Using High pH LCMS Conditions for Impurity Characterization of GLP-1 Therapeutics

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GLP-1 Agonist overview

FDA Approved

- **Exenatide** (Byetta®) – Approved in 2005 ; **Half-Life: 2.4 Hrs**
- **Liraglutide** (Victoza®) – Approved in 2010 ; **Half-Life: 11 Hrs ;**

- **Semaglutide** (Ozempic®) – Approved in 2017; **Half-life: 7 days ;**
- **Tirzepatide** (Zepbound®) – Approved in 2022; **Half-life: 5 days ;**

- **AiB - Inhibits dipeptidyl Peptidase – 4 (DPP-4)**

Modifiers

X = α-amino butyric acid
Z = α-methyl Leucine

K = AEEA-AEEA-γ-Glu-ODDA
None
K = γ-Glu-C16 FA
None
K = AEEA-AEEA-γ-Glu-C20 DA
K = AEEA-γ-Glu-C20 DA

AEEA = 2-[2-(2-aminoethoxy)ethoxy]acetic acid

In Development

- **Retatrutide** – in Ph 3 Trials (Expected 2026); **Half-life: 8 days ; C20 diacid at Pos 17 Lys, AiB at pos 2,20; α-metLeu pos 13**
- **Orforglipron** – Ph 3 Trials (Expected 2026); First orally available GLP-1 agonist.

Solid/Liquid Phase Peptide Synthesis

- Modern GLP-1 agonists require SPSS.
- SPSS yields are typically sub-optimal
- Challenges for purification and impurity analysis

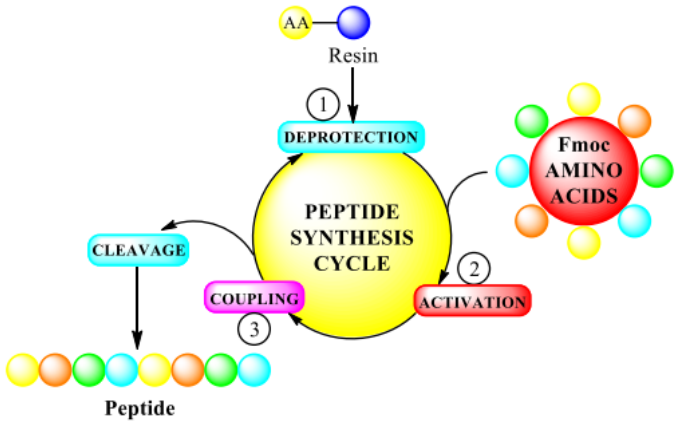
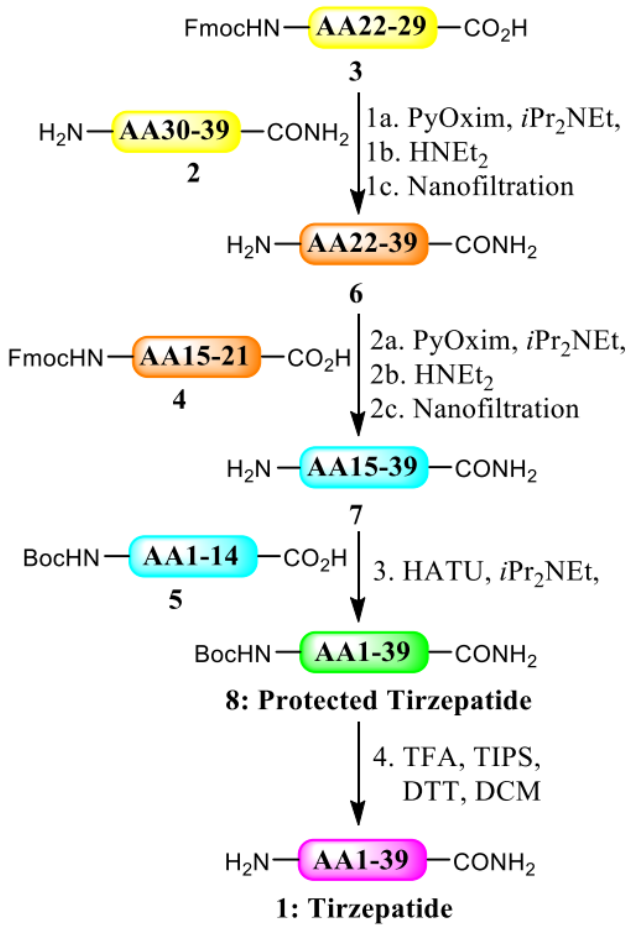


Figure 2. General SPPS methodology for the synthesis of peptides via (1) deprotection; (2) amino acid activation; and (3) coupling. Note: an optional capping step is often included in a SPPS process but is not included in the figure.

