Improved Bioseparations with a Novel Charged Surface Superficially Porous Silica Column

Stephanie Schuster, Ph.D., Joshua K. McBee, Ph.D., Conner McHale,
Peter Pellegrinelli, Barry E Boyes, Ph.D.

Advanced Materials Technology, Inc.

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Pittcon

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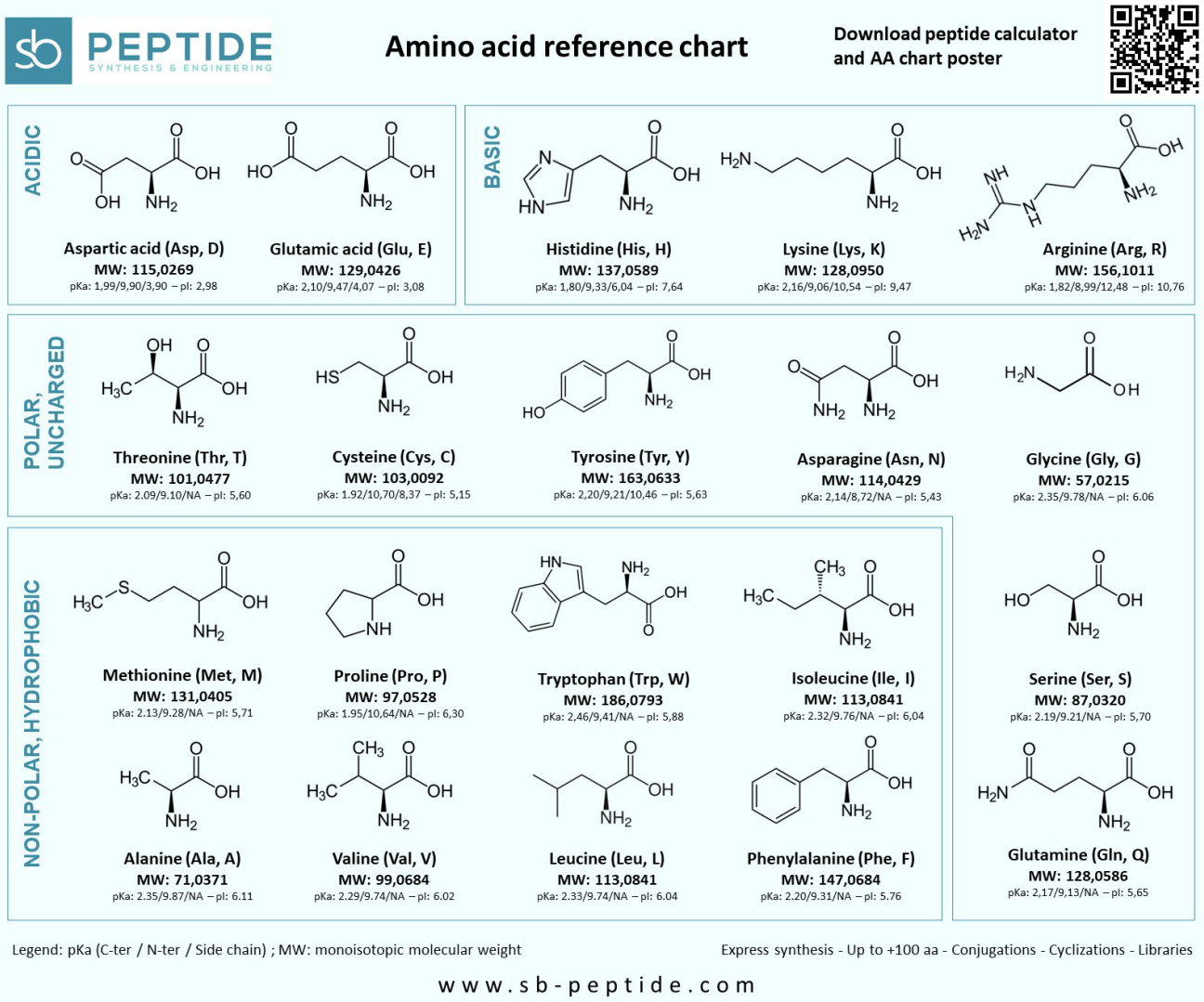
Peptides are Basic Compounds

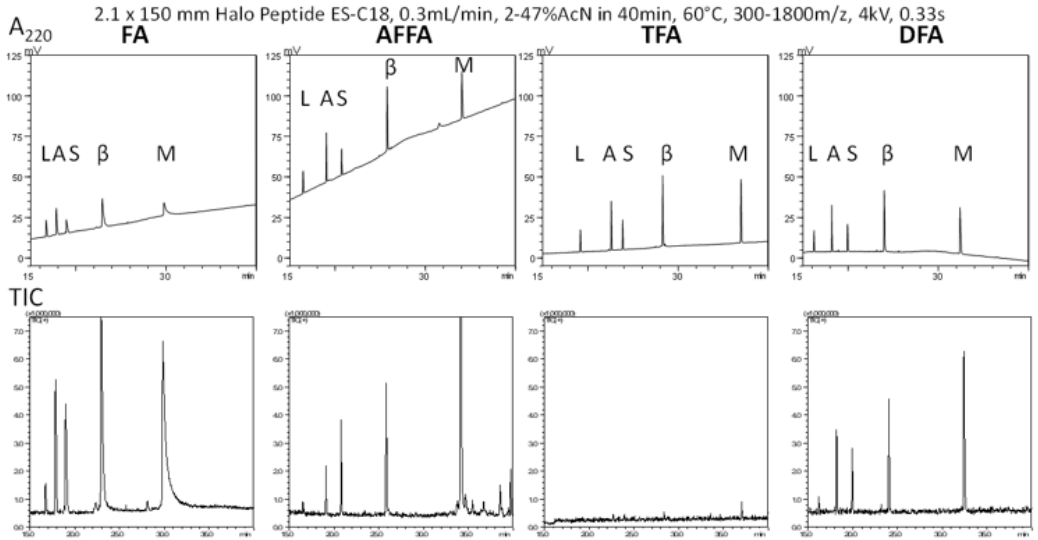
- In acidic conditions:
  - N-terminus protonated
  - Lysine/Arginine are protonated
  - C-terminal protonated
  - Carboxylic acids are neutral

