

High-Resolution Peptide Mapping Using Sub-Two Micron Columns

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Introduction

The analysis of proteins and peptides by RP-HPLC is important in the development of well-characterized biotechnology pharmaceuticals. Several factors influence sensitivity, resolution, and retention for LC and LC-MS applications: bonding chemistry, pore size, particle size, column configuration, and solvent composition. By decreasing the particle size of LC packings, column efficiency is increased. We demonstrate the use of 50 to 150mm length LC columns packed with 1.5µm, small pore C18 silica for high-resolution protein digest analysis performed on either conventional or high-speed/ultra-high pressure LC systems.

Protein Digest Protocol

1. Lyophilized sample solubilized in 6M urea, 100mM Tris buffer, pH 7.5
2. Reduced with 200mM dithiothreitol in 100mM Tris
3. Alkylated with 200mM iodoacetamide in 100mM Tris
4. Digested with trypsin solution (Sigma-Aldrich Trypsin Singles, Proteomics Grade)
5. Desalted using C18 SPE cartridges (Vydac® 218SPE1000 columns), concentrated to near dryness and dissolved in 5:95 Acetonitrile:Water containing 0.2% formic acid, 0.01% TFA

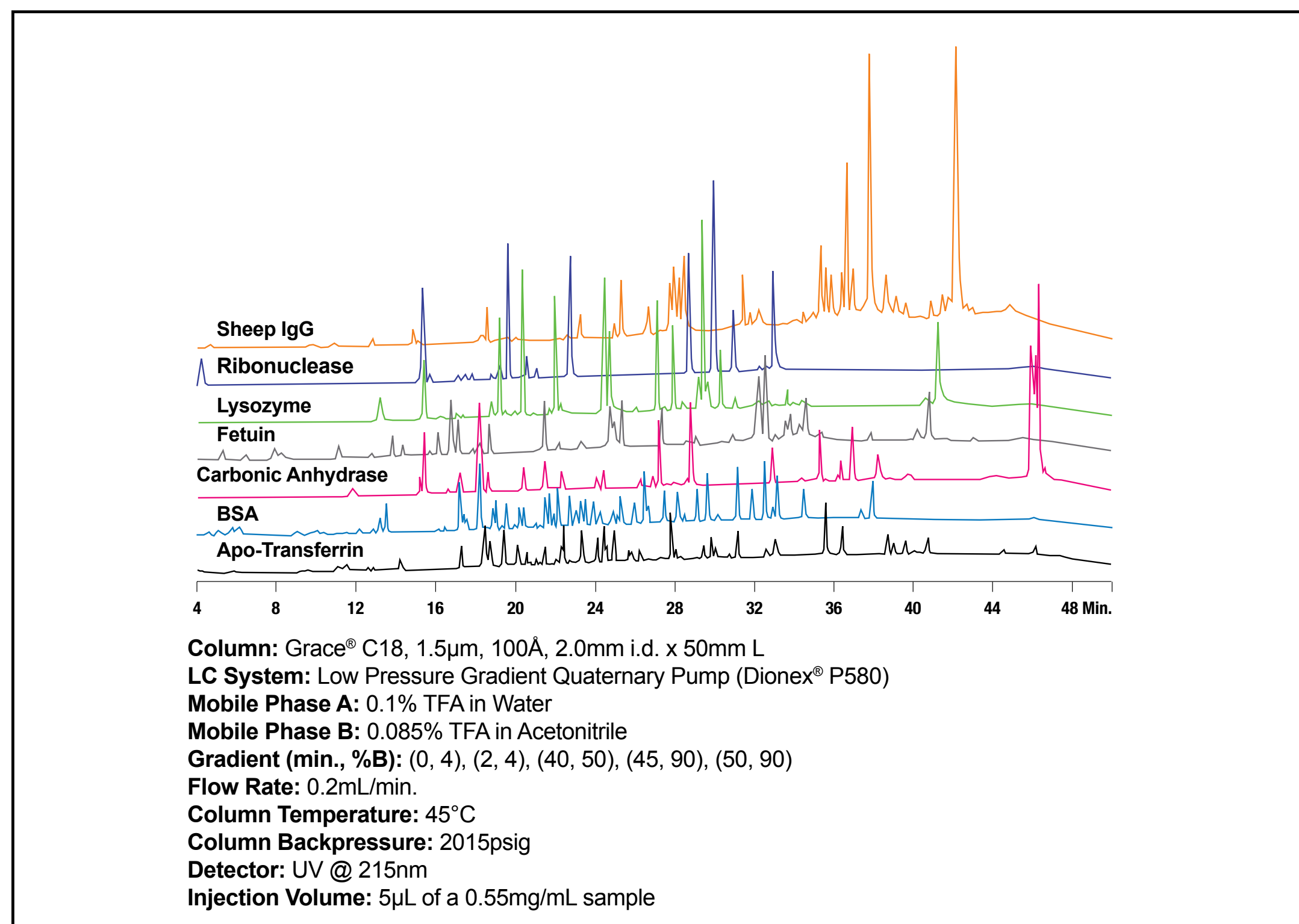
Proteins Digested

Apo-Transferrin (76 kDa)
Bovine Serum Albumin (66 kDa)
Carbonic Anhydrase, Bovine (29 kDa)
Fetuin (glycoprotein) (38 kDa)
Lysozyme (14.3 kDa)
Ribonuclease (13.7 kDa)
Sheep IgG Antibody (150 kDa)

