces MS ionization efficiency and phosphate buffer is not MS compatible. Another solution is to use a stationary phase with a positive charged ligand. The HALO® PCS (positive charged surface) product family incorporates a positively charged ligand in addition to a traditional stationary phase on superficially porous silica particles. This stationary phase enables improved peak shape, sample loading, and better impurity analysis.

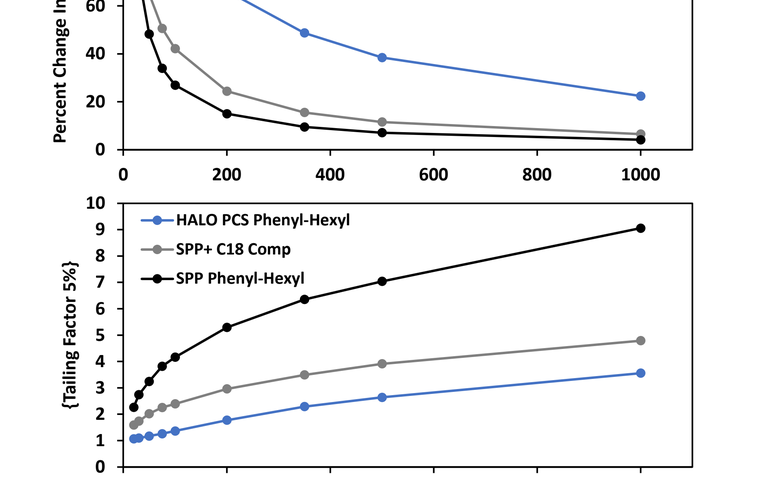
## HALO® PCS Stationary Phases




- 2.7 µm particle size with 0.5 µm thick shell
- 90 Å pore size for small molecules and 160 Å for peptides and tryptic fragments
- Excellent peak shape and increased loading capacity for basic compounds
- 100% aqueous compatible
- UHPLC and LCMS compatible

## Improvements to LC of Basic Molecules

2.1 x 100 mm, A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid; Flow Rate: 0.4 mL/min; Back Pressure: 242 bar; Temperature: 30 °C; Injection: 0.5 µL (31 µg)
Sample Solvent: 75/25 Water/ACN; Wavelength: PDA, 254 nm, LC System: Shimadzu Nexera X2




- Improved tailing factor and efficiency are obtained with HALO® PCS C18 when compared to a traditional uncharged C18 stationary phase for this mix of 4 tricyclic antidepressants

## Improved Sample Loading of Basic Compounds

Test Conditions:
Columns: 2.1 x100 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)
Flow Rate: 0.50 mL/min

MP A: Water/0.1% FA
MP B: ACN/0.1% FA
Injection: 1.0 µL
Temperature: 35 °C
Absorbance: PDA, 280 nm
Instrument: Shimadzu Nexera

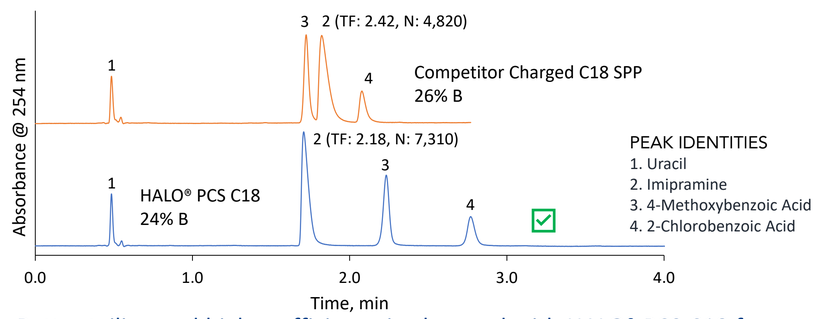



| Loading Results | | | |
|---|---|---|---|
| Column | 20 ng R-propranolol | | |
| | k' | TF 5% | Plates, N |
| HALO PCS Phenyl-Hexyl | 3.18 | 1.06 | 12357 |
| Competitor SPP Charged C18 | 2.99 | 1.59 | 10713 |
| SPP Uncharged Phenyl-Hexyl | 3.27 | 2.26 | 6317 |

- Lowest tailing factor and highest plates found with HALO® PCS Phenyl-Hexyl compared to a charged C18 phase
- Significant improvements are observed when compared to an uncharged Phenyl-Hexyl phase