- Basic compounds can be challenging
  - Reverse Phase



Transitioning from HPLC-UV to HPLC-MS

- Traditional HPLC-UV ion pairing uses Trifluoroacetic acid for good peak shape
- TFA causes significant ion suppression when using Mass Spectrometry as detector
- Weaker ion pairing agents
  - Formic Acid
  - Amm Formate/FA
  - Acetic Acid
  - DFA



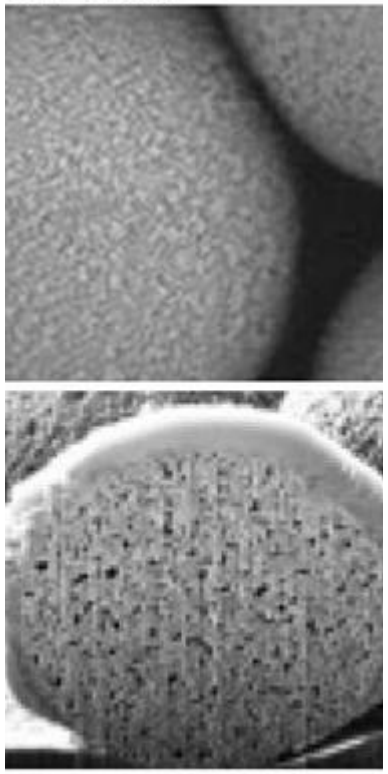
Peptide	Abbrev.	MW
[Leu5]-enkephalin	L	555.6
angiotensin I, human acetate hydrate	A	1297
substance P acetate salt hydrate	S	1348
Melittin, honey bee venom	M	2847
beta-endorphin, human	β	3465

- Weaker ion pairing agents cause degradation of peak shape on traditional C_{18}

The Rapid Development Cycle of Mass Spectrometry

- Chromatography has struggled to keep up with the rapid advances in MS
  - Faster electronics
  - Improved sensitivity
  - Additional dimensions of separation (Ion Mobility)
- Ionization is now the weakest link in the LCMS analytical workflow (Sorry Dr. Fenn!)
- Efficient Chromatography is more important than ever to minimize charge competition, ion suppression during ESI.
- Increasing demand for Chromatography columns specific for LCMS friendly conditions.

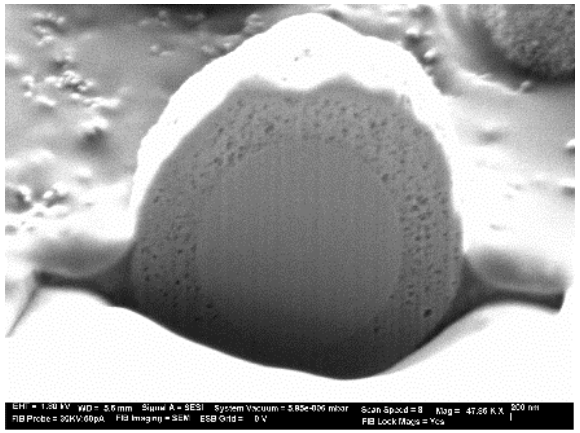
Fused-Core® Technology

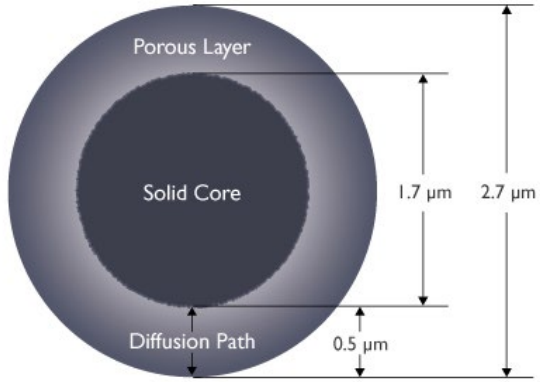


Fully Porous Particle (FPP)

- Improvements in silica and columns
  - Smaller column diameter
    - Increases sensitivity
    - Decreases Lifetime
    - More difficult to work with
    - Reduced loading capacity
- Smaller Silica Particles
  - $N \propto 1/d_p$
  - Increases Back Pressure
- Superficially Porous Silica Particles
  - Shorter diffusion distances
  - Sharper peak shapes
  - Reduced Back Pressures

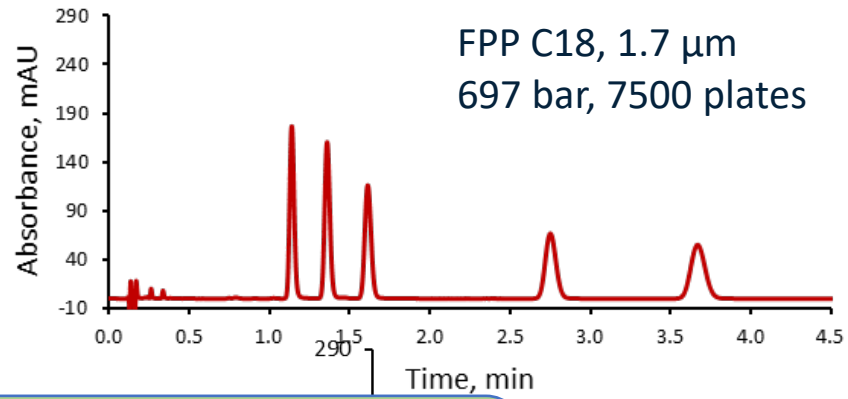
HALO 90 Å, 2.7 µm

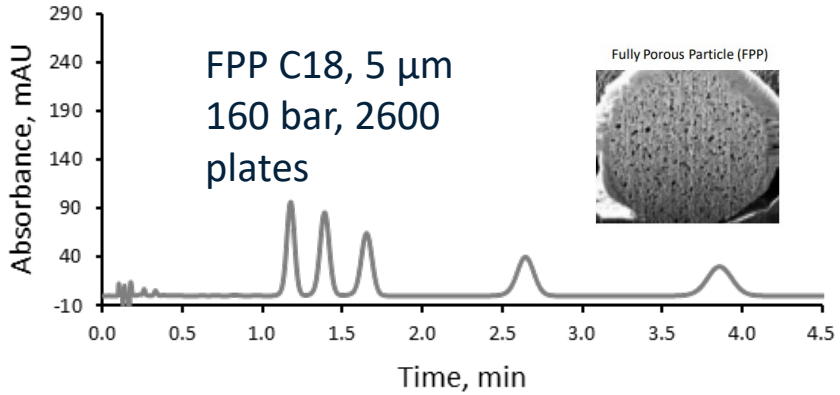




Superficially Porous Particle (SPP)