Conventional LC Setup

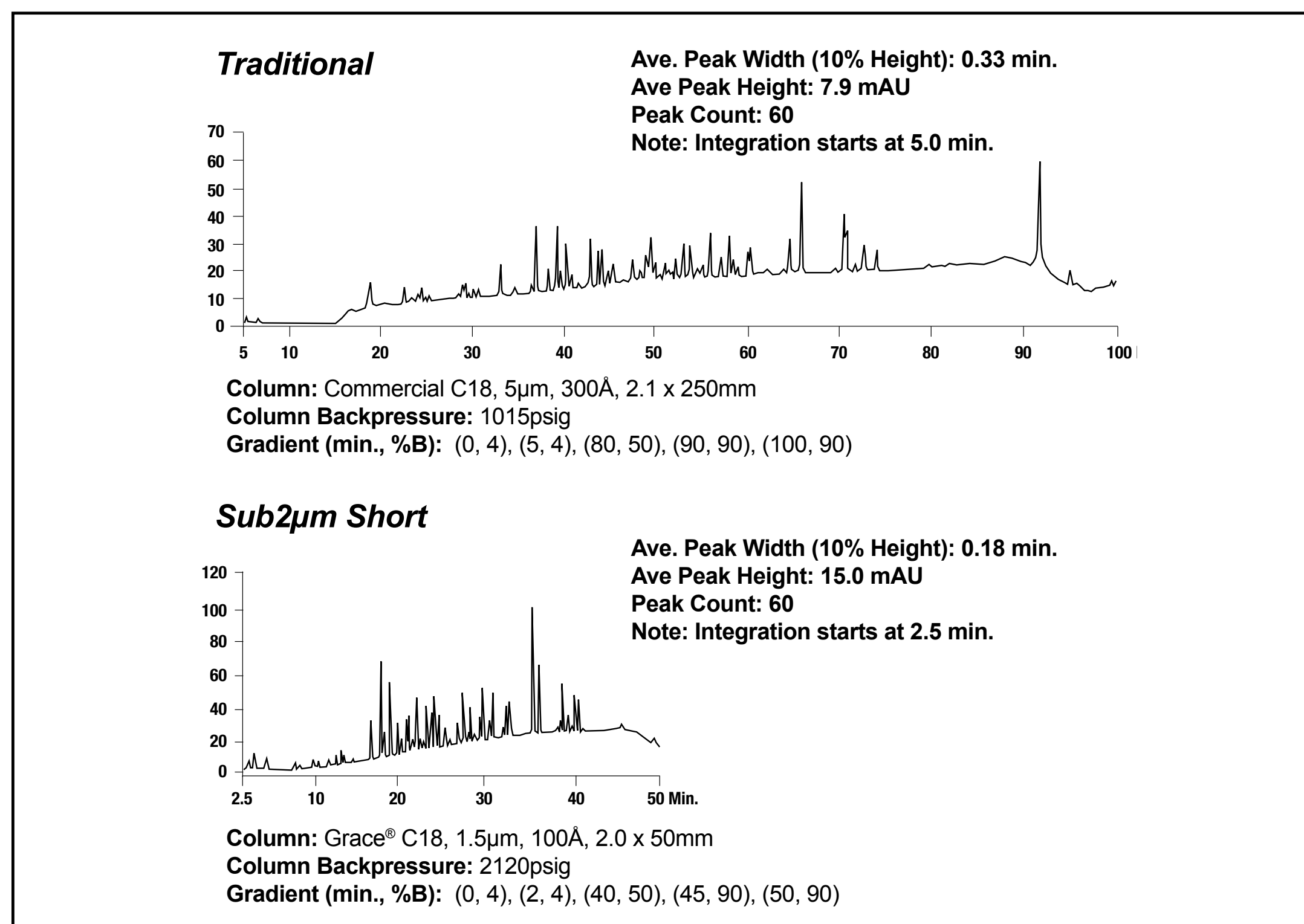
- No ultra-high pressure LC system was required for a 2 x 50mm column packed with 1.5µm media under the following conditions: (1) flow rate < 0.2mL/min.; (2) column heated to 45°C
- In this application, a quaternary pump system, commonly found in many “peptide mapping” laboratories, was used. A binary pump for precise, high-pressure mixing gradient formation and low delay volume is recommended over the quaternary pump for the fastest separations.
- Extra system volume was minimized by using the following:
 - Short tubing from injector-to-column and from column-to-detector with internal i.d. of 0.12mm (0.005")
 - Micro-capillary flow cell with volume of 0.14µL
 - 5µL injector loop

