Selection of Reversed-Phase Chemistries, Column Configurations, and Ion Pairing Agents for LC-MS Analysis of Peptides and Proteins

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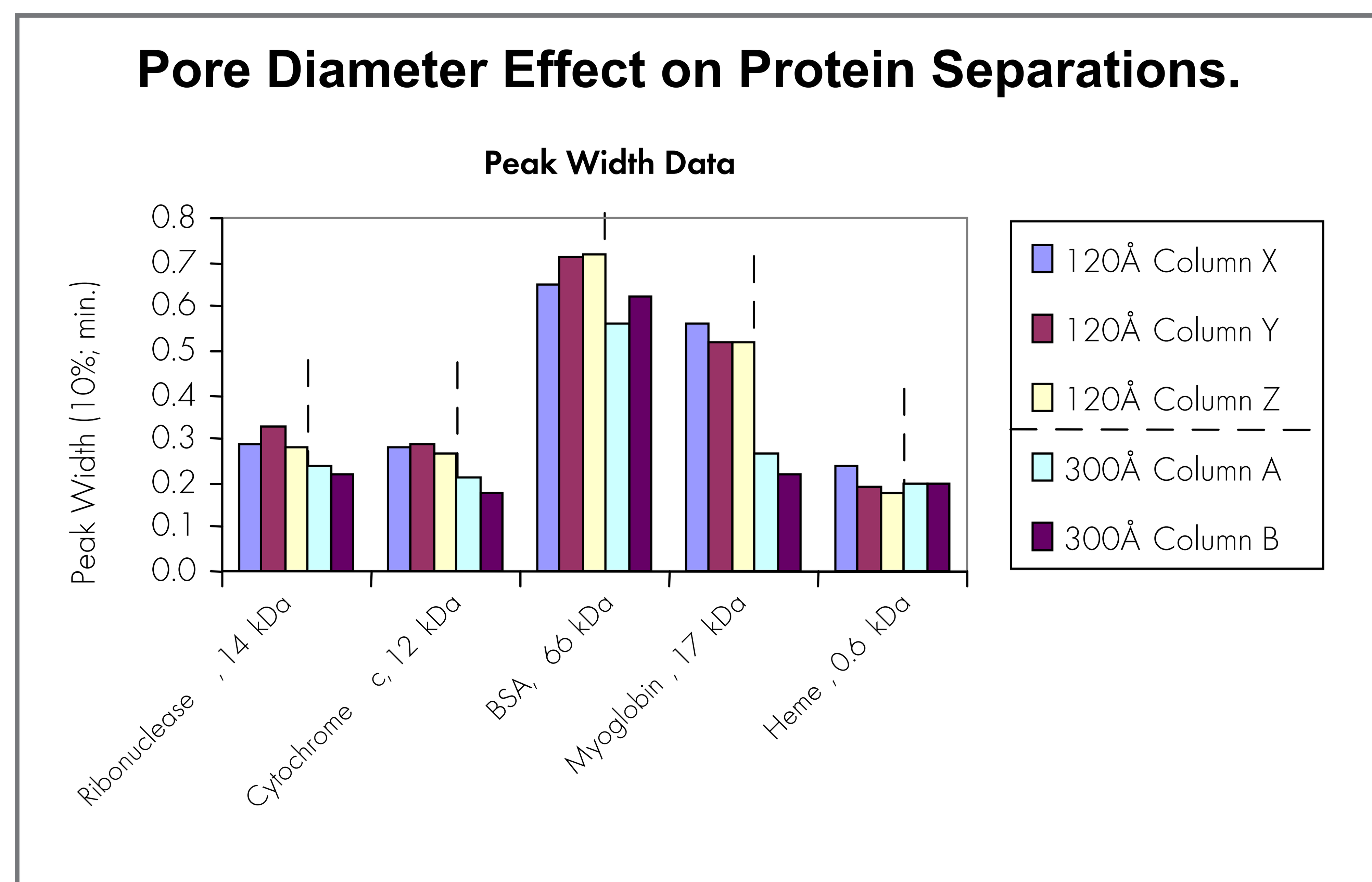
Introduction

The analysis of proteins and peptides by reversed-phase HPLC coupled to MS is important in the development of well-characterized biotechnology pharmaceuticals. Several factors influence sensitivity, resolution, and retention for LC-MS applications: bonding chemistry, pore size, particle size, column configuration, and solvent composition. These factors must be considered to ensure development of the best analytical methods.

