Breaking the speed limit: Fast, high-resolution peptide and tryptic digest separations using fused-core® particles

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Outline

- Problem: slow speed of biomolecule separations
  - Prior attempt to address
  - A better solution
- Brief history of superficially porous particles in HPLC
- Development and characterization of 160 Å fused-core particles
  - Particle size distribution
  - Pore size distribution
  - Stability
  - Efficiency
- Applications using HALO Peptide ES-C18
  - LC/MS of tryptic digests
  - Peak capacity
- Conclusions and summary

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Problem: Slow Speed of Biomolecule Separations

- Higher molecular weight has mass transfer limitations (diffusion)
- Complex: long shallow gradients used for increasing resolution of heterogeneous mixtures of similar structures (peptides and their PTMs)
- Less complex: not as difficult to resolve, but many samples to screen; need high throughput method
  - Example: one main peptide with minor components that MUST be quantified
  - Not all possible structures may be known (isobaric?)

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Prior Attempt to Address Problem

- N.D. Danielson and J.J. Kirkland, Anal. Chem.1987, 59, 2501-2506.
- 1-2-µm silica particles
- Bonded with C4
- Not Ideal
  - Inefficient (h = 3.5)
  - Unstable with repeated use of TFA-containing mobile phases (surface chemistry)
  - Required high pressure solvent delivery capability to provide necessary efficiency
  - Required special attention to extra column effects (band dispersion)
  - Required smaller frits
    - Increased chance that particulates in the sample / mobile phase will clog the frits
    - must filter both sample & mobile phase

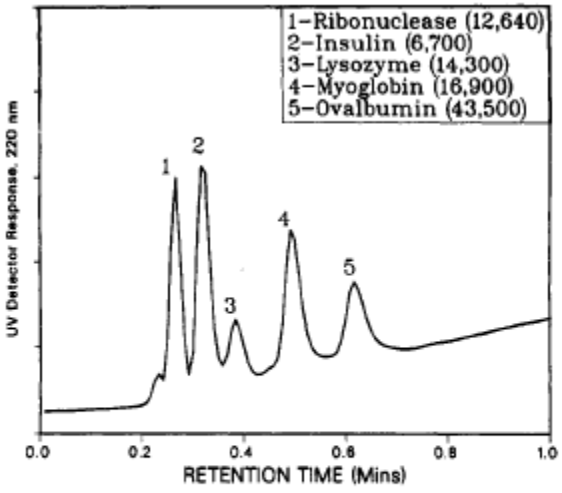

Figure 9. Separation of a five-component protein mixture: column, same as in Figure 3; mobile phase, 25:75 acetonitrile–water to acetonitrile (both with 0.1% trifluoroacetic acid); gradient, 0.3 min; flow rate, 3.3 mL/min; pressure, 340 bar; sample, 2 µg of each protein, except 0.6 µg of lysozyme; 35 °C; detection, 220 nm; molecular weight of proteins as shown.

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A Better Solution

- Use superficially porous particles
  - Advantages
    - Low back pressure due to the particle design (solid core with a porous shell)
    - No need for specialized HPLC equipment
    - Not necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm
    - High resolution is maintained at high flow rates (flat C-term in van Deemter plot)

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History of Superficially Porous Particles

- Superficially porous particles first used in GC by Golay
- 1967: Horvath, Preiss, and Lipsky described separation using ~50 µm cores with an anion exchange resin; called “pellicular” – a skin-like coating
- 1969: Kirkland, Zipax® ~30 µm Silica sol on glass beads
- Mid 70’s: Kirkland, Permaphase® permanently bonded stationary phase on SPP particles

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History of Superficially Porous Particles

Year	Who	Particle Name	Core (µm)	Shell (µm)	Total Size (µm)
2001	Agilent Technologies	Poroshell® 300	4.5	0.25	5.0
2007	Advanced Materials Technology	HALO®	1.7	0.5	2.7
2007	Supelco Sigma-Aldrich	Ascentis Express®	1.7	0.5	2.7
2009	Phenomenex	Kinetex®	1.9 1.25	0.35 0.23	2.6 1.7
2010	Agilent Technologies	Poroshell® 120	1.7	0.5	2.7

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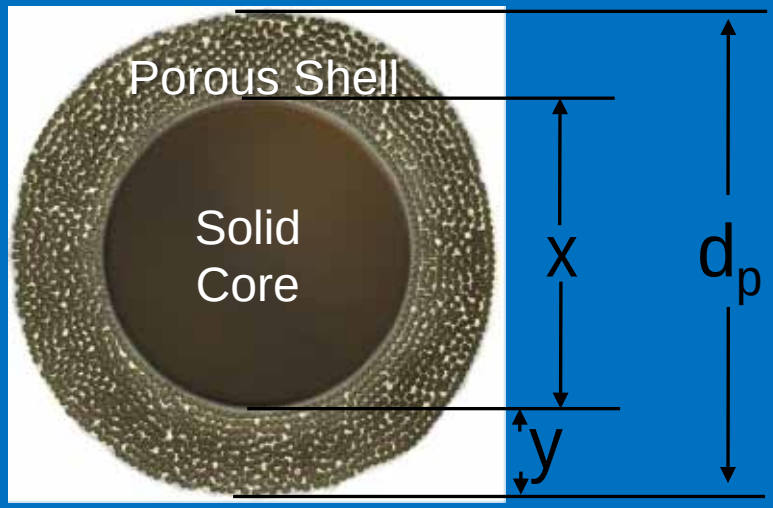
Fused-Core® Particles: Varying Pore Size



x (µm)	y (µm)	d_p (µm)	Pore Size (Å)	Surface Area (m^2/g)
1.7	0.5	2.7	90	150
1.7	0.5	2.7	160	80

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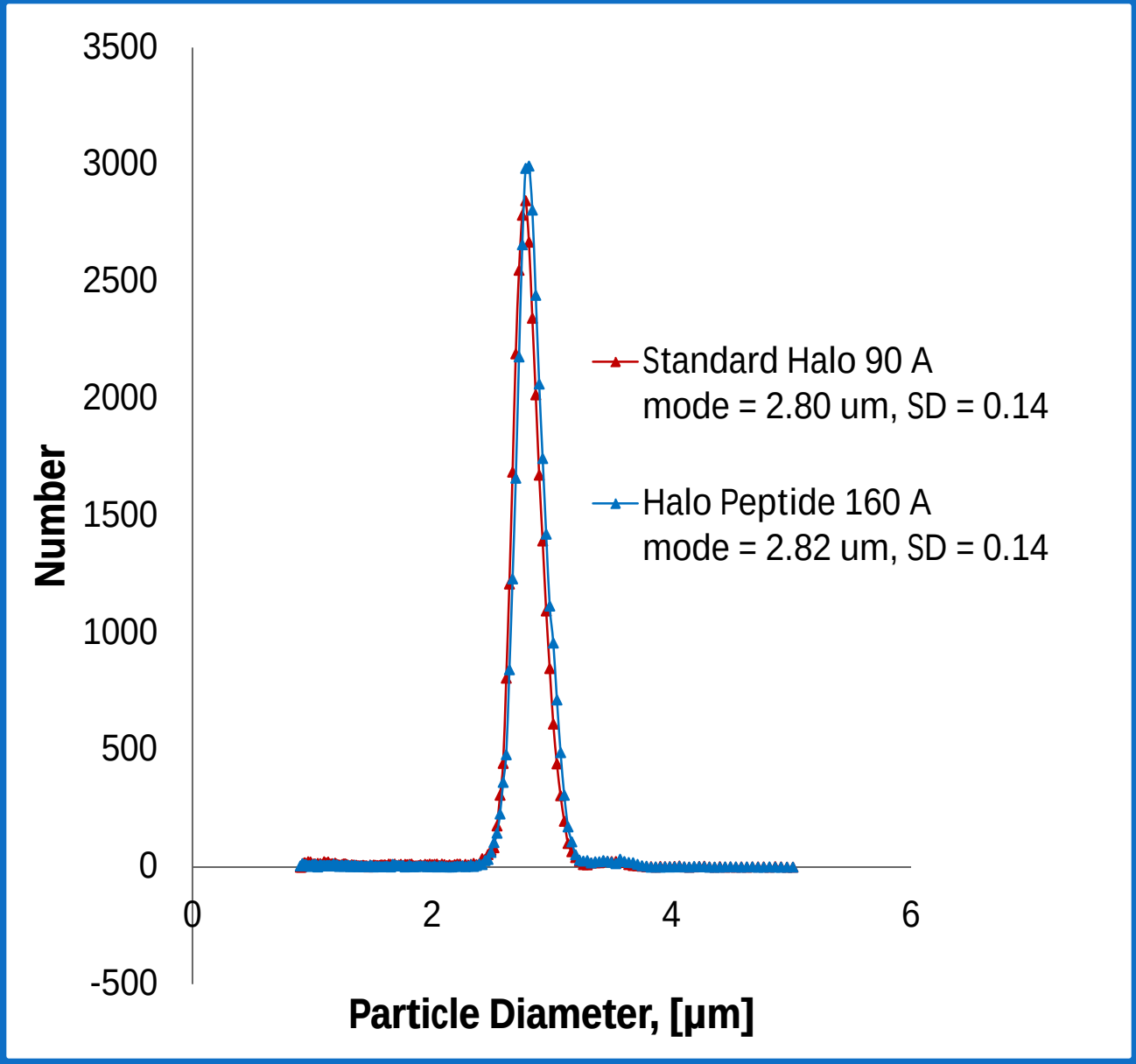
Particle Size Distributions