Peptide Mapping on Sub2µm using Conventional LC



Applying a temperature of 45°C, a conventional HPLC was used for high-resolution peptide mapping on a C18 1.5µm, 2 x 50mm column, with a maximum backpressure of 2000psig.

Apo-Transferrin Digest on Sub2µm Short vs. Traditional Column on Conventional LC

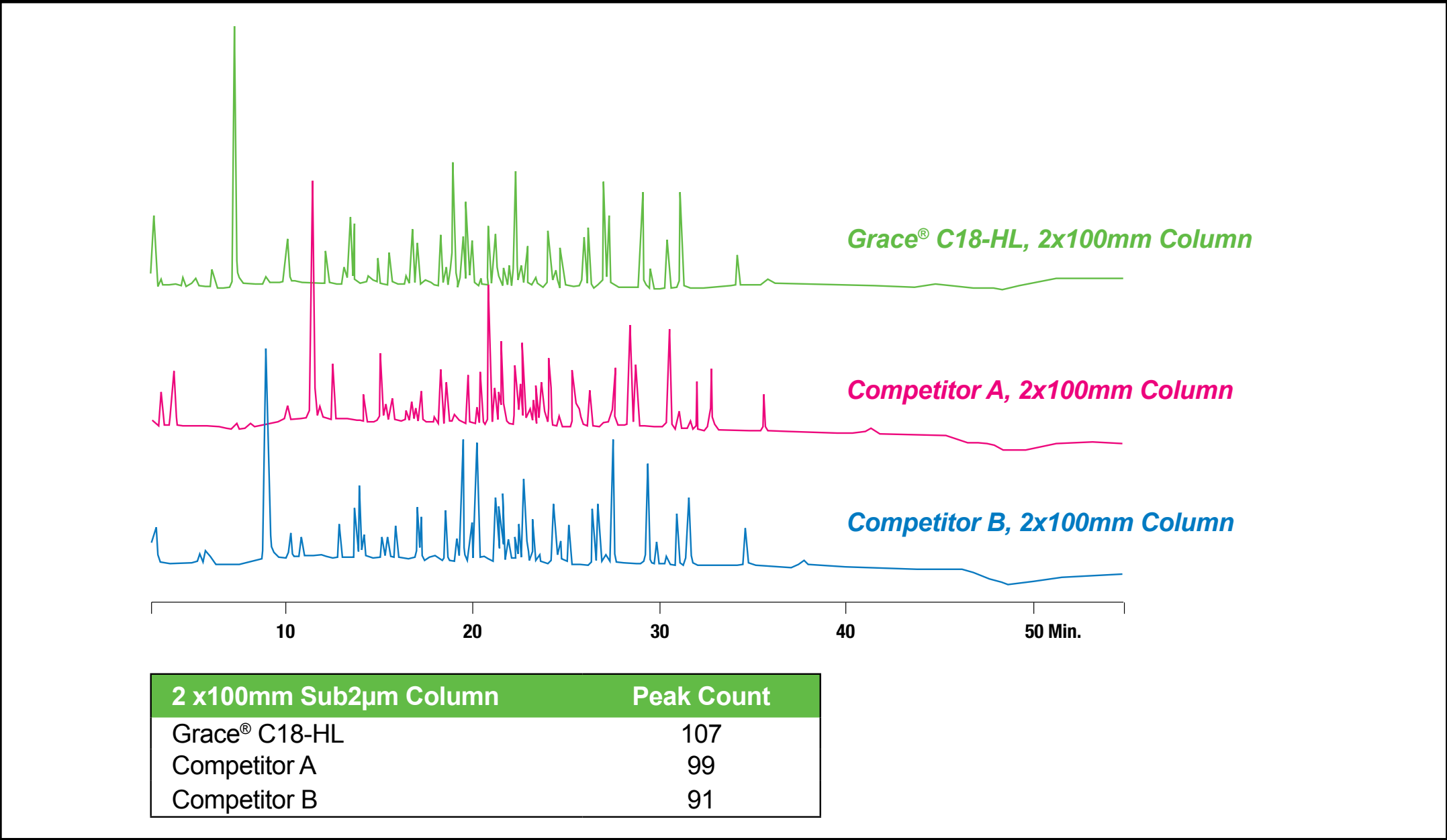


Equivalent peak count was observed for the 1.5µm, 2 x 50mm column in half the time typically used for a traditional 5µm, 2 x 250mm column. On the 1.5µm, 2 x 50mm column, peak width was almost half and peak height was about double, contributing to better sensitivity.