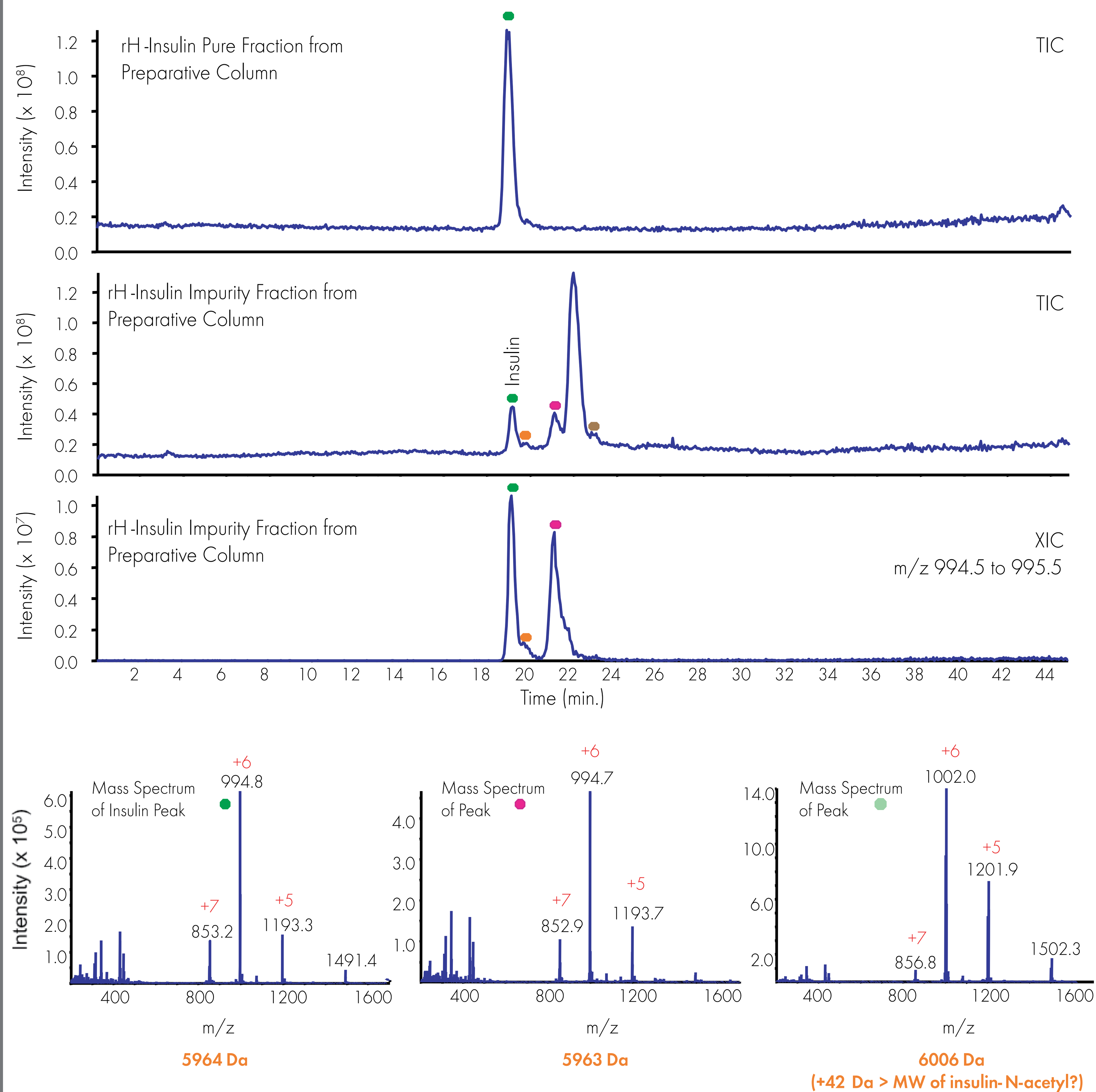
Experimental

Micro-bore (1.0 mm i.d.) to capillary (300 to 75µm i.d.) LC-MS analyses were performed on an AB/MDS-SCIEX Q TRAP® using a solvent system comprised of acetonitrile/water. Single or combinations of ion pairing agents (formic acid- FA, trifluoroacetic acid- TFA, heptafluorobutyric acid- HFBA) were used. C4 and C18 bonding chemistries on 120 or 300Å silica with particle sizes of 1.5 or 5µm were packed in various length columns. Sensitivity, resolution, and retention were assessed using a variety of samples including serum albumin and cytochrome tryptic digests, histone peptide fragment, snail venom peptides, human growth hormone, hemoglobin, recombinant human insulin, ribonuclease, ovalbumin, conalbumin, myoglobin, cytochrome c, oxytocin, bradykinin, angiotensin I & II, neurotensin, and eledoisin.



- 300Å provides narrowest peak width for proteins @ better mass transfer. This translates to better sensitivity in LC-MS.
- 120Å is okay for proteins < 15 kD.
- As expected, the small molecule heme exhibits good mass transfer in both pore diameters.

Fig. 2. 120Å C18 for Low MW Hormone



Column: VYDAC® DENALI® C18, 120Å, 5µm particles, 300µm x 150 mm (VYDAC® 238DE5.315)

Mobile Phase: **A:** 0.1% FA in H_20; **B:** 0.1% FA in CH_3CN

Gradient (%B, min.): (20, 0), (40, 30), (80, 45), (80, 55)

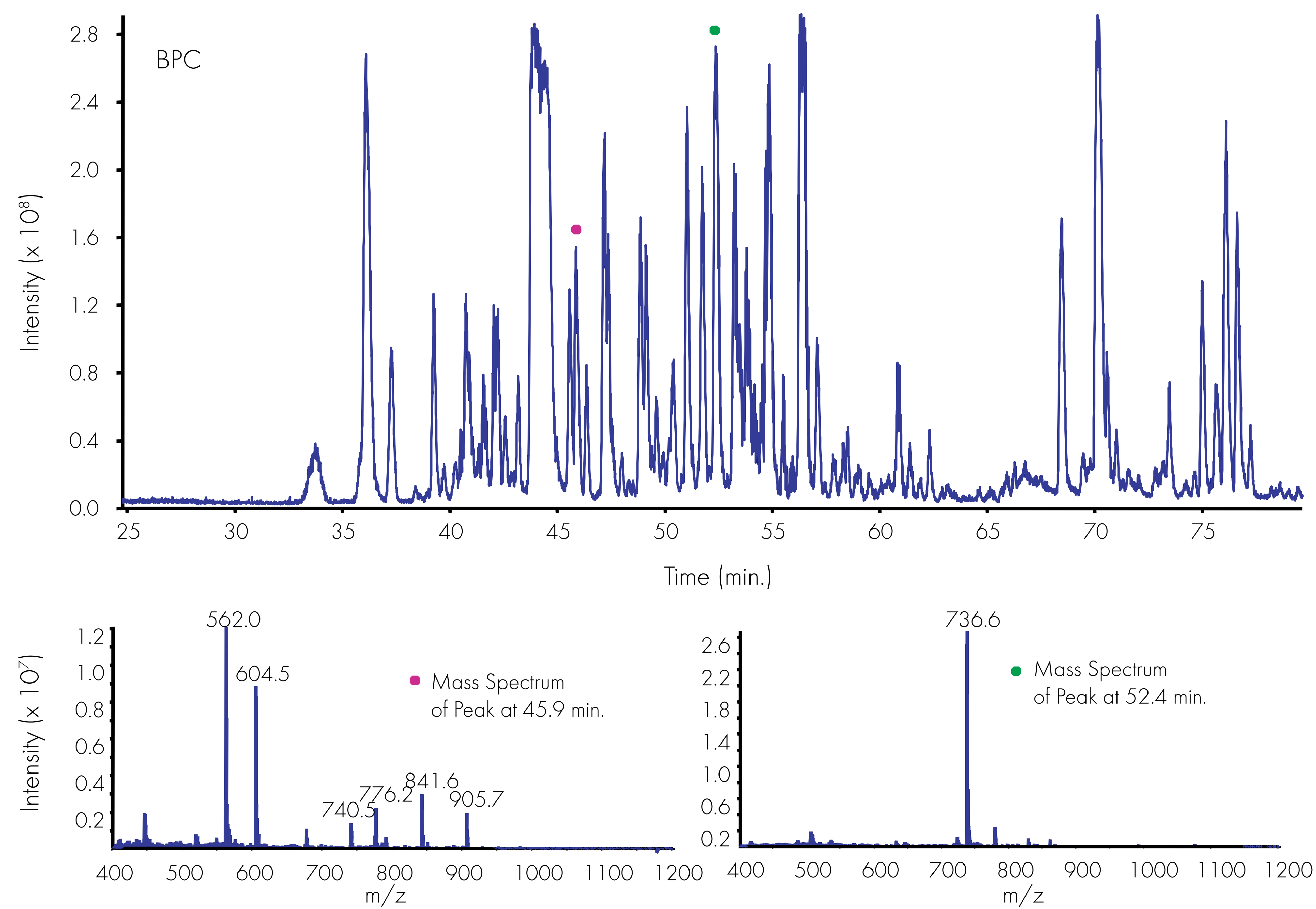
Flow Rate: 5µL/min.