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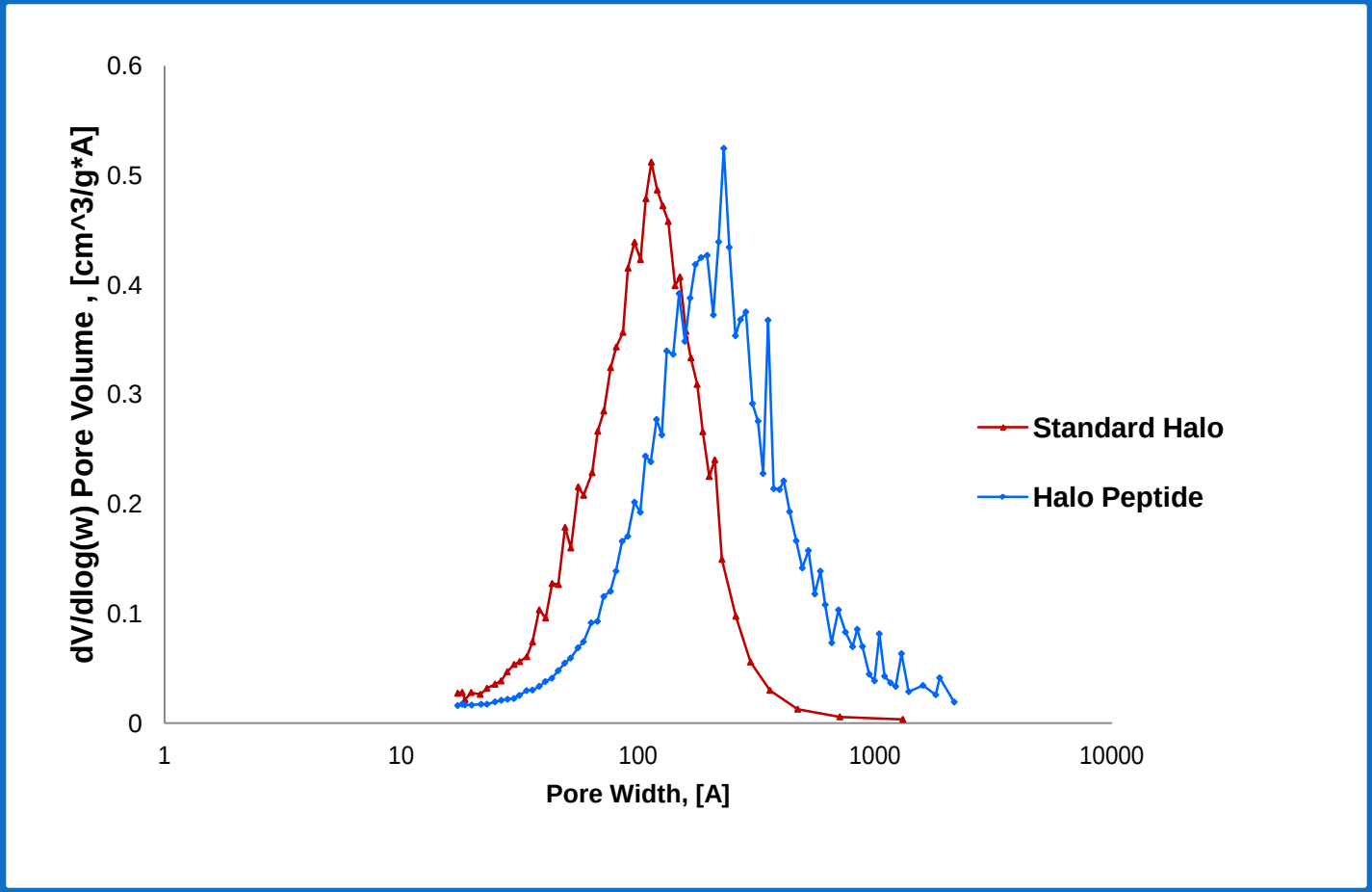
Pore Size Distributions



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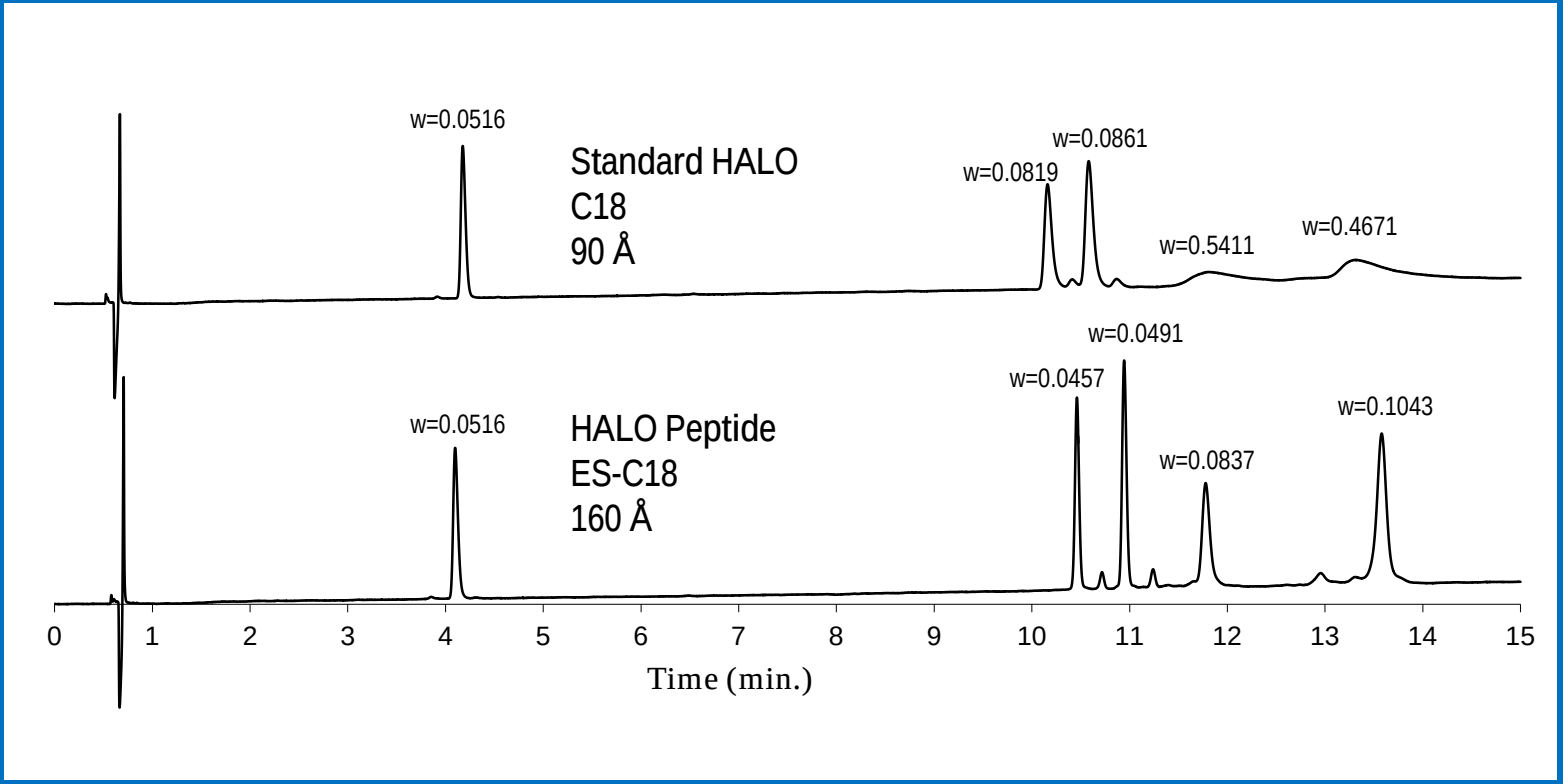
Effect of Pore Size on Peptide and Small Protein Separations



1. Leu-enk (555 g/mol)
2. Bovine Insulin (5733 g/mol)
3. Human Insulin (5808 g/mol)
4. Cytochrome C (12,400 g/mol)
5. Lysozyme (14,300 g/mol)

Columns: 4.6 x 100 mm
Flow rate: 1.5 mL/min
Temperature: 30° C
A: 0.1% TFA/10% ACN, B: 0.1% TFA/70% ACN
Gradient: 15% to 50% B in 15 min.
Injection volume: 5 µL
Detection: 220 nm