## HALO® PCS C18 Compared to Competitor Charged C18

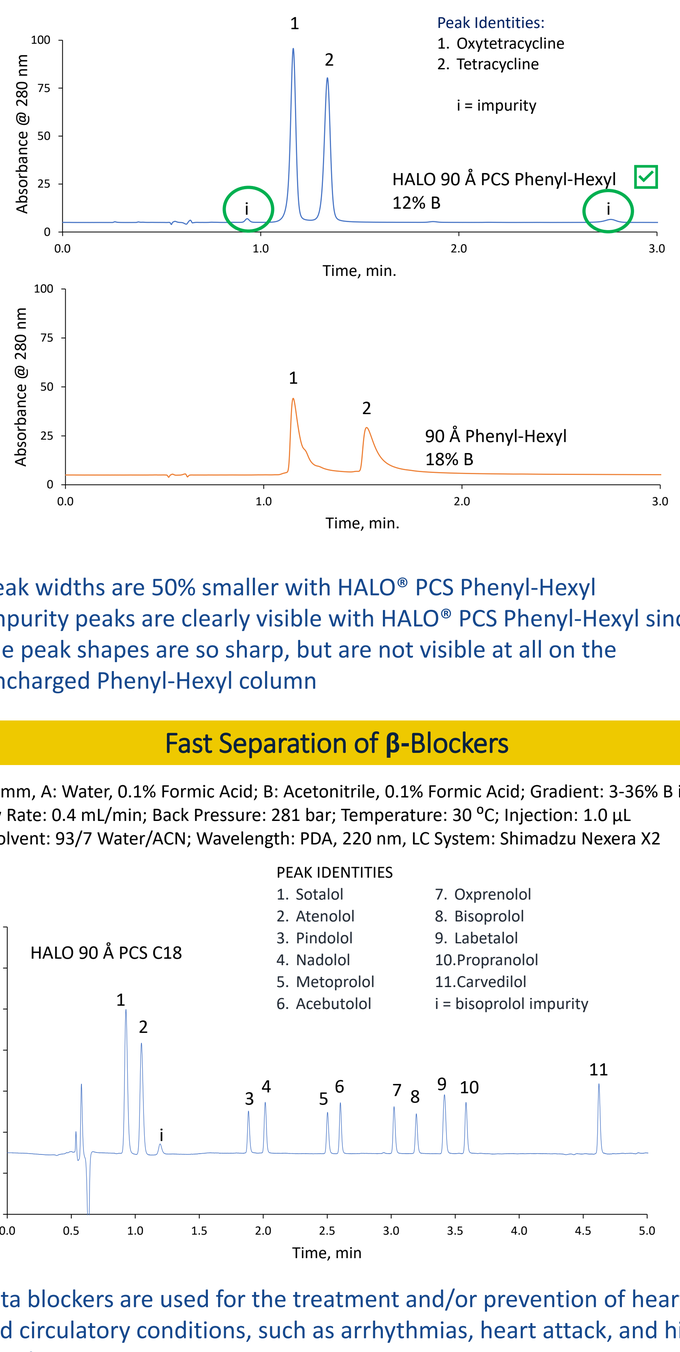
2.1 x 100 mm, A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid; Isocratic as listed; Flow Rate: 0.4 mL/min; Back Pressure: 238 bar; Temperature: 35 °C; Injection: 1.0 µL
Sample Solvent: 70/30 Water/ACN; Wavelength: PDA, 254 nm, LC System: Shimadzu Nexera X2




- Better tailing and higher efficiency is observed with HALO® PCS C18 for the basic compound (peak 2)
- Peaks 3 & 4 (acids) show symmetrical peak shape with HALO ® PCS C18

## Improved Impurity Analysis with HALO® PCS Phenyl-Hexyl

2.1 x 100 mm, A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid; Isocratic as listed; Flow Rate: 0.4 mL/min; Back Pressure: 206 bar; Temperature: 35 °C; Injection: 1.0 µL
Sample Solvent: 90/10 Water/ACN; Wavelength: PDA, 280 nm, LC System: Shimadzu Nexera X2




- Peak widths are 50% smaller with HALO® PCS Phenyl-Hexyl
- Impurity peaks are clearly visible with HALO® PCS Phenyl-Hexyl since the peak shapes are so sharp, but are not visible at all on the uncharged Phenyl-Hexyl column

## Fast Separation of β-Blockers

2.1 x 100 mm, A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid; Gradient: 3-36% B in 5 min; Flow Rate: 0.4 mL/min; Back Pressure: 281 bar; Temperature: 30 °C; Injection: 1.0 µL
Sample Solvent: 93/7 Water/ACN; Wavelength: PDA, 220 nm, LC System: Shimadzu Nexera X2