High-Speed/UHPLC Setup for Peptide Mapping

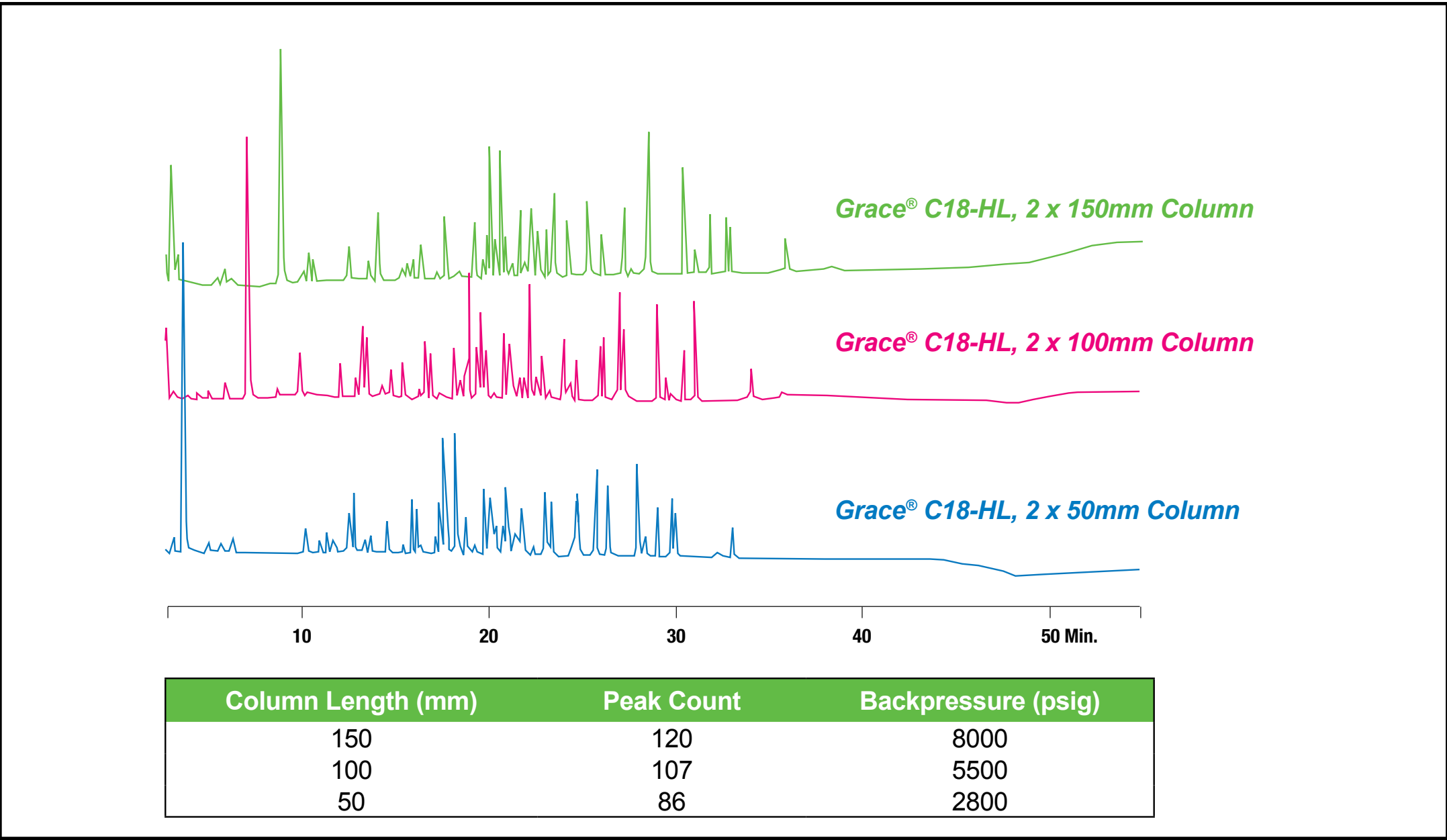
LC System: High-Speed Gradient Binary Pump (Agilent RRLC1200)
Mobile Phase A: 0.1% TFA in Water
Mobile Phase B: 0.085% TFA in Acetonitrile
Gradient (min., %B): (0, 4), (2, 4), (40, 50), (45, 90), (55, 90)
Flow Rate: 0.2mL/min.
Column Temperature: 30°C
Detector: UV @ 215nm
Injection Volume: 5µL of a 0.55mg/mL sample
Peak Integration: starts at 2.5 minutes