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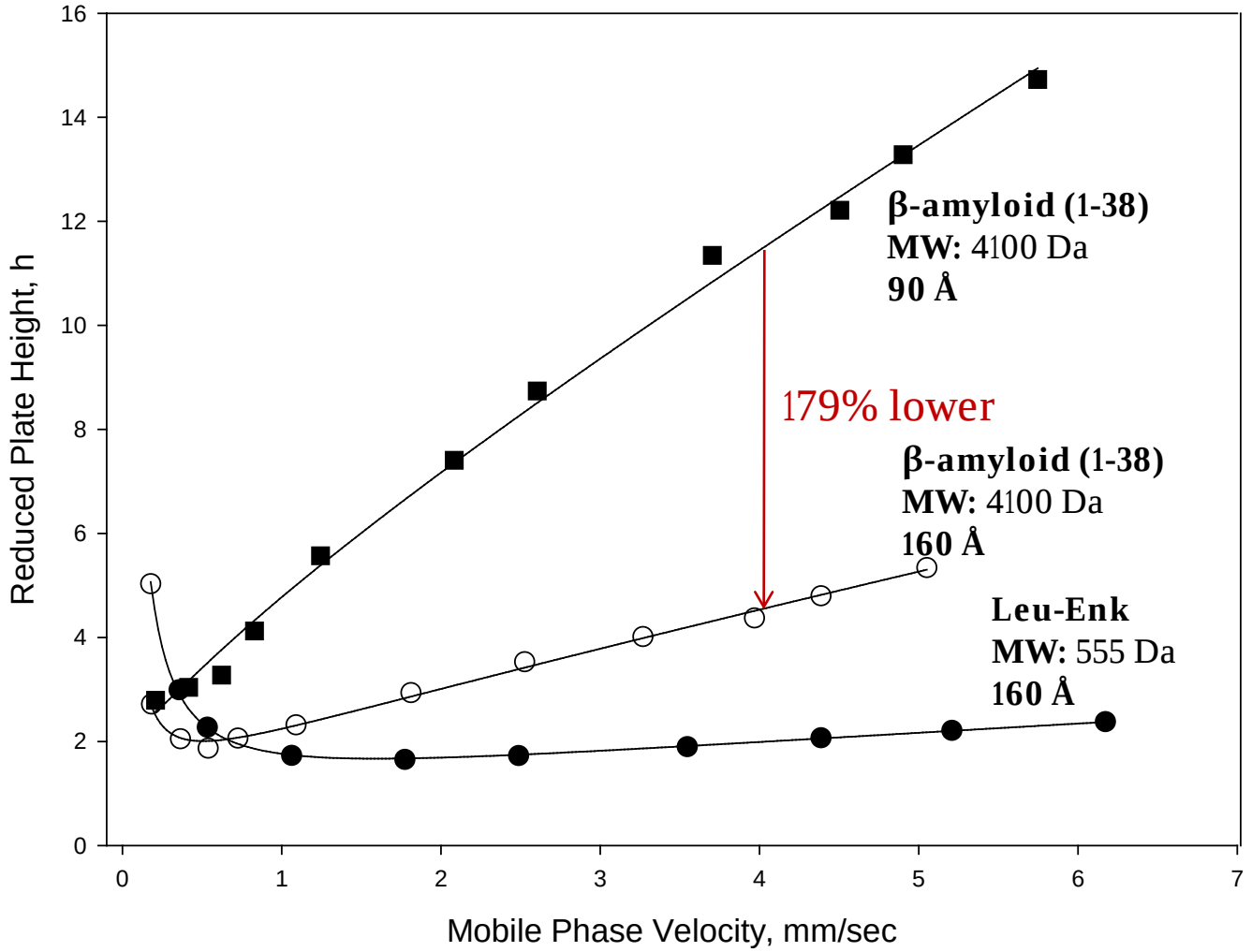
Effect of Pore Size on Efficiency



Columns: 4.6 x 100 mm HALO C18, 2.7 µm, 90 Å
4.6 x 100 mm HALO Peptide ES-C18, 2.7 µm, 160 Å

Mobile Phase: Leu-Enk: 21% ACN/79% Water/0.1% TFA
β-amyloid (1-38) 160 Å : 29% ACN/71% Water/0.1% TFA
β-amyloid (1-38) 90 Å : 27% ACN/73% Water/0.1% TFA

Temperature: 60 °C
Detection: 215 nm

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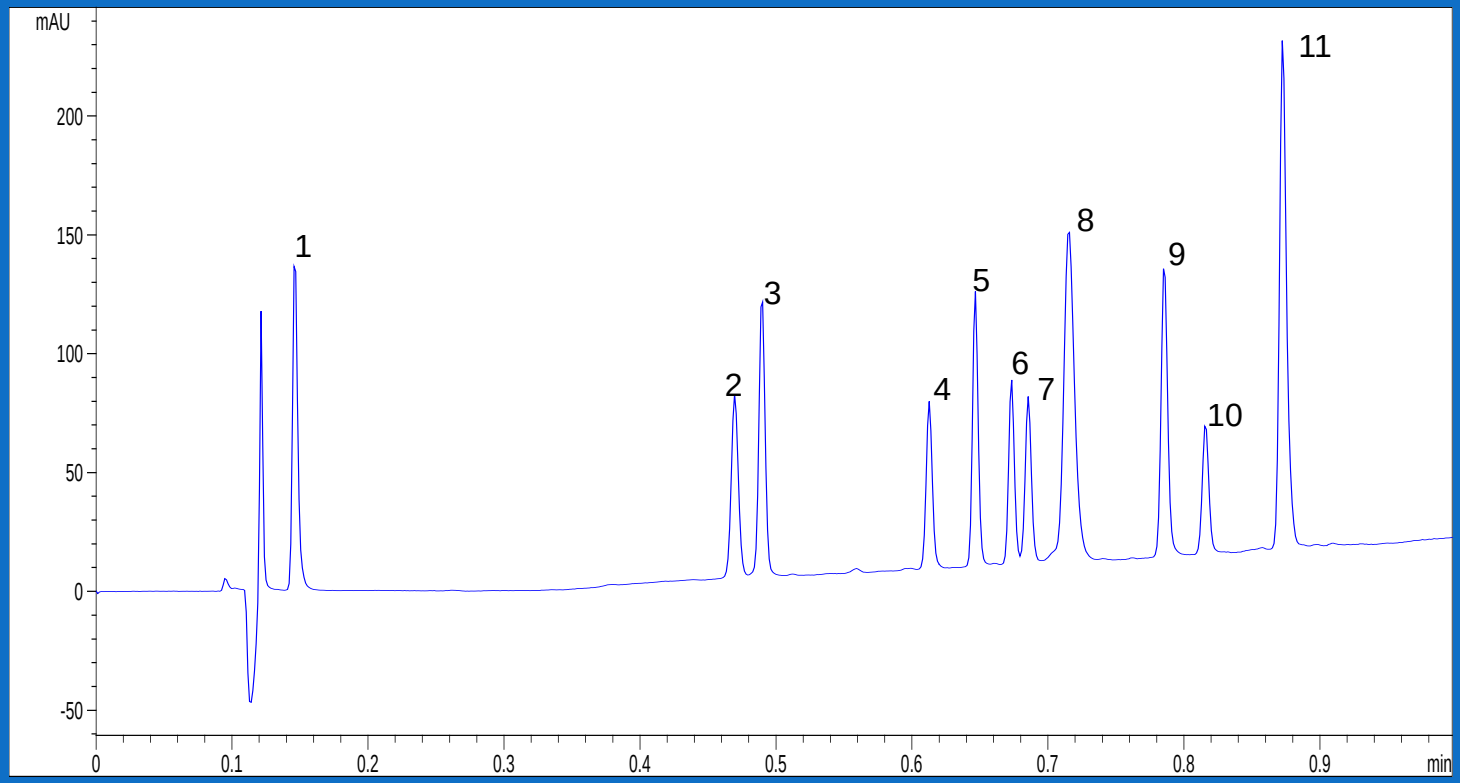
9 Peptides and 2 Proteins in < 1 minute

Column: HALO Peptide ES-C18, 4.6 x 50 mm;
Mobile Phase: A: 0.1% TFA/10% ACN; B: 0.1% TFA/70% ACN; 0% to 87.5% B in 1 min;
Flow Rate: 5.0 mL/min; Temperature: 60 C; Pressure: 330 bar;
LC System: Conventional HPLC, Agilent 1100;



Sample:

1. Gly-Tyr
2. Val-Tyr-Val
3. Angiotensin 1/2 (1-7) amide
4. Met-enk
5. Angiotensin 1/2 (1-8) amide
6. Angiotensin II
7. Leu-enk
8. Ribonuclease A
9. Angiotensin (1-12) (human)
10. Angiotensin (1-12) (mouse)
11. Porcine Insulin