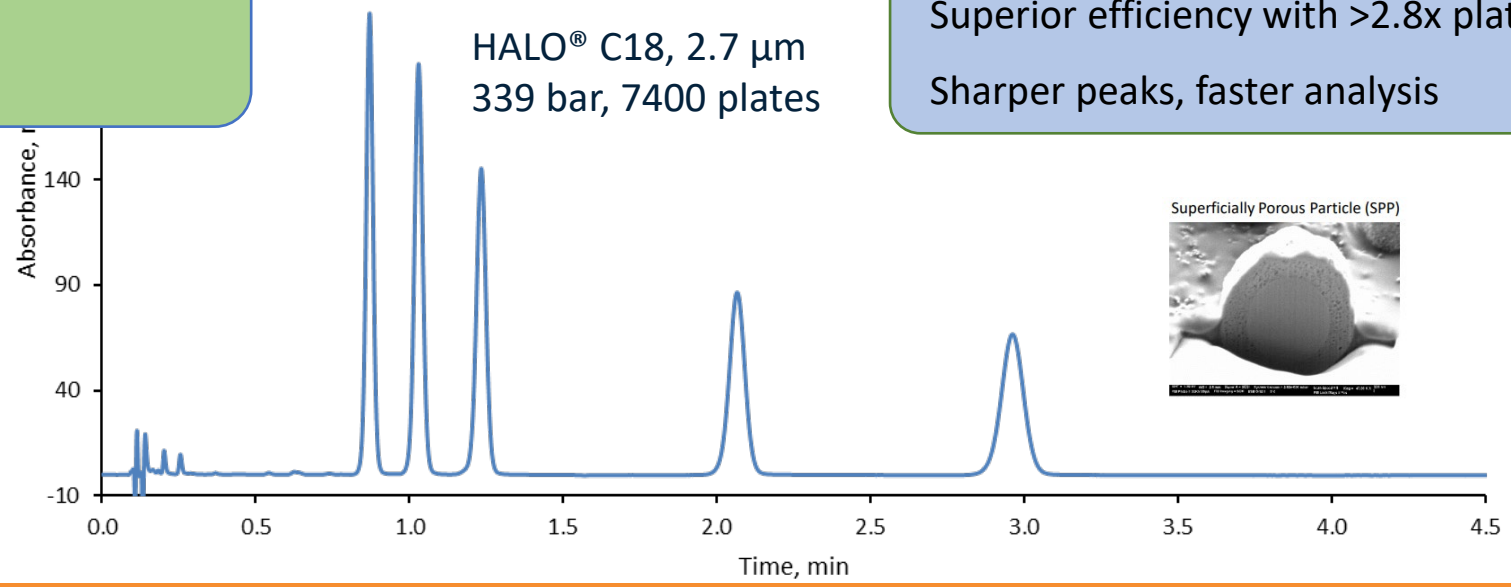
Power of Fused-Core® Technology





High performance with <1/2 the back pressure

Faster analysis



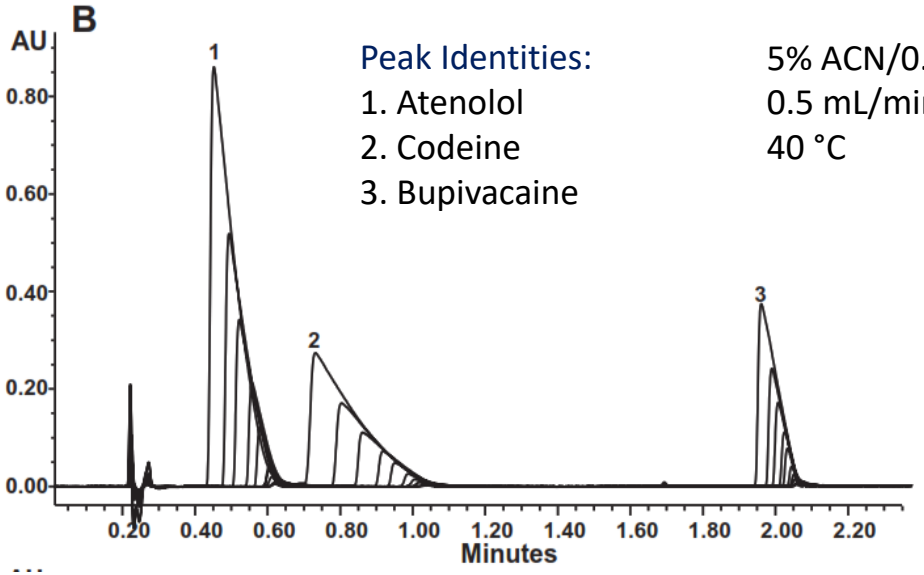
Superior efficiency with >2.8x plates!

Sharper peaks, faster analysis

Managing Basic Compound Separations (including peptides) in Reversed-Phase

Tailing Peak Shape of Basic Compounds

- When basic compounds are run at low pH, they gain a proton and become positively charged.
- At high sample loads, the tailing will become significant and the peak shape will suffer with weak acidic mobile phase modifers.

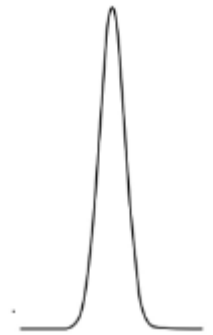


Peak Identities:
1. Atenolol
2. Codeine
3. Bupivacaine

5% ACN/0.1% formic acid for 1 min, then 5-95% ACN/0.1% formic acid in 3 min
0.5 mL/min; 230 nm
40 °C

Joséphine Ruta, Daria Zurlino, Candice Grivel, Sabine Heinisch, Jean-Luc Veuthey, Davy Guillarme, Evaluation of columns packed with shell particles with compounds of pharmaceutical interest, J. Chromatogr. A 1228 (2012) 221-231.