Grace® Column vs. Other UHPLC Columns: BSA Digest



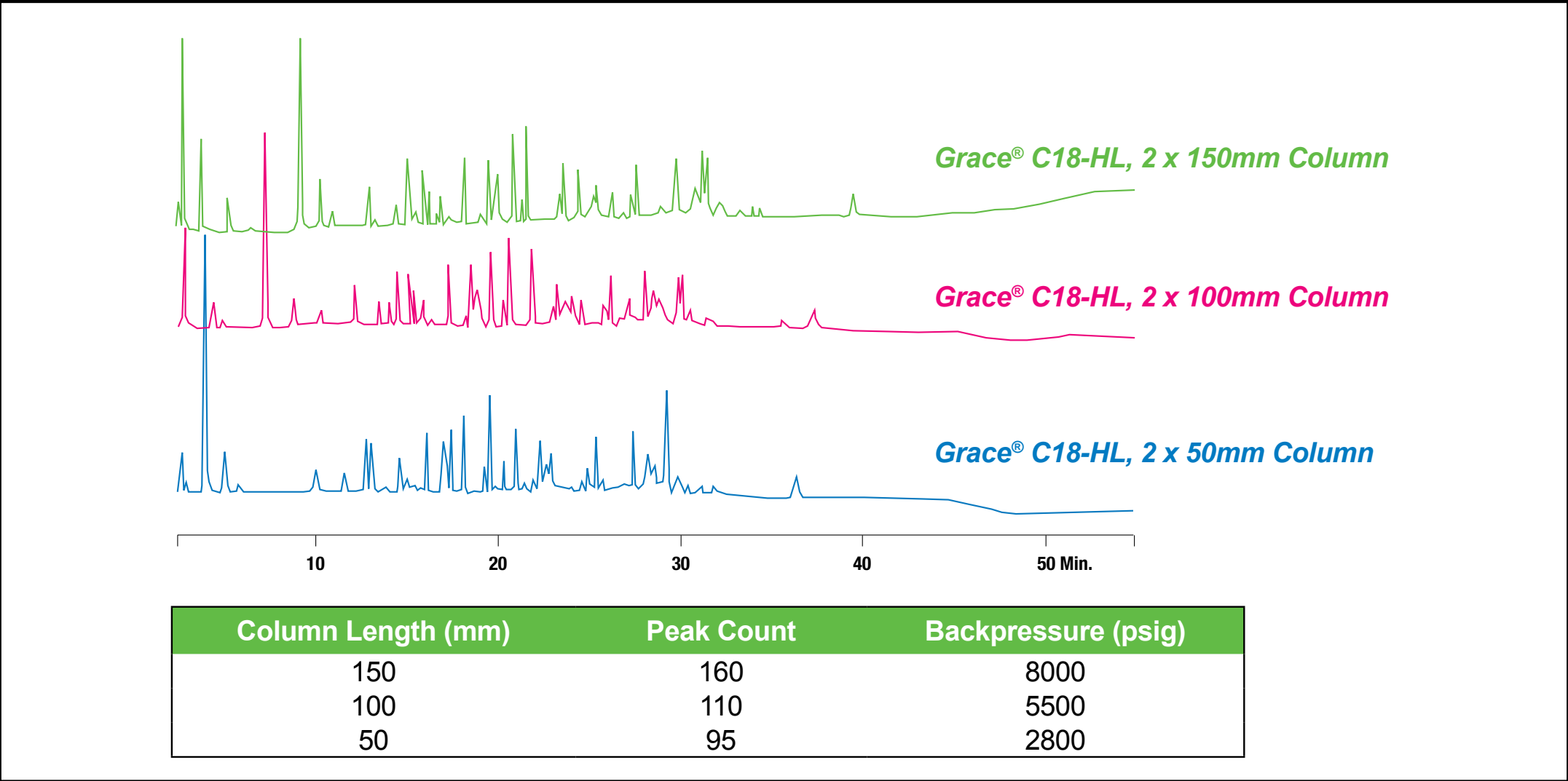
Excellent resolution for a BSA digest sample run on a Grace® column compared to other commercially available UHPLC columns.