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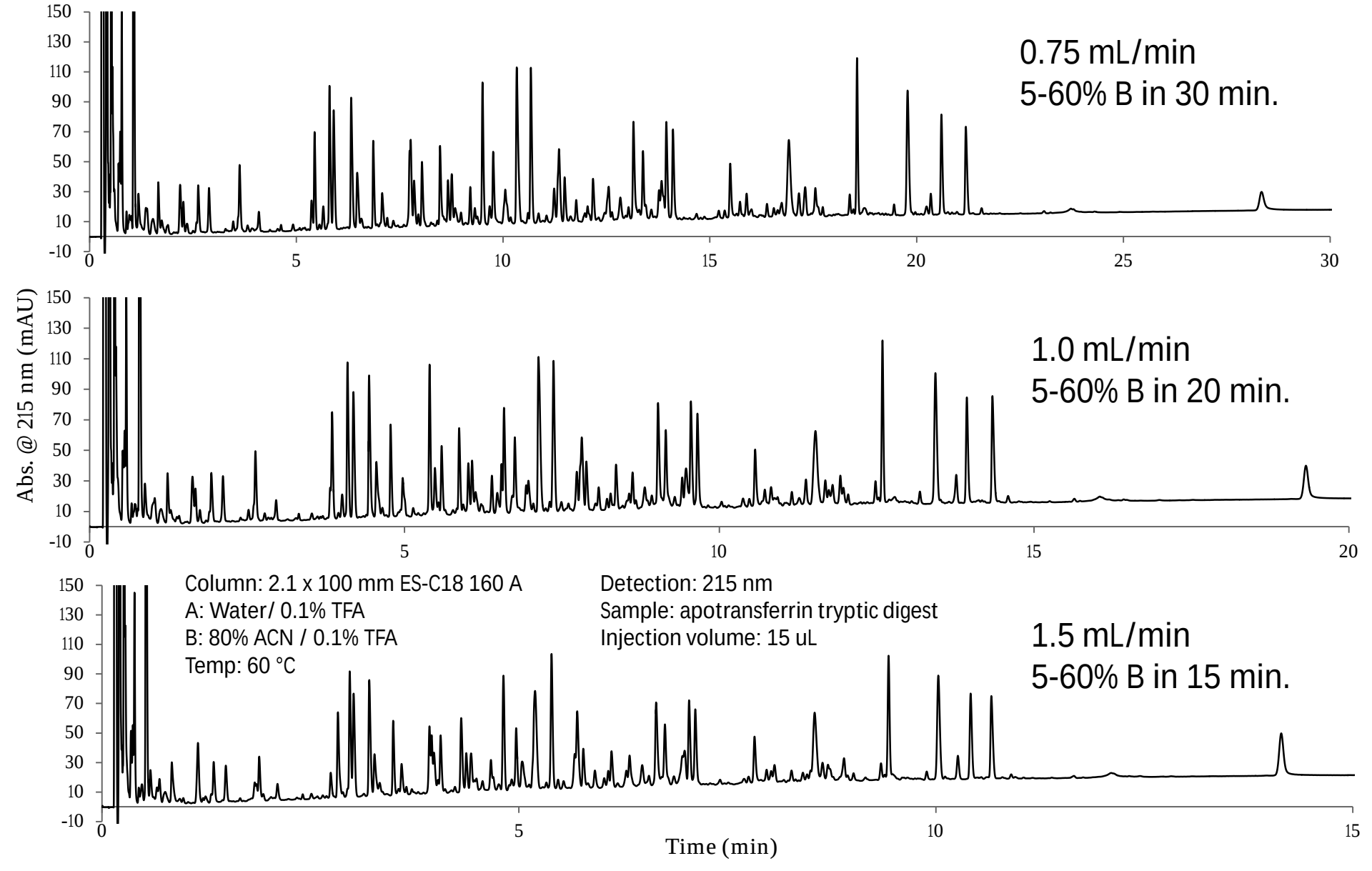
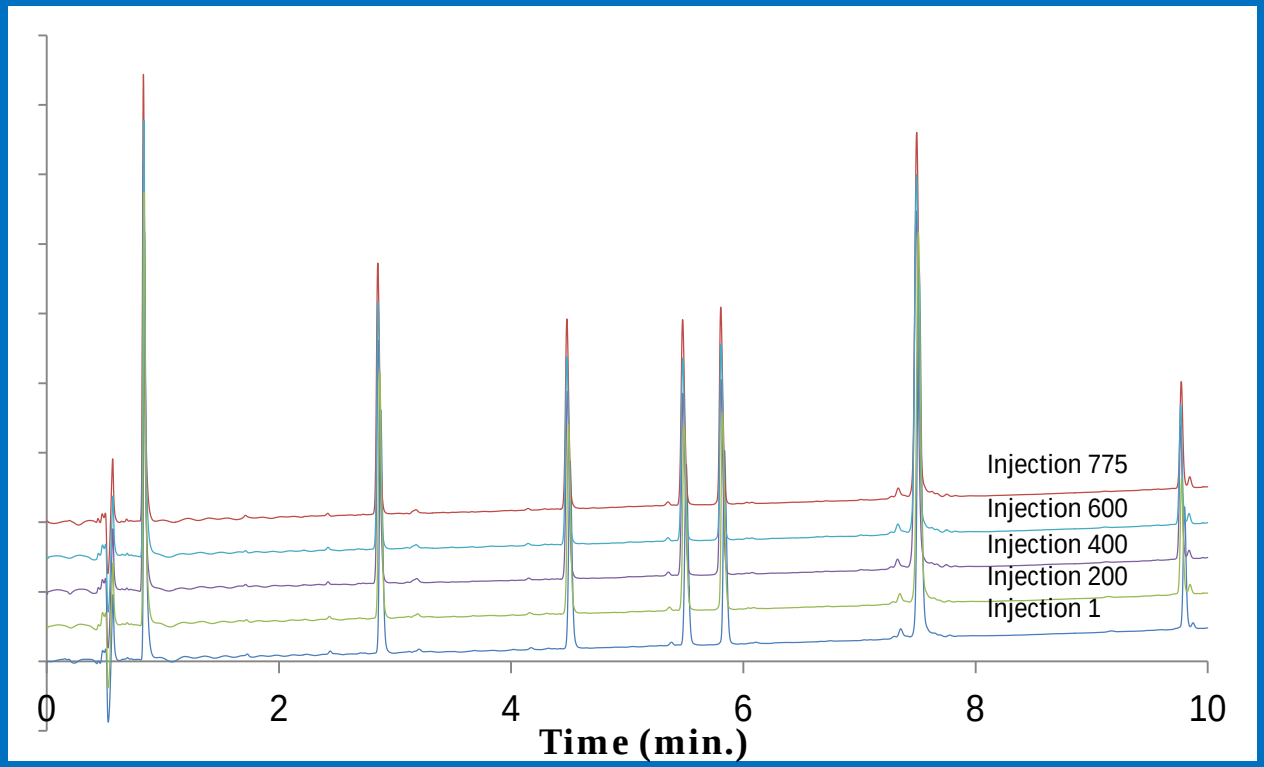
Ultra Fast High Resolution Separation of Apo Transferrin Tryptic Digest



Stability

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: 9-55% B in 10 min.; Injection volume: 5 µL;
Shimadzu Prominence UFLC XR



Sample:
Gly-Tyr, Val-Tyr-Val, Met-enk, Angiotensin II, Leu-enk
Ribonuclease, Porcine Insulin

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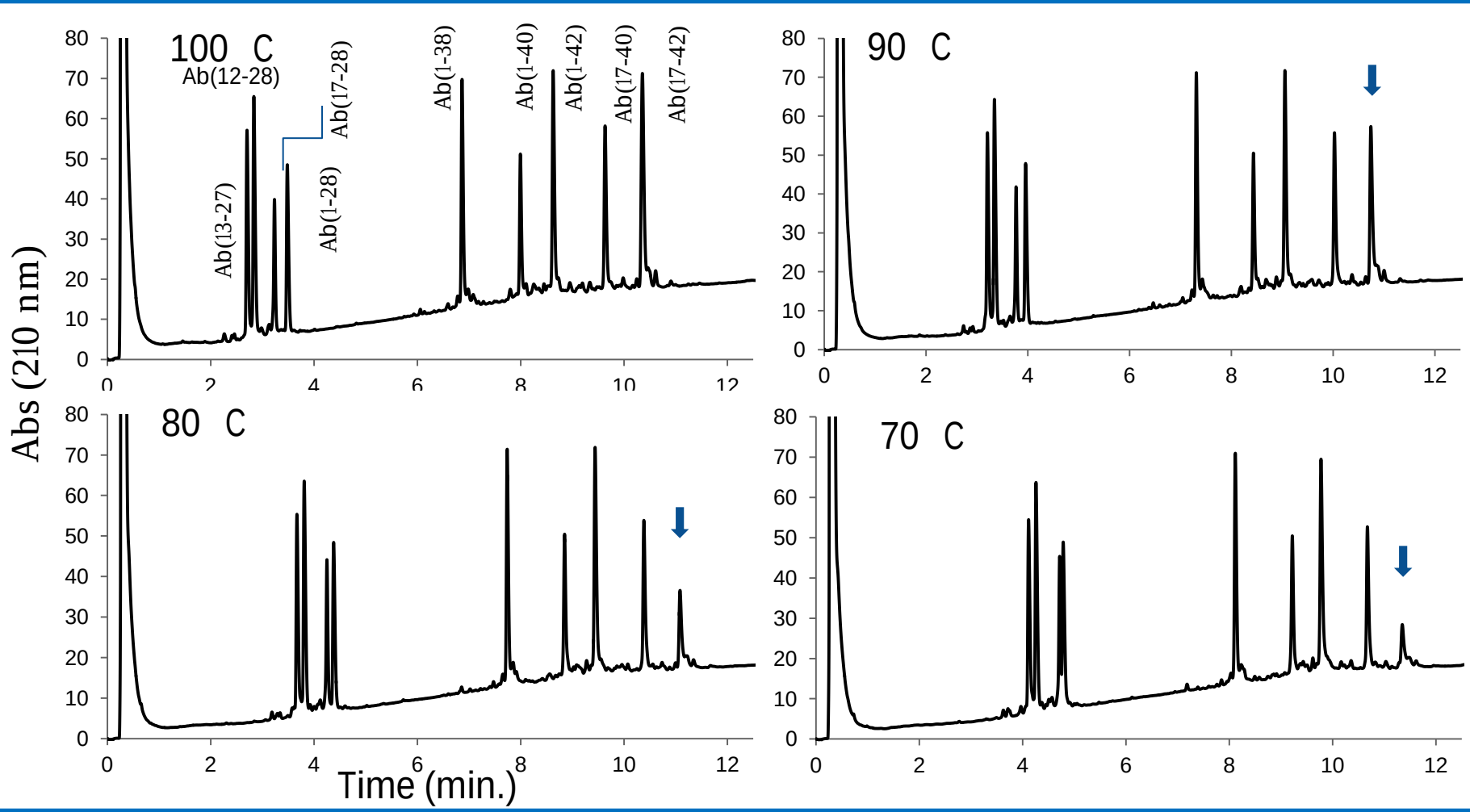
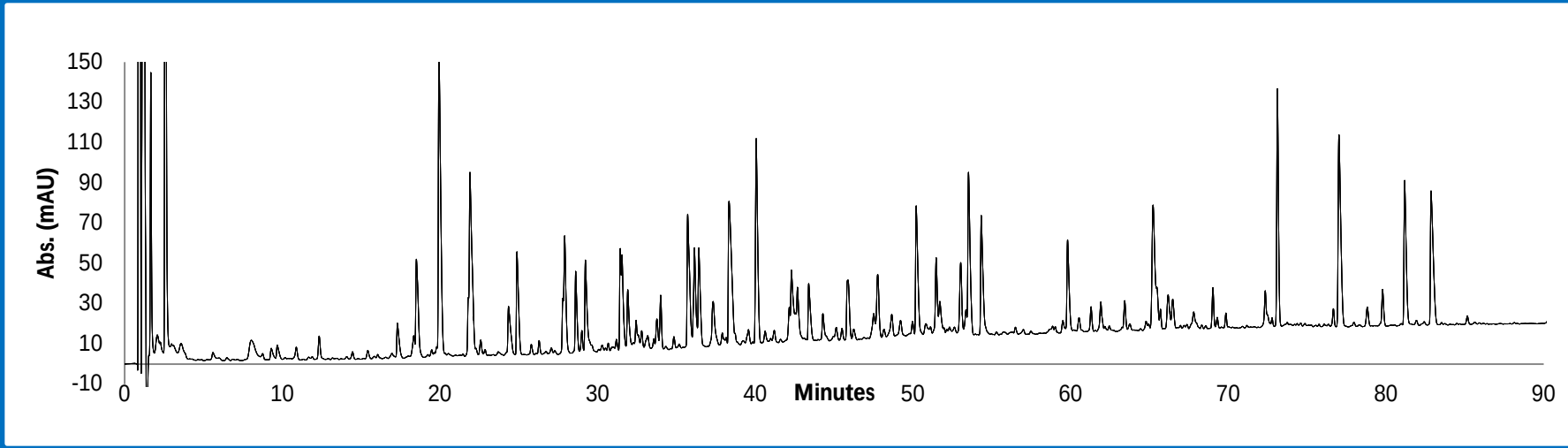
Rapid Separation at High Temperature

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 b 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 Ab(17-42).