Scheme 1. LPPS Portion of the Synthesis[a]



- **ANDA Guidelines for Peptide Impurities**
  - **NMT 0.5% of FLP that are characterized as "safe"**
  - **0.1-0.5% identified, characterized, and demonstrated safe**

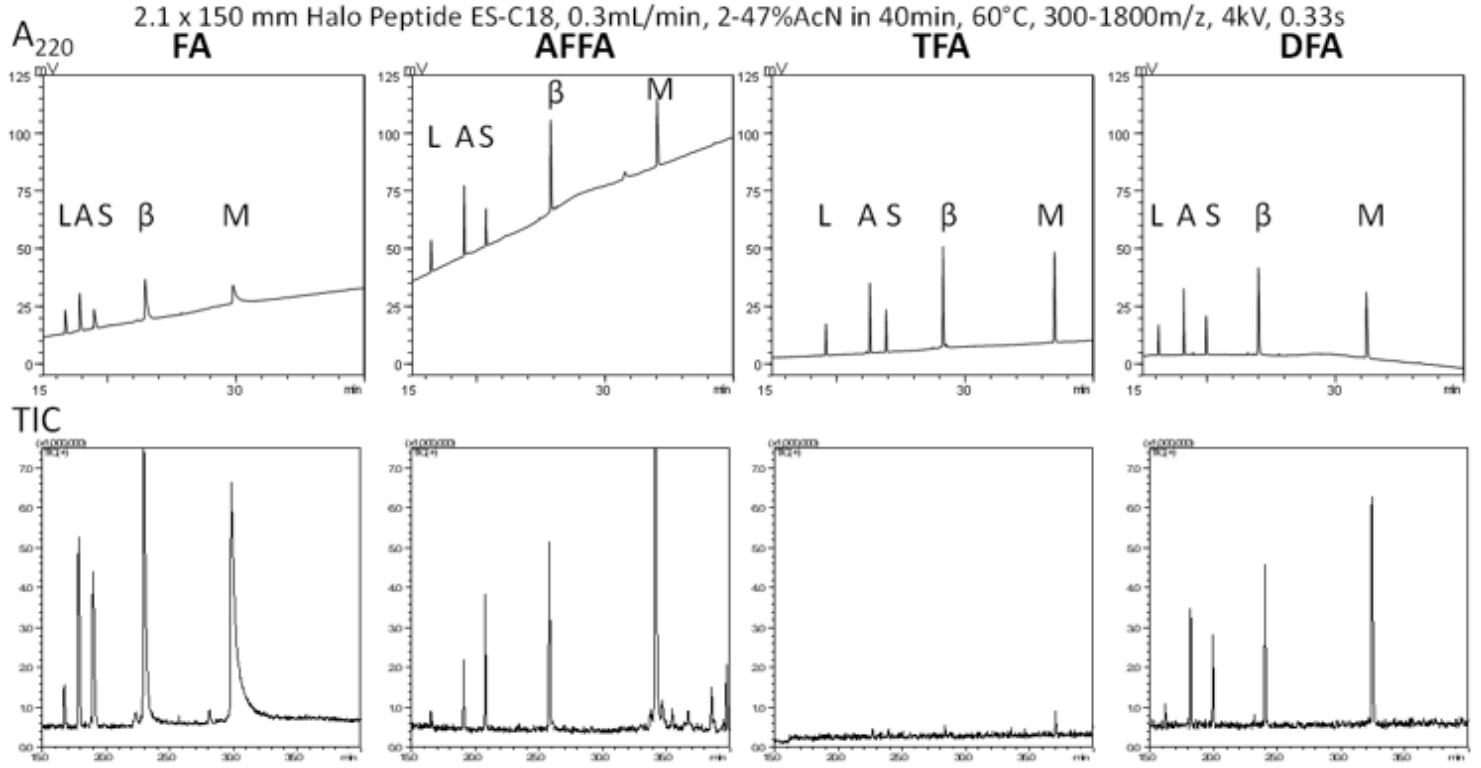
Frederick, MO *et al.* Kilogram-Scale GMP Manufacture of Tirzepatide Using a Hybrid SPPS/LPPS Approach with Continuous Manufacturing, **Org. Process Res. Dev. 2021, 25, pp1628-1636**

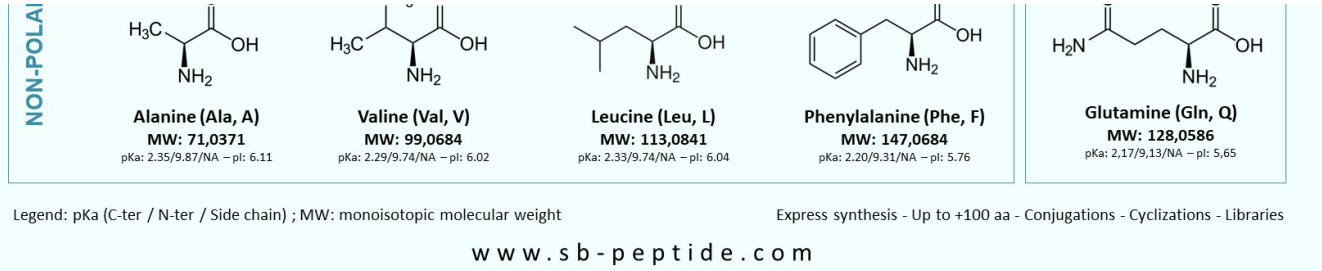
Peptides are Basic Compounds

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

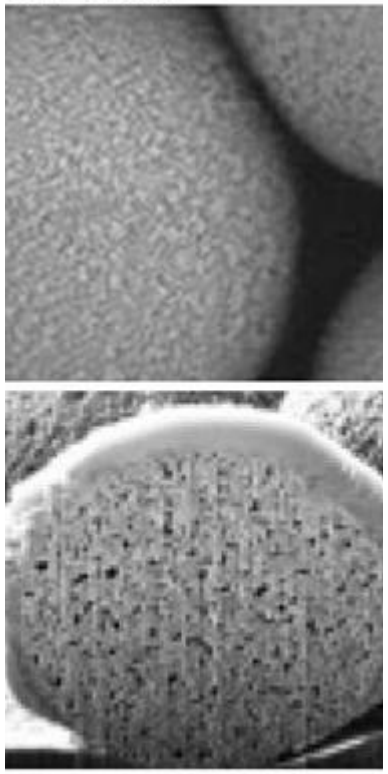
- Basic compound Separations can be Challenging via Reverse Phase
  - LC-UV – Strong Ion pairing agents e.g. TFA
  - LC-MS – Weaker Ion Pairing Agents e.g. Formic Acid