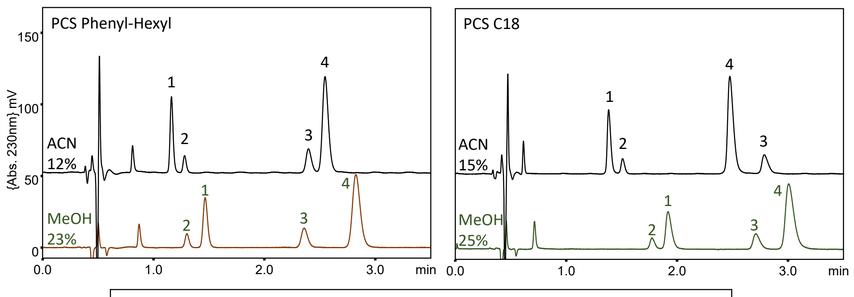



- Beta blockers are used for the treatment and/or prevention of heart and circulatory conditions, such as arrhythmias, heart attack, and high blood pressure
- Eleven different beta blockers are separated in under 5 minutes using a HALO® PCS C18 column with UV detection and a mobile phase that is MS compatible

## Effect of Organic Solvent on HALO® PCS Selectivity

2.1 x 100 mm, A: Water, 0.1% Formic Acid; B: Specified Solvent, 0.1% Formic Acid; Isocratic at specified % B; Flow Rate: 0.5 mL/min; Temperature: 30 °C; Injection: 1.0 µL
Wavelength: PDA, 230 nm, LC System: Shimadzu Nexera X2

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



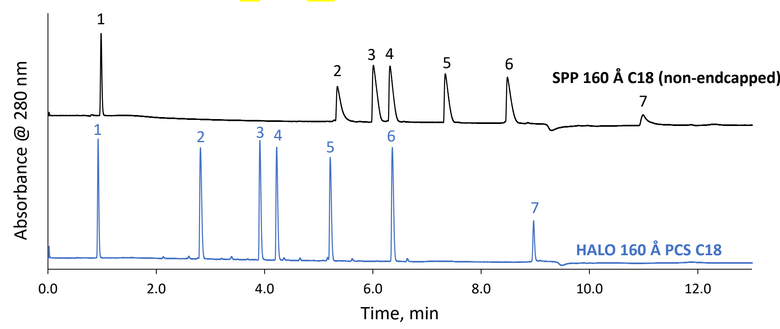

| Acetonitrile Mobile Phase | | | | |
|---|---|---|---|---|
| Bonded Phase | Peaks 1 and 2 | | Peaks 3 and 4 | |
| | Selectivity | Rs | Selectivity | Rs |
| PCS Phenyl-Hexyl | 1.17 | 2.22 | 1.08 | 1.56 |
| PCS C18 | 1.13 | 2.08 | 1.15 | 3.07 |
| **Methanol Mobile Phase** | | | | |
| Bonded Phase | Peaks 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 |

- Changing from acetonitrile to methanol gives elution order changes for both PCS C18 and PCS Phenyl-Hexyl
- Increased selectivity and resolution are observed with PCS Phenyl-Hexyl compared to PCS C18 when run with methanol

## Improvements to LC and LCMS of Peptides

2.1 x 100 mm, A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid; Gradient: 2-35 %B in 10 min.; Flow Rate: 0.3 mL/min; Temperature: 30 °C; Injection: 1.0 µL; Wavelength: PDA, 280 nm

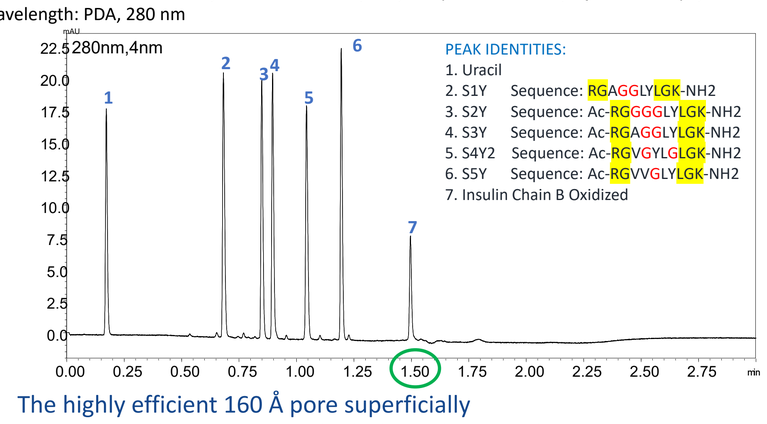
PEAK IDENTITIES:
1. Uracil
2. S1Y Sequence: RGAGGLYLGK-NH2
3. S2Y Sequence: Ac-RGGGGLYLGK-NH2
4. S3Y Sequence: Ac-RGAGGLYLGK-NH2
5. S4Y2 Sequence: Ac-RGVGYLGLGK-NH2
6. S5Y Sequence: Ac-RGVVGLYLGK-NH2
7. Insulin Chain B Oxidized




