Rapid Separations of Polypeptides

B.E. Boyes, S.A. Schuster, B.M. Wagner, and J.J. Kirkland
Advanced Materials Technology, 3521 Silverside Road, Suite 1-K, Wilmington, DE 19810 USA

OBJECTIVE

The objective of this work is to demonstrate the fast separations of polypeptides that are possible using new Fused-Core® silica particles with 160 Å pores and a highly stable C18 bonded phase.

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 (M.W.). 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. Guiochon and colleagues have also noted that such columns can be usefully applied to peptide separations, including moderately complex samples of protein tryptic digest fragments.[1,2] The available Fused-Core particles have been designed specifically to meet the needs of small molecule separations, with a pore size optimally centered at 90 Å. Separations of peptide and polypeptide mixtures should benefit from Fused-Core particles of larger pore size, which may exhibit improved mass transfer characteristics, as reflected by decreased resistance to diffusion of larger molecules. A variety of materials have been prepared, and various properties of performance for separations of biological molecules have been investigated. We have observed that for such Fused-Core column packing materials, a mean pore size of about 160 Å (BET) to be a good compromise for separation of complex samples (peak capacities), reduced resistance to mass transfer for larger molecules (small proteins), and acceptable load tolerance for analytical applications. Highly stable bonded phases are shown to be impervious to operation at elevated temperatures (> 60 °C) with low pH mobile phases usually required for separating peptides and for conducting LC-MS analyses.

As shown in this presentation, separations of peptides and polypeptides are improved for the larger pore sized Fused-Core material, exhibiting increased retention, decreased band widths, and for complex samples, overall improvement in gradient elution peak capacity for protein tryptic digests. Comparative analysis of these new materials with totally porous 3 µm particle size wide pore materials (300 Å), as well as mid-pore sized (130 Å) sub-two micron materials demonstrate highly efficient separations, while permitting high through-put operation (low back pressures, allowing high flow rates), or very high resolution (extended column lengths for maximizing peak capacity). The desirable properties of these new Fused-Core particles and new bonded-phase chemistry are shown for separations of complex peptide samples.

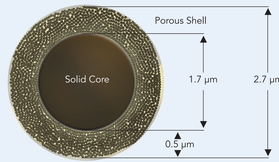
1.J. Chromatogr. A 1176 (2007) 206-216., 2.J. Chromatogr. A 1216 (2009) 2511-2518.

MATERIALS AND METHODS

Columns of HALO® C18 or HALO Peptide ES-C18 were obtained at Advanced Materials Technology Inc. (Wilmington, DE). The 3 µm particle diameter, 300 Å pore size Ace C18 column was obtained from Mac-Mod Analytical Inc. (Chadds Ford, PA), whereas the 1.7 µm particle diameter 130 Å pore size BEH C18 column was obtained from Waters (Milford, MA). Peptide and protein samples were obtained from AnaSpec (Freemont, CA) or Sigma Chemical (St. Louis, MO). Human aportransferrin tryptic digest was prepared by denaturation at 7.5 mg/mL in 6 M Urea/500 mM Tris-HCl, pH 7.6, alkylated with iodoacetamide, then diluted 10-fold into 100 mM ammonium bicarbonate, pH 8, for digestion with modified trypsin at 1/30 enzyme to protein by weight (Promega, Madison, WI). Equine myoglobin was rendered free of the haem group by acid-acetone precipitation, followed by dialysis against 10 mM ammonium bicarbonate. The resulting apomyoglobin was denatured, diluted and digested with modified trypsin at a 1/40 enzyme to protein ratio.

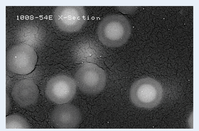
Digestions were terminated by adjusting the mixtures to 1% with respect to acetic acid, followed by storage at -25 °C. HPLC analyses were conducted using the Agilent 1100 LC, for 4.6 mm ID columns, or the Agilent 1200 LC for the 2.1 mm ID columns, in either case with data acquisition and instrument control using version 4.01 Chemstation Software. All analyses of tryptic digests employed the 2.1 mm ID column format. Mobile phases used Pierce trifluoroacetic acid, and acetonitrile from EMD. Tryptic digest fragments were identified in eluates off column by fraction collection, drying in the Savant rotary vacuum system, dissolved in 0.1% TFA in water then were infused into the Thermo Fisher LTQ FT ion-trap mass spectrometer (University of Georgia, CCRC, Dr. Ron Orlando). The identities of tryptic fragments on the accompanying chromatogram refer to sequences of the Asn 104 variant of MYG_HORSE (P68082).