HPLC System: LC Packings UltiMate® pump with FAMOS™ autosampler

MS System: AB/MDS SCIEX Q TRAP® MS; EMS scan 200-1700 amu; scan rate = 1000 amu/s; ion spray voltage = 5000 V; curtain gas = 20; nebulizer gas flow = 20; heater gas flow = 15; heated nebulizer off; LIT fill time = 20 ms; Q3 entry barrier = 8V

- 120Å C18 provides good peak width for small proteins using 0.1% v/v FA.
- 1pmol insulin loaded on 300µm i.d. column.
- A small amount of insulin of interest was detected in impurity fraction.
- Other variants of insulin were also detected.

Fig. 3. 300Å C18 for Crude Cone Snail Venom Peptides



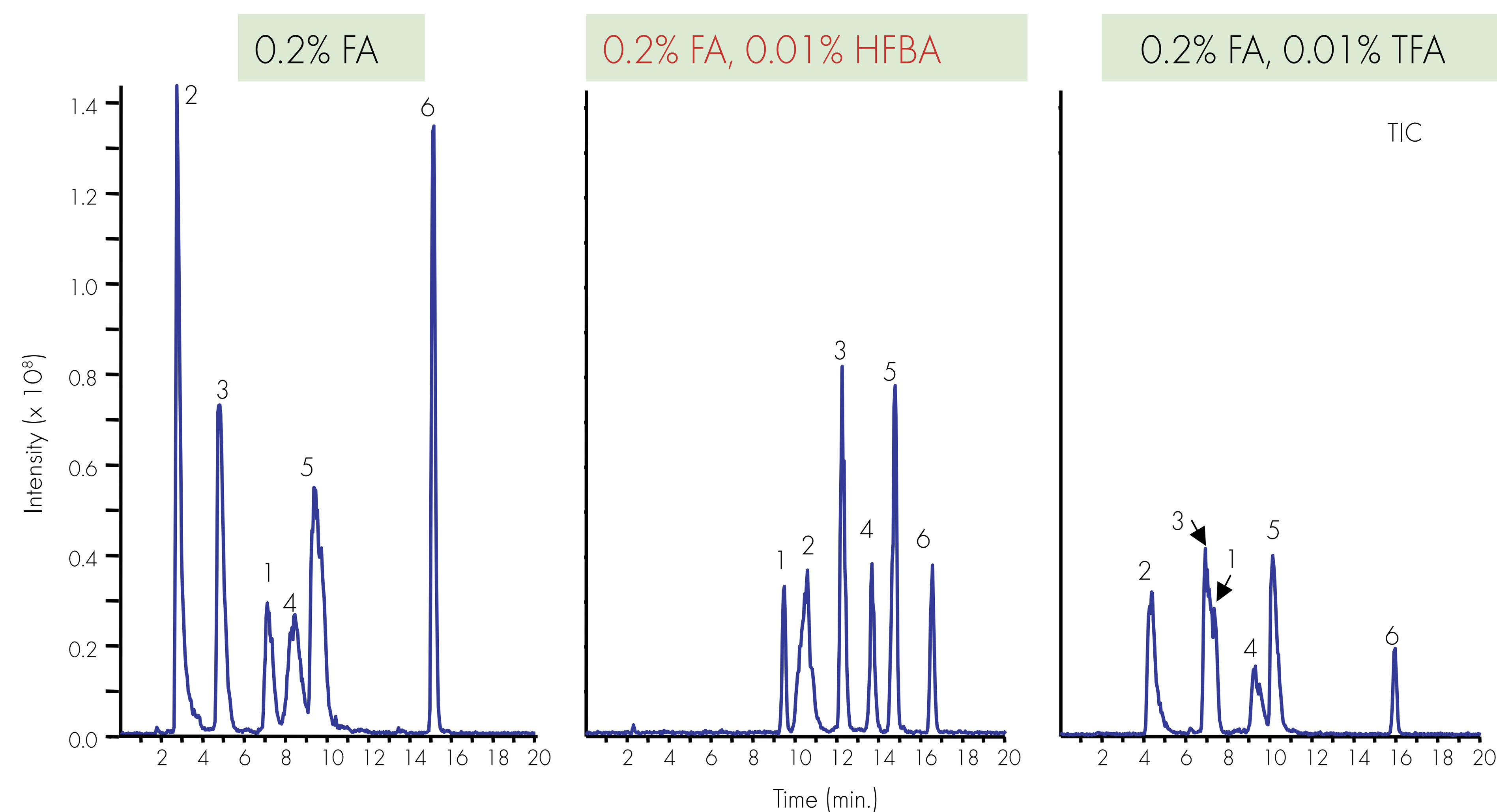
Column:	Vydac® Everest® C18, 300Å, 5µm, 75µm x 250mm (Vydac® 238EV5.07525)
Sample Preparation:	Dissolved in 2% CH_3CN with 0.1% FA.
Mobile Phase:	**A:** 98:2 H_2O:CH_3CN with 0.1% FA; **B:** 40:60 H_2O:CH_3CN with 0.1% FA
Gradient (%B, min):	(0,0), (100, 60), (100, 70)
Flow Rate:	250nL/min.
Q TRAP® settings:	EMS scan 400-1200 amu; scan rate = 4000 amu/s; ion spray voltage = 2300 V; curtain gas = 15; nebulizer gas flow = 0; heater gas flow = 0; LIT fill time = 50 ms; Q3 entry barrier = 8V; Qo trapping on; declustering potential = 20; collision energy = 10 eV

Sample and gradient conditions courtesy of Drs. Frank Mari and Victor Asirvatham, Dept. Chem. & Biochem., Florida Atlantic University, Boca Raton, FL 33431

- 300Å C18 in a 250-mm length hardware provides excellent resolution of a complex sample of peptides using 0.1% FA.
- Since the sample's CH_3CN content was the same as found in the mobile phase equilibration conditions, it was possible to obtain excellent resolution and retention of peptides with a large 10 µL injection on a column with volume of only 1.1µL.