- Gradient separation of 5 variant synthetic peptides + insulin $B_{ox}$
- Reduced retention time and increased resolution for HALO® PCS C18 Peptide compared to uncharged Peptide C18
- Improved peak widths and reduced tailing in formic acid

## High Speed Peptide Analysis

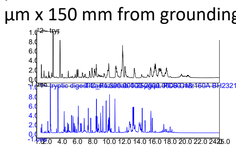
HALO 160 Å PCS C18, 2.1 x 50 mm, A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid; Gradient: 0-35 %B in 1.5 min.; Flow Rate: 1.0 mL/min; Temperature: 30 °C; Injection: 1.0 µL; Wavelength: PDA, 280 nm




- The highly efficient 160 Å pore superficially porous particle permits very high throughput analysis
- The example shows separation conducted in less than 2 minutes, with modest backpressure, even at moderate temperature

## Improved Peak Capacity with HALO® PCS C18 Peptide

A: Water, 0.1% Formic Acid; B: Acetonitrile, 0.1% Formic Acid; Gradient: 3-50 %B in 30 min.; Flow Rate: 0.4 mL/min; Temperature: 60 °C; ; Shimadzu NexeraX2 -> diverter valve -> QExactive HF (res=240,000)
MarvelXACT Post-Column Plumbing:
50 µm x 350 mm from column to diverter valve
50 µm x 350 mm from diverter valve to union
50 µm x 150 mm from grounding union to HESI I



**Peptide C18** 2.7 µm, 2.1 x 150 mm

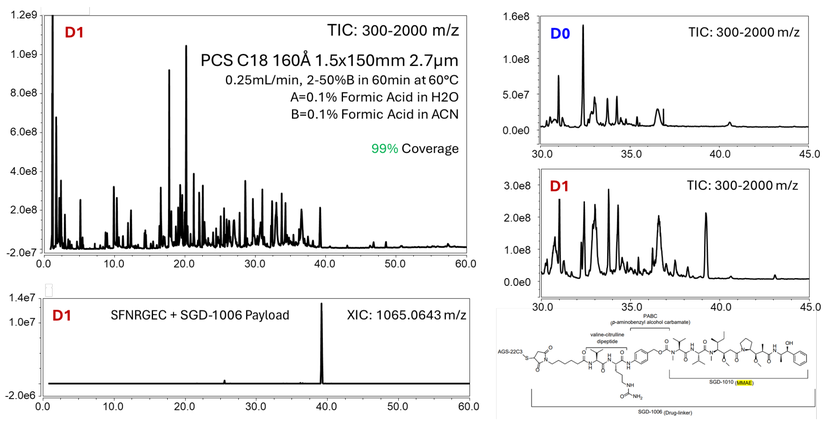
**Peak Capacity = 170**

**HALO® PCS C18 Peptide** 2.7 µm, 2.1 x 150 mm

**Peak Capacity = 488**

- Peak capacities ($n_{PC}$) measured with modest load (2 µg) of trastuzumab tryptic digest on a 2.1 mm ID column
- $n_{PC}$ based on 12 ID peptides measured using extracted ions (XICs) $PW_{1/2}$, $t_R$ and $\Delta t_G$ for this specific sample set
- Decreased peak widths effect notable increase in peak capacity

## Tryptic digest LCMS analyses to identify payload site for isolated DAR2 ADC




- HIC isolated mAb with 2 vedotin-ejfv payload conjugated to enfortumab was digested with trypsin
- A single L-chain cystine site was occupied by the payload, with retention identified in the XIC, verified by MS/MS

## Conclusions

- HALO® PCS phases improve load tolerance in formic acid vs. traditional uncharged stationary phases making PCS phases useful for analysis of less abundant impurities.
- HALO 160 Å PCS C18 exhibits favorable peak shape for peptides in weakly acidic mobile phase thus expanding the choice of mobile phase for effective LC/MS.
- HALO® PCS phases show symmetrical peak shape for basic, neutral, and acidic analytes.
- All HALO® PCS phases exhibit the speed and resolution advantages of Fused-Core® superficially porous particles.