Effect of Column Length using High-Speed/UHPLC: BSA Digest



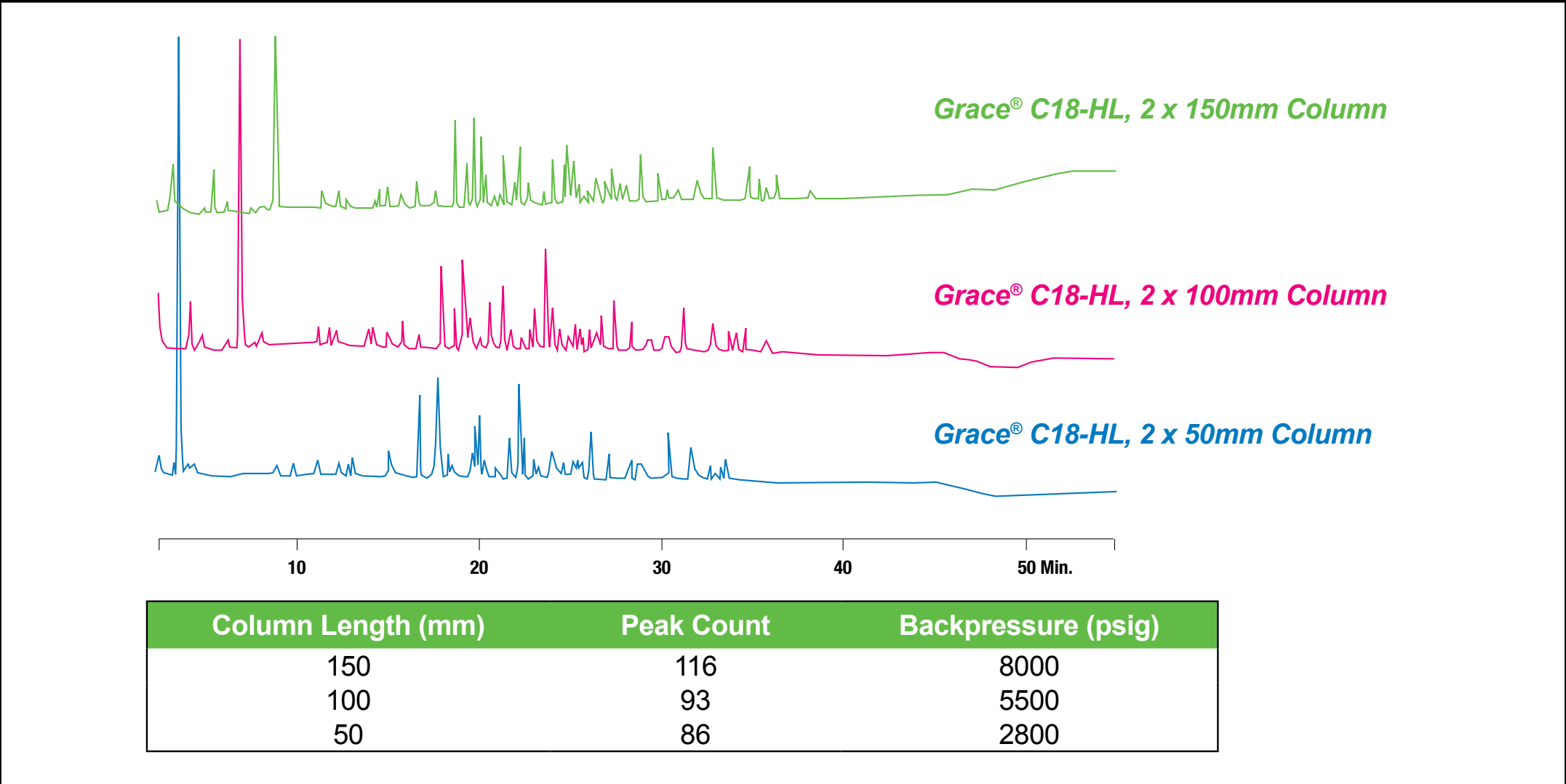
When a UHPLC system is available, the 2 x 150mm column is recommended. Resolution increased 40% when switching from a 50 to 150mm length column.

