

Advanced RP-HPLC Techniques for Peptide Analysis

From column chemistry to mobile-phase modifiers

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Applications Engineer

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Examples of Biotherapeutic Peptides

Generic	AA	MW	Route
Insulin and variants	51	5807.6	Extracted; Recombinant
Exenatide	39	4186.6	Synthetic
GLP-1 agonists (e.g. semaglutide, liraglutide)	31	~ 3700 – 4100	Synthetic; Recombinant (chemically modified)
Nesiritide	32	3464.0	Recombinant (E coli)
Goserelin	10	1269.4	Synthetic
Ceruletide (Cerulein)	10	1352.4	Synthetic
Bentiromide	1 (X-Tyr-Y)	404.4	Chemical
Enfuvirtide	36	4492.1	Synthetic
Vancomycin	7 (highly modified)	1449.3	Enzymatic
Leuprolide	9	1269.5	Synthetic
Oxytocin	9	1007.2	
Gramicidin	Mixture		Fermentation
Corticotropin	39	4541.1	Extracted
Icatibant	10	1304.5	Synthetic
Bivalirudin	20	2180.3	Synthetic
Gramicidin D	Mixture		Fermentation
Bacitracin	Mixture	1422.7	Fermentation
Cetorelix	10	1431.0	Synthetic
Pramlintide	37	3951.4	Recombinant
Teduglutide	33	3752.1	Recombinant (E coli)
Lepirudin*	65	6979.5	Recombinant (Yeast)
Octreotide	8	1019.2	Synthetic
Aprotinin	58	6511.5	Extracted

Therapeutic peptides can be produced in a variety of ways:

- Extraction (natural sources)
- Recombinant (wholly natural sequences)
- Fermentation (e.g., antimicrobial)
- Synthetic (solution phase, solid phase. etc.)

Impurities can arise that are:

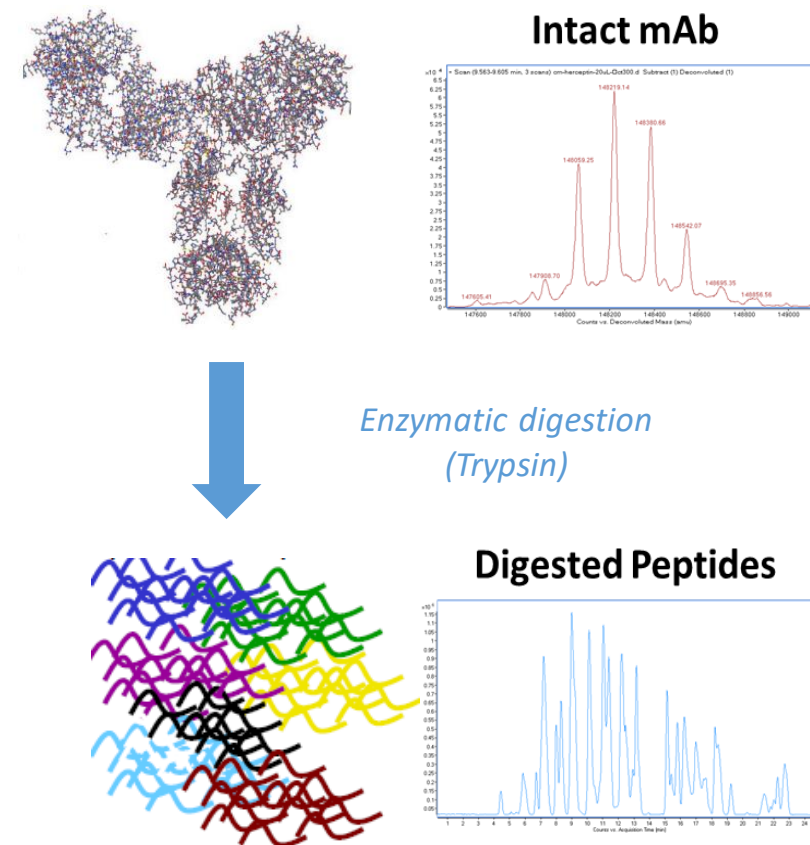
- Product related
- Process related
- Storage related

Peptide Mapping

- Comprehensive protein characterization is crucial for the quality control of biotherapeutics. Characterization includes powerful methods that detect and monitor single amino acid changes, modifications, and degradation products.
- A peptide map is a fingerprint of a protein.

