Development of Column Packing Materials and Methods for Proteomics Applications Using LC/MS Detection

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Objectives

- Evaluate utility of a novel mid-pore size (160 Å) 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 throughput 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.
- Application of the new column packing material to a rapid proteomic analytical workflow.

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 sub-2 µm totally porous particles, but with less than one-half of the operating back pressure. Separations of peptides, protein digests and proteomic samples should benefit from using Fused-Core particles of appropriate pore size. Our analyses indicate that 16 nm (160 Å) pore size is 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.

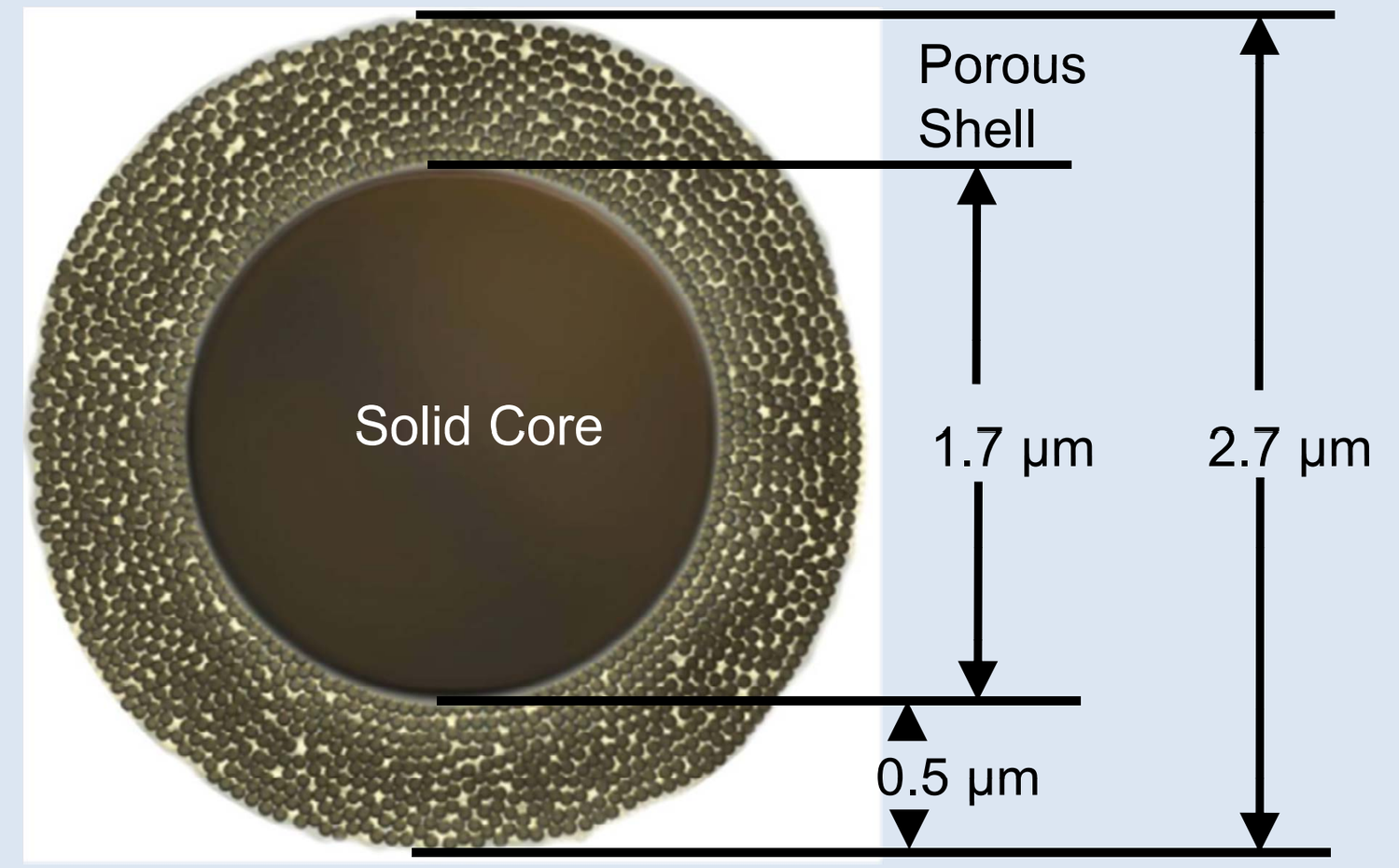
The Halo Peptide ES-C18 column is prepared using fused core particles that are surface modified with an extremely stable steric-protected C18 bonded phase, appropriate for the low pH conditions preferred for peptide separations. We show, consistent with many previous observations, that TFA is a most 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.