Fig. 4. LC-MS of Peptides, Combining Ion Pairing Agents

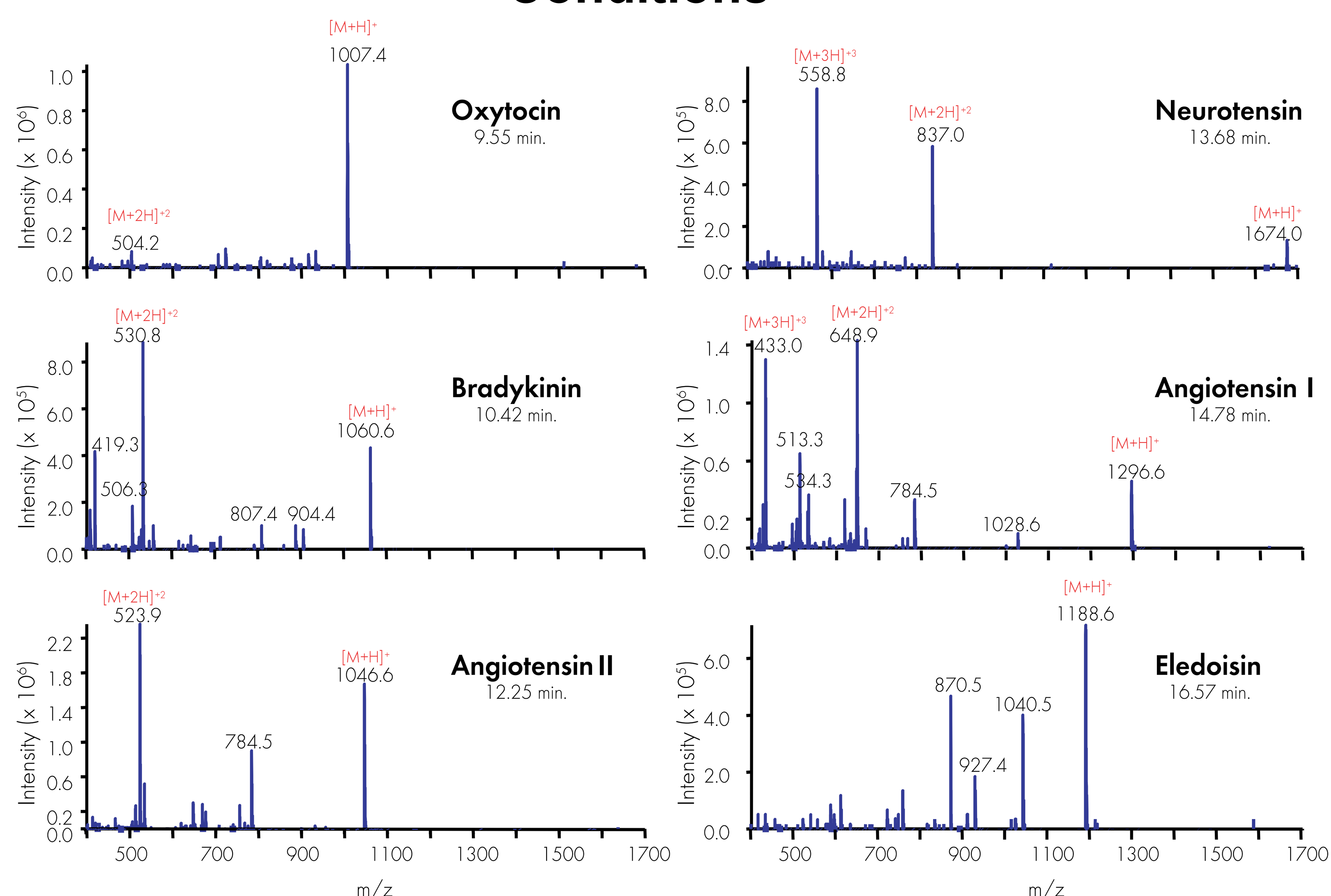
1. **Oxytocin,** 1007 Da (Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-NH_2)
2. **Bradykinin,** 1060 Da (Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg)
3. **Angiotensin II,** 1046 Da (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)
4. **Neurotensin,** 1672 Da (pGlu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu)
5. **Angiotensin I,** 1296 Da (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)
6. **Eledoisin,** 1188 Da (pGlu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2)0.4 µg ea. (200 to 400 pmol ea.)



Column:	Vydac® Everest® C18, 300Å, 5µm, 1.0mm x 150mm column (Vydac® 238EV5115)
Mobile Phase:	**A:** 95:5 H_20:CH_3CN with ion pairing agent(s); **B:** 20:80 H_20:CH_3CN with ion pairing agent(s)
Gradient (%B, min.):	(12.5, 0), (50, 30), (75, 35), (75, 40)
Flow Rate:	50µL/min.
Q TRAP® settings:	EMS scan 400-1700 amu; scan rate = 1000 amu/s; ion spray voltage = 5000 V; curtain gas = 20; nebulizer gas flow = 20; heater gas flow = 15; LIT fill time = 20 ms; Q3 entry barrier = 8V; declustering potential = 20; collision energy = 10 eV