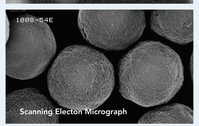
SUPERFICIALLY POROUS PARTICLE



HALO Peptide Particles

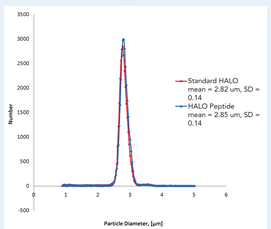
Silica	High Purity Type B
Ave. pore diameter	**16 nm**
Surface area, nitrogen	**80 sq.m/g**
Pore volume	0.30 mL/g
Particle density	1.3 cc/g





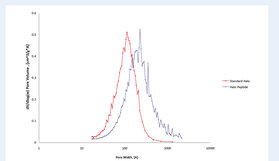
CHARACTERISTICS OF HALO® PEPTIDE ES-C18 PARTICLES

Standard HALO vs. HALO Peptide



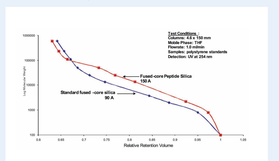
Particle sizes and distribution data were collected on a Multisizer 3 Coulter Counter (Beckman Coulter, Fullerton, CA) using a standard procedure. The results show that the particle size and distribution of HALO Peptide is very similar, if not identical, to Standard HALO. The extremely narrow particle size distribution of both Standard HALO and HALO Peptide have standard deviations that are 5% of the mean.

Pore Size Distribution



- The pore size distribution of the new particles centers at 160 Å, enabling efficient separations of peptides and peptide tryptic digest fragments.

Size-Exclusion Comparisons of Fused-Core Silicas

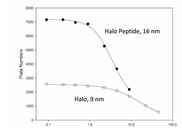


- SEC analysis confirms interpretation of pore size analysis above.

CHROMATOGRAPHIC PERFORMANCE OF HALO PEPTIDE ES-C18

Effect of Pore Size on Sample Loading

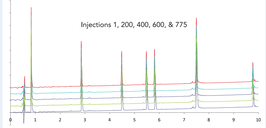
Column: 4.6 x 100 mm Halo Peptide ES-C18; T= 60 °C
Sample: Bovine insulin, MW= 5733; Inj vol = 10 µL
Mobile Phase: 31.5% ACN/68.5% Water/0.1% TFA, Flow Rate: 0.5 mL/min



- Useful sample loading is observed up to at least 1 µg under isocratic test conditions.

Stability at Low pH and High Temperature

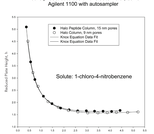
Column: 2.1 x 100 mm; Flow rate: 0.5 mL/min; Temperature: 60° C
A: Water/ 0.1% TFA;B: 70% ACN/30% Water/0.1 % TFA
Gradient: 9% to 55% B in 10 min.; Injection volume: 5 µL; Sample 1



- The steric-protected octadecyldiisobutylsilane bonded phase is stable when used with TFA-containing mobile phases and can withstand temperatures up to at least 90 °C.

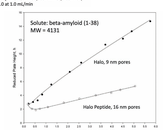
Effect of Pore Size on Efficiency

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



Beta-amyloid van Deemter plot

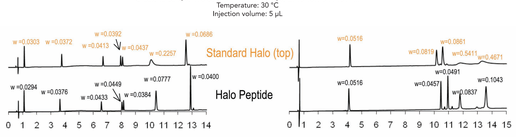
Columns: 4.6 x 100 mm; **Particle size:** 2.7 µm
Standard Halo: 27% ACN/73 % water /0.1% TFA; h = 3.0 at 1.0 mL/min
Halo Peptide: 29% ACN/71% water /0.1% TFA; h = 3.0 at 1.0 mL/min
Temperature 60°C
Instrument: Agilent 1100 with autosampler using I heat exchangers



- The 160 Å pores of HALO Peptide ES-C18 allow increased mass transfer for peptides while maintaining high efficiency for small molecules.

Effect of Pore Size on Peptide and Protein Retention and Peak Width

Columns: 4.6 x 100 mm
Flow rate: 1.5 mL/min
Mobile Phase: A: 10% ACN/90% Water/0.1% TFA B: 70% ACN/30% Water/0.1% TFA
Gradient: Sample 1: 0-50% B in 15 min. Sample 2: 15-15% B in 15 min.
Temperature: 30 °C
Injection volume: 5 µL



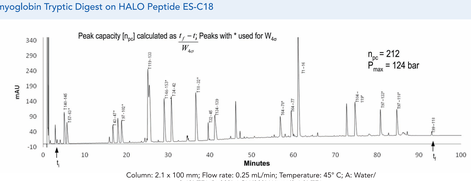
Sample 1
Gly-Tyr
Val-Tyr-Val
Met Enkephalin
Angiotensin II
Leu Enkephalin
Ribonuclease
Porcine Insulin

Sample 2
Leu Enkephalin
Bovine Insulin
Human Insulin
Cytochrome C
Lysozyme

- For peptides > 5 aa residues, retention is increased on the HALO Peptide ES-C18 column.
- For larger molecules, peak widths are smaller due to improved mass transfer.

