Superficially-Porous Fused-Core® Particles for the Rapid Separation of Polypeptides and Small Proteins

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Introduction

Studies of columns packed with fused-core® 2.7-µm silica particles with 90 Å pores previously have been shown to be highly efficient for separating small molecules in the range of up to about 5000 molecular weight (MW). Columns of such particles demonstrate unusually high efficiencies that are comparable to those for sub-2-µm totally porous particles, but with about one-half of the operating back pressure. This presentation will present new data on the effects on column performance of reducing the shell thickness and increasing the pore size of fused-core particles, especially for higher molecular weight solutes. Rapid and high-resolution separations of polypeptide and small protein mixtures of up to about 20,000 Dalton will be shown. Separations of these larger molecules typically can be performed faster using columns packed with superficially porous particles because of the superior mass transfer properties of core-shell particles at higher mobile phase velocities. Gradient elution peak capacity studies for columns of the new fused-core particles demonstrate the high-resolution utility obtained with superficially porous particles, without disadvantages previously seen with non-porous particles. The fused-core particles show good sample loading properties, since the effective surface area per column is nearly equivalent to that in columns packed with comparable totally porous particles. Optimal bonded-phase selection and surface modification approaches lead to exceptional retention and peak shape properties for peptides with mass-spec-friendly mobile phases.

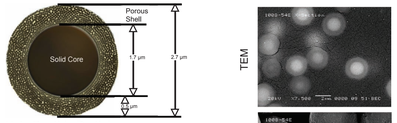
Materials and Methods

Columns of HALO® C18 or HALO Peptide ES-C18 were produced at Advanced Materials Technology Inc. (Wilmington, DE). HPLC analyses used the quarternary Agilent 1100, binary 1200 SL or capillary 1100 LC systems controlled with ChemStation software. The capillary LC was connected to the ThermoFisher LTQ ion-trap mass spectrometer via the Michrom Bioresource Advance spray source. Samples from the autoinjector were captured on the EXP Stem Trap (2.6 µL) cartridge packed with Halo Peptide ES-C18 (Optimize Technologies), using the LTQ automated valve.

Synthetic peptides were obtained from AnaSpec (Freemont, CA) or from ThermoFisher, in the case of the Retention Standard Mix (Mant and Hodges), the S1-S5 sequences are:

S1	RG**AG**GLGLGK-Am
S2	Ac-RG**GG**GLGLGK-Am
S3	Ac-RG**AG**GLGLGK-Am
S4	Ac-RG**VG**GLGLGK-Am
S5	Ac-RG**VV**GLGLGK-Am

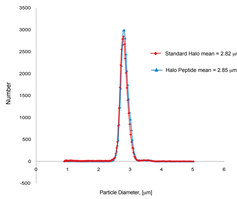
Fused-Core Column Packing Materials



Halo Peptide Particles

Silica ..... High Purity Type B
Ave. pore diameter..... 16 nm
Surface area, nitrogen.....80 sq.m/g
Pore volume ..... 0.30 mL/g
Particle density .....1.3 cc/g

Halo Particle Distribution

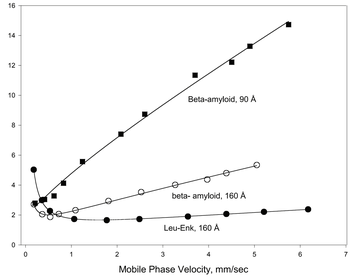


Efficiency Increases with HALO Peptide ES-C18

- A short diffusion path in the particle, combined with very narrow particle distributions, yields efficient separations at high flow rates. Halo Peptide extends use to larger analytes.

Comparison of Halo and Halo Peptide Columns

Columns: 4.6 x 100 mm; Particle size 2.7 µm
Mobile Phase: 0.1% TFA/Acetonitrile/Water; Acetonitrile content adjusted to maintain k' ~ 3; 60°C;
Agilent 1100 with autosampler

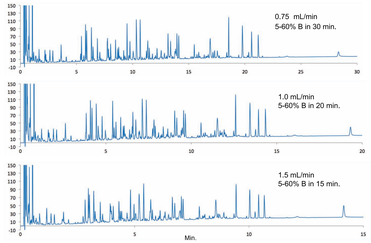


Tryptic Digest Separations Using Halo Peptide ES-C18

- Efficient columns and high mass transfer allows high flow rates for rapid separations without loss of resolution.