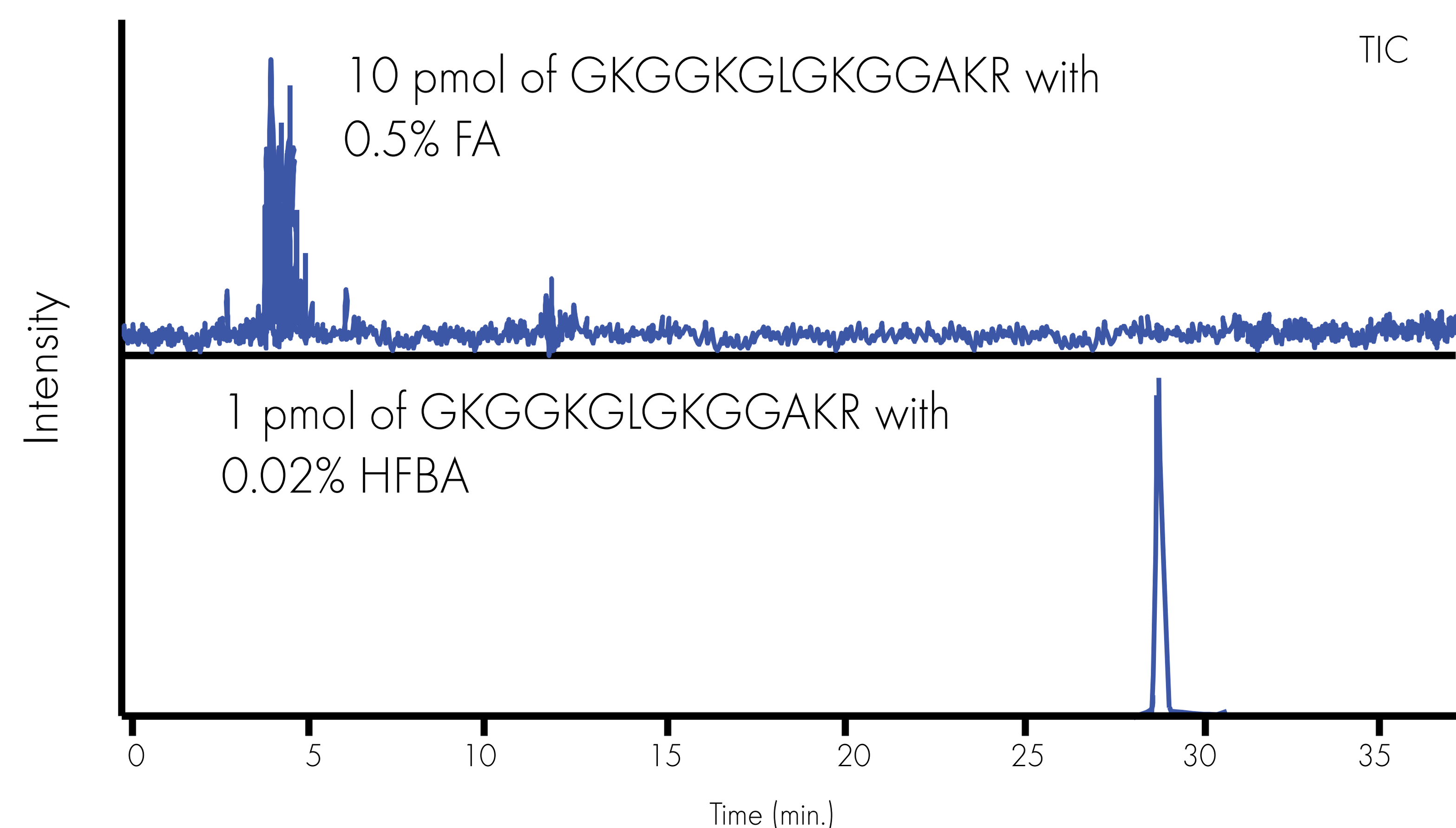
- Peptides 2, 3, and 4 with basic residues exhibit the longest retention with the hydrophobic, strong ion pairing agent HFBA added at 0.01% v/v.
- Narrowest peak width and best resolution obtained with FA, HFBA combination.
- Peptides 2 and 6 exhibit some ion suppression with the stronger HFBA ion pairing agent. However, if additional retention is necessary, HFBA is an excellent additive.

Fig. 5. Mass Spectra for 0.2% FA, 0.01% HFBA Conditions



- Good mass spectra obtained with FA, HFBA combination.

Fig. 6. HFBA for Highly Basic Peptide



Sample: Histone H4 digested with endoproteinase Arg-C to peptide H4 (4-17)

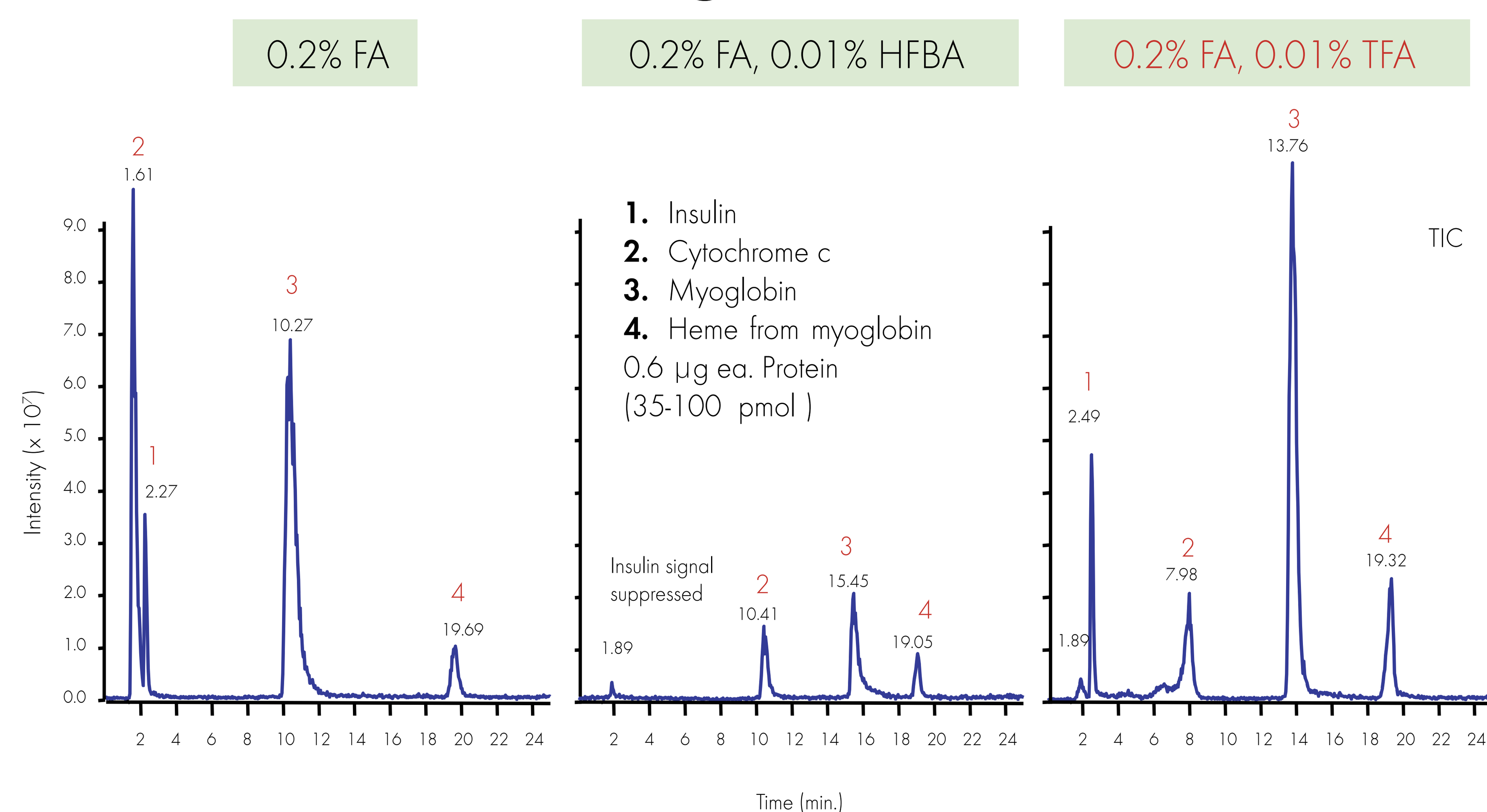
Column: C18, 300Å, 5µm, 300µm i.d. x 150mm (VYDAC® 218MS5.315)

MS System: Finnigan LCQ-Deca

Data courtesy of J.R. Crowley, Washington Univ. School of Medicine and Baylor College of Medicine.