Effect of Column Length using High-Speed/UHPLC: Fetuin Digest



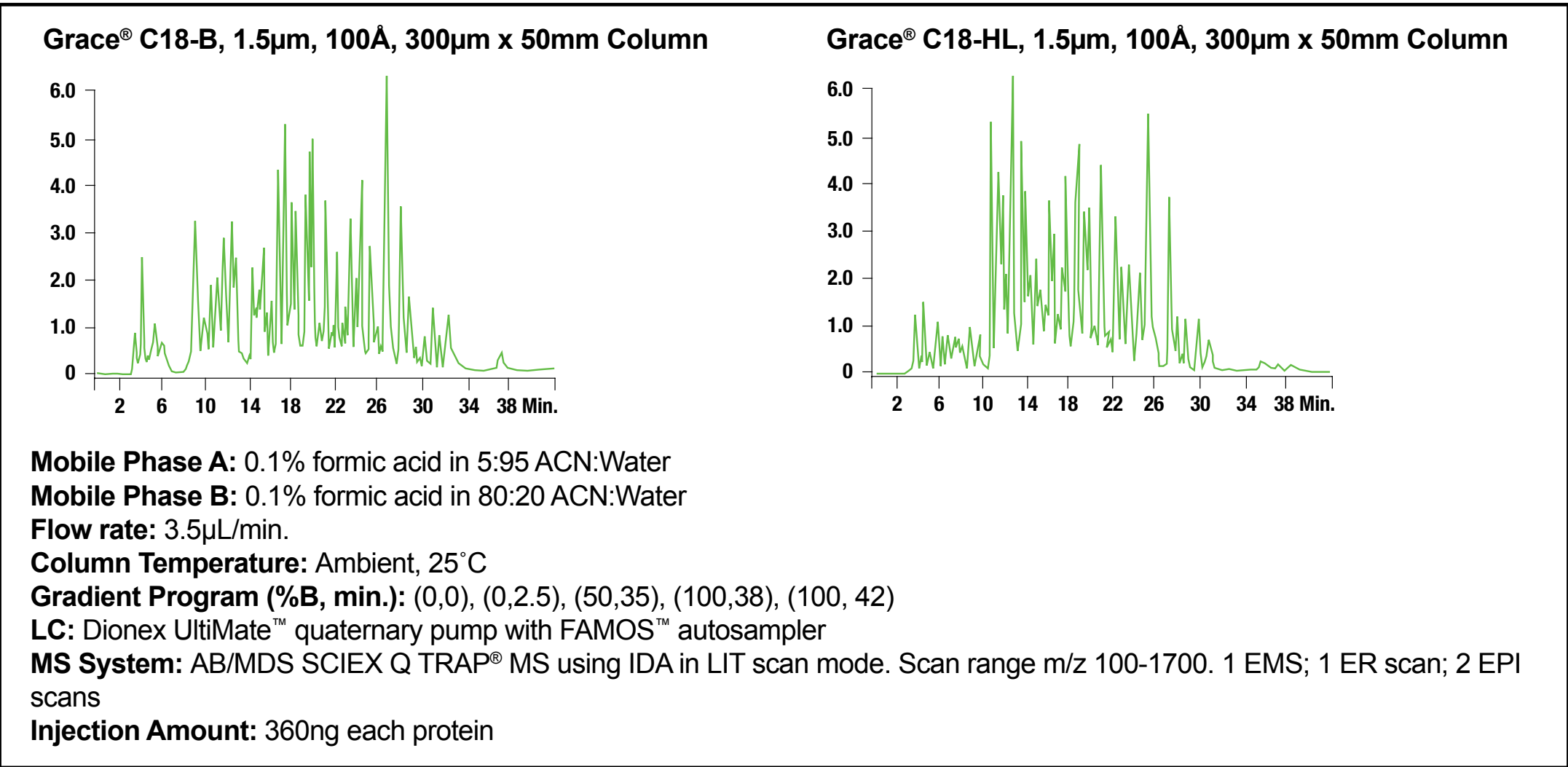
Resolution increased 68% when switching from a 50 to 150mm length column.