Column: 2.1 x 100 mm Halo Peptide ES-C18;
A: Water/ 0.1% TFA,
B: 80% ACN / 0.1% TFA.,

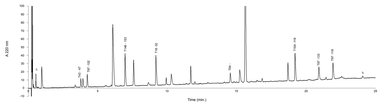
Detection: 215 nm,
Sample: apotransferrin tryptic digest,
Injection of 15 µL (15 µg),
Temp: 60 °C



Peak Capacity Comparisons for Digest Separations

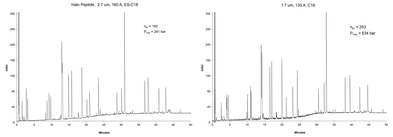
- Comparable peak capacity of Halo Peptide ES-C18, at less than ½ back pressure of sub-2 µm particle columns.

Column: 4.6 x 50 mm; Flow rate: 2.4 mL/min; T= 60°C;
A: Water/ 0.1% TFA; B: 80% ACN/0.1 % TFA
Gradient: 5% to 60% B in 30 min.; Injection: 5 µL (5 µg)



- Peak capacity was determined to be 229 calculated by averaging the peak widths of the labeled tryptic digest peaks using the expression:

Column: 2.1 x 100 mm; Flow rate: 0.5 mL/min; T= 45°C;
A: Water/ 0.1% TFA;B: 80% ACN/0.1 % TFA
Gradient: 5% to 65% B in 60 min.; Injection: 15 µL (15 µg)



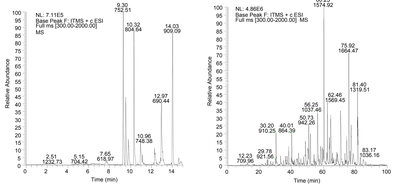
High Linear Velocity LC/MS Analyses of Digests

- Efficient separations allow selection of conditions matching sample complexity (long or short columns and gradients).

A: 0.1% Formic Acid/Water;B: ACN/0.1% Formic Acid

Flow Rate 9 uL/min; 0.2 mm ID x 50 mm Halo Peptide ES-C18, 330 Bar Max Pressure; 2-45% B in 15 minutes, 3 pmol apoMyoglobin Digest

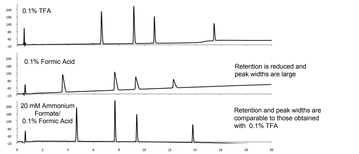
Flow Rate 4 uL/min; 0.2 mm ID x 150 mm Halo Peptide ES-C18, 320 Bar Max Pressure; 2-45% B in 85 minutes, 1.5 µg Mixed Protein Digest



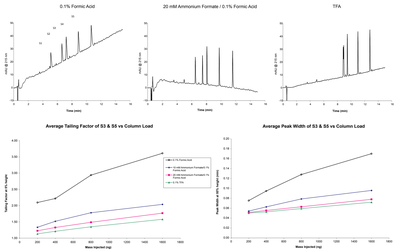
Mobile Phase Modifiers for LC/MS Analyses

- TFA and Formic acids have disadvantages for LC/MS. A mix of Ammonium Formate/Formic Acid is an alternative mobile phase.

Column: Halo Peptide ES-C18, 4.6 x 100 mm; Flow rate: 2.0 mL/min;
T=30°C; A: Water/acid modifier; B: ACN/0.1% TFA or Formic Acid;
Gradient: 5% to 40% B in 20 min.; Injection: 32 µL (3200 ng) of
Angiotensin 1-7 amide, Met-Enk, Leu-Enk, and Angiotensin (1-12) human



Column: Halo Peptide ES-C18, 4.6 x 100 mm; Flow rate: 2.0 mL/min;
T= 30°C; A: Water/acid modifier; B: ACN/0.1% TFA or Formic Acid
Gradient: 1.5% to 26% B in 15 min.; Injection: 8 µL (800 ng) of
synthetic peptides S1-S5



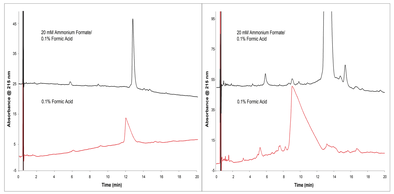
Enhanced Detection of Trace Impurities with Ammonium Formate/Formic Acid Mobile Phase

- Detection of trace impurities is enhanced with Ammonium Formate/Formic Acid mobile phase.

