Novel Superficially Porous HILIC Column Packing Material for High Speed Separations of Biological Molecules

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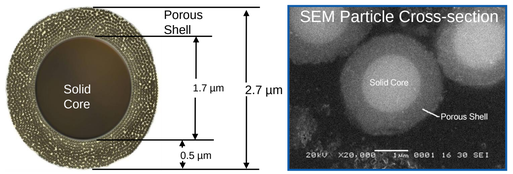
Introduction

During the past few years superficially porous silica particles have emerged as preferred materials for high efficiency and high speed separations in HPLC. Superficially porous (also known as core-shell or Fused-Core®) silica particles can be obtained with a variety of pore sizes, particle sizes, and shell thicknesses. Columns packed with 2.7 µm diameter Fused-Core particles (Halo®) have been shown to exhibit surprising efficiency. Columns packed with Fused-Core bonded phase particles can demonstrate performance superior to smaller-sized porous particles, or at performance equivalence, can be operated at lower column back pressures. High column efficiencies have been observed for the use of bare silica Fused-Core particle columns operated in hydrophilic interaction liquid chromatography separations (HILIC). Our objective has been to develop highly polar bonded-phase surface modifications of Fused-Core silica particles for HILIC separations that maintain the excellent efficiencies previously observed with bare Fused-Core silica, but that exhibit the many potential advantages of covalently-modified surface HILIC packing materials. To this end, a variety of highly polar covalent bonded-phases were applied to Fused-Core silica particles. Novel hydroxylated bonded-phases were synthesized which are observed to be highly hydrophilic, exhibit typical HILIC retention properties, but with notably reduced ionic interactions. We demonstrate applications of such highly polar bonded phases for a variety of HILIC separations of small molecules, synthetic peptides, tryptic digests and small proteins. We compare the retention properties and column efficiencies obtained for these hydroxylated bonded Fused-Core HILIC packing materials using small and larger pore materials. These novel HILIC column packing materials are shown to exhibit advantages for very rapid and high resolution separations of small polar molecules, as well as larger molecules, including polypeptides, glycopeptides and enzymatically-released protein N-glycans.

Superficially Porous Particles (Fused-Core®)

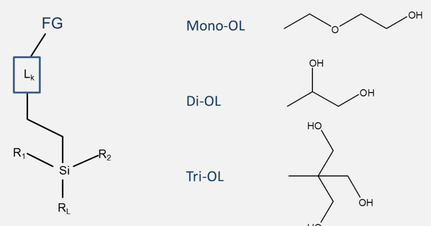


Selection of HILIC Bonded Phases

A range of functional group types were immobilized on Fused-Core silica particles. Based on performance properties, the Penta-HILIC structure has been selected for further development.

- Supports effective HILIC retention
- Exhibits desired kinetic advantages of SPP morphology
- Reduces unfavorable ionic/coloumbic interactions

Hydroxylic Functional Groups



Mono-OL

Di-OL

Tri-OL

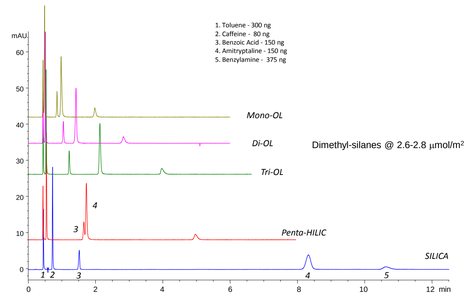
Pentan-OL
AKA
Halo Penta-HILIC

R_L = silanol reactive leaving
$R_1 = R_2$ or $R_1 \neq R_2$, $R_1 = R_L$
Eg. -Me, -OMe, -Cl, -DiiPr
L_k is a proprietary linker

Features of Halo Penta-HILIC

Effect of Surface Functional Groups on a HILIC Separation

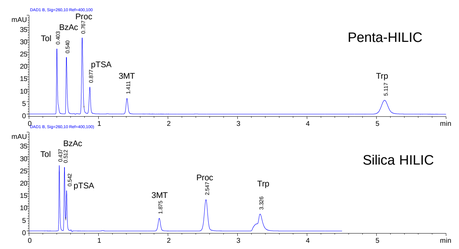
95% Acetonitrile/5 mM NH_4OAc, pH 4.0: 2.1 mm ID x 100 mm, 25 °C, 0.5 mL/min



- The hydroxylic functional group family of bonded-phase surfaces demonstrates increasing retention with chain length (number of hydroxyls).