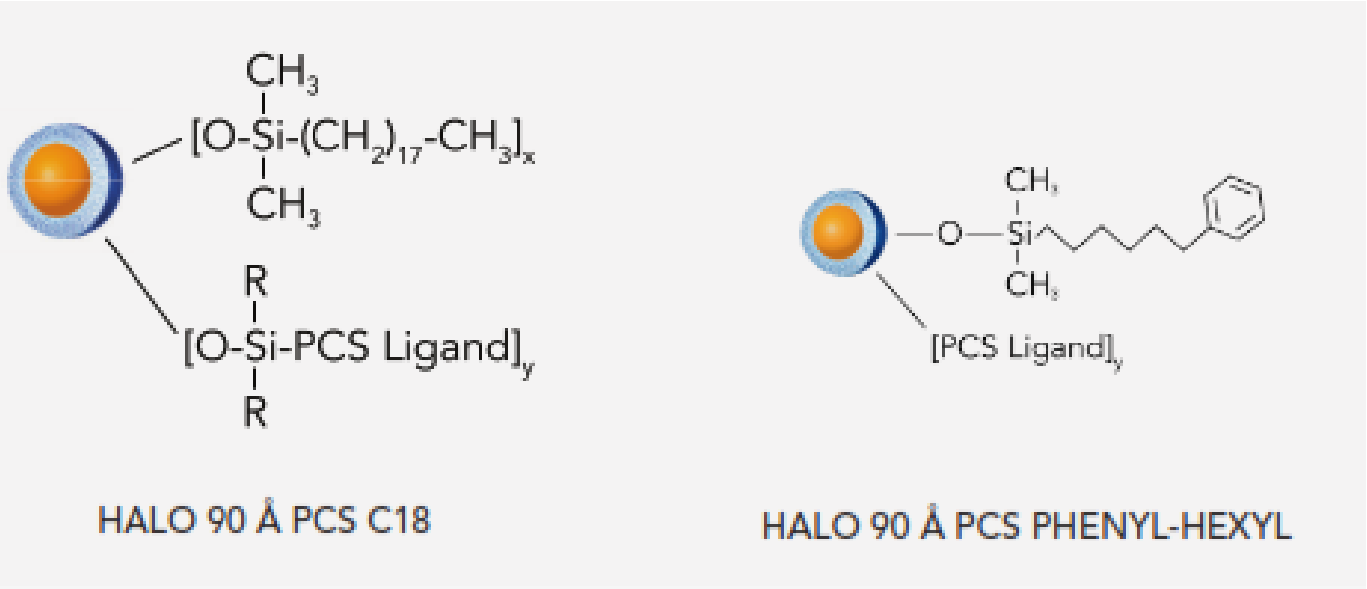
McCalley, D.V. Rationalization of Retention and Overloading Behavior of Basic Compounds in Reversed-Phase HPLC Using Low Ionic Strength Buffers Suitable for Mass Spectrometric Detection. Anal. Chem. 2003 ,75,3404-3410.



Goal is to have symmetrical peak shapes across a wide range of sample concentrations

Using a modified silica stationary phase

Introducing the HALO® PCS Phases: **P**ositively **C**harged **S**urface



90 Å, 2.7 µm for Small Molecule Analyses

- Excellent peak shape and increased loading capacity for basic compounds
- Alternate L1 selectivity (PCS C18)
- Alternate L11 selectivity (PCS Phenyl-Hexyl)
- Built upon Fused-Core® technology for fast, efficient and reliable separations

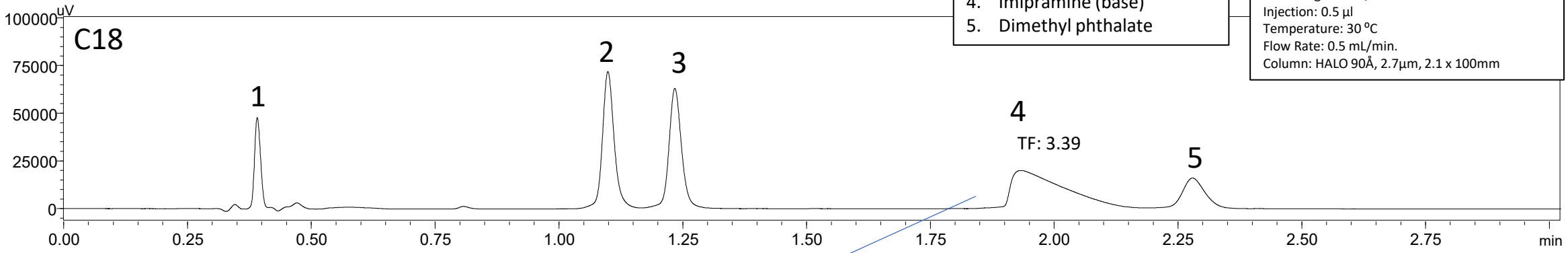
160 Å, 2.7 µm for Peptide Separations

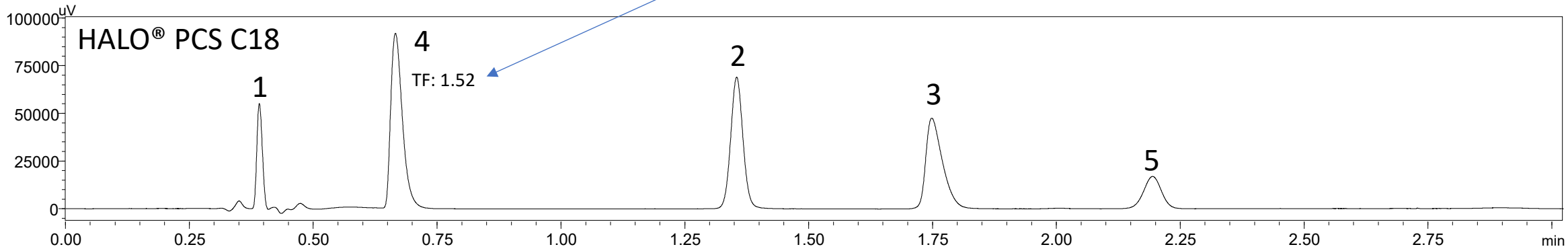
- Significantly improved peak widths and symmetry for basic peptides compared to traditional peptide C18 stationary phases
- Designed for performance with formic acid avoiding LCMS signal suppression from TFA
- Alternate L1 selectivity with optimized pore size for peptide separations

C18 vs. HALO® PCS C18

Peak Identities:
1. Uracil
2. 4-Methoxybenzoic acid
3. 2-Chlorobenzoic acid
4. Imipramine (base)
5. Dimethyl phthalate