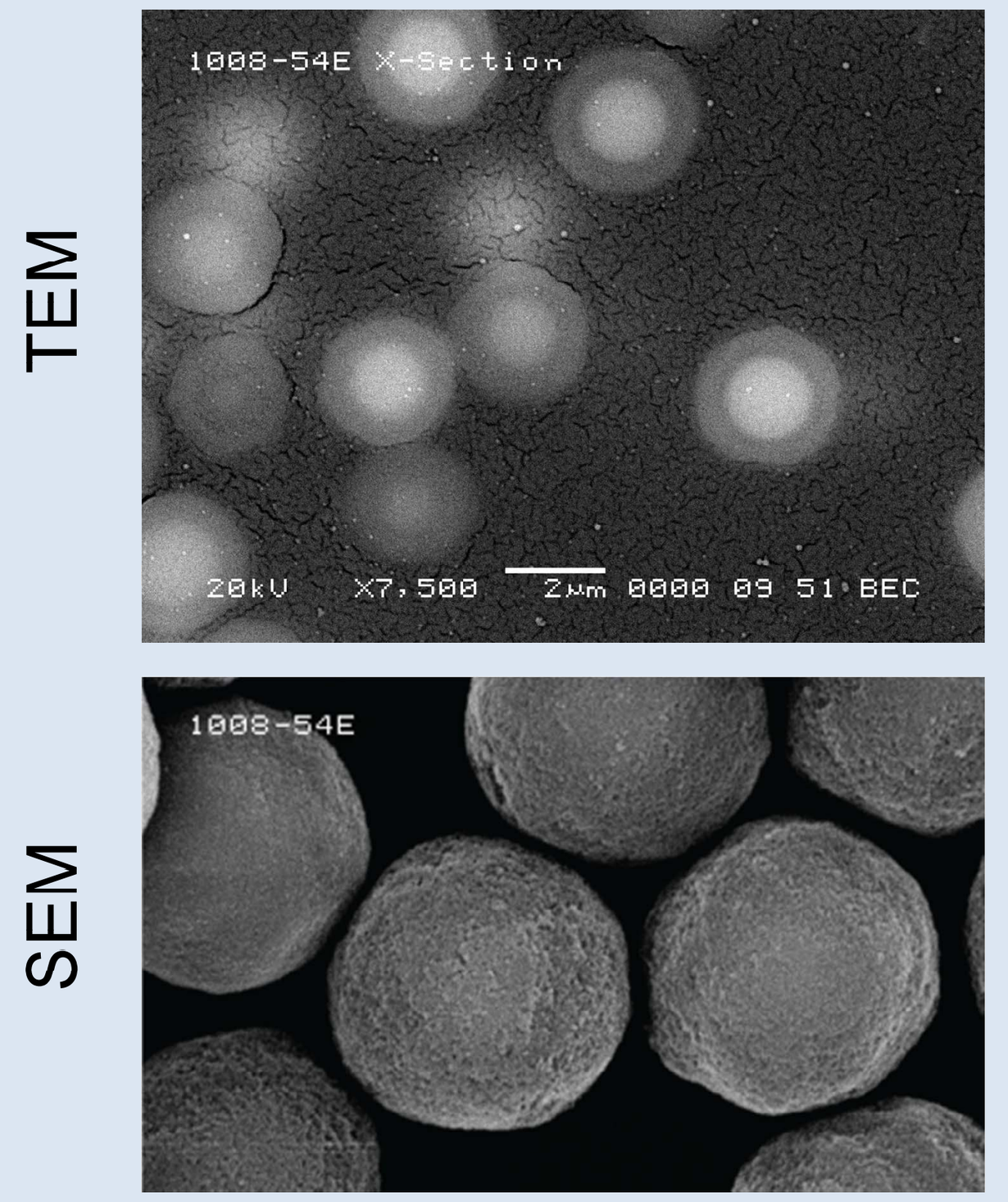
Materials and Methods

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 quaternary 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. The mass spectrometer was set to scan m/z 400 – 2000. Repeat count was set to 2, repeat duration 10s, exclusion duration 30s, and set to fragment the top 5 peaks for MS/MS.
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:

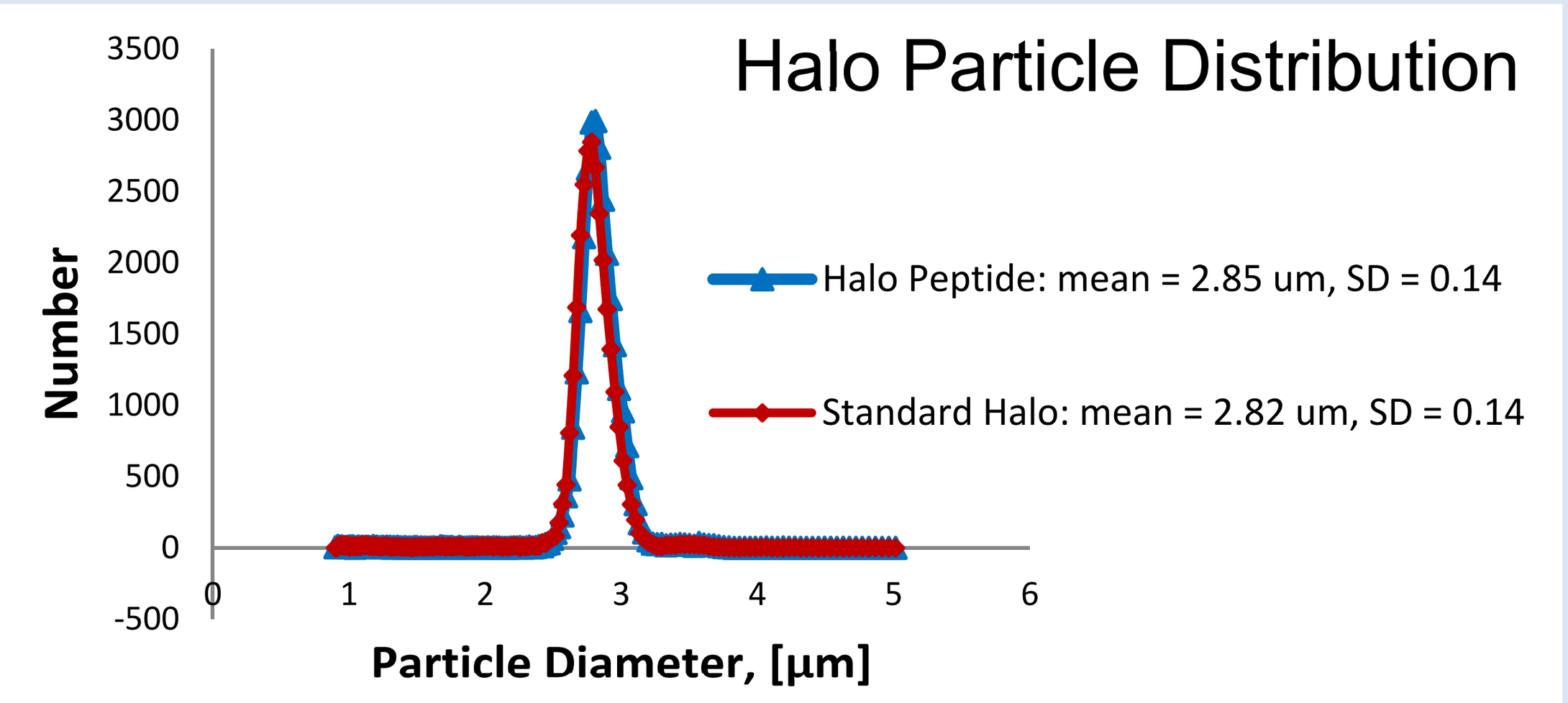
S1	RGAGGLGLGK-Am
S2	Ac-RGGGGLGLGK-Am
S3	Ac-RGAGGLGLGK-Am
S4	Ac-RGVGGLGLGK-Am
S5	Ac-RGVVGLGLGK-Am