- HFBA dramatically improves retention and ion intensity for highly basic peptide comprised of 1 arginine and 4 lysine residues.

Fig. 7. LC-MS of Proteins, Combining Ion Pairing Agents



Column: C4, 300Å, 5µm, 1.0mm x 150mm column (Vydac® 214MS5115)

Mobile Phase: **A:** 95:5 H_2O:CH_3CN with ion pairing agent(s);
B: 20:80 H_2O:CH_3CN with ion pairing agent(s)

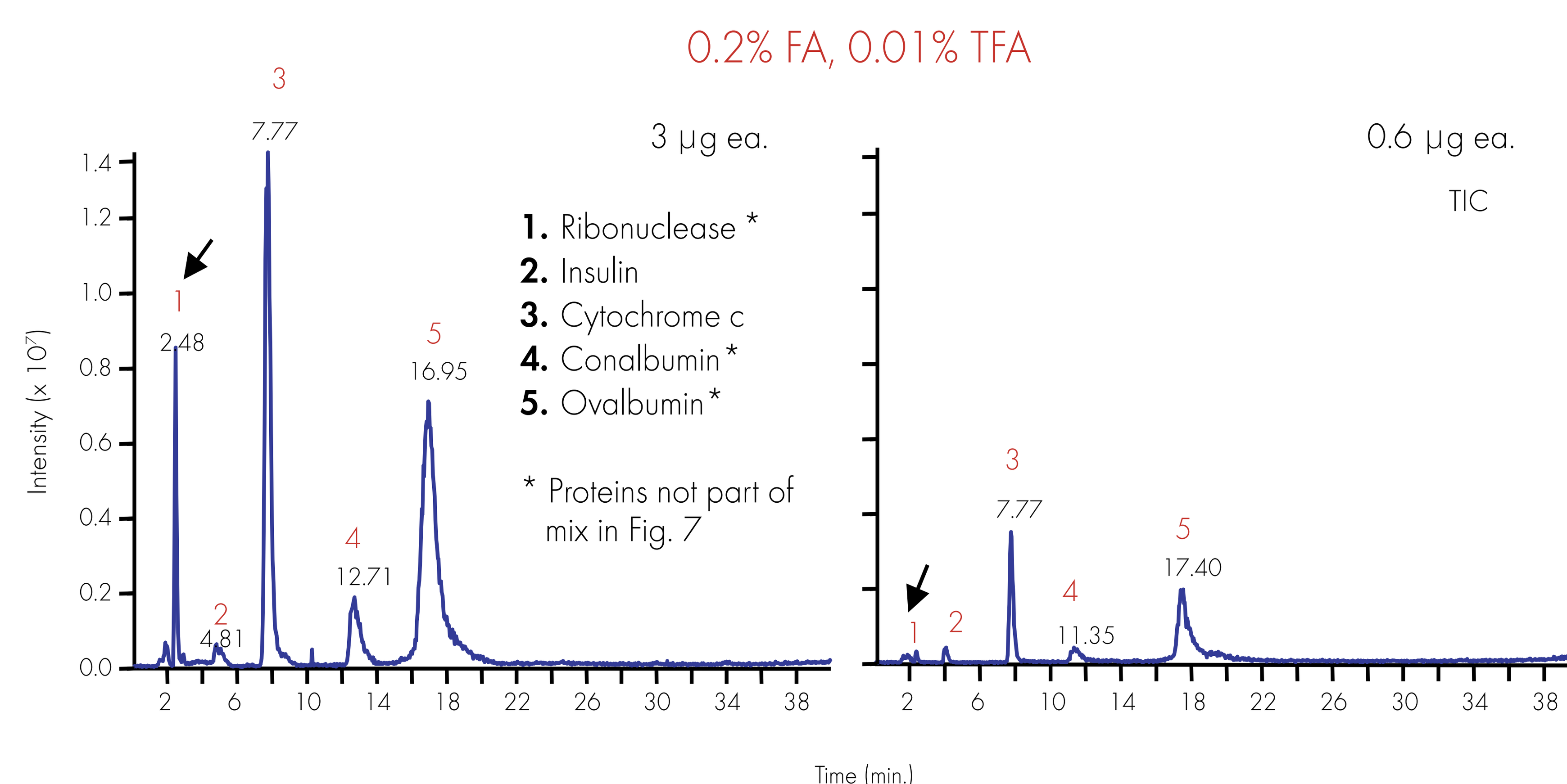
Gradient (%B, min.): (25, 0), (75, 30), (100, 35), (100, 40)

Flow Rate: 50µL/min.