Low Back Pressure Permits Coupled Columns for Increased Resolution



Columns: 2 @ 2.1 x 100 mm (200 mm total) Halo Peptide ES-C18; Flow rate: 0.5 mL/min.; Gradient: 5-65% B in 120 min.; 0.1% TFA; B: 80% acetonitrile/0.1% TFA; Temperature: 45 ^{o}C; Injection volume: 15 µL (15 µg).

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Typical Analysis Times for Peptide and Tryptic Digest Separations

- Sample Dependent
  - Simple proteomics sample: 30 - 45 min gradients
  - Complex tryptic digest: 45 min - 3 hour gradients
    - Many tens to thousands of proteins, leading to hundreds of thousands of components

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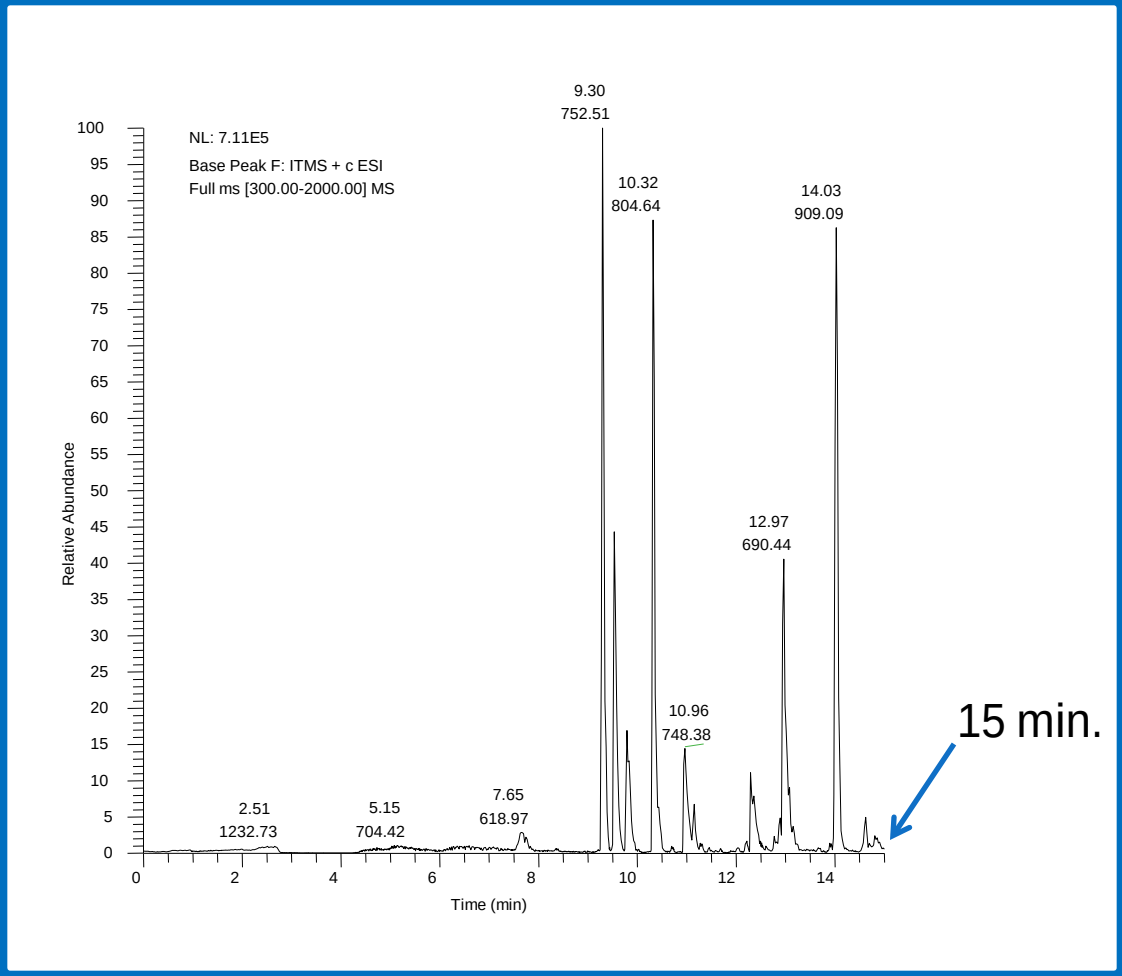
High mobile phase velocity LC/MS analysis of an apomyoglobin tryptic digest

Column: 0.2 x 50 mm Halo Peptide ES-C18; Flow rate; 9 µL/min; Gradient: 2 - 45% B in 15 min; A: 0.1% formic acid/water; B: acetonitrile/0.1% formic acid; Maximum pressure: 330 bar; Sample- 3 pmol apomyoglobin digest.



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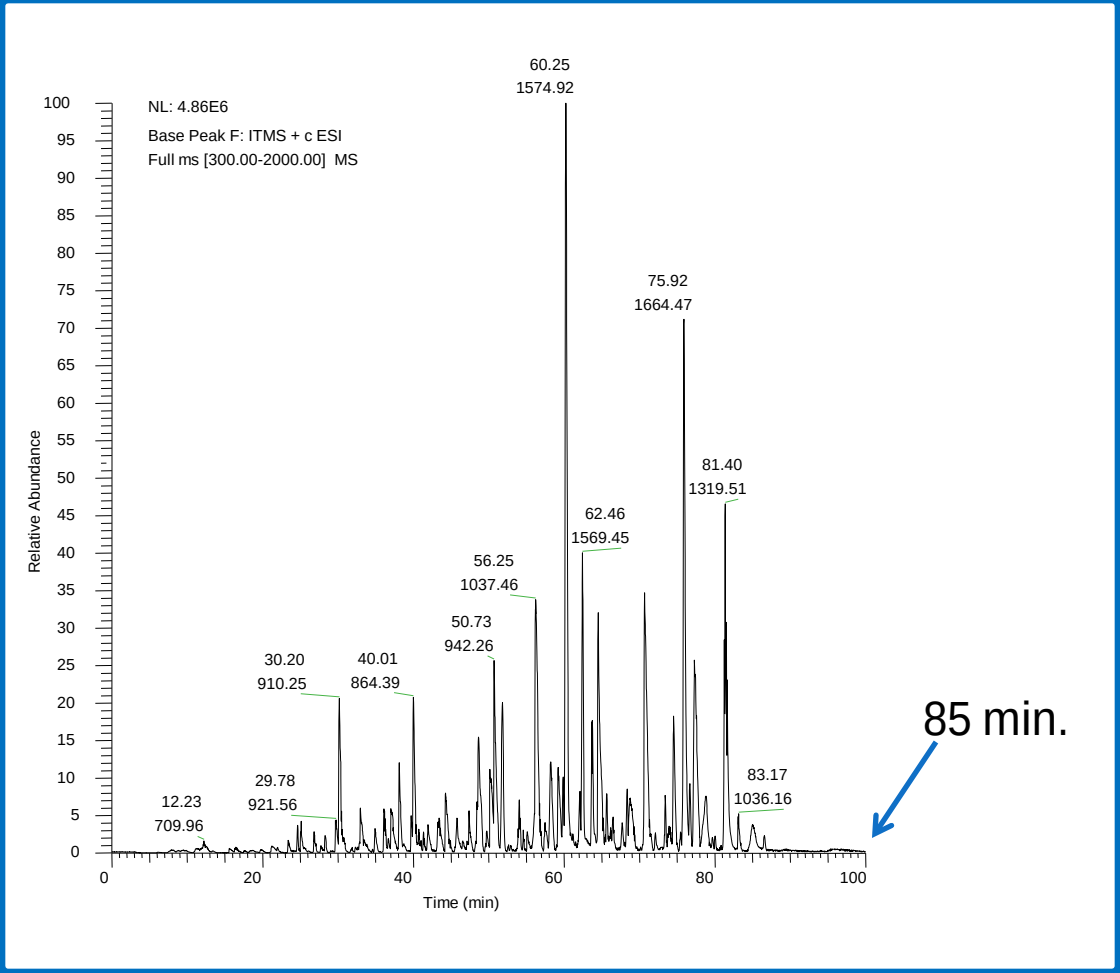
High mobile phase velocity LC/MS analysis of mixed protein digests

Column: 0.2 x 150 mm Halo Peptide ES-C18; Flow rate: 4 µL/min; Gradient: 2 - 45% B in 85 min; A: 0.1% formic acid/water; B: acetonitrile/0.1% formic acid; Maximum pressure: 320 bar; Sample: mixed protein digest (5 pmol total of transferrin, carbonic anhydrase, and apomyoglobin).