Halo® Fused-Core Column Packing Materials

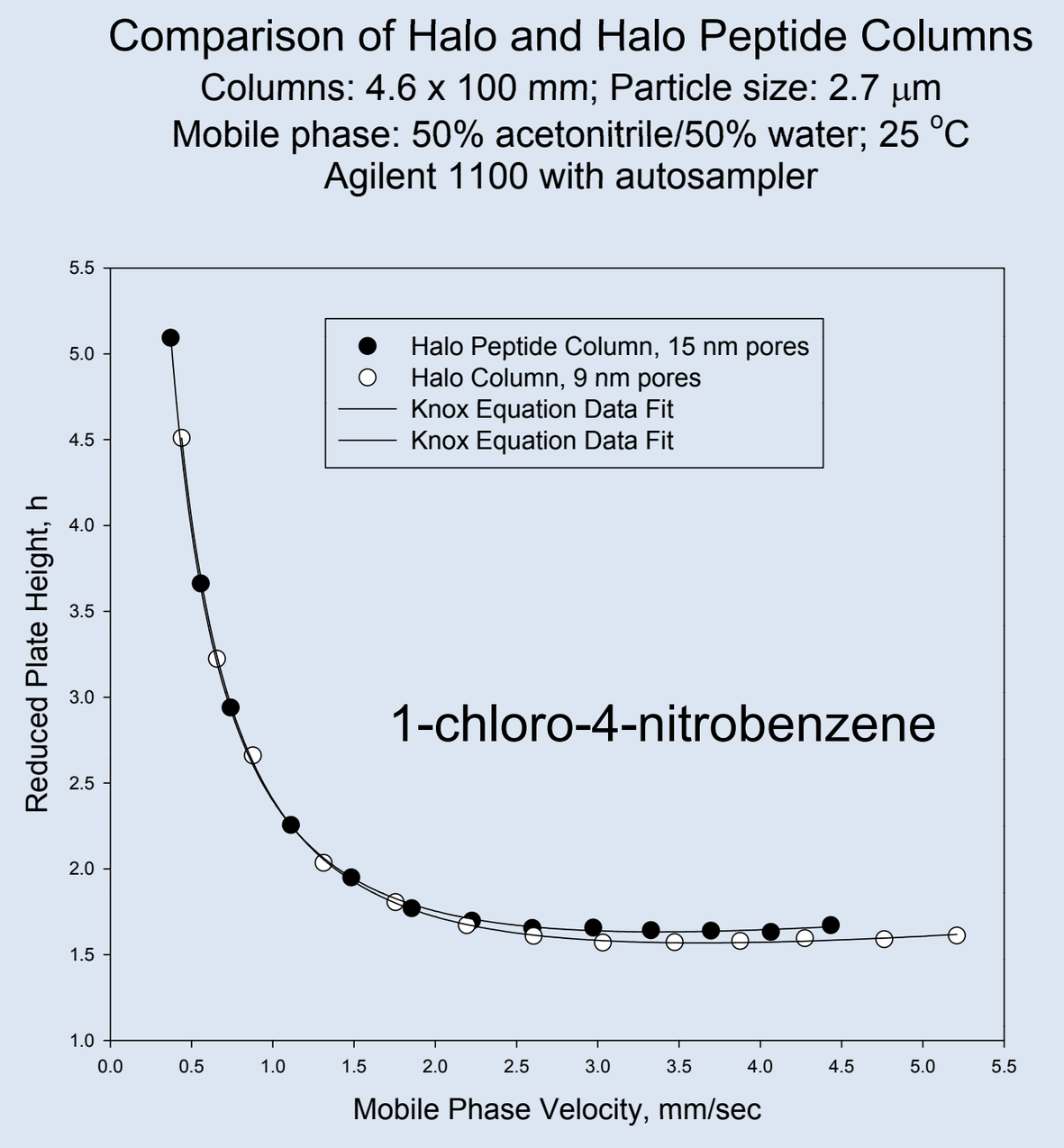


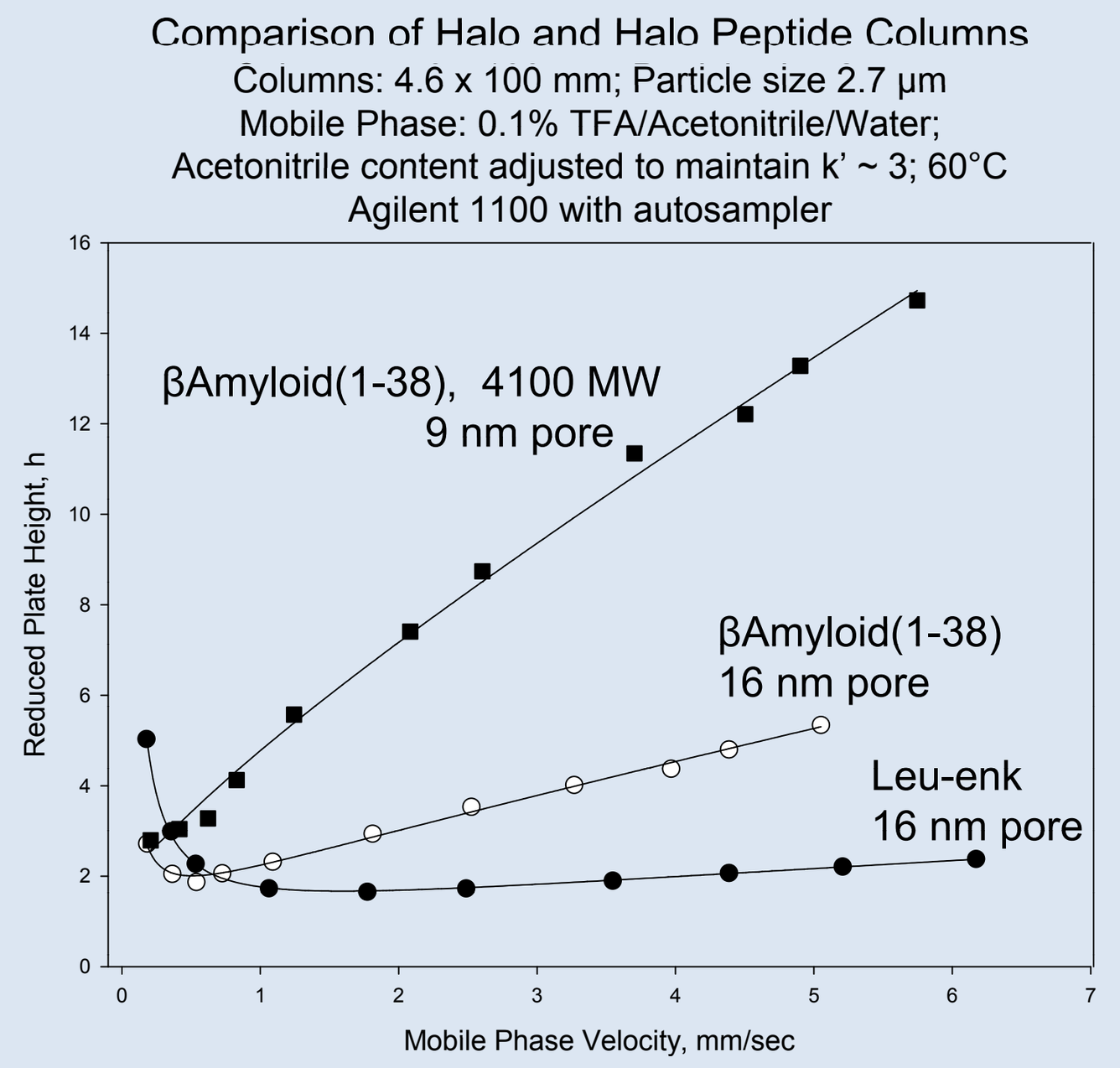


Halo Peptide Particles
Silica --------------------High Purity Type B
Ave. pore diameter--------------------16 nm
Surface area, nitrogen----------80 sq.m/g
Pore volume -----------------------0.30 mL/g
Particle density ----------------------1.3 cc/g



- 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 molecules.

