New HILIC Bonded-Phase Fused-Core® Columns Demonstrating Highly Efficient Separations

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Introduction

Recent developments in HPLC instruments and column packing materials are permitting faster separations, particularly for reversed-phase analyses. The recent popularity of sub-2 µm diameter particles and the new development of small diameter superficially porous particles is leading to ever improving analytical separations. The availability of very high performance superficially porous HILIC packing materials has been limited to bare silica materials. Recent studies of Fused-Core Silica HILIC columns have demonstrated high utility for fast and high resolution separations (McCalley, Journal of Chromatography A, 1171 (2007) 46–55), but the use of unmodified silica has limited broader adoption of Fused-Core technology for HILIC separations.

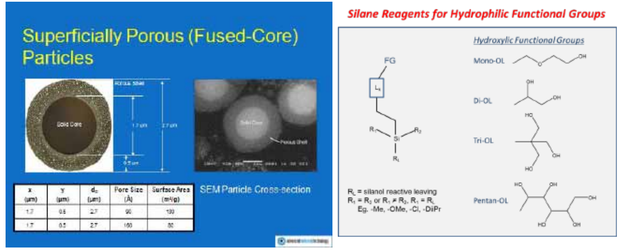
Current understanding of HILIC retention mechanisms, and the factors that impact chromatographic materials performance, remain incomplete compared to other modes of separation. Nevertheless, there is an increasing demand for high performance HILIC separations materials, particularly to address needs in analytical biochemistry, where high utility has been noted for structural analyses of proteins and protein post-translational modifications, as well as for resolution of very complex metabolomic samples. Our goal has been to develop improved HILIC column packing materials for analytical separations, taking advantage of the high performance capabilities of Halo Fused-Core particle technologies pioneered by Advanced Materials Technology. These new materials utilize directed design of stable and robust silica surface chemical modifications. For a number of reasons, both ourselves and others have conjectured that highly hydroxylated organosilane surface structures could well suit the needs for high performance HILIC separations. Our developments have centered on reagents that yield monomeric silane ligands, intended to maintain the superior mass transfer kinetics inherent to superficially porous particles. We describe a new column packing material, Halo Penta-HILIC, that exhibits many desired properties for high performance HILIC separations.

Materials and Methods

Columns of HALO Peptide ES-C18, various prototype modified Fused-Core silica materials, as well as Halo Penta-HILIC were produced at Advanced Materials Technology Inc. (Wilmington, DE). Separations used the quaternary Agilent 1100 or binary 1200 SL LC systems controlled with ChemStation software, or the Shimadzu Nexera UHPLC system. LC/MS for peptide and tryptic digests were conducted using the Nexera system coupled to the LCMS-2020 single quadruploe mass spectrometer, operated in positive ion mode using the ESI interface. Protein digestion was accomplished using Promega Gold modified bovine trypsin, after reduction and carboxyamidomethylation of sulfhydryls. Synthetic peptides were obtained from AnaSpec (Freemont, CA) or from ThermoFisher, using the so-called "Alberta" Retention Standard Mix (Mant and Hodges); thus the S1-S5 sequences are:

S1	RGAGGLGLGK-Am
S2	Ac-RGGGGLGLGK-Am
S3	Ac-RGAGGLGLGK-Am
S4	Ac-RGVGGLGLGK-Am
S5	Ac-RGVVGLGLGK-Am

New Fused-Core Particle Surfaces for HILIC Separations



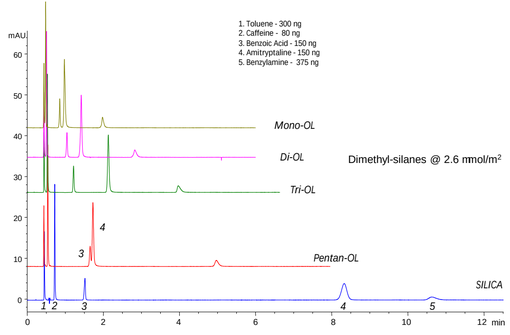
x (µm)	y (µm)	d_p (µm)	Pore Size (Å)	Surface Area (m²/g)
1.7	0.5	2.7	90	150
1.7	0.5	2.7	160	90

- The well defined Fused-Core superficially porous particles offer proven advantages for efficient LC separations. Over 6 years of manufacturing have proven the basic technology.

- To broaden applications beyond Halo Silica HILIC separations, unique new chemistry for highly polar surface modifications has been created for Fused-Core Halo. Examples of highly hydroxylated ligands are above, and example HILIC separations below.