Q-TRAP settings: see **Fig. 4**

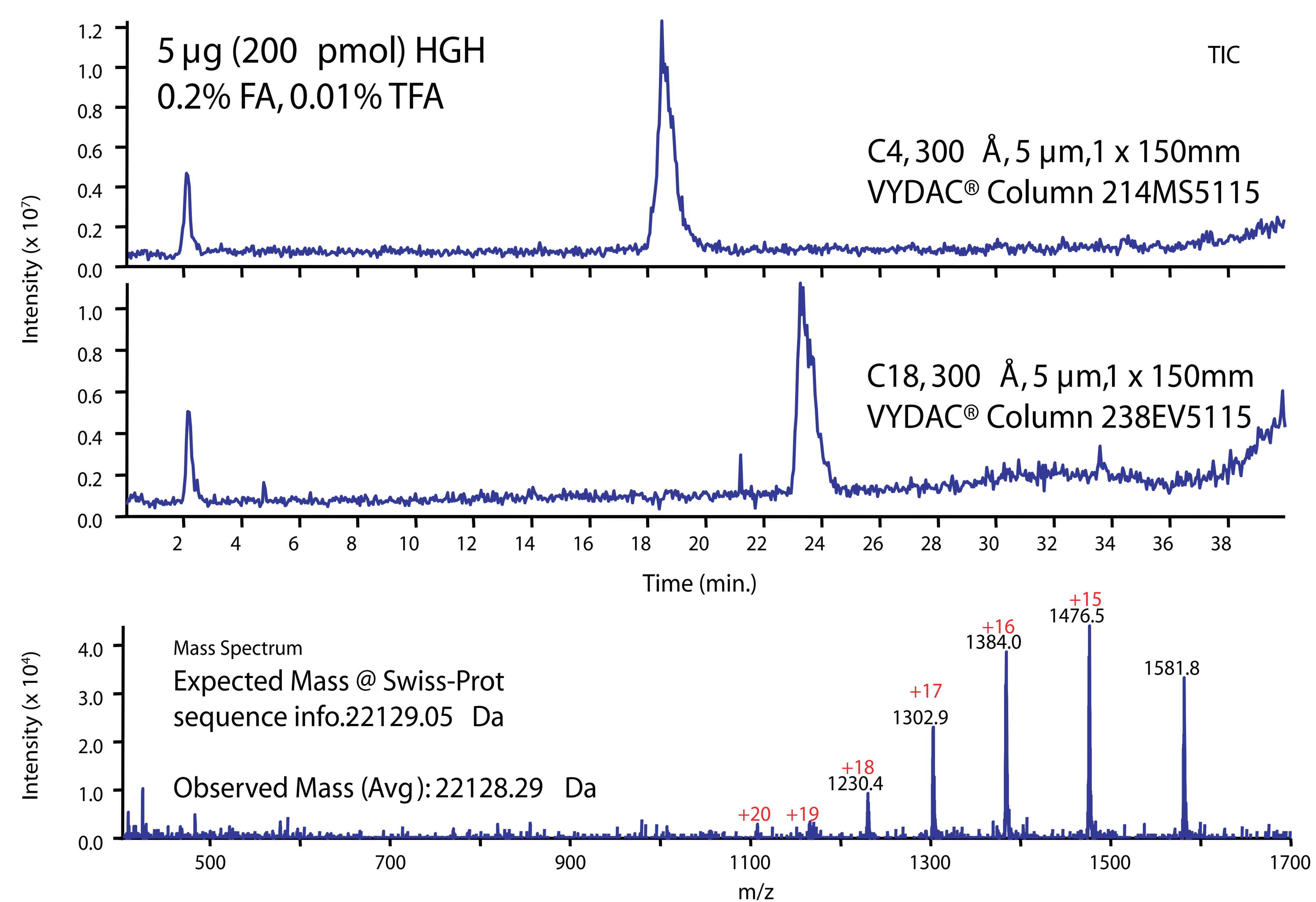
- FA, TFA combination provides best retention and good ion intensities for proteins.

Fig. 8. LC-MS of Additional Proteins



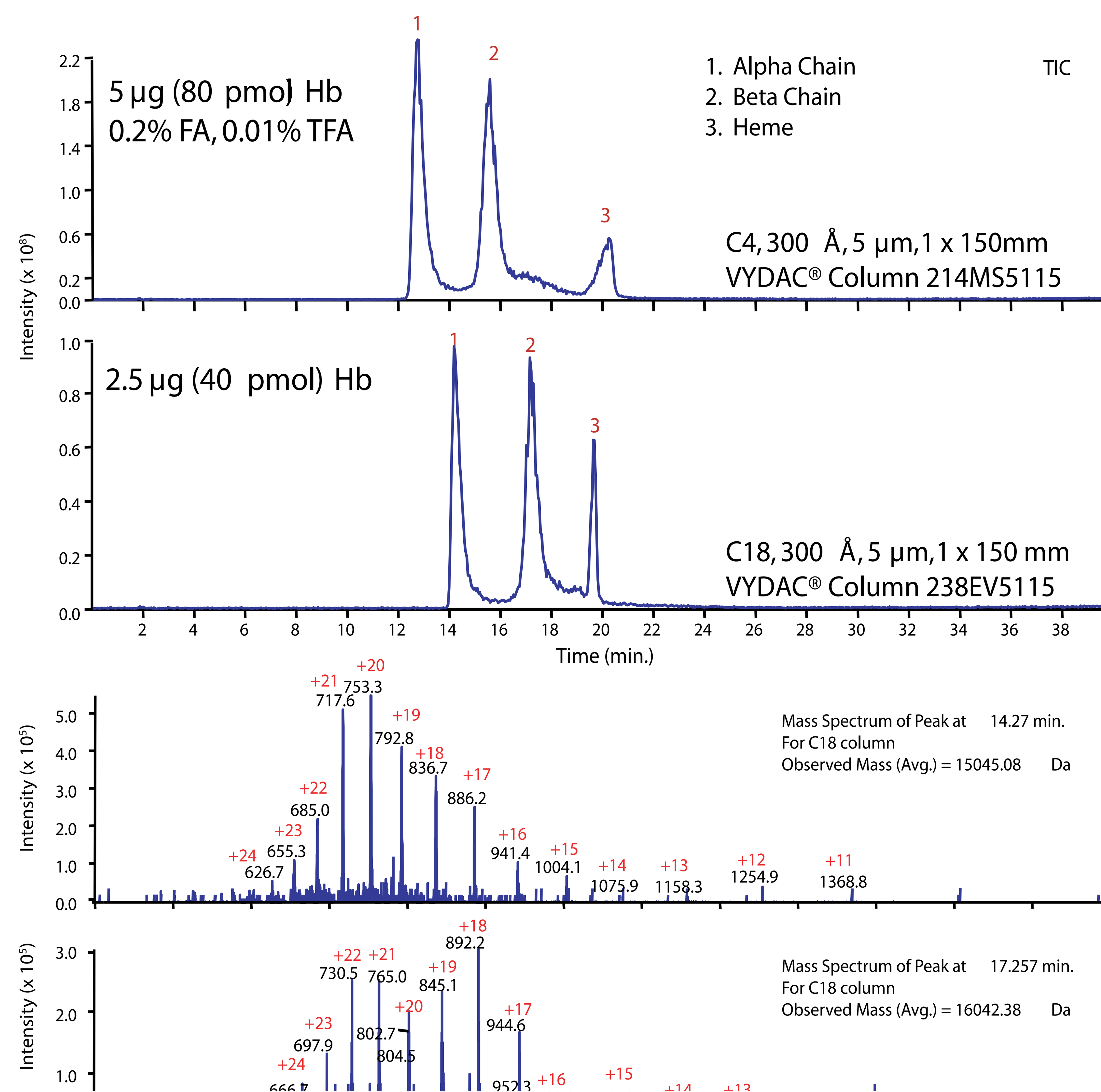
- Ion suppression may occur at lower loading.