- Ways to improve peptide separations via RP





Fused-Core® Technology

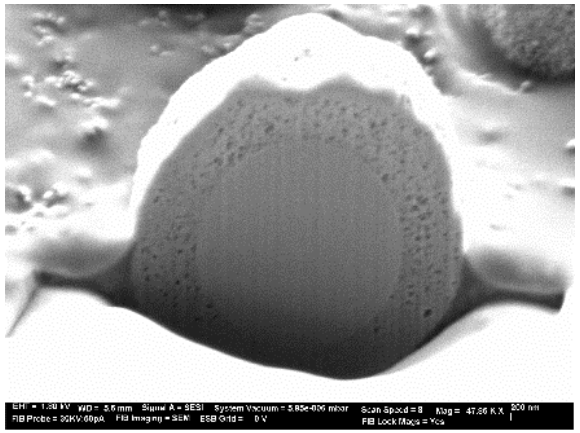


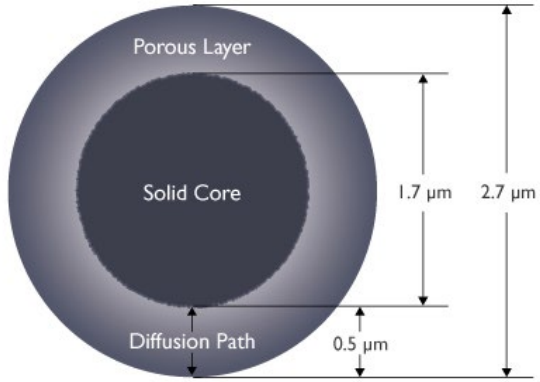
Fully Porous Particle (FPP)

Silica Technology Improvements

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

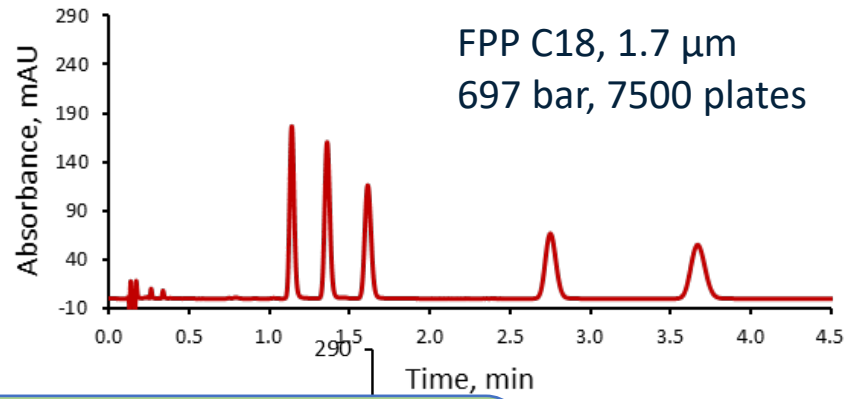
HALO 90 Å, 2.7 µm

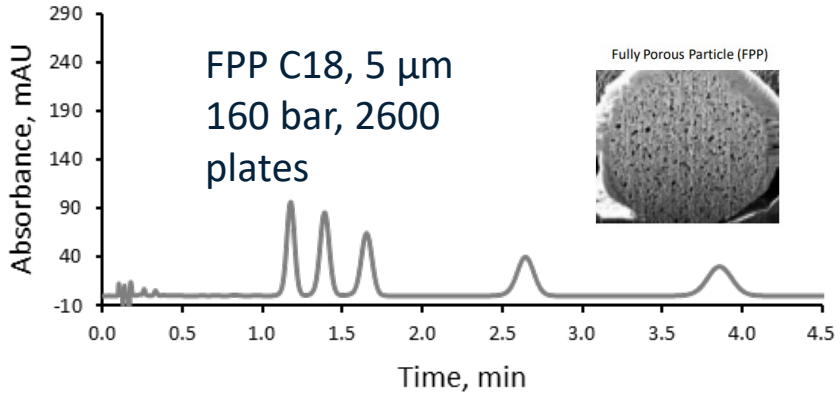




Superficially Porous Particle (SPP)