Peptide mapping as identity test:

- Confirms primary structure and detects alterations in structure
- Demonstrates process consistency
- Used to identify **multiple critical quality attributes**



Peptide-Mapping Workflows for Biopharm

LC/UV

- Used for routine work such as in process measurements, QA/QC, and some stability studies
- Relies on retention time, peak area, and peak area ratio measurement
- Utilizes TFA in the mobile phase
- Some requirement to use LC/MS (same column and conditions) to confirm peak assignments

LC/MS

- Used in discovery/early development (and other R&D areas) to determine the primary sequence of an antibody/protein, to identify post-translational modifications, and to confirm protein identity
- Accurate results require good peak shape and MS sensitivity, so formic acid is the preferred additive as TFA is known to suppress ionization

Peptide Impurities

Chemical synthesis impurities

- Deletions
- Additions
- Racemisation
- Incomplete side-chain protection removal
- Side-chain removal by-products

General impurities

- Deamidation
- Oxidation
- Isomerisation
- Aggregation
- Salts (e.g., trifluoroacetate)

Peptide API impurity level

~ 0.1% Report

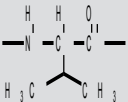
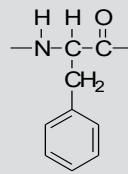
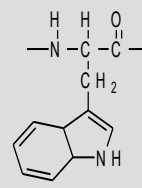
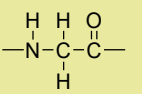
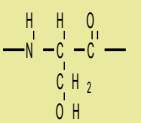
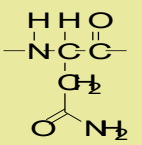
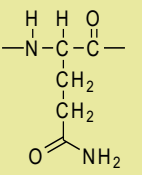
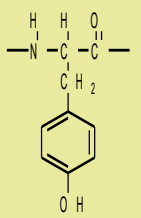
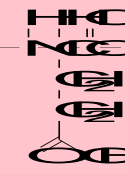
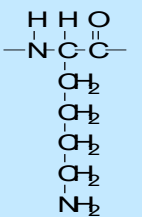
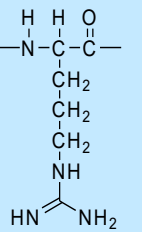
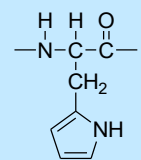
~ 0.5% Identify

> 1% Qualify

(Total impurities < 5%)

Hydrophobic, Neutral/Polar, Acid and Basic Amino Acids

Hydropathy values

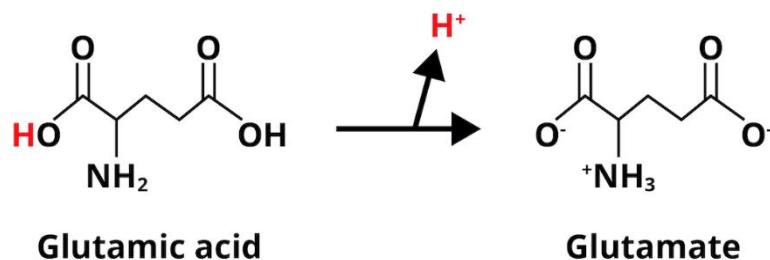
Ala  1.8	Val  4.2	Leu  3.8	Ile  4.5	Pro  -1.6
Met  1.9	Phe  2.8	Trp  -0.9	Gly  -0.4	Ser  -0.8
Thr  -0.7	Cys  2.5	Asn  -3.5	Gln  -3.5	Tyr  -1.3
Asp  -3.5	Glu  -3.5	Lys  -3.9	Arg  -4.5	His  -3.2

Kyte, J.; Doolittle, R. F. A Simple Method for Displaying the Hydropathic Character of a Protein. *J. Mol. Biol.* **1983**, 157 (1), 105–132.