Fig. 9. 300Å C4 vs. C18 for Human Growth Hormone



- C18 provides significantly increased retention vs. C4.
- Same peak response is obtained on either phase, indicating no recovery issue on more hydrophobic C18.

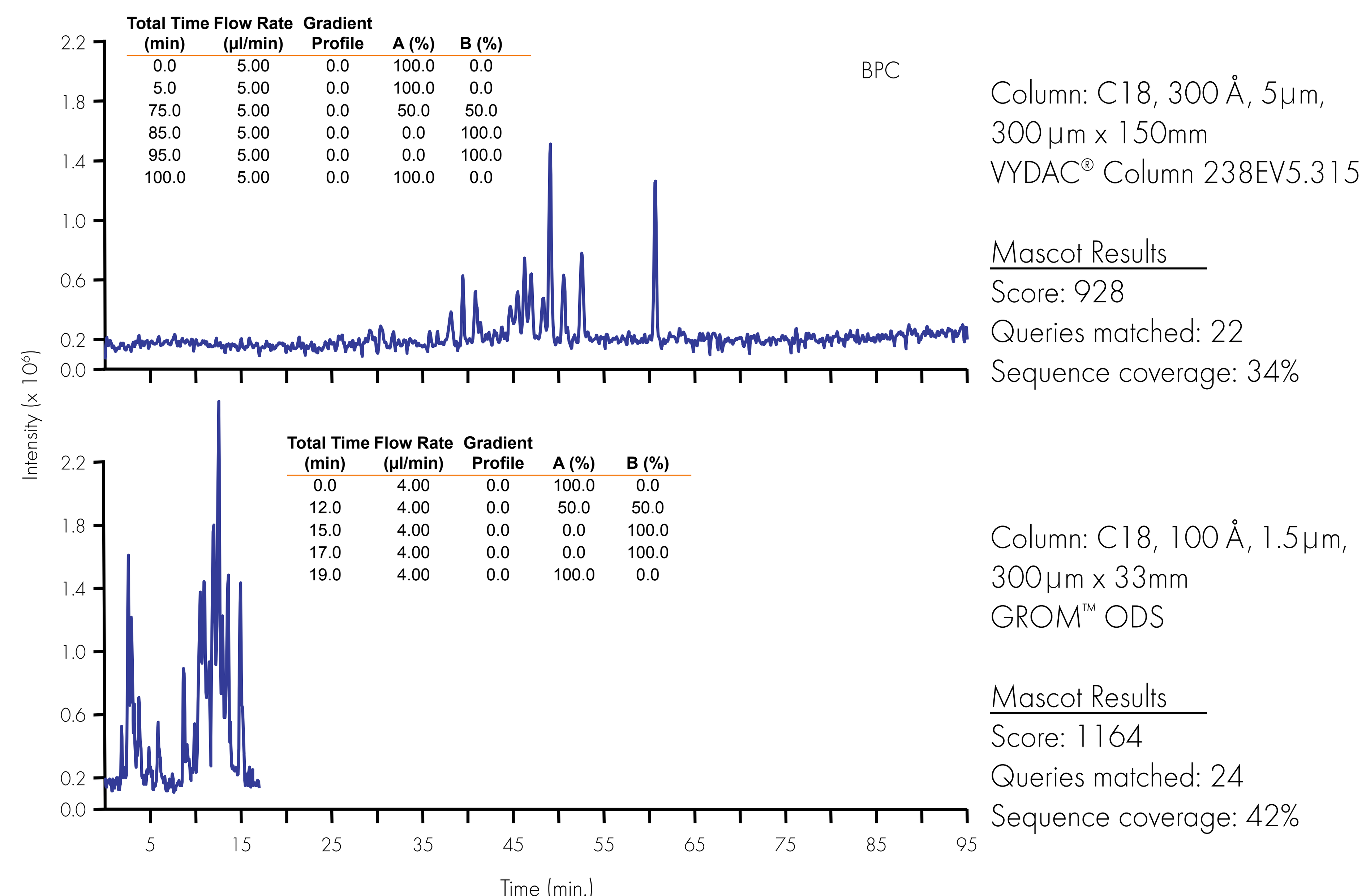
Fig. 10. 300Å C4 vs. C18 for Hemoglobin



- C18 provides significantly increased retention vs. C4.
- Same peak response is obtained on either phase, indicating no recovery issue on more hydrophobic C18.

Fig. 11. 5 µm, 300Å vs. Fast 1.5 µm, 100Å Column:

Tryptic Digest of BSA, 0.2% FA, 0.01% HFBA



Sample: 500 ng (7 pmol) tryptic digest of BSA, denatured, reduced, & alkylated (Michrom Bioresources)

Mobile Phase: **A:** $H_2$0:CH_3CN with 0.2%FA, 0.01%HFBA (95:5);
B: 20:CH_3CN with 0.2%FA, 0.01%HFBA (20:80)

Q TRAP® settings: IDA in LIT scan mode; 1 EMS, 1 ER, and 2 EPI scans; scan 400-1700 amu; ion spray voltage = 5000 V; curtain gas = 20; nebulizer gas flow = 20; heater gas flow = 15; declustering potential = 20; collision energy = 10 eV

- Better Mascot score and higher sequence coverage obtained using fast 1.5 µm, 100Å column with FA, HFBA combination.

Conclusions

The choice of ion pairing agent may have a significant effect on sensitivity, resolution, and retention. While formic acid generally provides good sensitivity, its weak ion pairing nature often results in broader peak width and short retention, especially for basic peptides. A combination of 0.2% formic acid plus 0.01% HFBA may provide the best peak width and retention, without sacrificing sensitivity for a many peptides < 2 kDa. Without the addition of HFBA, some basic peptides elute essentially in the void volume, and the sensitivity is lower almost by ~ 10-fold. For proteins > 10 kDa, 0.2% formic acid plus 0.01% TFA may be the best combination of ion pairing agents for a number of proteins such as human growth hormone and ribonuclease.

Generally, 300Å, C4 silica is best for polypeptides > 10 kDa, as mass transfer and recovery are improved for such large biomolecules. 300Å, C18 silica has also been shown to work for polypeptides, and small peptides such as those derived from complex digest samples, with the larger pore size being less prone to clogging effects. However, 100Å silica has benefits for single protein digests, especially when 1.5µm silica is used. With FA, HFBA ion pairing agents, MASCOT ion scores and sequence coverage were higher for 19 min. runs on the small particle size, short (33mm) length column compared to 100min. runs on the conventional (150mm) length column of 5µm particles. The former column allows for high-throughput analysis of samples in LC-MS/MS applications.

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