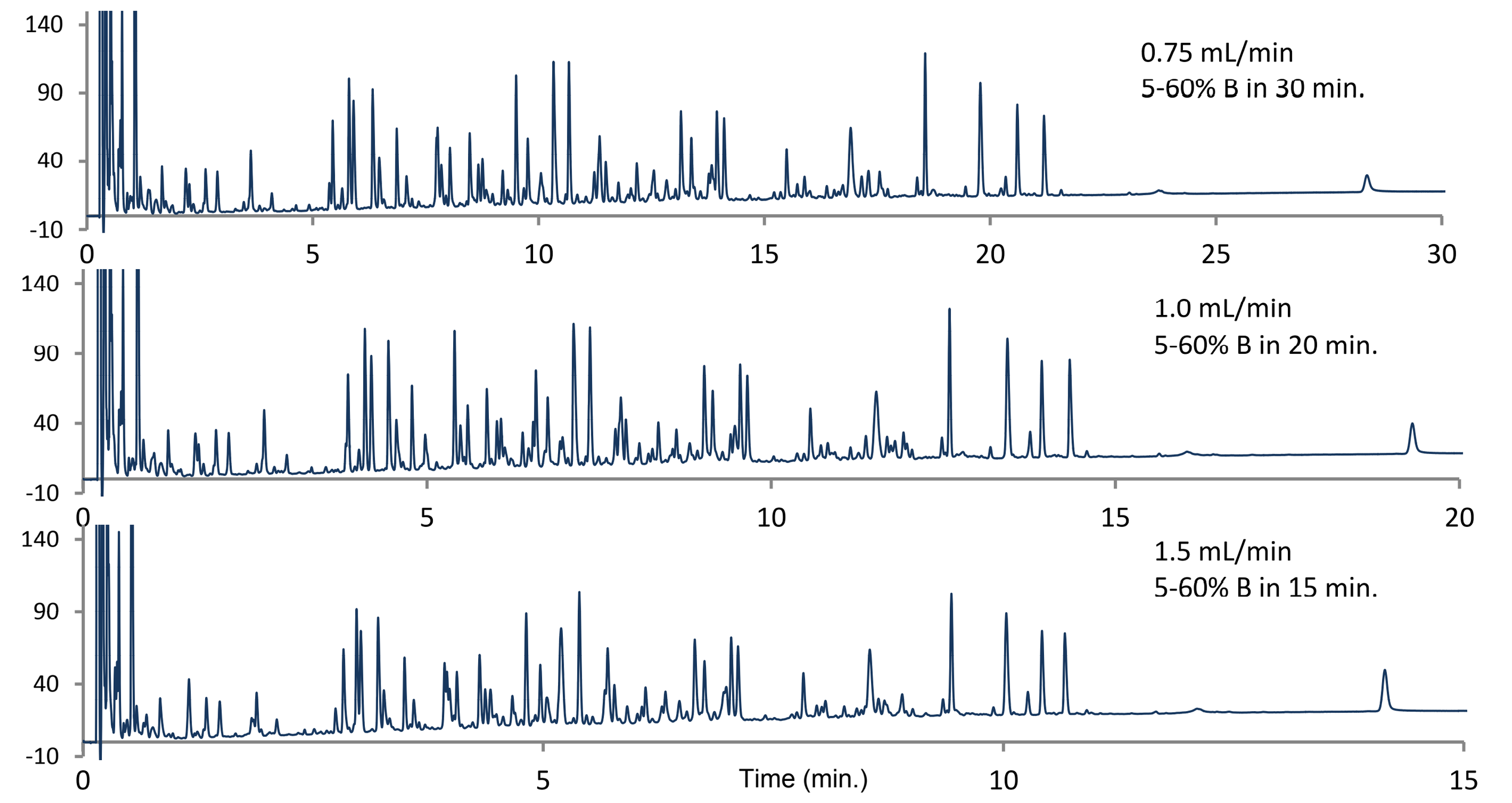



Comparison of Halo and Halo Peptide Columns
Columns: 4.6 x 100 mm; Particle size 2.7 µm
Mobile Phase: 0.1% TFA/Acetonitrile/Water;
Acetonitrile content adjusted to maintain k' ~ 3; 60°C
Agilent 1100 with autosampler
βAmyloid(1-38), 4100 MW
9 nm pore
βAmyloid(1-38)
16 nm pore
Leu-enk
16 nm pore
Reduced Plate Height, h
Mobile Phase Velocity, mm/sec

Tryptic Digest Separations Using Halo Peptide ES-C18

- Efficient columns and fast mass transfer allows very high flow rates for tryptic digest separations with high resolution.

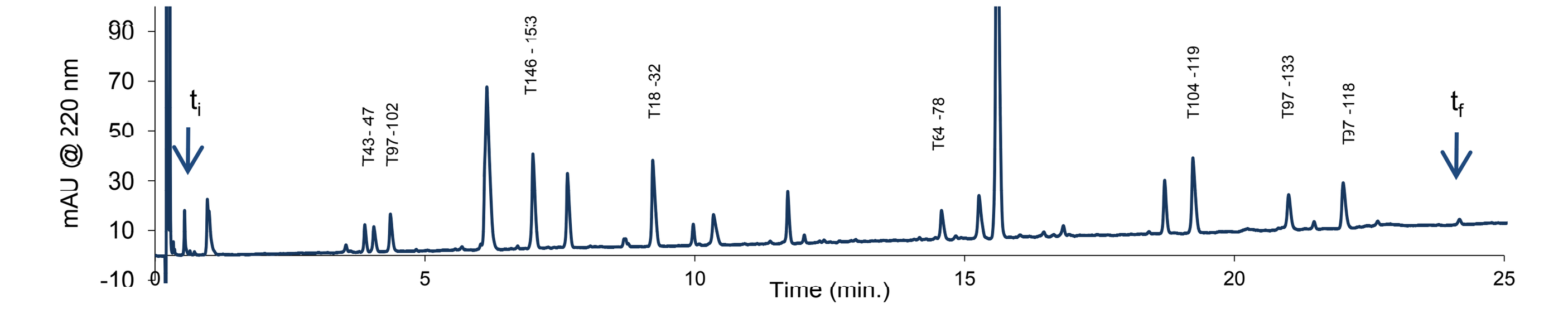
Column: 2.1 x 100 mm Halo Peptide ES-C18; A: Water/ 0.1% TFA, B: 80% ACN / 0.1% TFA., Detection: 215 nm, Sample: apotransferrin tryptic digest, Injection of 15 uL (15 µg), Temp: 60 C



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)



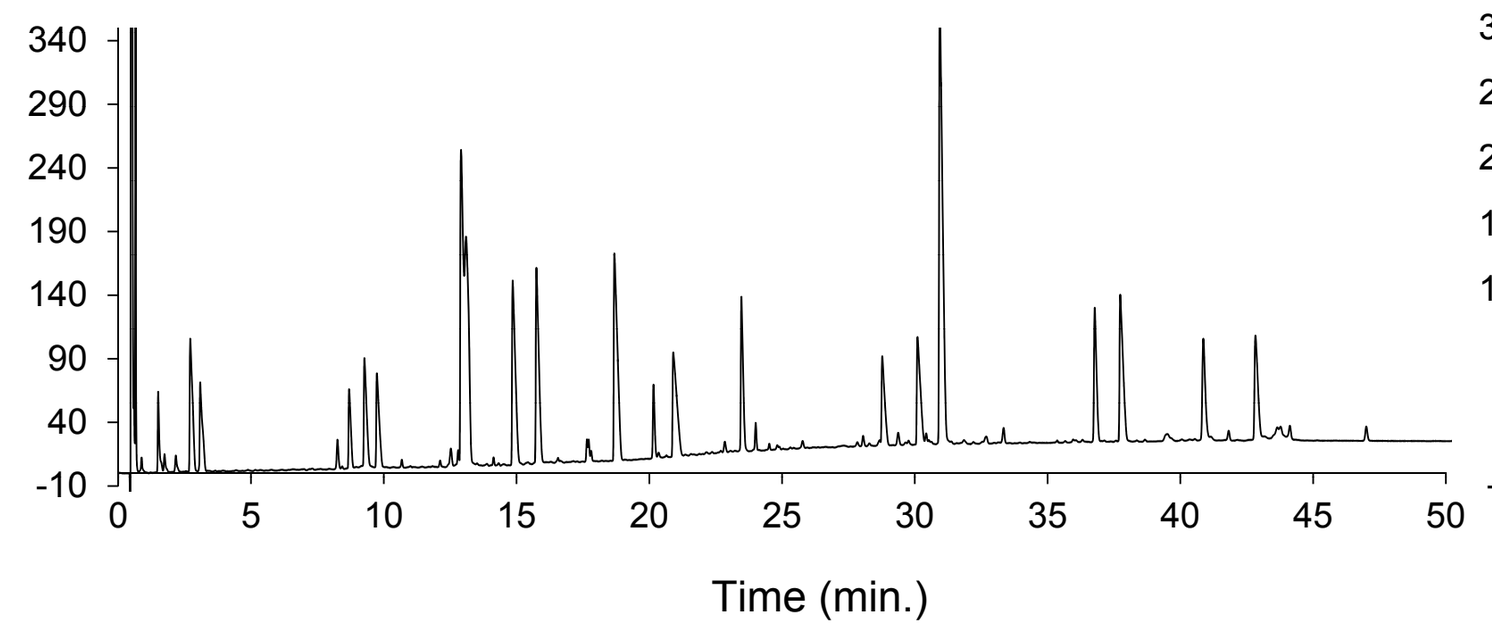
Peak capacity was determined to be 229 calculated by averaging the peak widths of the labeled tryptic digest peaks using the expression:

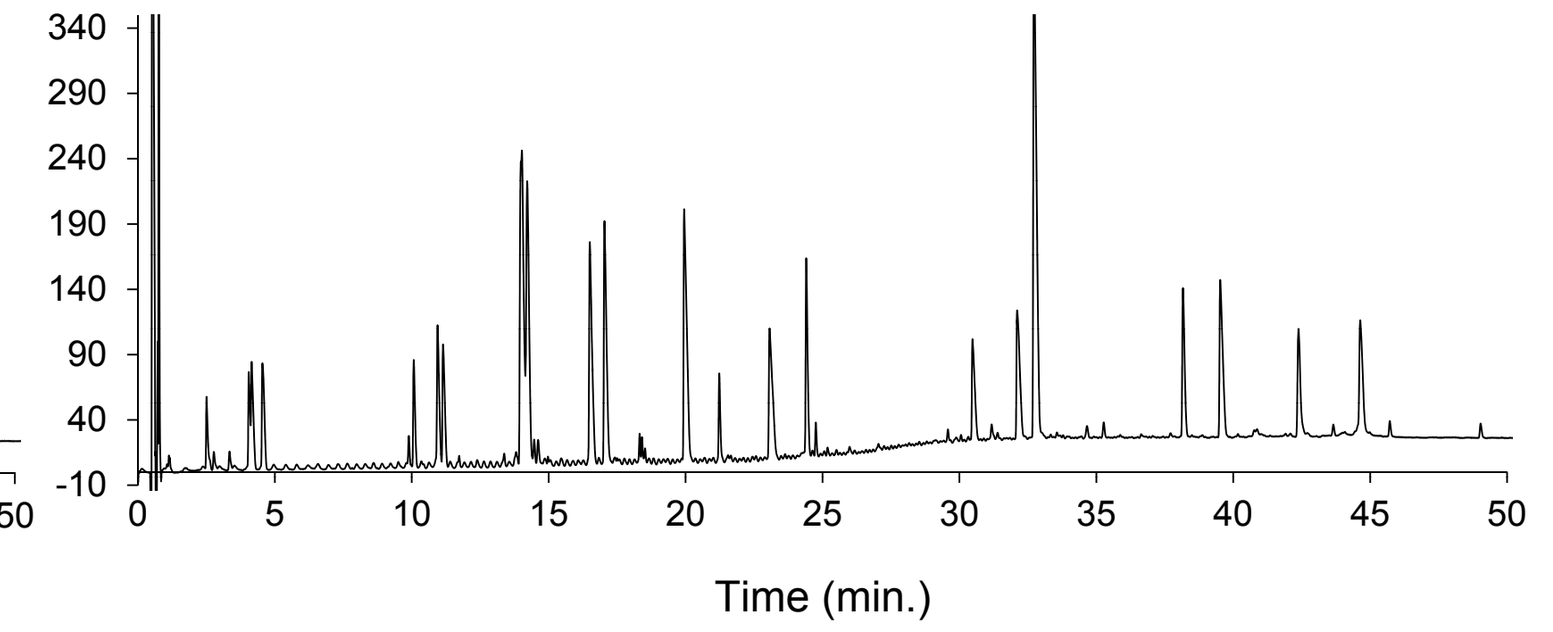
$$n_{pc} = \frac{t_f - t_i}{W_{4\sigma}}$$