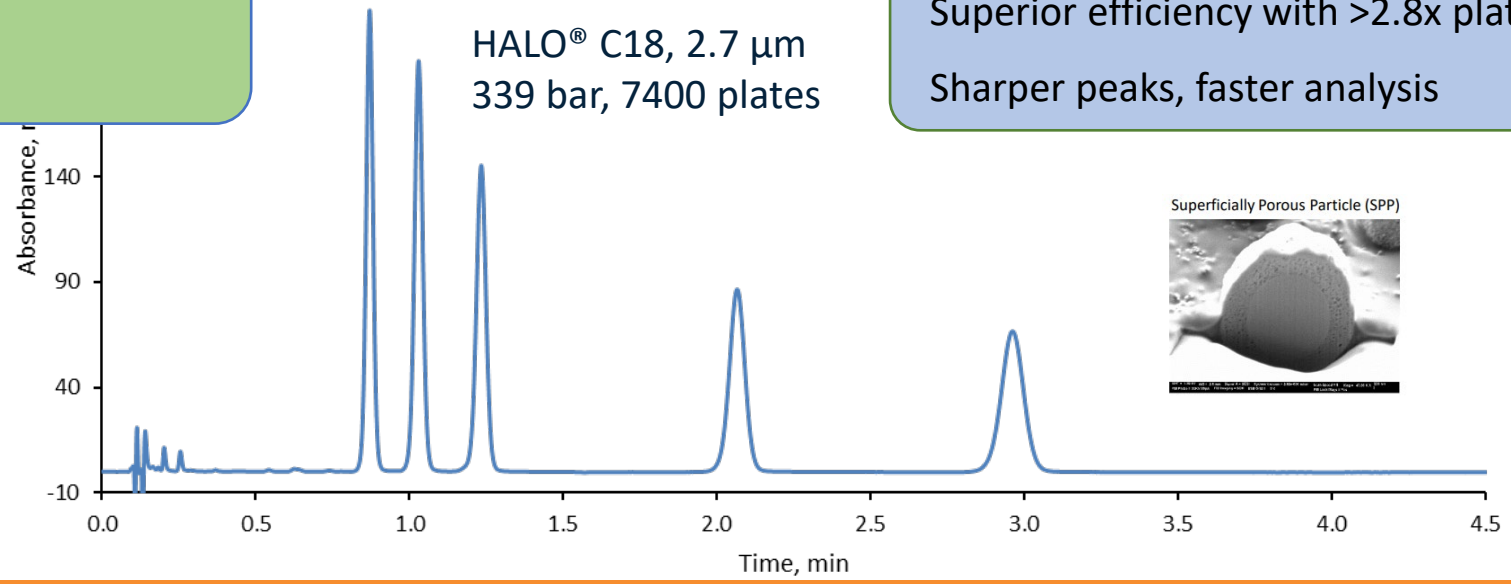
Power of Fused-Core® Technology





High performance with <1/2 the back pressure

Faster analysis



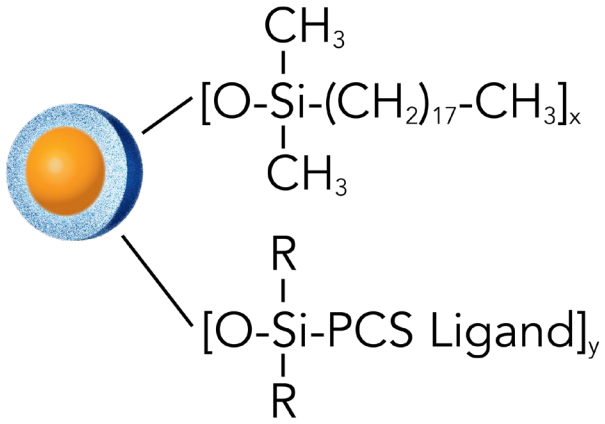
Superior efficiency with >2.8x plates!

Sharper peaks, faster analysis

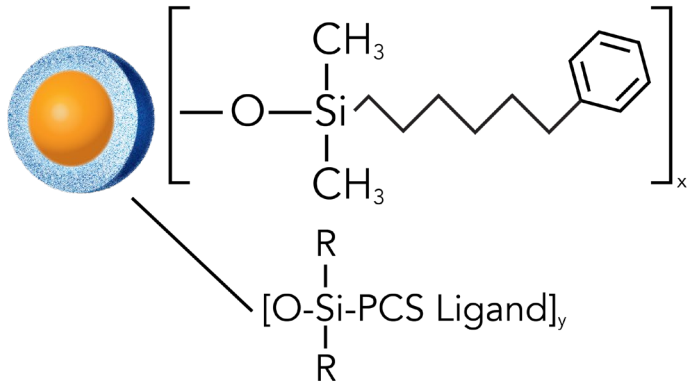
Superficially Porous Particle (SPP)

Using a modified silica stationary phase

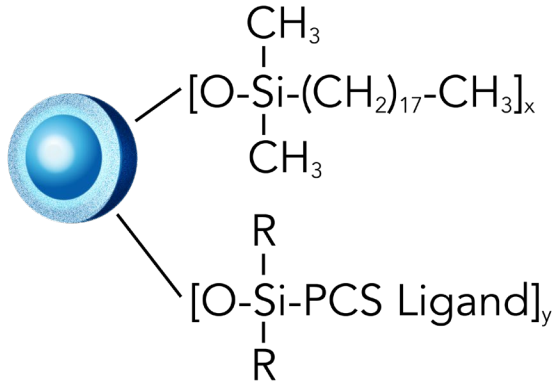
Introducing the HALO® PCS Phases: **P**ositively **C**harged **S**urface