Testing Conditions:
Mobile Phase: A: Water/ 0.1% FA
B: Acetonitrile/ 0.1% FA
Isocratic Separation: 30% B
Instrument: Shimadzu Nexera X2
Wavelength: PDA, 254 nm
Injection: 0.5 µl
Temperature: 30 °C
Flow Rate: 0.5 mL/min.
Column: HALO 90Å, 2.7µm, 2.1 x 100mm



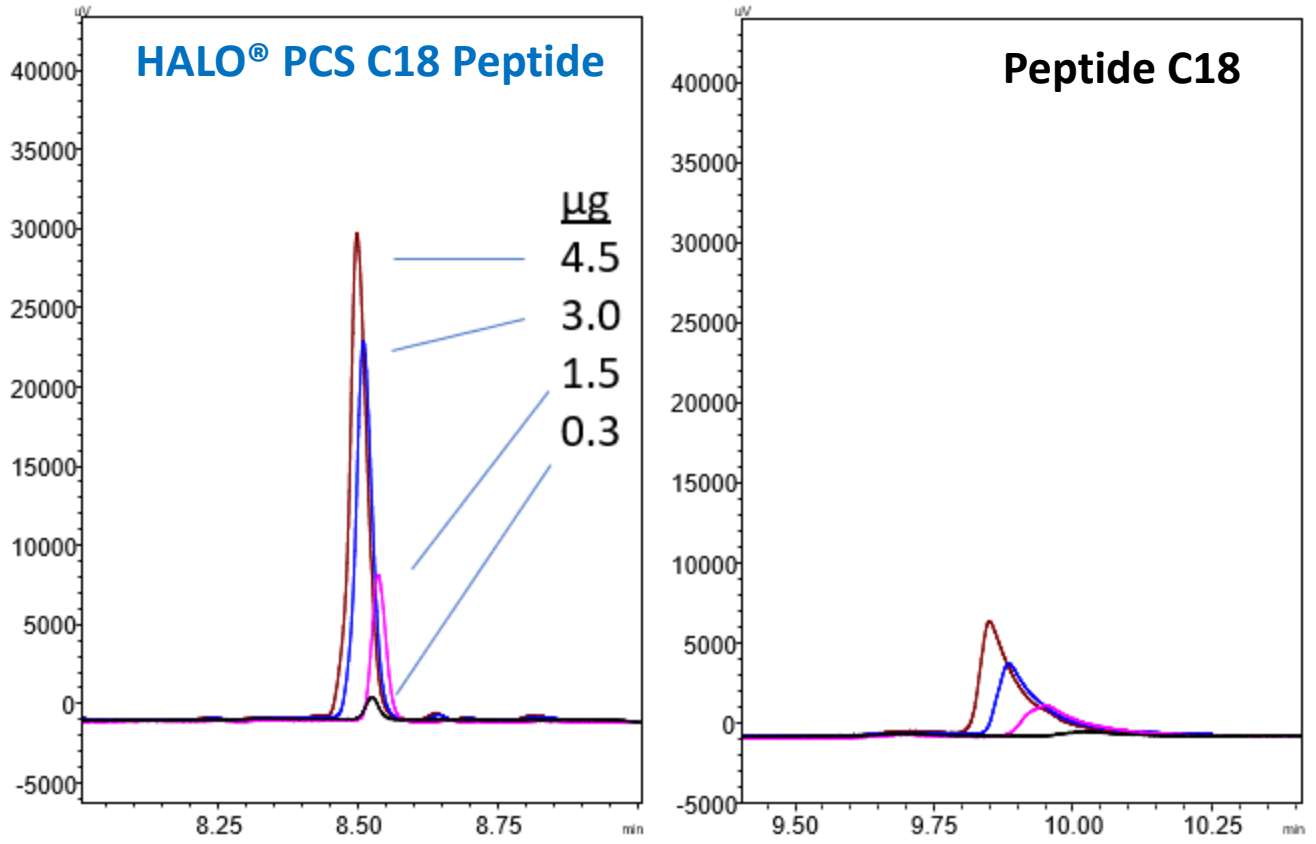


Peptide Load Tolerance

1, 5, 10, and 15µL injections of polypeptide (0.3µg/µL peptides) on 4.6x100mm

Insulin Chain B_{ox}; 3496 Da

Sharp, narrow Insulin B chain peaks on HALO® PCS C18 compared to broad and tailed peaks on standard Peptide C18



With increased load:

- Rt shifts forward
- Peak asymmetry increase

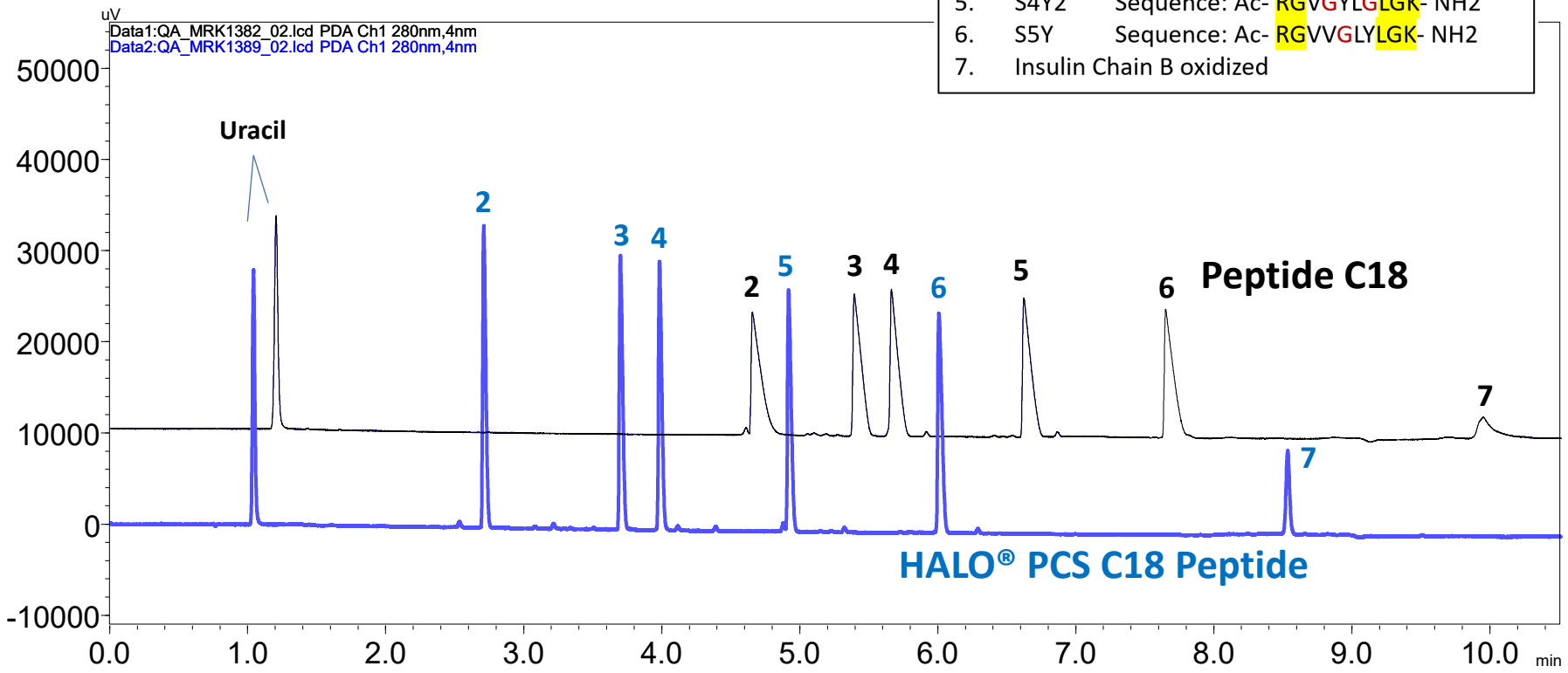
1.5mL/min, 0-35%B in 10min, 30°C; 280nm A=0.1%Formic; B=0.1%Formic in ACN