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

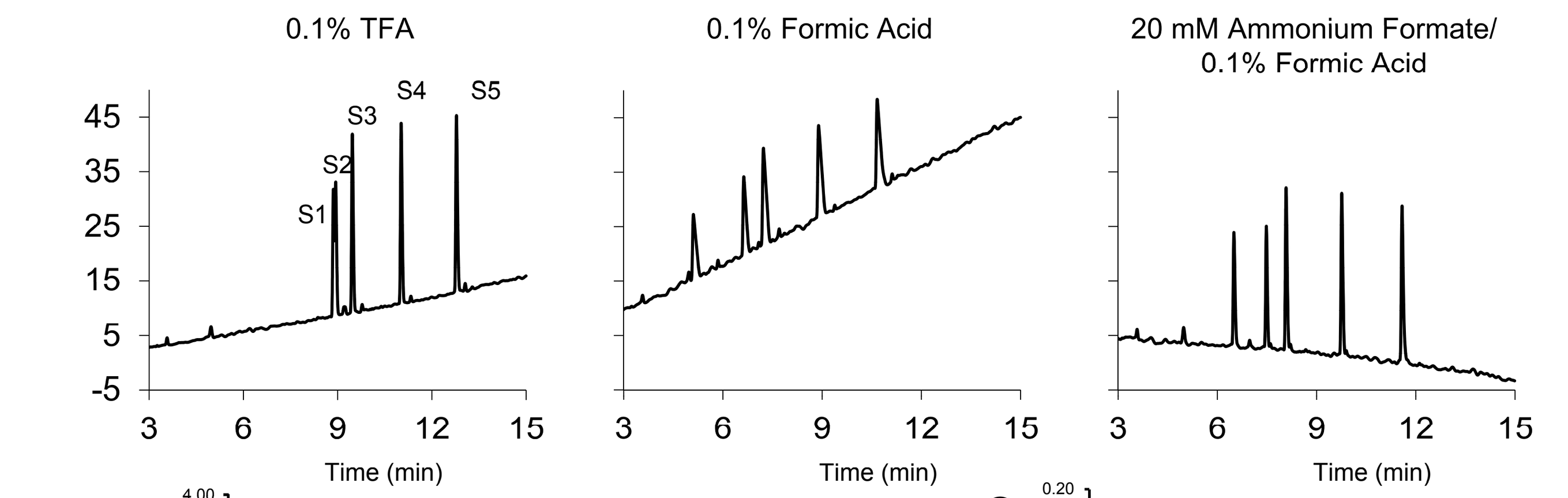


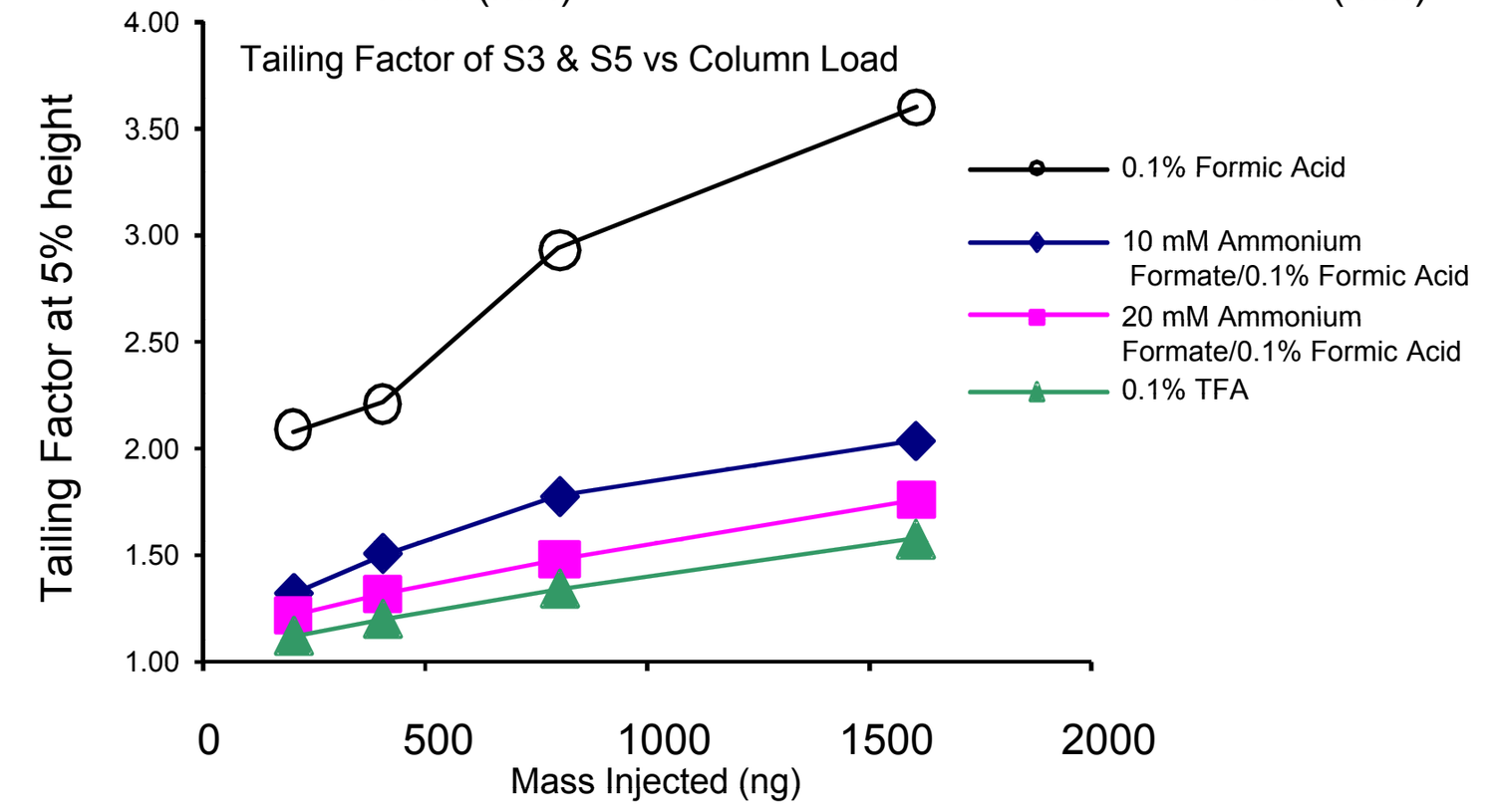


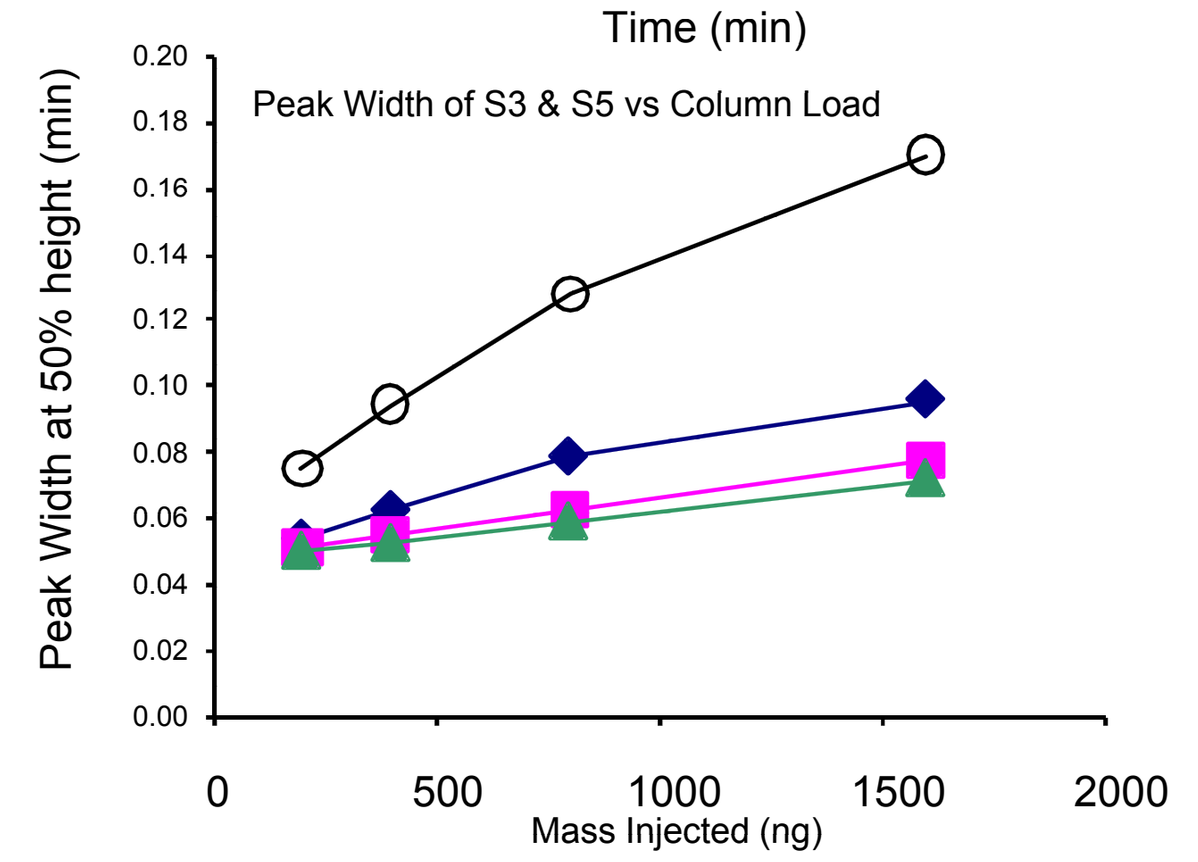
Mobile Phase Modifiers for LC/MS Analyses