HILIC Separation of A/B/Z on Silica and Penta-HILIC Silane Surfaces

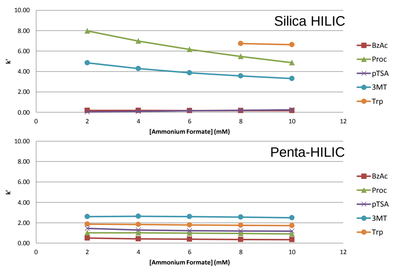
90% Acetonitrile/10 mM NH_4Form, pH 3.0: 2.1 mm ID x 100 mm, 25 °C, 0.5 mL/min



- The Halo Penta-HILIC particle exhibits good peak symmetry with various small molecules that varying in functional groups.

Effect of Buffer Concentration on HILIC Separations

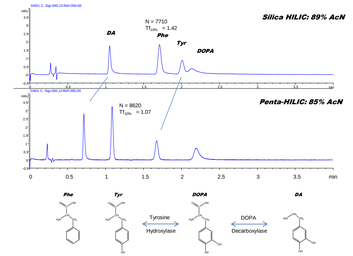
90% Acetonitrile/NH_4Form, pH 3.0: 2.1 mm ID x 100 mm, 25 °C, 0.5 mL/min



- Unlike bare Silica HILIC , the bonded-phase Halo Penta-HILIC surface exhibits little dependence of retention on ionic strength of the mobile phase.

High Speed HILIC Separation of Catecholamines and Amino Acids

4.6 mm ID x 50 mm; 2 mL/-min., 85% AcN/10 mM NH_4Form 3.0, 25 °C; 3 µL inj

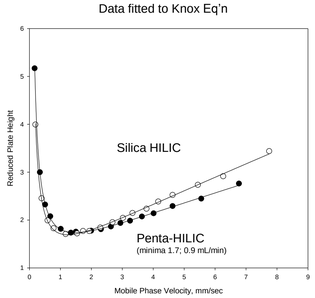


Note: at 85% AcN all k' lower on Silica than on Penta.

- Separations of catecholamines and related amino acids are accomplished at high speed, with good efficiency and peak shape, with modest ionic strength mobile phase modifier.

Effect of Linear Velocity on Penta-HILIC Column Efficiency

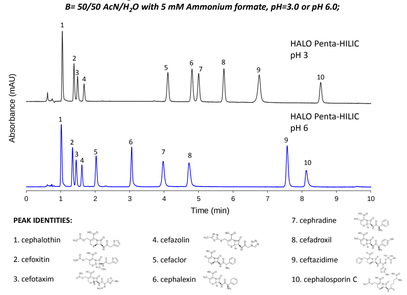
4.6 mm ID x 50 mm; 90% AcN/10 mM NH_4Form 3.0, 25 °C; 1 µL, 50 ng Adenosine



- The desirable mass transfer kinetics of the superficially porous particle are preserved by appropriate selection of HILIC bonded phase characteristics. Mass transfer properties for bare silica and Penta-HILIC are not significantly different with a small molecule probe.

Cephalosporin Analysis on Penta-HILIC

2.1 x 150 mm, 30°C, 0.5 ml/min., UV 254 nm, 85-65% B in 10 min.,
A- 95/5 AcN/H_2O with 5 mM Ammonium formate, pH=3.0 or pH 6.0
B= 50/50 AcN/H_2O with 5 mM Ammonium formate, pH=3.0 or pH 6.0;

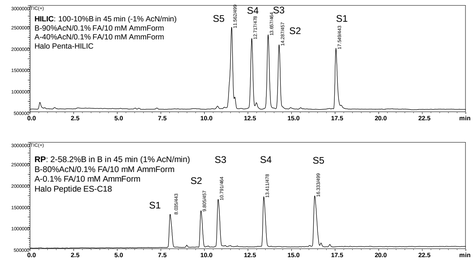


- Separations of this 10 component mixture can be conducted under conditions that manipulate selectivity, while demonstrating excellent peak shape and column efficiency.