HALO 90 Å PCS C18



HALO 90 Å PCS Phenyl-Hexyl



HALO 160 Å PCS C18

90 Å, 2.7 µm for Small Molecule Analyses

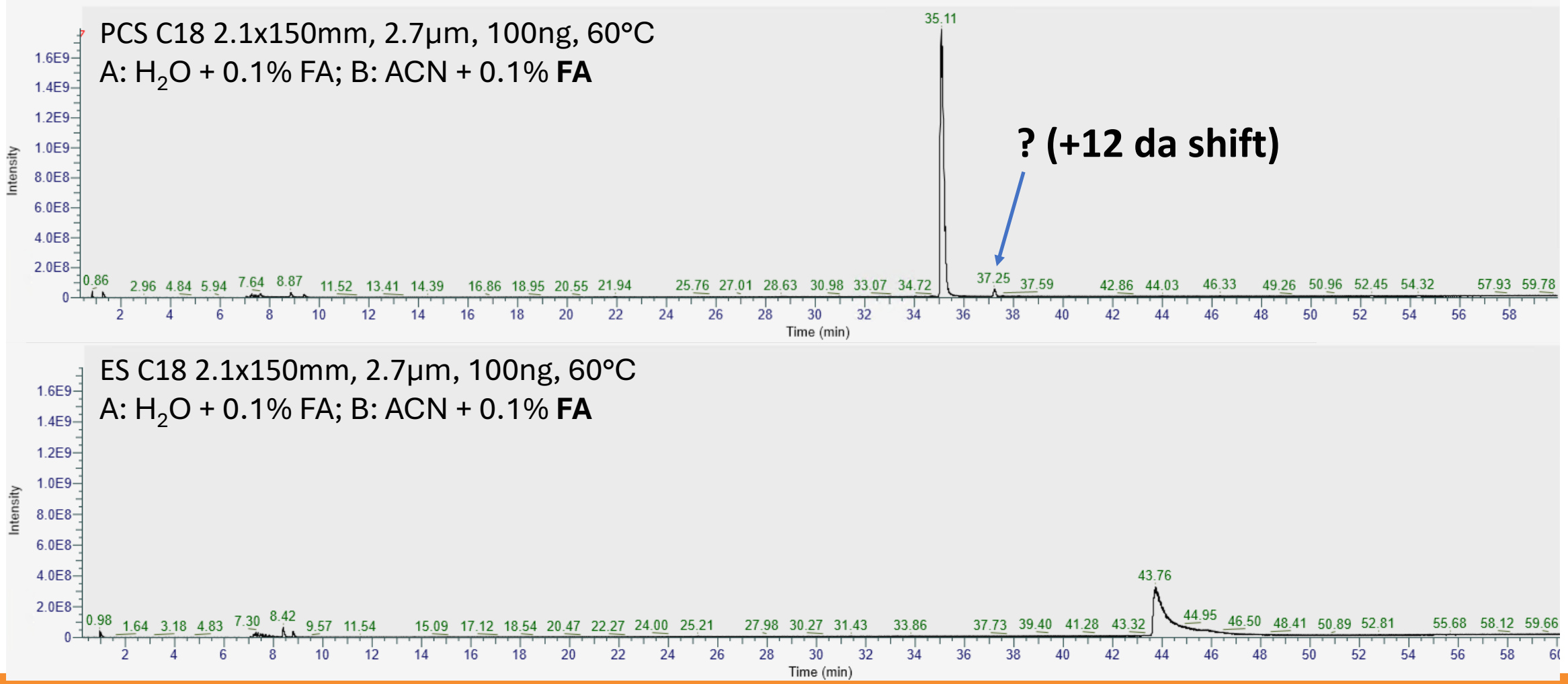
- Excellent peak shape and increased loading capacity for basic compounds
- Alternate L1 selectivity (PCS C18)
- Alternate L11 selectivity (PCS Phenyl-Hexyl)
- Built upon Fused-Core® technology for fast, efficient and reliable separations