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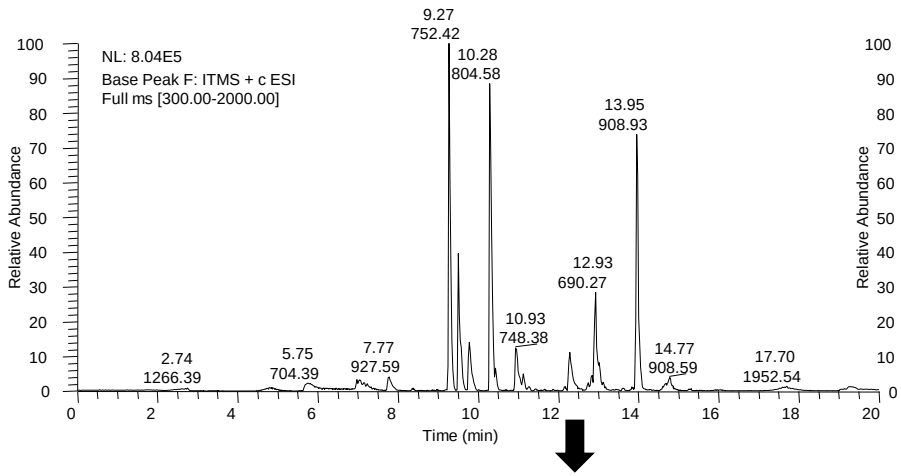
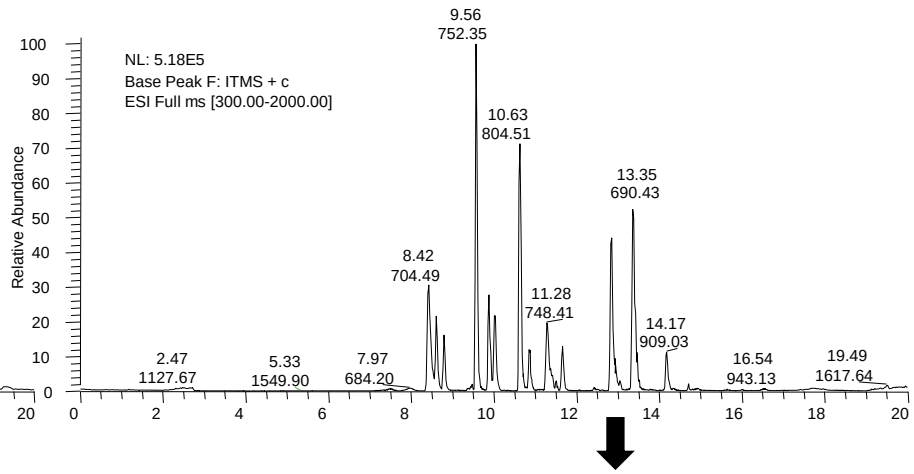
Ammonium formate as an additive for LC/MS separations

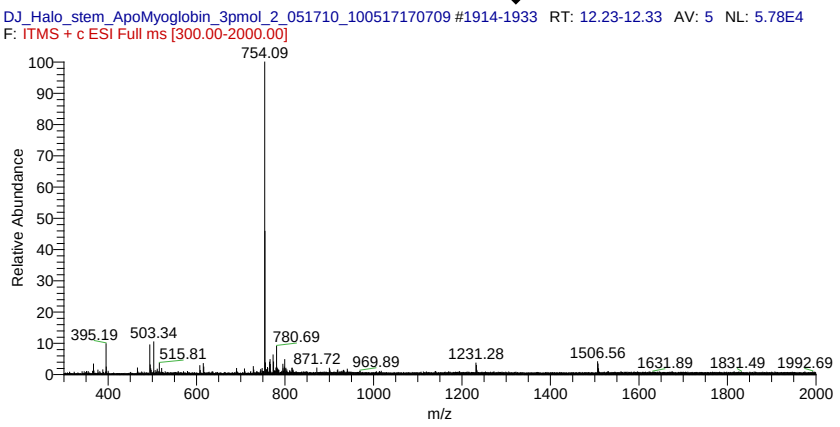
Column: 0.2 x 50 mm Halo Peptide ES-C18; Flow rate: 9 µL/min; Gradient: 2 - 45% B in 15 min; Mobile phases as shown; Sample: 2 µL (3 pmol) apomyoglobin digest.

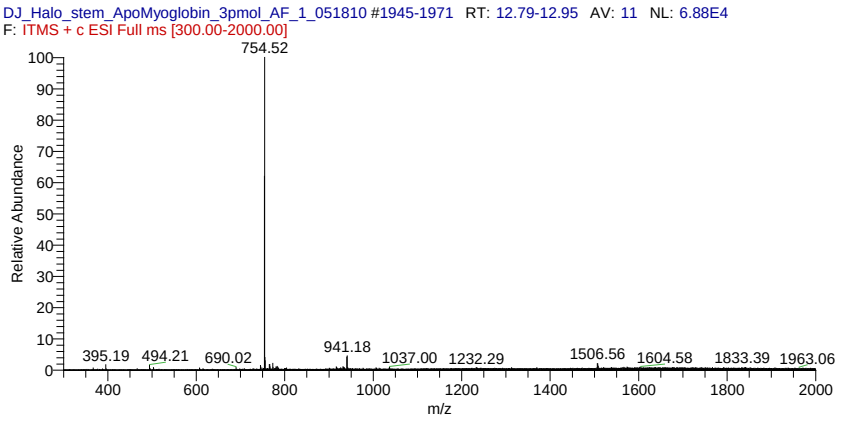
A: 0.1 % Formic Acid



B: 0.1 % Formic Acid/10 mM Ammonium Formate







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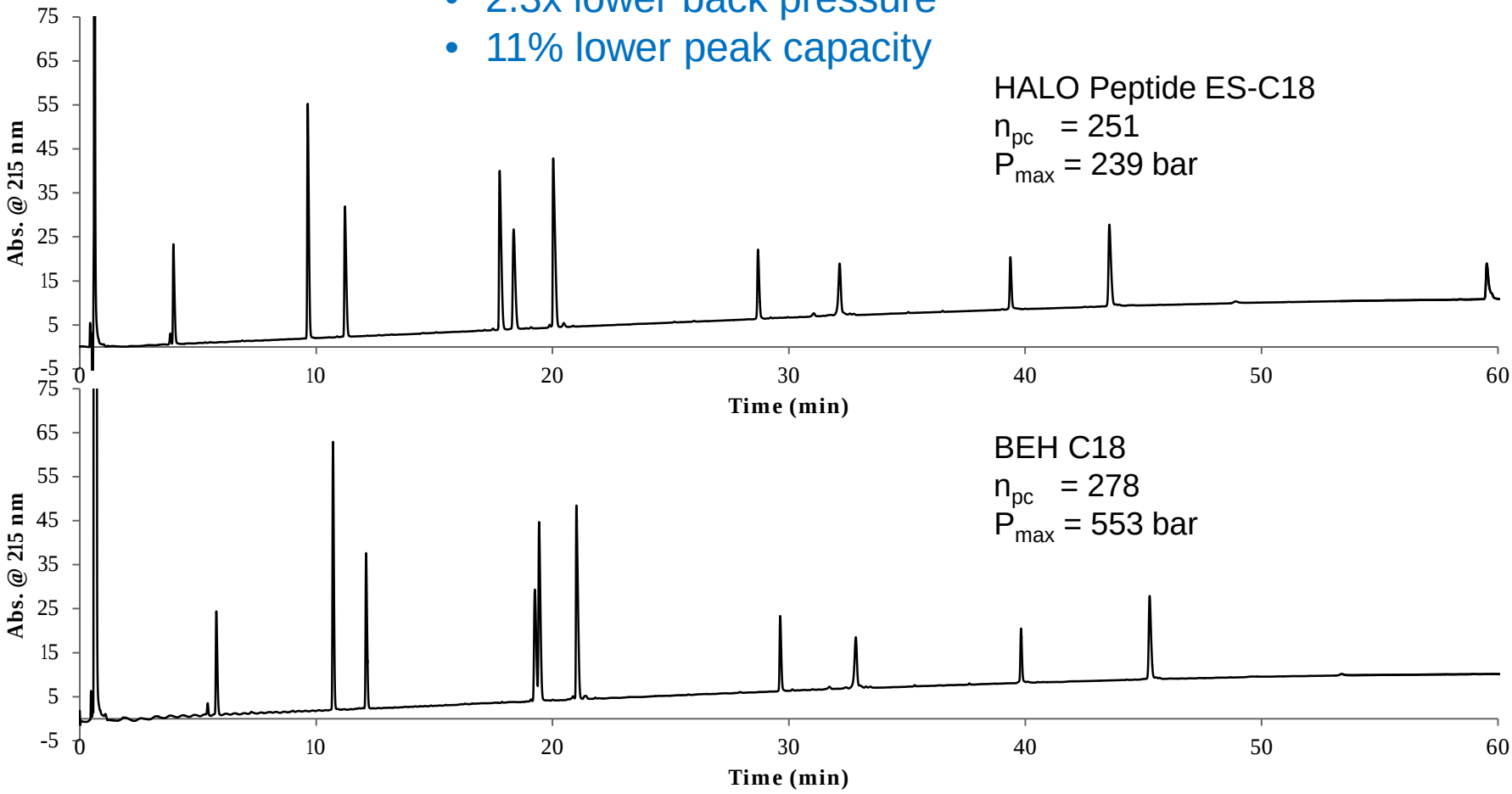
Peak Capacity