Biomolecules Are Full of These Metal-Sensitive Functional Groups

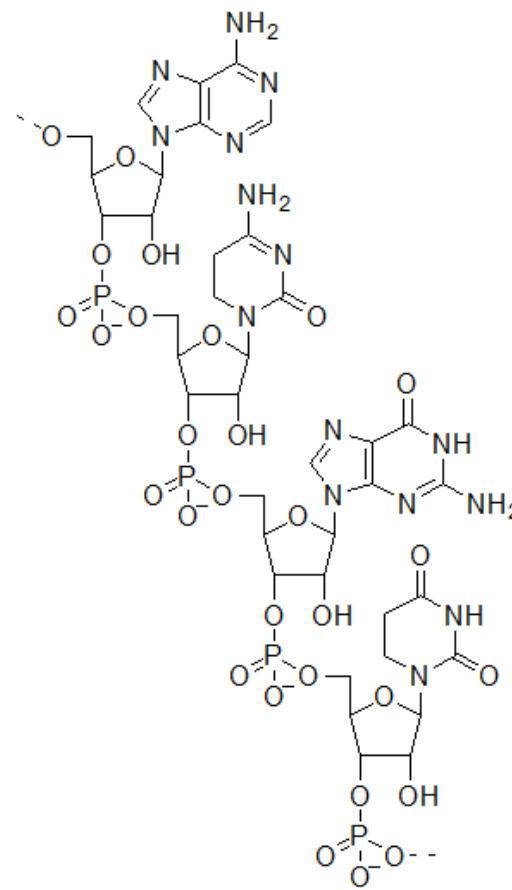
Peptides and proteins

Acidic amino acids and phosphorylation modifications

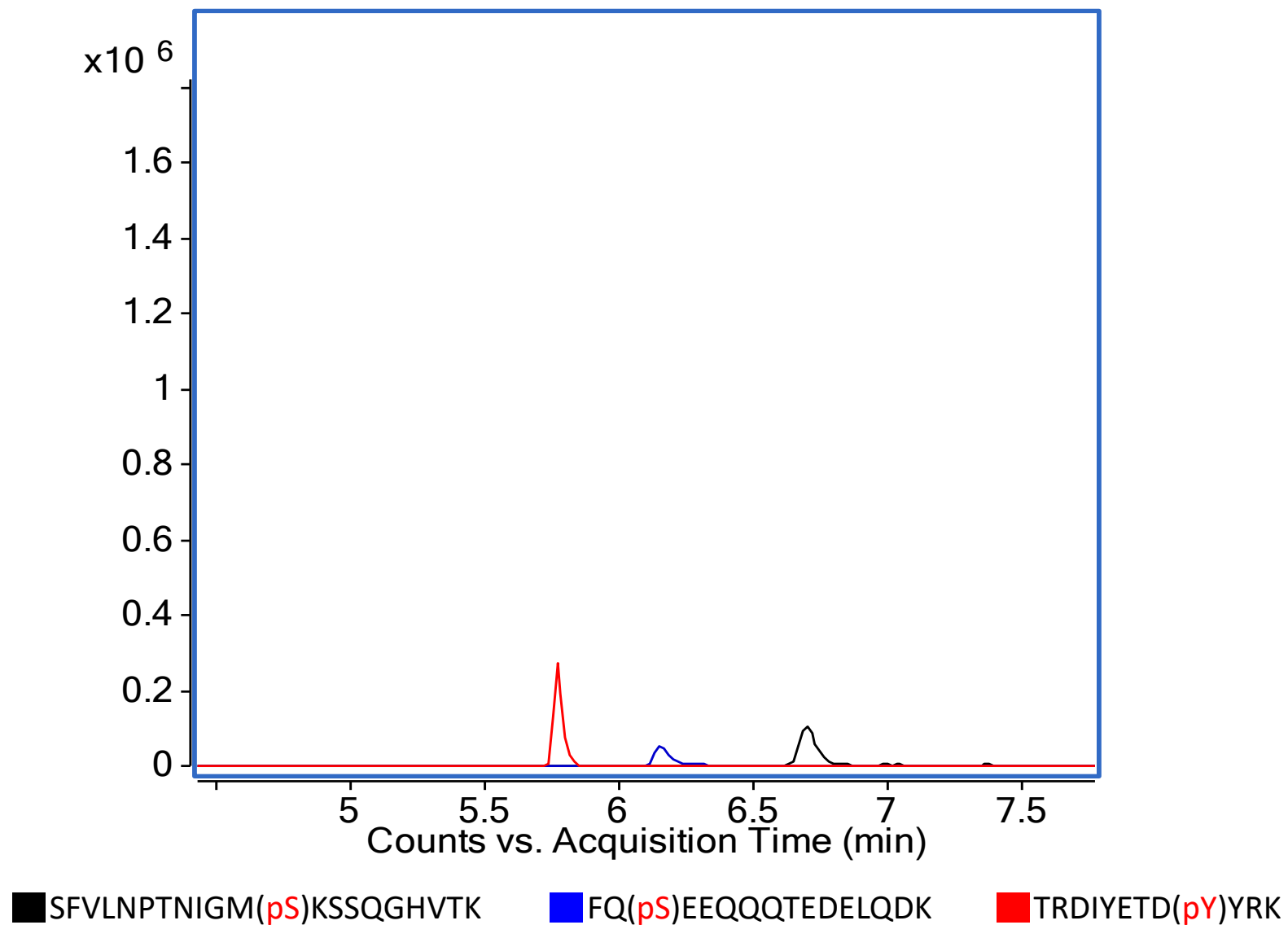


Oligonucleotides

Negatively charged phosphate backbone



Phosphopeptide: Stainless-Steel Flow Path



LC Separation Techniques for Peptides

Non-denaturing

Size exclusion chromatography

- Aggregation analysis

Ion exchange chromatography

- Cation exchange most common

Denaturing

Reversed phase

- Ion pairing

HILIC

- Primarily used for very hydrophilic peptides

Reversed Phase for Impurity Analysis

Separation based on hydrophobicity effectively separates different sequence lengths as well as impurities that change hydrophobicity:

- Oxidation
- Deamidation
- Loss of water