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

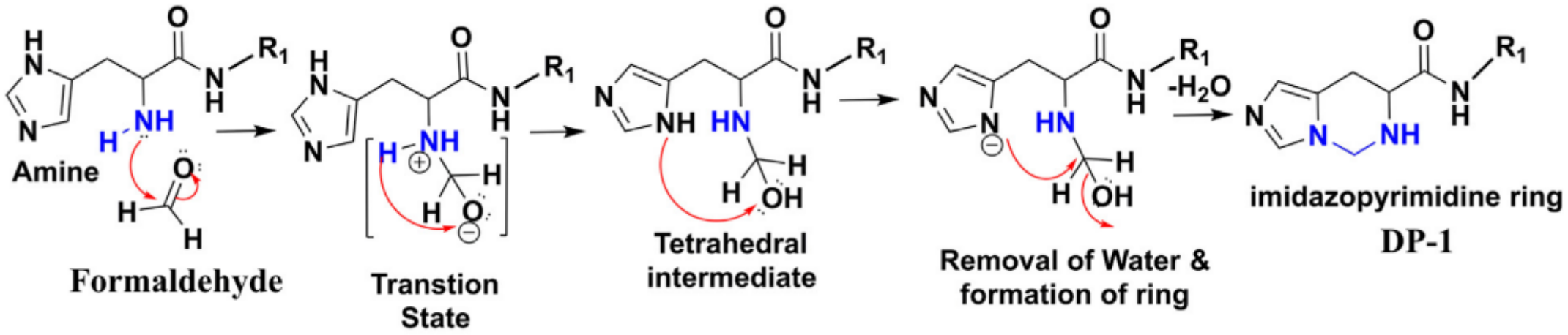
Semaglutide ES-C18 vs PCS C18

Compounded Semaglutide Sample



N-Terminal Modification of Liraglutide

- **N-terminal Histidine sensitive to Formaldehyde exposure in Liraglutide**



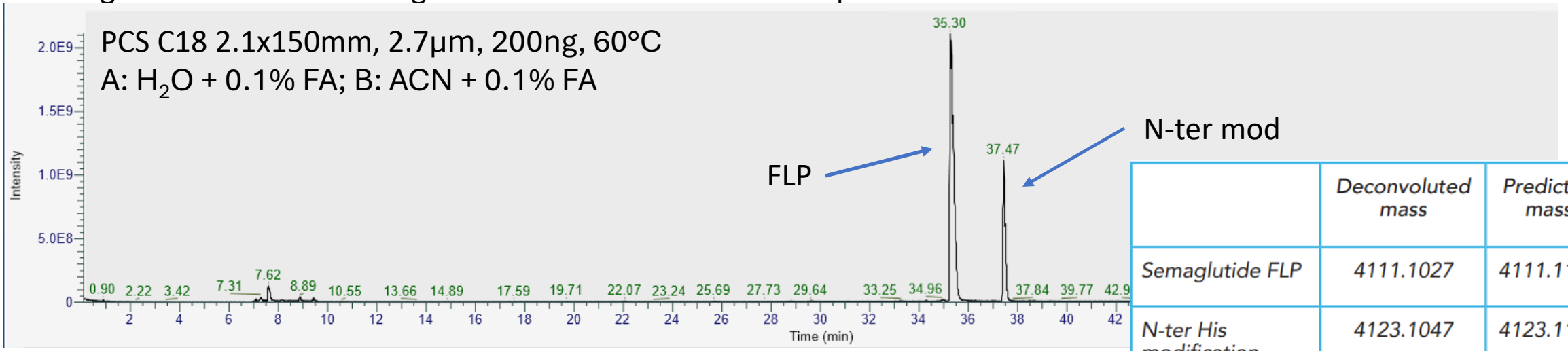
- **Semaglutide also has N-terminal Histidine**
- **Is Semaglutide also sensitive to formaldehyde?**

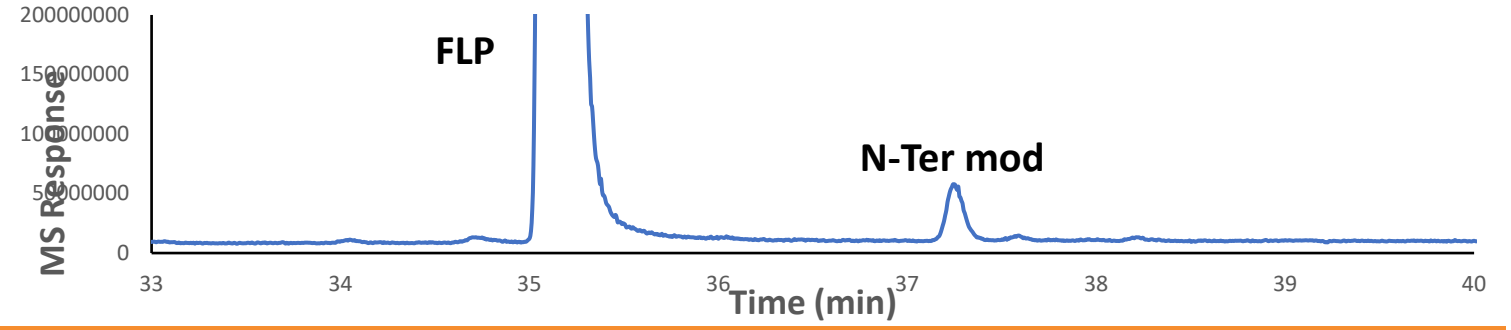
Sheikh, AR *et Al.* **J. Pharmaceutical Sciences** 113(2024) pp3246-3254

Semaglutide in Tris buffer

- Tris buffer manufactured from Nitromethane and Formaldehyde in a 1:3 Molar Ratio
- Tris buffer can also thermally degrade back into formaldehyde

