Rapid High-Resolution Reversed-Phase LC-MS Analyses of Peptides and Tryptic Digests using New Fused-Core Particle Columns

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Objectives

- Evaluate utility of a novel fused-core column packing material for separation of peptides and tryptic digests.
- Demonstrate the capability of these new columns to conduct rapid separations of synthetic peptides and digests.
- Define the operational conditions for high thoughput use of the columns with broadly available LC/MS instruments.
- Explore the use of ammonium formate as an additive to formic acid mobile phase commonly used for LC-MS analysis of tryptic digests..

Introduction

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 2000 molecular weight. Several recent studies have noted that columns of such particles demonstrate efficiencies that are comparable to those for sub-2 µm totally porous particles, but with less than one-half of the operating back pressure. Separations of peptides and protein digests should benefit from the Fused-Core particles of 16 nm (160 Å) pore size shown in this poster. Our analyses support this pore size to be optimal to promote rapid mass transfer kinetics for peptides and small proteins, allowing fast separations (up to 8-10 mm/s) with minimal resolution loss.

The Halo Peptide ES-C18 column is prepared using fused core particles that are surface modified with an extremely stable steric-protected C18 bonded phase, appropriate for the low pH conditions preferred for peptide separations. We show, consistent with many previous observations, that TFA is a most effective acid modifier for separations, and that formic acid exhibits significant band broadening when used with the Halo Peptide ES-C18 columns; this is nearly universally observed for high performance column packing materials of use for peptide separations. Ammonium formate is an effective modifier to reduce peptide band broadening, but the compatibility of this additive with LC-MS has not been fully explored for proteomic applications.

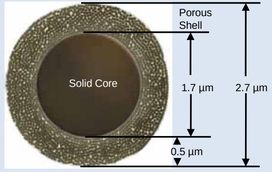
Materials and Methods

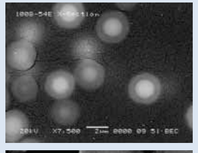
Columns of HALO® C18 or HALO Peptide ES-C18 were produced at Advanced Materials Technology Inc. (Wilmington, DE). The 1.7 µm particle diameter 130 Å pore size BEH C18 column was obtained from Waters (Milford, MA). HPLC analyses used the quarternary Agilent 1100, binary 1200 SL or capillary 1100 LC systems 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.

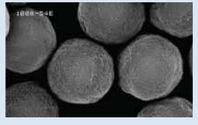
Synthetic peptides were obtained from AnaSpec (Freemont, CA) or from ThermoFisher, in the case of the Retention Standard Mix (Mant and Hodges), the S1-S5 sequences are:

S1	RGAGGLGLGLGK-Am
S2	Ac-RGGGGLGLGLGK-Am
S3	Ac-RGAGGLGLGLGK-Am
S4	Ac-RGVGGLGLGLGK-Am
S5	Ac-RGVVGLGLGK-Am

Fused-Core Column Packing Materials







Halo Peptide Particles

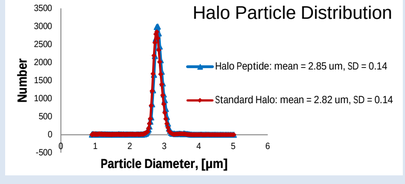
Silica ----------High Purity Type B

Ave. pore diameter----------16 nm

Surface area, nitrogen----------80 sq.m/g

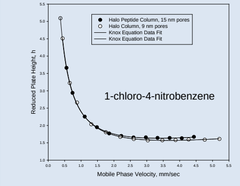
Pore volume ----------0.30 mL/g

Particle density ----------1.3 cc/g

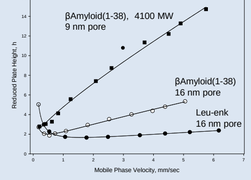


- Short diffusion path in the particle, combined with very narrow particle distributions yield efficient separations at high flow rates. Halo Peptide extends use to larger analytes.