Reversed phase won't reliably separate or measure:

- Sequence isomers
- Racemization
- Aggregates

AdvanceBio Peptide Plus is the first choice for synthetic peptide impurity analysis.

Deletion sequence



Full-length product



Insertion (n + 1)



Stationary Phase Variables

Particle size → Smaller particle size = higher efficiency, *and* much higher pressure

Morphology → Superficially porous particles give higher efficiency than fully porous particles

Pore size → Large pore size gives faster mass transfer, but lower capacity

Surface area → Higher surface area = greater interaction

pH range → Change pH to control selectivity? Is the stationary phase stable?

Ligand → C18, C8, C4/C3, Diphenyl, or PS/DVB?

How about Carbon load and Endcapping?

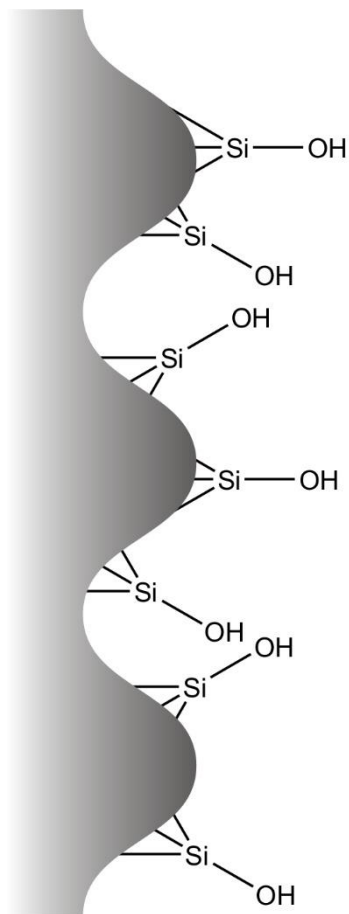
Stationary Phase Physical Characteristics