HALO® PCS C18 Peptide vs. Traditional Peptide C18

4.6 x 100 mm, 1.50 mL/min, 280 nm, 5.0 µL inj.
Gradient Separation, 0-35%B in 10min
Mobile Phase A = H_2O + 0.1% formic acid
Mobile Phase B = ACN + 0.1% formic acid

Peptide QA **4.6x100mm 2.7µm**

1. Uracil
2. S1Y Sequence: RGAGGLYLGK-NH2
3. S2Y Sequence: Ac- RGGGGLYLGK- NH2
4. S3Y Sequence: Ac- RGAGGLYLGK- NH2
5. S4Y2 Sequence: Ac- RGVGYLGLGK- NH2
6. S5Y Sequence: Ac- RGVVGLYLGK- NH2
7. Insulin Chain B oxidized

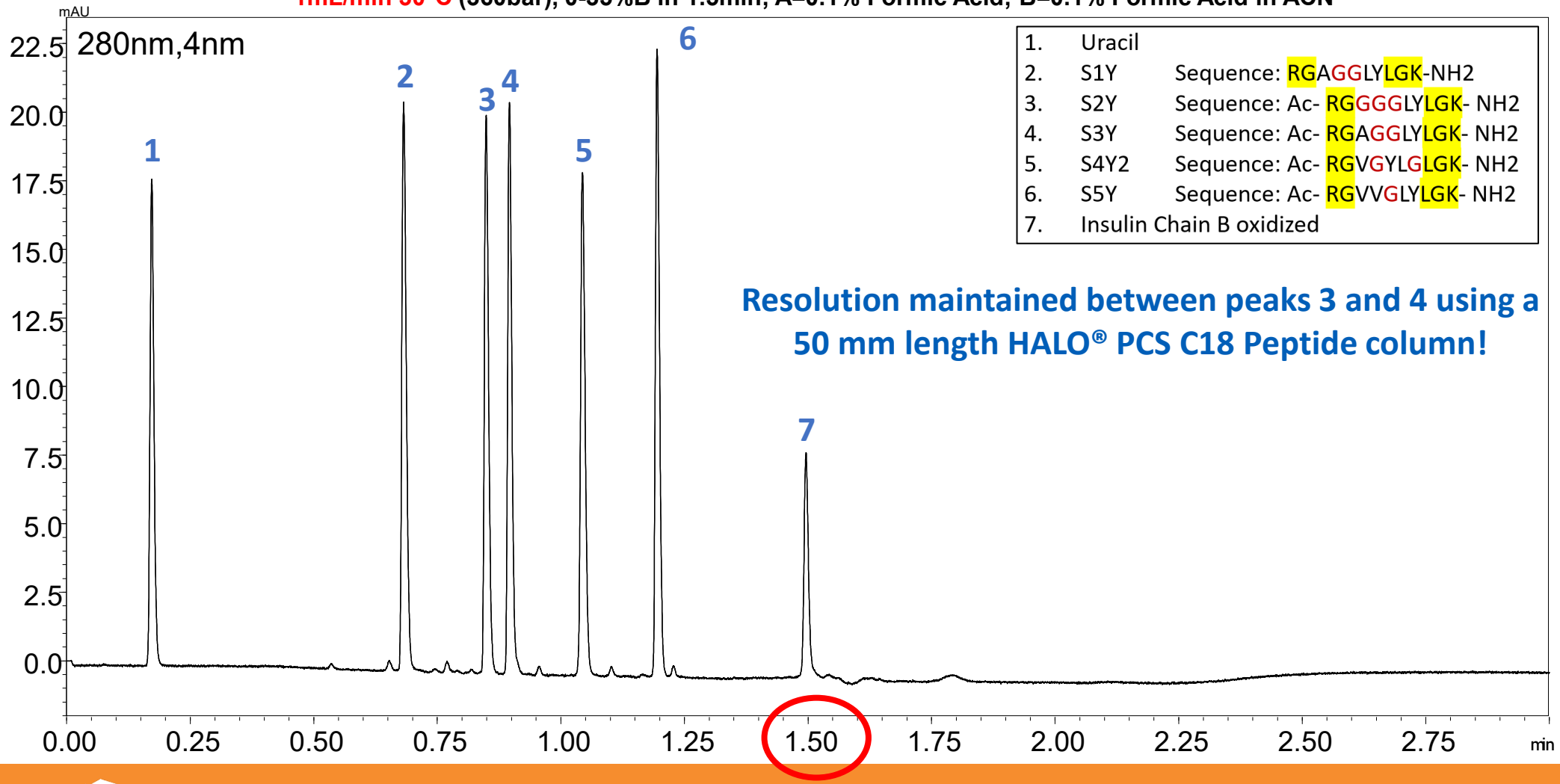


- Gradient separation of 6 peptides
- Decrease in retention time for HALO® PCS C18 Peptide
- Improved peak widths and reduced tailing