Column: Halo Peptide ES-C18, 2.1 x 100 mm; Flow rate: 0.5 mL/min;
T= 60°C; A: Water/acid modifier; B: ACN/0.1% Formic Acid
Gradient: 20 mM Ammonium Formate/0.1% Formic Acid: 24% to 27%
B in 20 min.; 0.1% Formic Acid: 20% to 24% B in 20 min.

1 µg of β-amyloid (1-38) injected

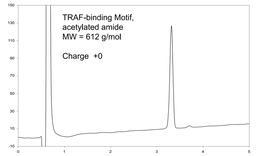
Column overloaded to show impurities
10 µg of β-amyloid (1-38) injected

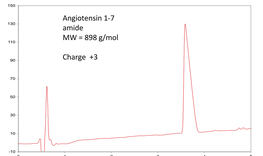


Peptide Peak Shape in Formic Acid Containing Mobile Phases

- A peptide with basic amino acid residues and a free amino group at the N-terminus has broad peak shape and increased tailing at high sample loads compared to a peptide without basic residues and with an acetylated N-terminus.

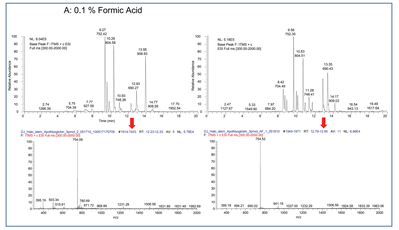
Column: Halo Peptide ES-C18, 4.6 x 100 mm; Flow rate: 2.0 mL/min;
T= 30°C; A: Water/formic acid B: ACN/0.1% Formic Acid; Gradient: 5% to 40% B in 20 min.; Injection: 32 µL (3200 ng) of TRAF-binding motif or
32 µL (3200 ng) of Angiotensin 1-7 amide.





Ammonium Formate as an Additive for LC/MS Analyses

Halo Peptide ES-C18, 0.2 mm ID x 50 mm, Flow Rate 9 µL/min.,
2-45% B in 15 minutes, 3 pmol apoMyoglobin digest in 2 µL;
B: 0.1% Formic acid in Acetonitrile



Comparing Formic Acid PLUS Ammonium Formate versus Formic Acid for LC/MS analyses of synthetic peptides and tryptic digests reveals:

- Concentration dependent retention increase, selectivity shifts, and improvement of peak shape for many peptides
- Improved sample mass load tolerance at 10 or 20 mM ammonium formate
- IT-MS signal differences are limited, but for a small percentage of peptides (c. 15%) up to 10-fold differences in either direction of relative signal strength are observed (+/- AmmForm). Analysis of peak intensities and areas in SIM for >30 tryptic and synthetic peptides has not revealed specific composition or structural features to explain ionization differences for the 5 peptides that show >2-fold shifts
- 10 mM ammonium formate is a good compromise for improved LC performance, while reducing the ionization differences with formic acid alone
- Systematic analysis of peak shapes for digest complex mixtures is ongoing. Initial indications support better peak capacities with the ammonium formate/formic acid mixture, driven by overall lower peak tailing and reduction in peak widths.

Conclusions and Future Directions

- A new fused-core column packing material, Halo Peptide ES-C18, was observed to perform well at high flow rates for separations of peptides and tryptic digests.
- Modest back pressures permit high throughput separations at very high linear velocity, or the use of longer capillary columns at more moderate flow rates.
- Ammonium formate addition to formic acid eluent was compatible with ion trap LC-MS operation, improving load limitations for complex samples.
- Additional work will examine the practical benefits of this additive for proteomic samples, and also the potential problems that could arise with identifying post translational modifications.