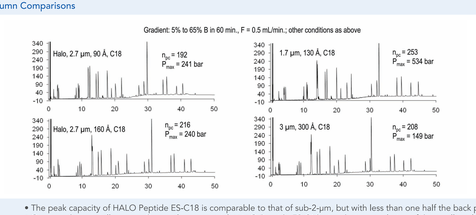
APPLICATION TO PEPTIDE SEPARATIONS

Apomyoglobin Tryptic Digest on HALO Peptide ES-C18



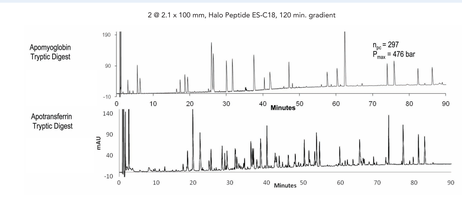
Column: 2.1 x 100 mm; Flow rate: 0.25 mL/min; Temperature: 45° C; A: Water/ 0.1% TFA;B: 80% ACN/20% Water/0.1 % TFA
Gradient: 5% to 65% B in 120 min.; Injection volume: 15 µL [15 µg]

Column Comparisons



- The peak capacity of HALO Peptide ES-C18 is comparable to that of sub-2-µm, but with less than one half the back pressure.
- Compared to a totally porous 3 µm column material, HALO Peptide ES-C18 gives better resolution of the small, early eluting tryptic digest fragments, and high flow rate tolerance.

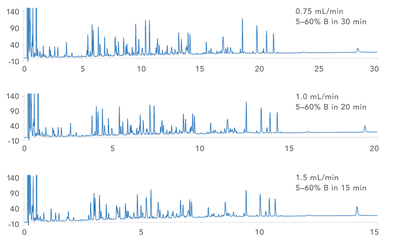
High Resolution Separations Using Longer Columns



- For very high resolution, low back pressure permits the use of longer columns.

High Speed Tryptic Digest Separations

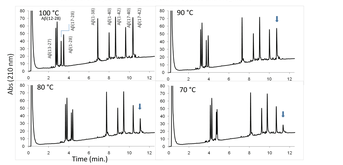
Column: HALO Peptide ES-C18, 2.1 x 100 mm
Mobile Phase: A: water/0.1% TFA; B: 80% ACN/0.1% TFA; gradient as indicated
Temperature: 60 °C
Detection: 215 nm
Injection Volume: 15 µL
Sample: apotransferrin tryptic digest



- The short diffusion path of Fused-Core particles allows the use of high mobile phase velocities for fast separations, without sacrificing resolution.

Rapid Separation at High Temperature

Column: 2.1 x 50 mm Halo Peptide ES-C18; Flow: 0.5 mL/min;
A: 0.1% TFA; B: 0.1% TFA/80% AcN; Gradient: 15-50% B in 12.5 min.;
Sample: 5 µL (250-500 ng) Amyloid β Peptides and Fragments



- Efficient separation of a difficult sample accomplished in less than 15 minutes.
- Note requirement for high temperature to achieve full recovery of Aβ(17-42).

CONCLUSIONS

A new material for highly efficient peptide and polypeptide separations has been described, the HALO Peptide ES-C18 column. We observe that this material provides improved performance for separations of simple and complex mixtures of peptides and small proteins. Relative to the 90 Å pore size HALO C18 column, the 160 Å pore size material shows lower resistance to diffusion into the pores for larger molecules, as reflected by increased column efficiency at both high and low loads, and improved peak capacities for complex mixtures of the peptides and glycopeptides derived from proteolytic digests. This improvement in material properties (pore size) did not result in compromise in the desired particle size distribution, which is indistinguishable from the smaller pore material, nor is there an increase in column resistance to flow (back pressure) or loss of column physical or chemical stability. In a manner similar to the beneficial features enjoyed for the smaller pore HALO columns, highly efficient and robust separations can be obtained with very modest back pressures for columns of HALO Peptide ES-C18, permitting the use of longer columns or higher flow rates, depending on whether high through-put or ultimate resolution are the limiting factors for a specific application.