HALO® PCS C18 Peptide: Rapid Separation

1µL of Synthetic Peptide Standard (0.3µg/µL) PCS C18 Peptide 2.1x50mm 2.7µm

1mL/min 30°C (360bar), 0-35%B in 1.5min; A=0.1% Formic Acid; B=0.1% Formic Acid in ACN

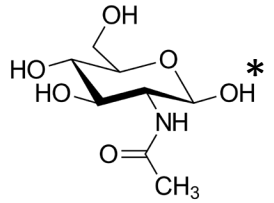


1.	Uracil	
2.	S1Y	Sequence: RGAGGLYLGK-NH2
3.	S2Y	Sequence: Ac- RGGGGLYLGK- NH2
4.	S3Y	Sequence: Ac- RGAGGLYLGK- NH2
5.	S4Y2	Sequence: Ac- RGVGYLGLGK- NH2
6.	S5Y	Sequence: Ac- RGVVGLYLGK- NH2
7.	Insulin Chain B oxidized	

Resolution maintained between peaks 3 and 4 using a 50 mm length HALO® PCS C18 Peptide column!

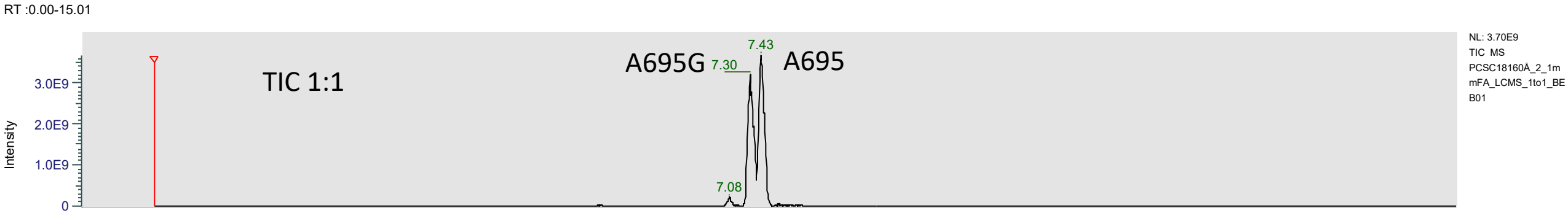
Peptide Impurity Characterization

	Structure	Mass (mi.)	$[M+2H]^{+2}$
APP695-14Pep	VP$T^{291}T^{292}$AASTPDAVDK	1371.6881	686.8513
APP695-14GPep	VPTT(GlcNAc)AASTPDAVDK	1574.7675	788.3910

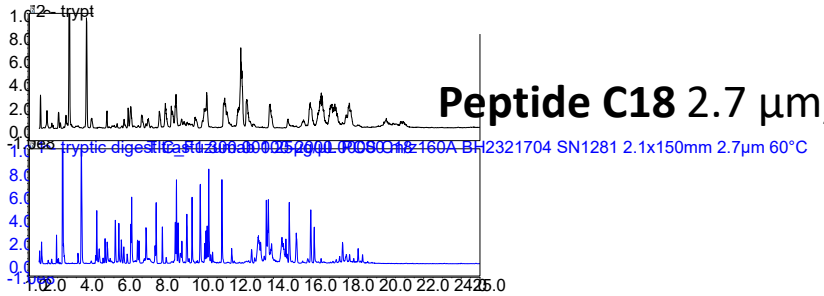


Synthetic peptide of 14 aa, glycosylated at position 4 (Thr292), purity by LC/UV = 98+%
What Impurities are present? How much Parental peptide is in the Glycosylated Peptide sample?

HALO® PCS C18 Peptide, 2.1 x 100 mm
0.5 mL/min, 2-12%B in 15 min, 30°C; A=0.1%Formic; B=0.1%Formic in AcN
QExactive HF, 200 ng each injected (0.4 µL)



Trastuzumab Tryptic Digest: Higher Peak Capacity with HALO® PCS C18 Peptide



Peptide C18 2.7 µm, 2.1 x 150 mm

n_{PC} = 170

0.4mL/min; 60°C; 3-50% in 30 min;
2 µg tryptic digest
A=0.1% Formic Acid in H_2O
B=0.1% Formic Acid in ACN
Shimadzu NexeraX2 -> diverter valve ->
QExactive HF (res=240,000)