- Peak capacity is the maximum number of peaks that can be resolved with $R_s = 1$ in a given chromatographic space
- The higher the peak capacity of a given separation, the more likely it is that the peaks will be resolved in a complex mixture
- Peak capacity [n_{pc}] calculated as $\frac{t_f - t_i}{W_{4\sigma}}$
  - t_i = time of the initial measurable peak in the gradient
  - t_f = time of the final peak
  - W_{4s} = average of the 4s widths of the peaks in the chromatogram
- 11 peptides used for the calculation of peak capacity
  - Span a wide range of hydrophobicities to mimic an actual tryptic digest
  - Asp-Phe, Tyr-Tyr-Tyr, angiotensin (1-7) amide, bradykinin, Leu-Enk, angiotensin II, angiotensin 1-12 (human), beta-endorphin, sauvagine, melittin, and Hel 11-7

Peak Capacity Comparisons

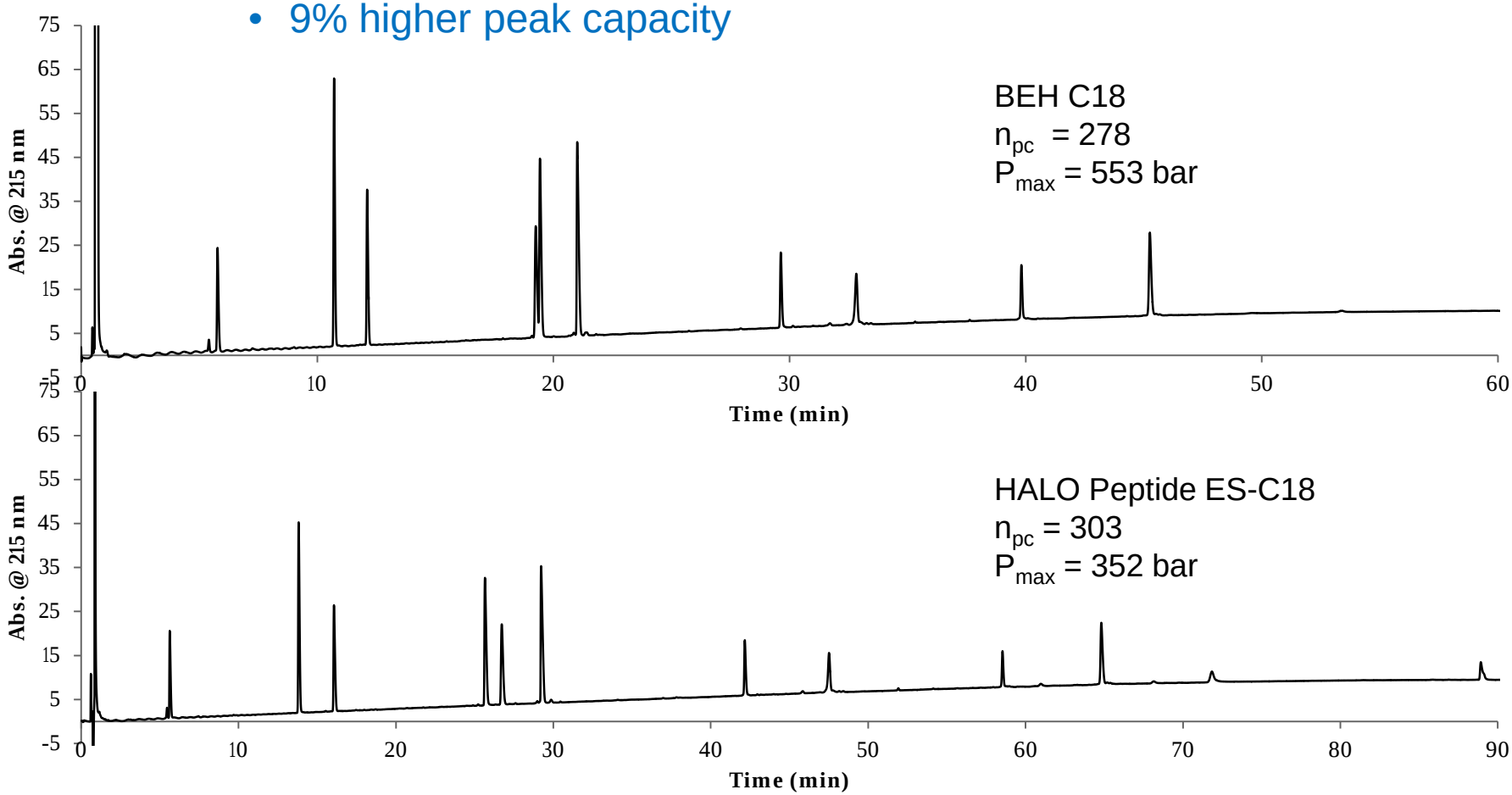
- 2.3x lower back pressure
- 11% lower peak capacity



Columns: 2.1 x 100 mm Halo Peptide ES-C18, 2.1 x 100 mm BEH C18; Flow rate: 0.5 mL/min; Temperature 45 C; Gradient: 5 - 70% B in 60 min; A: water/0.1% trifluoroacetic acid; B: 80/20 acetonitrile/water/0.1% trifluoroacetic acid; Sample: 15 µL of 11 synthetic peptides (50 ng each).

Peak Capacity Comparisons

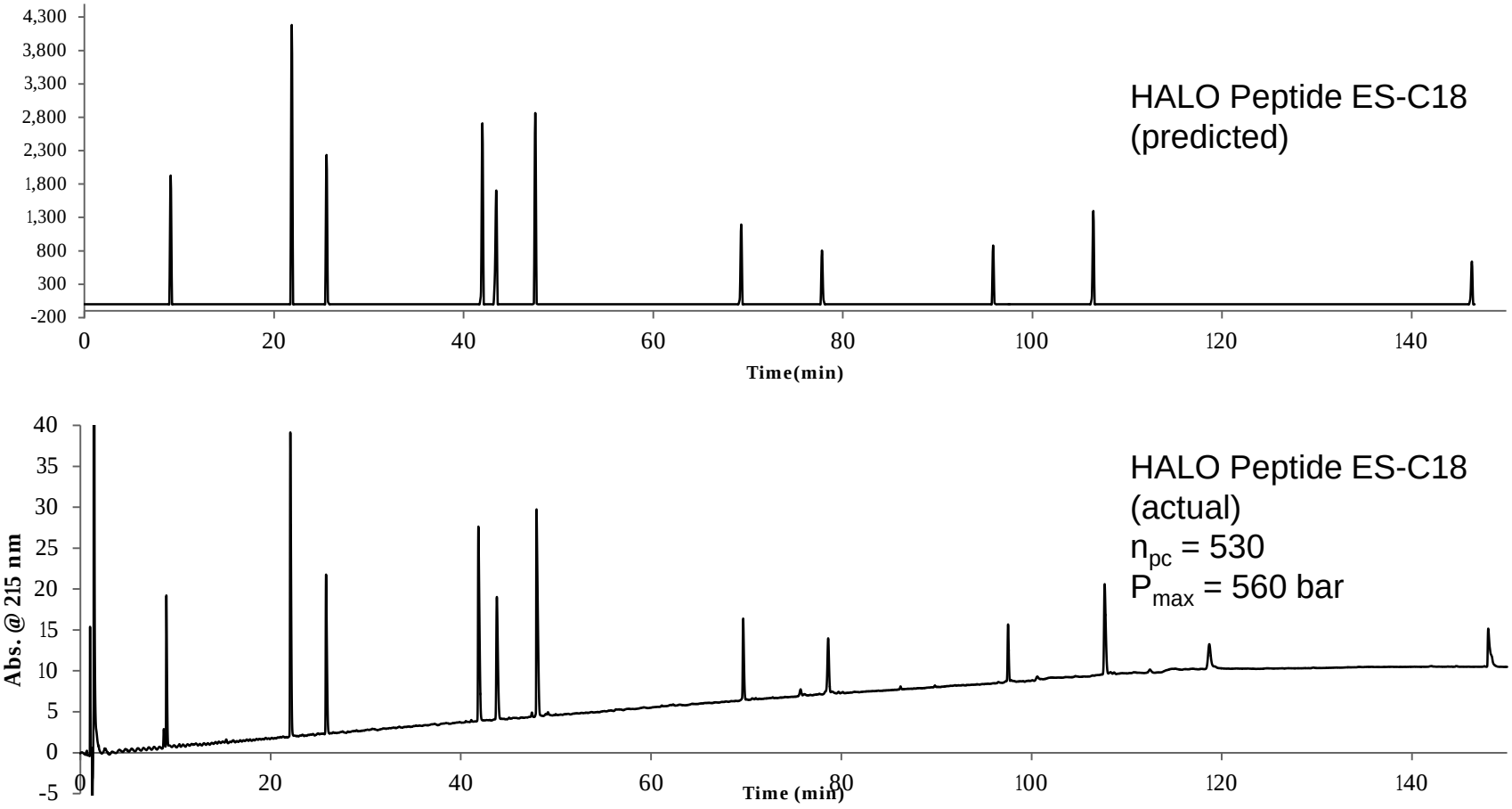
- Increased column length and gradient time by 1.5x
- 1.57x lower back pressure
- 9% higher peak capacity



Columns: 2.1 x 100 mm BEH C18, 2.1 x 150 mm HALO Peptide ES-C18; Flow rate: 0.5 mL/min; Temperature 45 C; Gradient: 5 - 70% B in 60 or 90 min; A: water/0.1% trifluoroacetic acid; B: 80/20 acetonitrile/water/0.1% trifluoroacetic acid; Sample: 15 µL of 11 synthetic peptides (50 ng each).