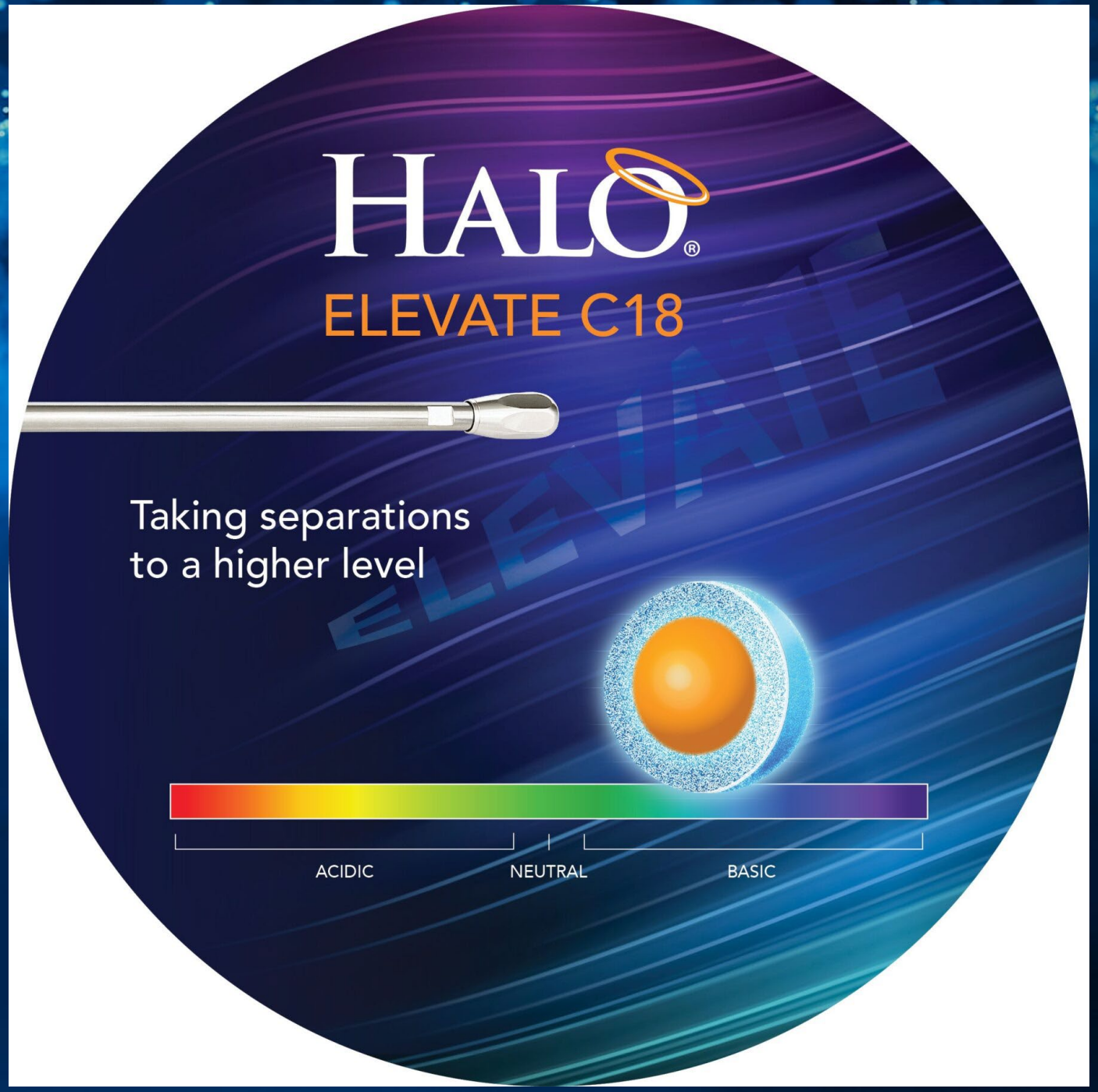
- 1mg Research Grade Semaglutide in 1ml 10mM Tris-HCl at pH 8.0. Heated 24hr at 40°C





	Deconvoluted mass	Predicted mass	ppm mass error
Semaglutide FLP	4111.1027	4111.1154	3.09
N-ter His modification	4123.1047	4123.1154	2.60

	Deconvoluted EIC	Integrated TIC
Compounded Semaglutide	1.42%	1.77%
Ozempic	0.24%	0.47%

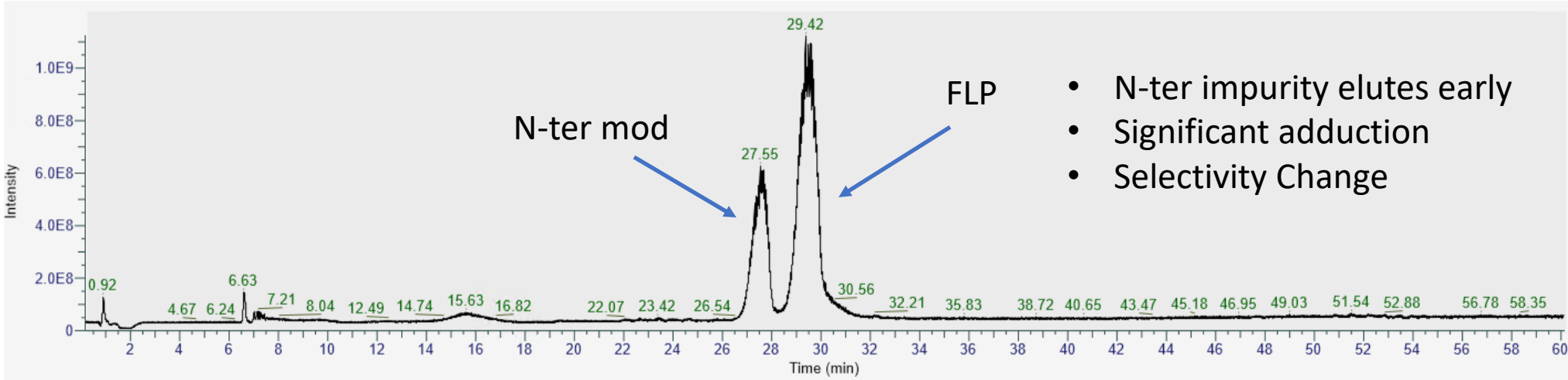


- Modified Organo-Silane Technology
- Excellent Lot to Lot Reproducibility and Stability in Alkaline Environments
- pH 2-12 Enables Wide Operational Use Range for Robust Method Development
- Reliability of Proven Fused-Core® Technology for Highest Efficiencies and Speed
- 2.7 µm particle size in 120 Å
- *1000 Å OLIGO Now available!*