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

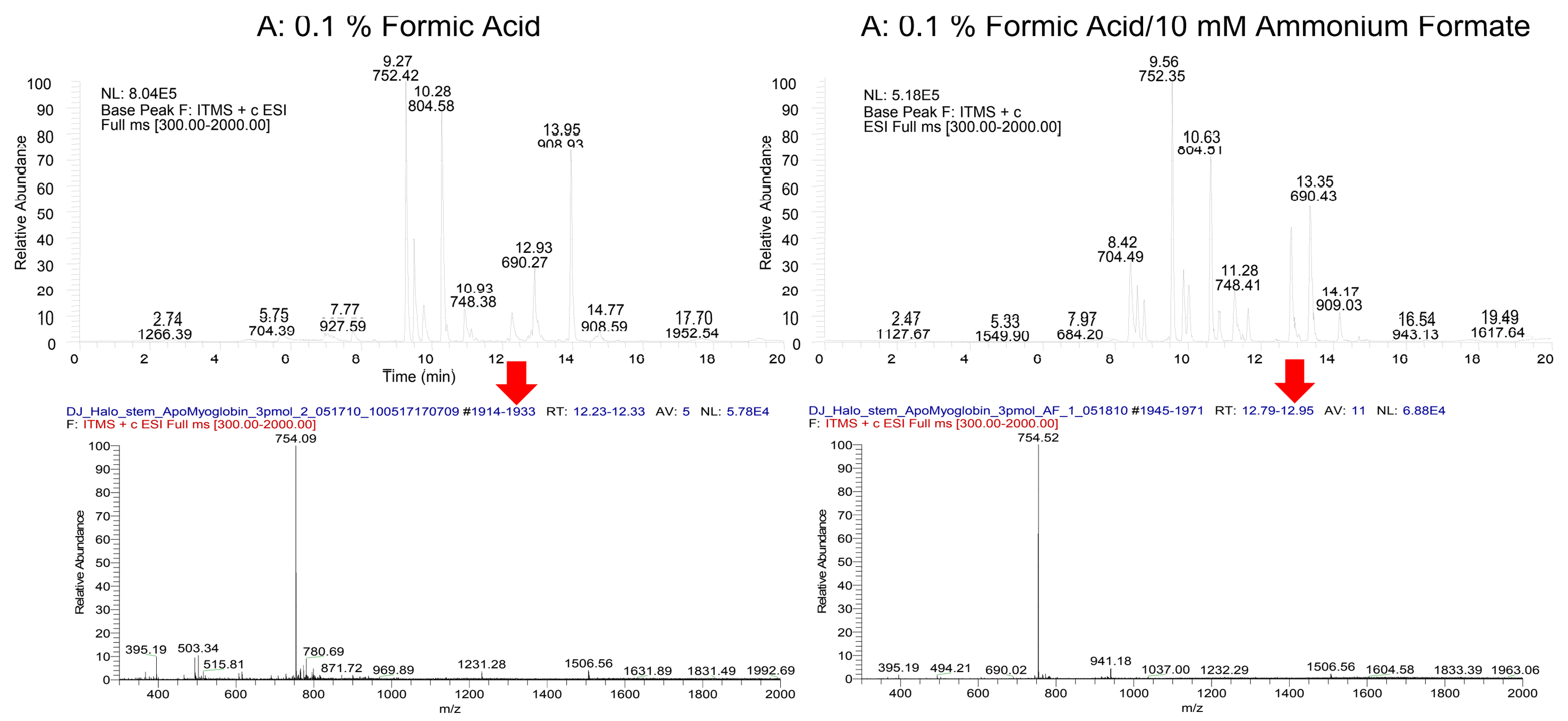






Ammonium Formate as an Additive for LC/MS Analyses

Halo Peptide ES-C18, 0.2 mm ID x 50 mm, Flow Rate 9 µL/min., T=25C, 3 pmol apoMyoglobin digest in 2 µL; 2-45% B in 15 minutes, B: 0.1% Formic acid in Acetonitrile



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

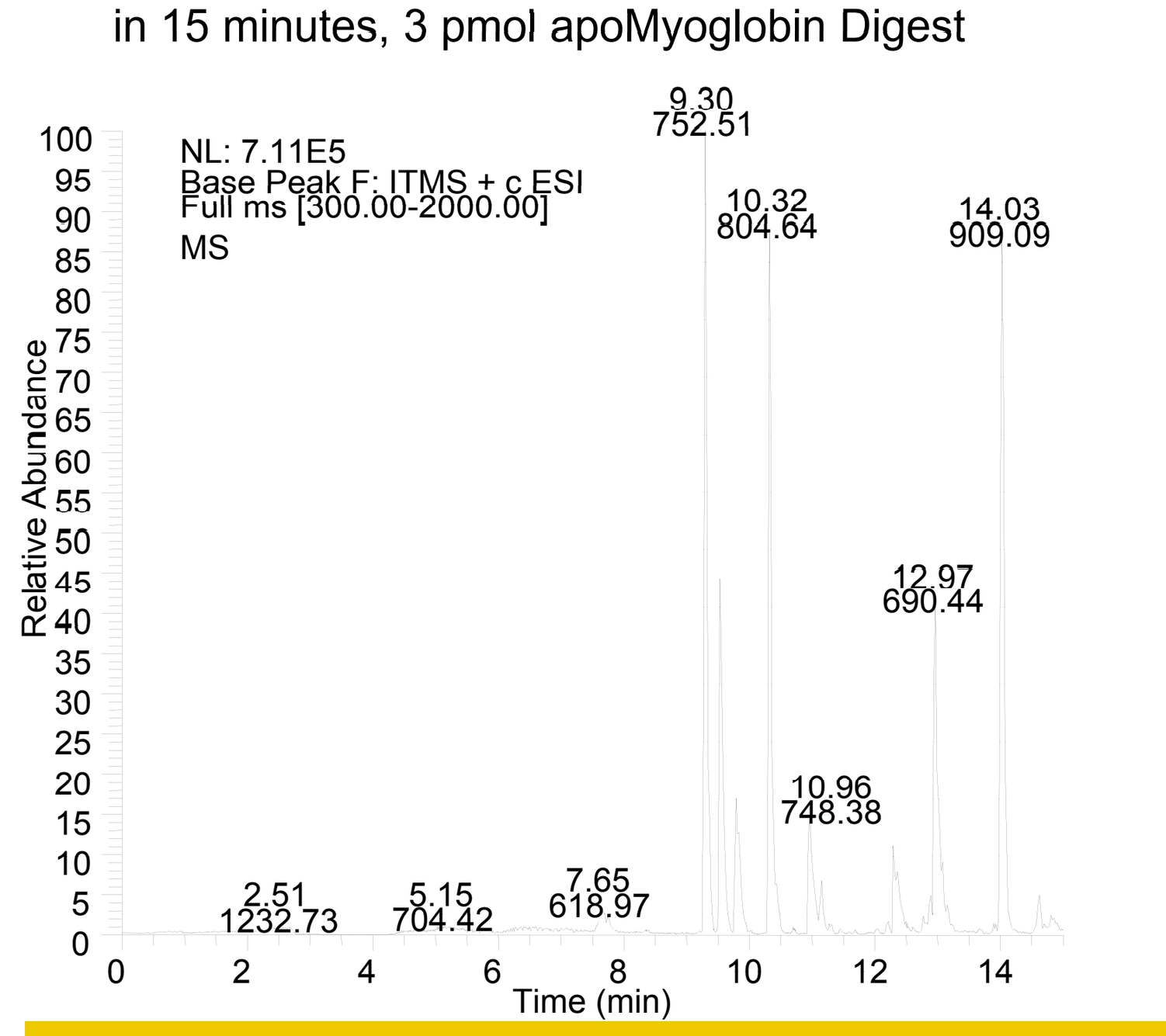
- Concentration dependent retention increase, selectivity shifts, and improved peak shape.
- Improved sample mass load tolerance at 10 or 20 mM ammonium formate
- IT-MS signal differences are limited, but for a small percentage of peptides (c. 15%) up to 10-fold differences in either direction of relative signal strength are observed.