Comparison of Halo and Halo Peptide Columns
Columns: 4.6 x 100 mm; Particle size: 2.7 mm
Mobile phase: 50% acetonitrile/50% water; 25 °C
Agilent 1100 with autosampler



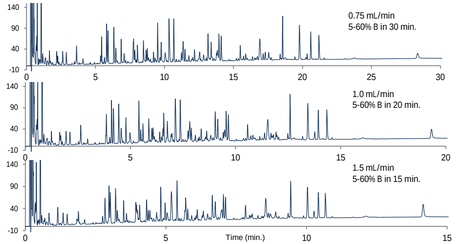
Comparison of Halo and Halo Peptide Columns
Columns: 4.6 x 100 mm; Particle size 2.7 µm
Mobile Phase: 0.1% TFA/Acetonitrile/Water;
Acetonitrile content adjusted to maintain k' ~ 3; 60 C
Agilent 1100 with autosampler



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.

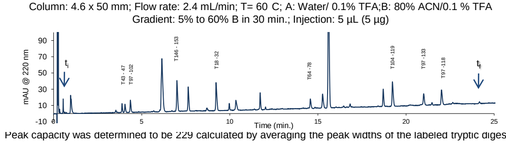
Column: 2.1 x 100 mm Halo Peptide ES-C18; A: Water/ 0.1% TFA, B: 80% ACN / 0.1% TFA.,
Detection: 215 nm, Sample: apotransferrin tryptic digest, Injection of 15 uL (15 µg), Temp: 60 C



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)

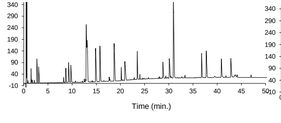


Peak capacity was determined to be 229 calculated by averaging the peak widths of the labeled tryptic digest peaks using the expression:

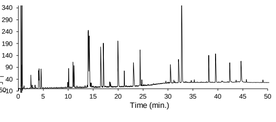
$$n_{pc} = \frac{t_f - t_i}{W_{4s}}$$

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)

Halo, 2.7 µm, 160 Å, ES-C18 n_{pc} = 216 P_{max} = 240 bar



Porous 1.7 µm, 130 Å, C18 n_{pc} = 253 P_{max} = 534 bar

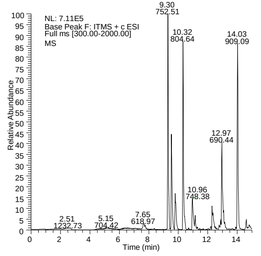


High Linear Velocity LC/MS Analyses of Digests

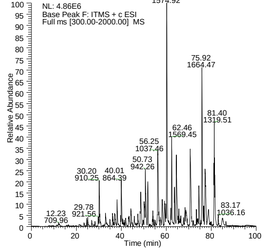
- Efficient separations allow selection of conditions matching sample complexity (long or short columns and gradients).

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



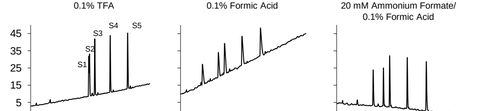
Flow Rate 4 uL/min; 0.2 mm ID x 150 mm Halo Peptide ES-C18, 320 Bar Max Pressure; 2-45% B in 85 minutes, 1.5 µg Mixed Protein Digest

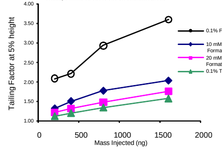


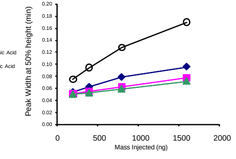
Mobile Phase Modifiers for LC/MS Analyses

- 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: 1.5% to 26% B in 15 min.; Injection: 8 µL (800 ng) of synthetic peptides S1-S5

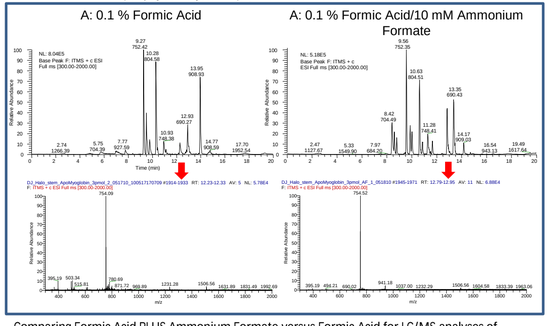






Ammonium Formate as an Additive for LC/MS Analyses

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.