Separations of Biomolecules

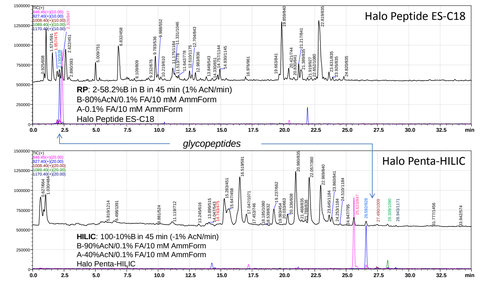
Comparing RP and HILIC Columns for Peptide Separations

2.1 mm ID x 100 mm, 0.35 mL/min, 40 C, MS 2020: SQ TIC (+ 300-2000 m/z) @ 0.35 scan/s
"Alberta" Peptide Mix (Mant and Hodges)



Comparing RP and HILIC Columns for Tryptic Digest Separations

2.1 mm ID x 100 mm, 0.35 mL/min, 40 C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s
20 µg Bovine Ribonuclease B digest (reduced, CAM modified)

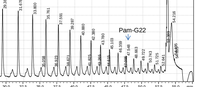


Analysis of PNGase Released and Labeled N-Glycans

Release of protein N-linked glycans using PNGase F releases oligosaccharides with a free reducing terminus (alditol) that is readily labeled by amines via formation of a Schiff's base, which can be reduced readily.

Many amines have been applied to labeling glycans, (Harvey, 2011, J. Chrom. 879).
In the current work Procainamide is favored due to reported improvements in ESI-MS detection (Klapoetke, et al., 2010, J. Pharm. Biomed. Anal. 53)

Typical Labeling Conditions
Glycan in water (up to 10% volume)
90+% volume of:
0.4 M procainamide
1M sodium cyanoborohydride
in 30% acetic acid/70% DMSO
12-16 hr reaction at 37°C
SEC cleanup on Sephadex G-10 minicolumn
Absorbance Detection 300 nm or Fluorescence Ex 330/Em 380 nm



Mass: glycan + 219.32

Penta-HILIC Separations of Labeled Oligosaccharides and Glycans

2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min.
Detection: 300 nm Abs; ESI-MS (MS-2020, (+) 4.2 kV, 400-2000 with SIM)

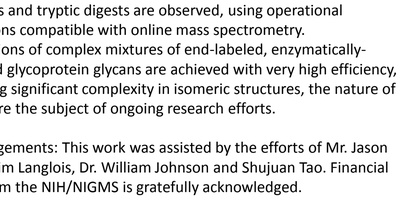
Penta-HILIC Separations of Abundant bov. α1-AGP N-glycans

2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min.

MALDI of Permethylated Glycans

Column Efficiency Comparisons using Pam-G_5

2.1 (2.0 mm) ID x 150 mm, 60°C, k' = 6,
50 mM Ammonium Formate Aqueous, pH 4.4
0.5 uL Injection (50 pmol), Abs. 300 nm



* Pam-G_5 denotes a maltopentaose (5 glucose unit) end-labeled saccharide.

Penta-HILIC Retention of Labeled Oligosaccharides and Glycans

2.1 mm ID x 150 mm; 50 mM Ammonium Formate, pH 4.4, 77.5-56.5% AcN (B) in 52.5 min., 60°C; 600 µL/min.
Detection: 300 nm Abs; ESI-MS (MS-2020, (+) 4.2 kV, 400-2000 with SIM)

* Retention of glycans by HILIC is well described by a 5th order polynomial, consistent with previous literature for an amide bonded phase material.

Conclusions

- A variety of highly polar bonded phase superficially porous silica particles were investigated for operation in HILIC separations.
- The Penta-HILIC material was selected for commercial development, based on high retention, good peak symmetry, limited apparent mixed-mode interactions, and desirable mass transfer properties.
- The Halo Penta-HILIC column packing material demonstrates superior separations at high flow rates for a variety of small molecule types, including acids, bases and zwitterions.
- High performance HILIC separations of mixtures of synthetic peptides and tryptic digests are observed, using operational conditions compatible with online mass spectrometry.
- Separations of complex mixtures of end-labeled, enzymatically-released glycoprotein glycans are achieved with very high efficiency, revealing significant complexity in isomeric structures, the nature of which are the subject of ongoing research efforts.

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