High Linear Velocity LC/MS Analyses of Digests

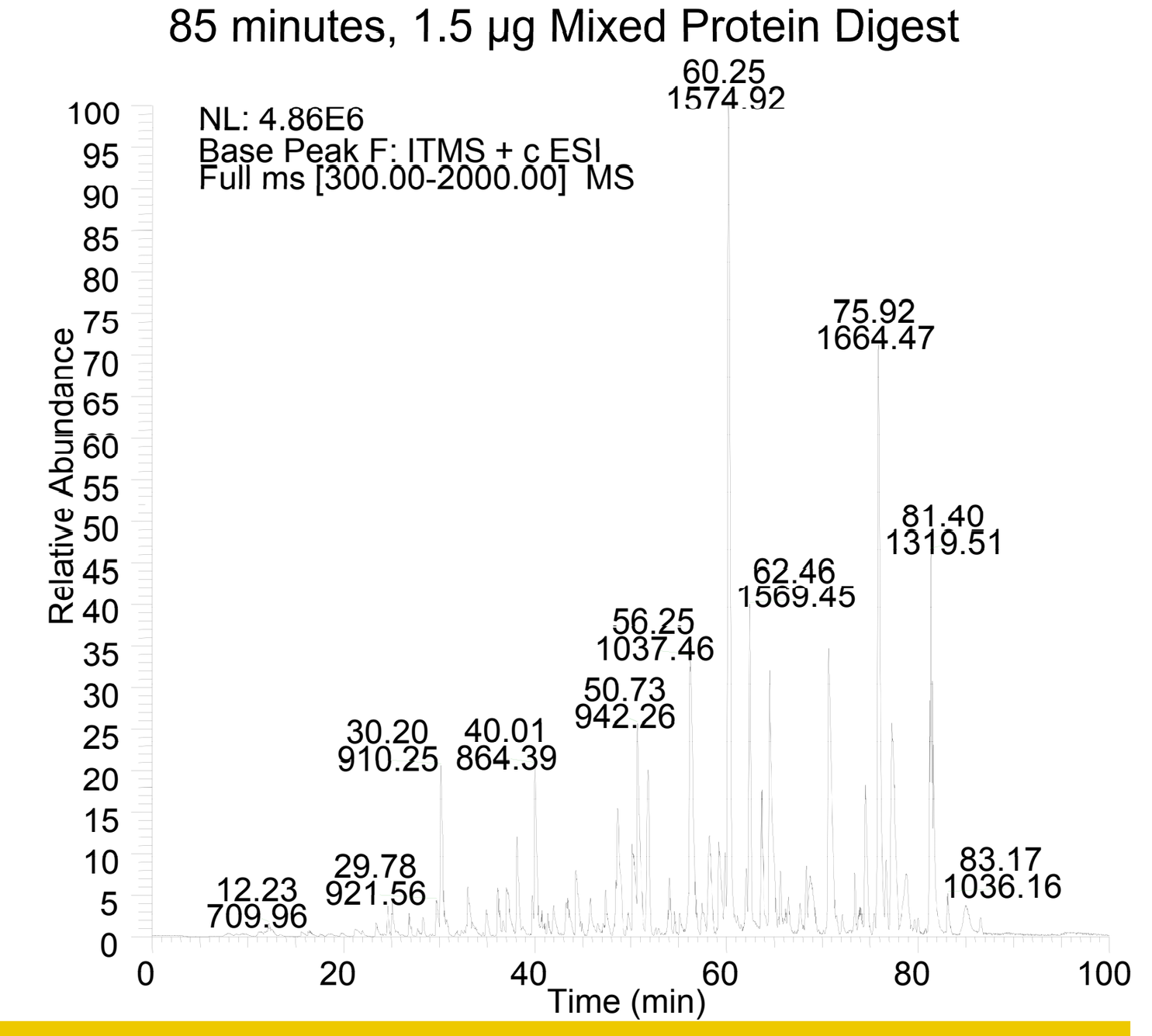
- Efficient separations allow selection of conditions matching sample complexity (long or short columns and gradients).

A: 0.1% Formic Acid/10 mM Ammonium Formate in 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



Rapid LC/MS Analyses of *Trypanosoma brucei* Proteins

- Proteins in a sample of procyclic *T. brucei* were identified by LC/MS using the ion trap. Capillary separations used a 0.2 mm ID x 50 mm Halo Peptide ES-C18 column at 9 uL/min, as shown above. Data were searched using Mascot and ProteoIQ (5% FDR), identifying 183 peptides, and 54 proteins. The top 20 protein IDs are shown below in the table.

	Sequence Id	Sequence Name	Length (AA)	Weight (kDa)	Protein Probability	Mascot Score	No. of Peptides	% Seq Coverage
1	gi|115504283|ref|XP_001218934.1|	alpha tubulin [Trypanosoma brucei]	451	49.737	0.99	894.93	20	46.78
2	gi|115504281|ref|XP_001218933.1|	beta tubulin [Trypanosoma brucei]	442	49.653	0.92	551.48	14	34.16
3	gi|72390503|ref|XP_845546.1|	glyceraldehyde 3-phosphate dehydrogenase, glycosomal [T. brucei TREU927]	402	43.823	1	563.62	14	43.28
4	gi|74025264|ref|XP_829198.1|	heat shock protein 70 [Trypanosoma brucei TREU927]	690	75.301	1	517.28	12	21.01
5	gi|72386615|ref|XP_843732.1|	ATP synthase beta chain, mitochondrial precursor [Trypanosoma brucei]	519	55.722	1	478.16	11	26.97
6	gi|71755837|ref|XP_828833.1|	glucose-regulated protein 78 [Trypanosoma brucei TREU927]	653	71.373	0.98	370.86	9	24.2
7	gi|71755425|ref|XP_828627.1|	triosephosphate isomerase [Trypanosoma brucei TREU927]	250	26.784	1	391.46	7	30.8
8	gi|71749346|ref|XP_828012.1|	glycosomal malate dehydrogenase [Trypanosoma brucei TREU927]	323	33.671	1	276.85	6	25.08
9	gi|84043670|ref|XP_951625.1|	NUP-1 protein [Trypanosoma brucei TREU927]	3647	406.638	1	167.19	4	0.8
10	gi|72390395|ref|XP_845492.1|	heat shock 70 kDa protein, mitochondrial precursor [Trypanosoma brucei TREU927]	657	71.412	0.65	235.37	8	16.44
11	gi|71748862|ref|XP_823486.1|	mitochondrial carrier protein [Trypanosoma brucei TREU927]	304	33.092	1	122.19	2	6.91
12	gi|72392767|ref|XP_847184.1|	glycerol-3-phosphate dehydrogenase [NAD+], glycosomal [T. brucei TREU927]	354	37.762	1	174.43	5	16.1
13	gi|71746820|ref|XP_822465.1|	elongation factor 1-alpha [Trypanosoma brucei TREU927]	449	49.056	0.75	159.66	4	14.25
14	gi|71747654|ref|XP_822882.1|	chaperonin Hsp60 [Trypanosoma brucei TREU927]	562	59.447	0.89	206.45	5	12.46
15	gi|71745578|ref|XP_827419.1|	Gim5A protein [Trypanosoma brucei TREU927]	243	26.372	0.78	171.17	3	12.35
16	gi|71744678|ref|XP_826969.1|	tryparedoxin peroxidase [Trypanosoma brucei TREU927]	199	22.392	0.84	136.84	4	18.59
17	gi|71748504|ref|XP_823307.1|	heat shock protein 83 [Trypanosoma brucei TREU927]	704	80.694	1	98.32	2	4.4
18	gi|74025780|ref|XP_829456.1|	hypothetical protein [Trypanosoma brucei TREU927]	262	28.740	0.93	172.03	4	18.32
19	gi|71747546|ref|XP_822828.1|	translation elongation factor 1-beta [Trypanosoma brucei TREU927]	201	21.927	1	106.83	2	21.89
20	gi|71754859|ref|XP_828344.1|	hypothetical protein [Trypanosoma brucei TREU927]	461	50.838	1	149.33	3	13.67

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 tolerance 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.
- Capillary columns packed with Fused-core particles can be operated at very high linear velocities to permit rapid and efficient proteomic analyses using conventional LC/MS instruments.

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