DryLab Optimization of Peak Capacity

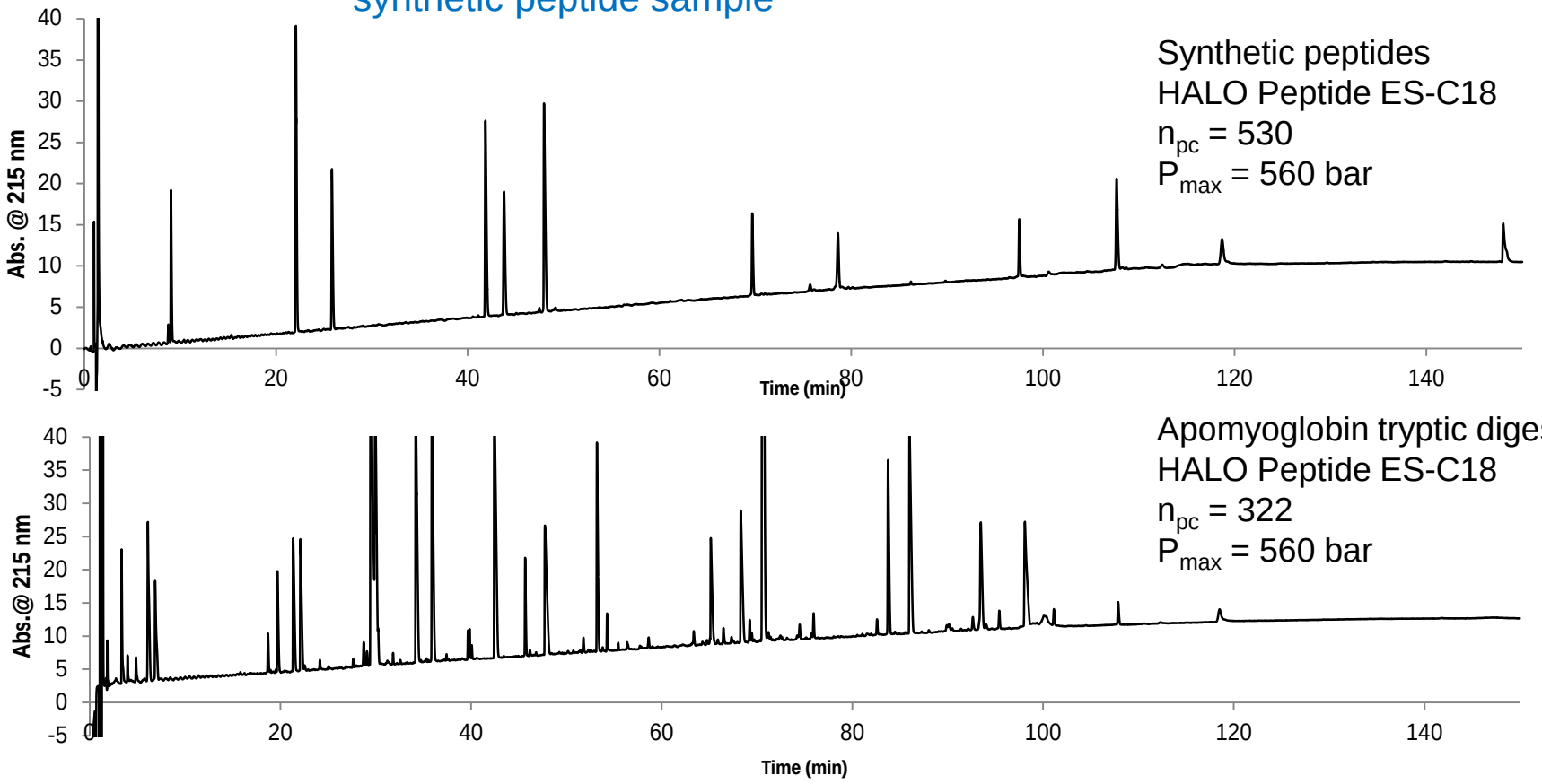
- Predicted chromatogram matches well with the actual



Columns: 2.1 x 100 mm Halo Peptide ES-C18 and 2.1 x 150 mm Halo Peptide ES-C18; Flow rate: 0.5 mL/min; Temperature 45 C; Gradient: 5 - 70% B in 150 min; A: water/0.1% TFA; B: 80/20 acetonitrile/water/0.1% trifluoroacetic acid; Sample: 15 µL of 11 synthetic peptides (50 ng each); Detection: 215 nm.

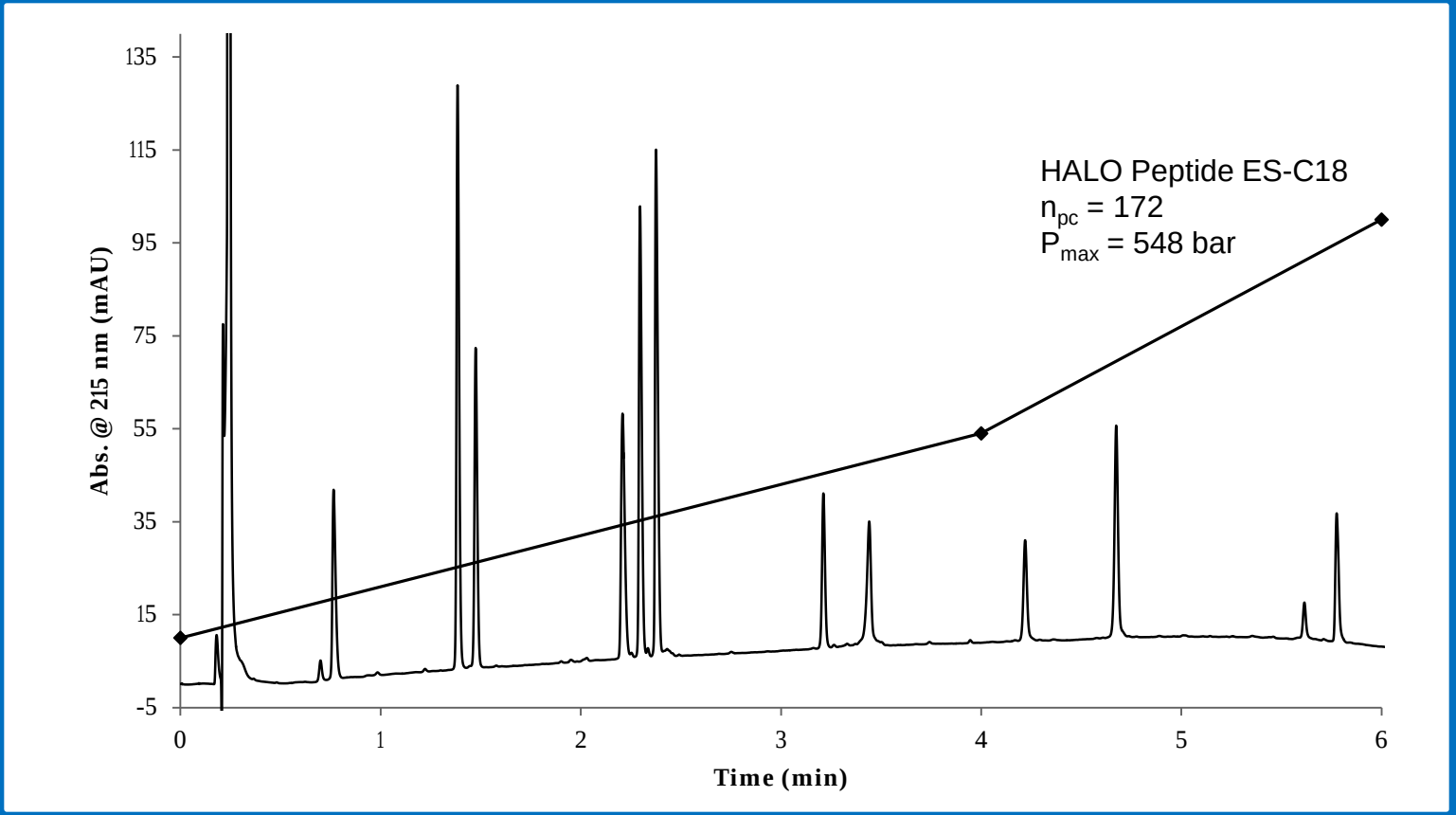
DryLab Optimization of Peak Capacity: Synthetic Peptides vs. Tryptic Digest

- Tryptic digest sample has ~ 60% of the peak capacity of the synthetic peptide sample



Columns: 2.1 x 100 mm Halo Peptide ES-C18 and 2.1 x 150 mm Halo Peptide ES-C18; Flow rate: 0.5 mL/min; Temperature 45 C; Gradient: 5 - 70% B in 150 min; A: water/0.1% TFA; B: 80/20 acetonitrile/water/0.1% trifluoroacetic acid; Sample: 15 µL of 11 synthetic peptides (50 ng each), or 15 µL apomyoglobin digest (15 µg); Detection: 215 nm.

172 Peak Capacity in < 6 Minutes



Fast separation of 11 peptides in 6 minutes with 2.9 minimum resolution. Column: 2.1 x 100 mm Halo Peptide ES-C18; Flow rate: 1.25 mL/min; Temperature 45 C; Gradient: 10 - 54% B from 0-4 min. 54-100% B from 4-6 min; A: water/0.1% TFA; B: 80/20 acetonitrile/water/0.1% trifluoroacetic acid; Sample: 15 µL of 11 synthetic peptides, 50 ng each; Detection: 215 nm.

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Conclusions and Summary

- Fused-core HALO Peptide provides a better solution for increasing the speed of biomolecule separations
  - Particle size distribution remains sharp as compared to the smaller pore size fused-core particles
  - Stability is high at low pH and elevated temperatures due to the nature of the sterically protected bonded phase
  - Increased pore size material shows lower resistance to diffusion into the pores for larger molecules, as reflected by increased column efficiency at high mobile phase velocity
  - Provides ultra fast, high resolution data with low back pressure
  - High throughput methods are possible due to the faster gradients that can be employed while still maintaining high resolution separations
  - Peak capacity is within 10% of sub-2-µm particle column performance, but with less than half the back pressure

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