HALO® PCS C18 Peptide 2.7 µm, 2.1 x 150 mm

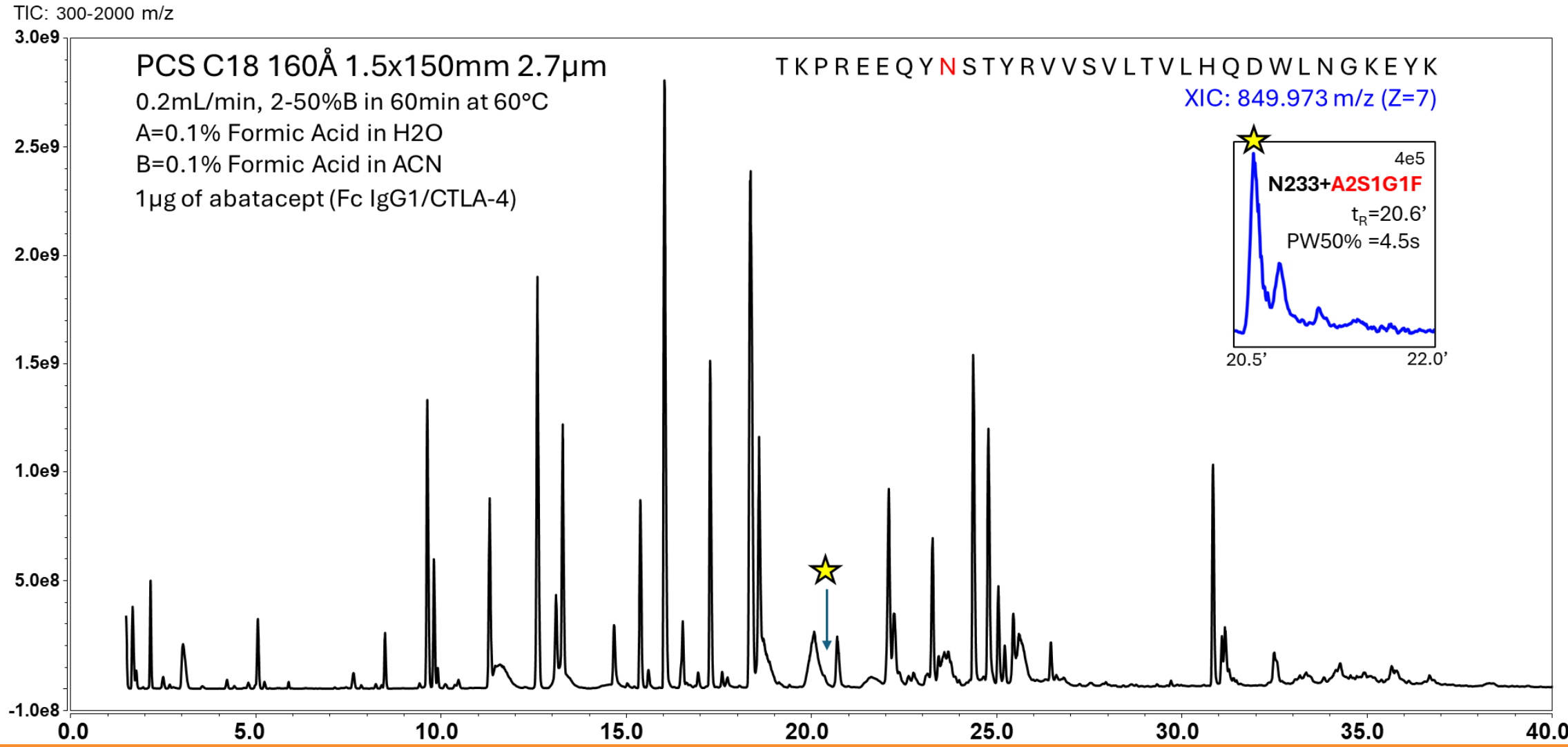
n_{PC} = 488

MarvelXACT Post-Column Plumbing:

1. 50 µm x 350 mm from column to diverter valve
2. 50 µm x 350 mm from diverter valve to union
3. 50 µm x 150 mm from grounding union to HESI II

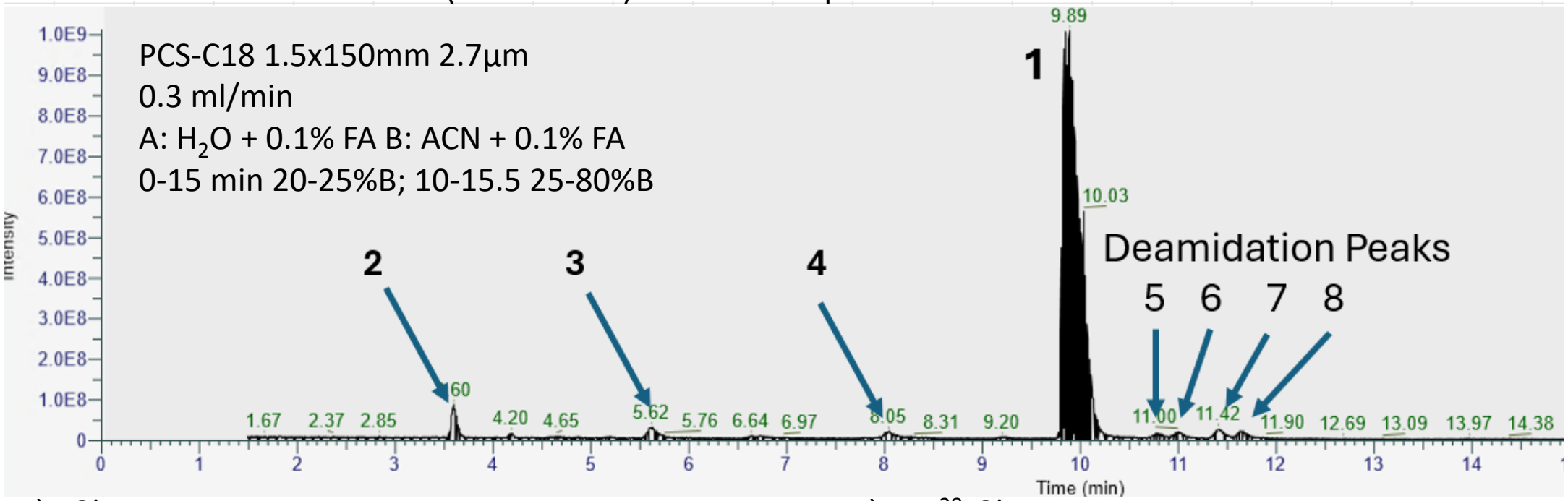
n_{PC} based on ID peptides

HALO® PCS C18 Peptide: 1.5 mm ID Columns for Fusion Protein Tryptic Digest



USP Glucagon Suitability Test

- Incubate Glucagon in 0.01N HCl @50°C for 48 Hours
- >7% of total peak area desamido glucagons
- USP Guidelines: L1 column (3.0x150mm) 45 minute separation



1) Glucagon
2) Glucagon Fragment [SRRSQDFVQWLMNT]
3) Glucagon Fragment [YSKYLDSRRAQDFVWLMNT]
4) Glucagon – H_2O
5) Asp^{28}-Glucagon
6) Glu^{3}-Glucagon
7) Glu^{20}-Glucagon
8) Glu^{24}-Glucagon

Conclusions

- Ion pairing agents are frequently not MS friendly
- Ion pairing agents that work well with MS generally degrade chromatographic separation
- Chromatographic separations have struggled to keep up with the speed of MS development
- MS specific silica is needed for separations in weak ion pairing agents and for basic compounds
- HALO PCS-C18 has a surface charge to improve separation of basic compounds in weak ion pairing
- HALO PCS-C18 outperforms C18 in peptide separations in 0.1% formic acid
  - Improved Peak Shapes
  - Faster Separations
- Moving PCS-C18 to smaller diameter columns further improves separation
  - Narrower peak widths
  - Higher Peak Capacities
  - Greater Sensitivity

Thank You!