Effect of Column Length using High-Speed/UHPLC: Apo-Transferrin Digest



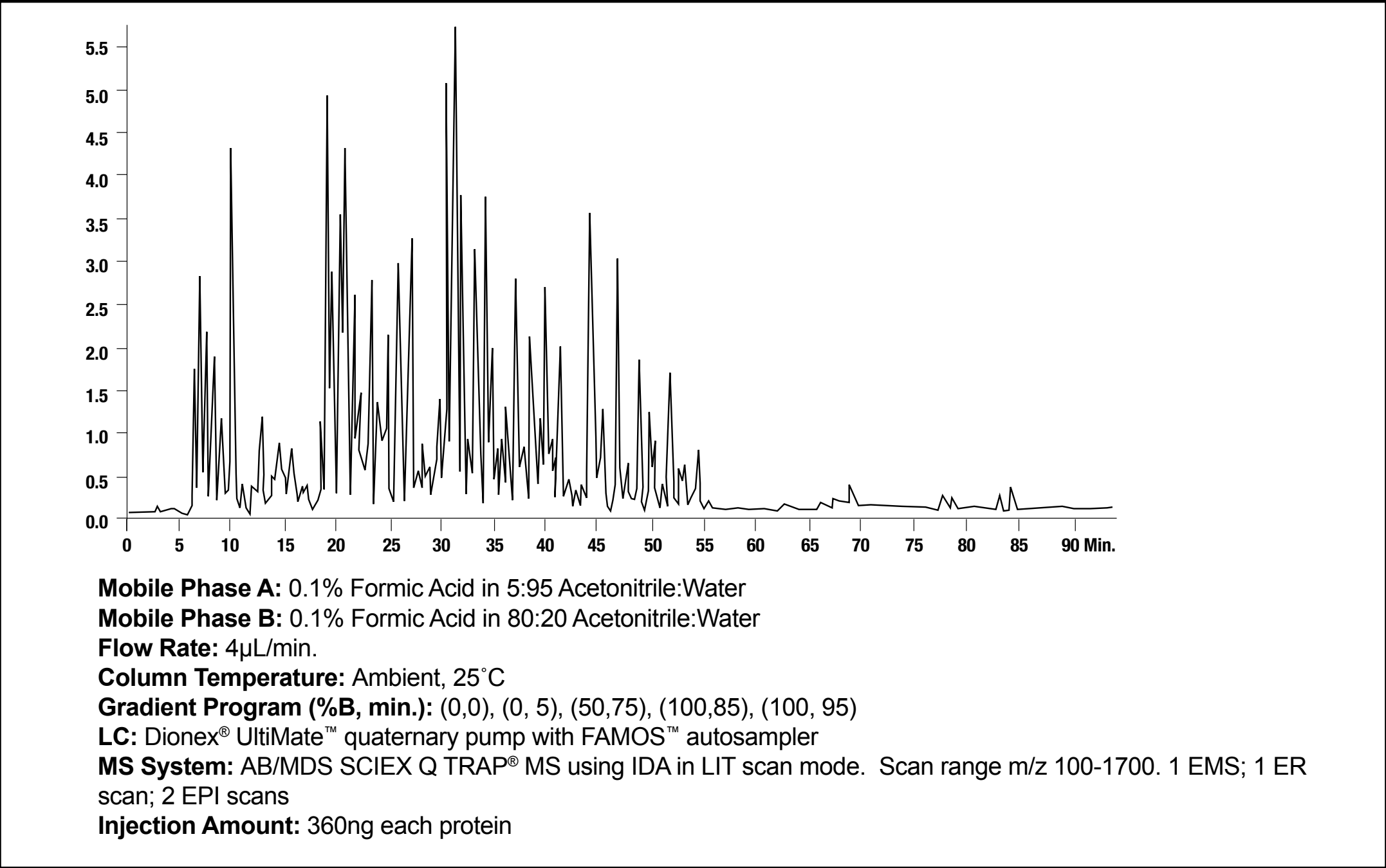
Resolution increased 35% when switching from a 50 to 150mm length column.

LC-MS/MS of Digest of Six Proteins (Mixed) using Conventional Capillary LC



Fast, high-resolution separation on capillary sub2µm columns was achieved with a quaternary HPLC interfaced to a MS.

Commercial C18, 5µm, 300Å, 300µm x 150mm



Mascot Results for Sub2µm, Short Capillary Columns vs. Traditional Column

Protein Hits	Grace® C18-B, 1.5µm, 100Å, 300µm x 50mm Column			Grace® C18-HL, 1.5µm, 100Å, 300µm x 50mm Column			Commercial C18, 5µm, 300Å, 300µm x 150mm		
	Score	Queries Matched	Sequence Coverage (%)	Score	Queries Matched	Sequence Coverage (%)	Score	Queries Matched	Sequence Coverage (%)
Serum Albumin Precursor – Bovine	923	19	35	984	22	39	1066	23	36
Lysozyme (EC 3.2.1.17) C Precursor – Chicken	508	9	69	486	9	59	556	11	74
Carbonic Anhydrase 2 (EC 4.2.1.1) – Bovine	414	10	50	505	10	53	359	10	50
Fetuin Precursor - Bovine	382	8	37	323	7	23	339	12	25
Transferrin Precursor – Human	371	9	14	393	11	16	613	16	24
IgG Heavy Chain C Region - Sheep (fragment)	98	4	21	90	3	16	82	3	34
Ovis Aries Immunoglobulin Lambda Light Chain Constant Region	58	2	25	145	3	39	119	4	44

Good Mascot scores and sequence coverage with 1.5µm, 100Å, 300µm x 50mm columns was possible in half the time applied for a 5µm, 300Å, 300µm x 150mm column.

Conclusion

- No ultra-high pressure LC system was required for a 2 x 50mm column packed with 1.5µm media under the following conditions: flow rate of 0.2mL/min.; and column heating at 45°C. Although the binary pump is recommended for fastest separations, a quaternary pump may be used.
- Using a quaternary HPLC, we observed the same resolution (based on equivalent peak count) for a 1.5µm, 2 x 50mm column in half the time generally applied for a traditional 5µm, 2 x 250mm column. On the 1.5µm, 2 x 50mm column, peak width was almost half and peak height was about double, contributing to better sensitivity.
- For protein digests, the best resolution was achieved with a high-speed/UHPLC system using a column length of 150mm. Resolution was observed to increase significantly (35 to 65% higher) with an increase in column length (from 50 to 150mm).
- For 300µm x 50mm capillary LC-MS columns packed with 1.5µm media, it was possible to achieve relatively fast separations with good sequence coverage of proteins at 3.5µL/min. at room temperature, using a conventional capillary HPLC system.

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