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

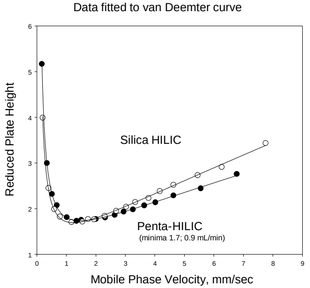


Characteristics of Halo Penta-HILIC

- The 5-carbon hydroxylated ligand (aka Penta-HILIC) has shown the desired combination of high HILIC retention, mass transfer properties indistinguishable from bare silica, very low ionic interactions, as well as stability under both low and moderately elevated pH (data not shown). Zwitterions exhibit excellent peak shape.

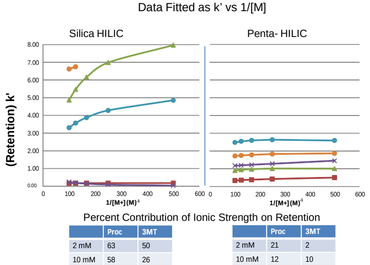
Effect of Linear Velocity on Column Efficiency

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



Contribution of Ionic Strength on HILIC Separations

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



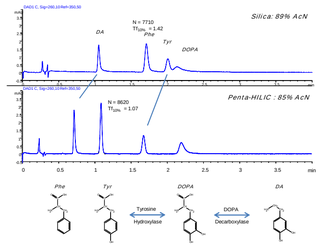
Percent Contribution of Ionic Strength on Retention

	Proc	3MT
2 mM	63	50
10 mM	58	26

	Proc	3MT
2 mM	21	2
10 mM	12	10

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



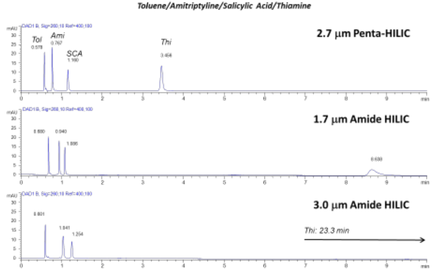
Note: at 85% ACN all k' lower on Silica than on Pentanol.

Comparative Retention of HILIC Columns

2.1 mm ID x 150 mm

Test: 90% ACN/10 mM NH_4Form pH 3.0, 0.5 mL/min, 23 °C

Toluene/Amitriptyline/Salicylic Acid/Thiamine

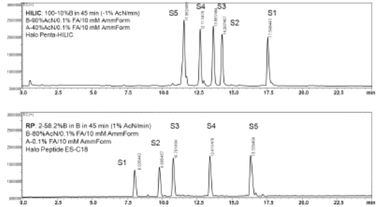


Biomolecule Separations using Halo Penta-HILIC

- The Penta-HILIC column material demonstrates high performance for separations of peptides and glycopeptides using MS-friendly mobile phases, as well as with TFA and phosphoric acid modified mobile phases. Peak shape and column efficiency are comparable to reversed-phase separations. Similar comparisons of retention on Halo Peptide ES-C18 and the Penta-HILIC column were completed for more than 65 synthetic and tryptic digest peptides, supporting the conclusion that these modes of separation are highly orthogonal.

Performance of RP and HILIC Columns for Peptide Separations

2.1 mm ID x 100 mm, 0.35 mL/min, 40 C, MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s

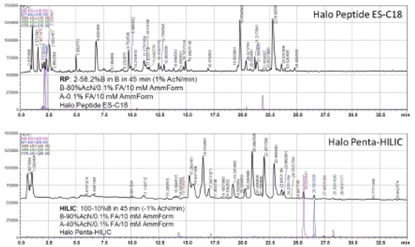


- Both glycopeptides and enzymatically-released N-glycans are strongly retained on Penta-HILIC.

Penta-HILIC Strongly Retains Glycopeptides

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 (CAM)



Conclusions and Future Directions

- The densely bonded hydroxylated ligand exhibits HILIC retention of small molecules insensitive to ionic strength, not exhibiting typical silanol anionic character
- Zwitterions exhibit excellent peak shape, particularly compared to bare silica HILIC
- Column stability is high, operation is at modest back pressures
- Column efficiency is as good, and sometimes better than, RP superficially porous materials
- Retention and performance is comparable or better than, amide-type bonded phases
- Fast separations of peptides, glycopeptides and glycans can be conducted.

Acknowledgements

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