Altering Selectivity using high pH

Goal: Find HPH conditions for GLP-1 that are also LC/MS friendly

20mM NH_4OH pH 9
1µg Semaglutide
Elevate C18 2.1x150mm



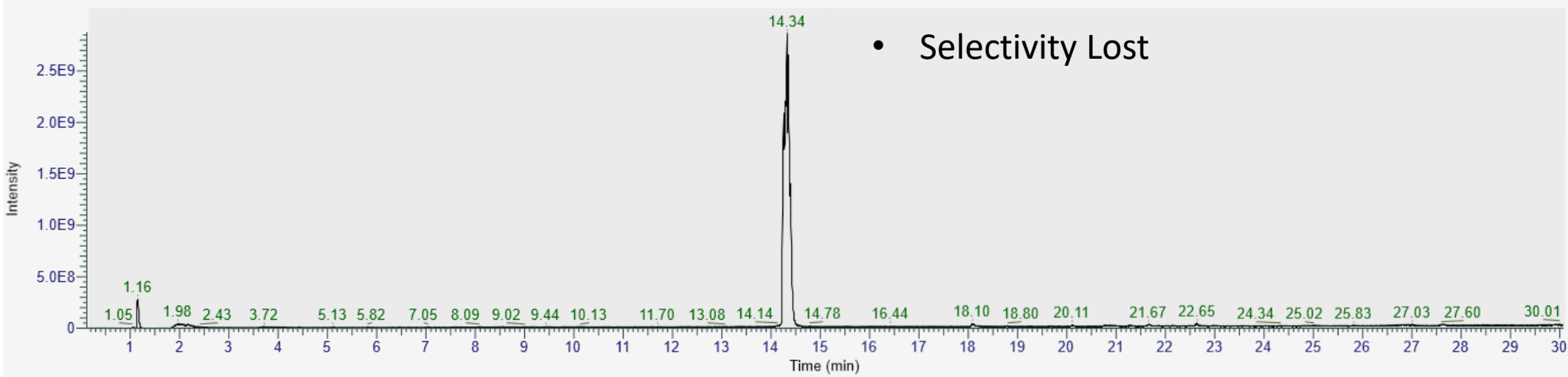
- N-ter impurity elutes early
- Significant adduction
- Selectivity Change

- Higher pH in NH_4OH showed peak shape degradation

- Other high pH buffers that are MS friendly.
  - Ammonium bicarbonate
  - N-Methyl Piperidine

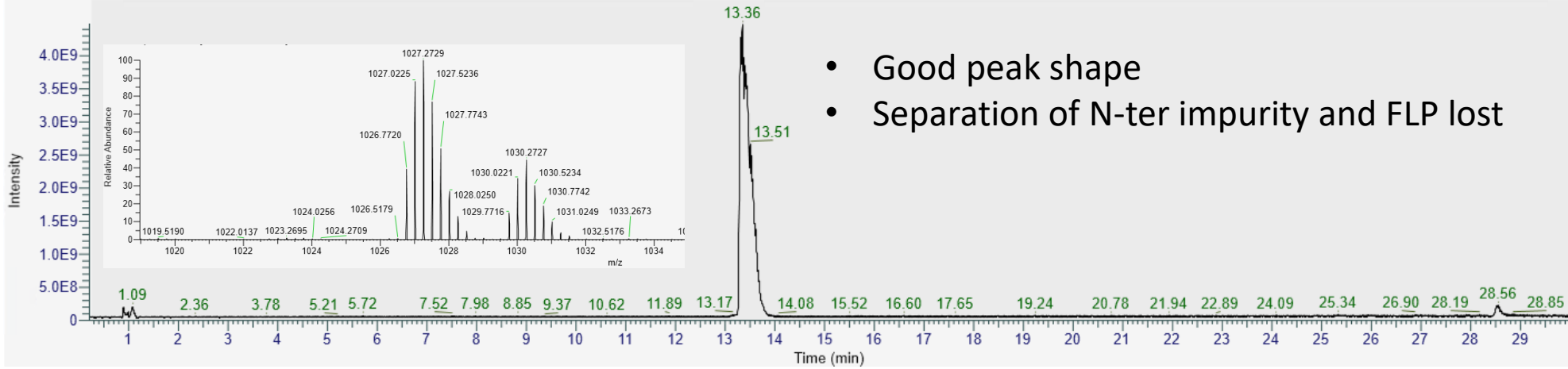
HPH Separation of Semaglutide

10mM AmBic pH 7.5
1µg Semaglutide
Elevate C18 2.1x150mm
Positive Mode



- Selectivity Lost

10mM N-methyl piperidine
pH 11.5
1µg Semaglutide
Elevate C18 2.1x150mm
Negative Mode



- Good peak shape
- Separation of N-ter impurity and FLP lost

- **N-ter modification comes out slightly earlier than FLP**
- **Sensitivity comparable to Formic Acid conditions on PCS C18**

Summary

- GLP-1's are a rapidly growing business
- Manufacturing presents significant challenges for Analytical QC processes
- Identification of a CQA in Liraglutide/Semaglutide
- HALO® PCS C18 ideal for evaluating impurities in GLP-1's at low pH
- HPH LC/MS presents methodology challenges
  - MS friendly buffers
- HALO® Elevate C18 shows robust separation conditions up to pH 11.5
  - Selectivity changes using N-terminal modification of Semaglutide as a marker
  - Provides a variety of conditions for analytical method development

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