Phase	Pore Size (Å)	Surface Area (m ² /g)	pH Range	Carbon Load (%)	Endcapped	USP Designation
Eclipse Eclipse Plus C18 ●	80	180	2.0–9.0	10	Double	L1
ZORBAX StableBond SB-C18	80	180	0.8–8.0	10	No	L1
Agilent Prep C18	100	400	2.0–10.0	24	Yes	L1
Pursuit XRs C18	100	440	2.0–8.0	22	Yes	L1
PLRP-S 100Å (PS/DVB)	100	500	1–14	N/A	N/A	L21
Poroshell 120 EC-C18 ●●	120	130	2.0–8.0	10	Double	L1
Polaris C18-A	180	200	2.0–8.0	13.80	Yes	L1
Pursuit C18	200	200	2.0–8.0	12.90	Yes	L1
ZORBAX 300StableBond C18	300	45	1.0–8.0	2.8	No	L1
PLRP-S 300Å (PS/DVB)	300	400	1–14	N/A	N/A	L21

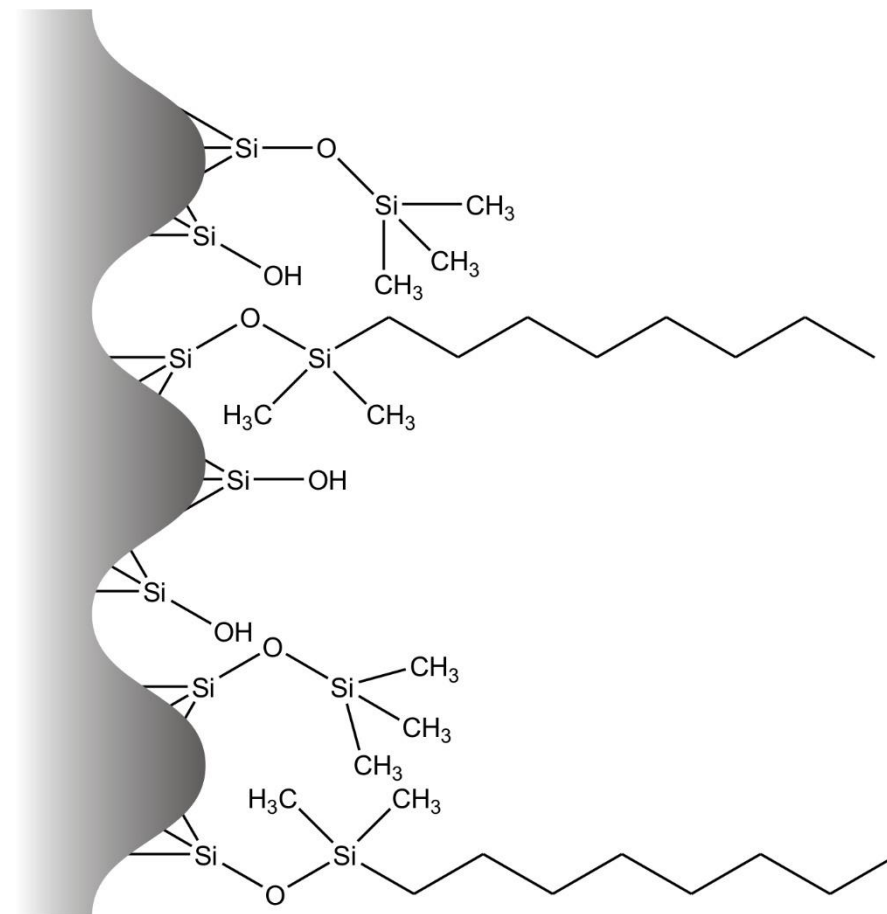
● Ultra Inert column hardware ● Solid PEEK or PEEK-lined SS (bio-inert) hardware

Manufacture of Reversed-Phase Silica

Silica/silanol surface

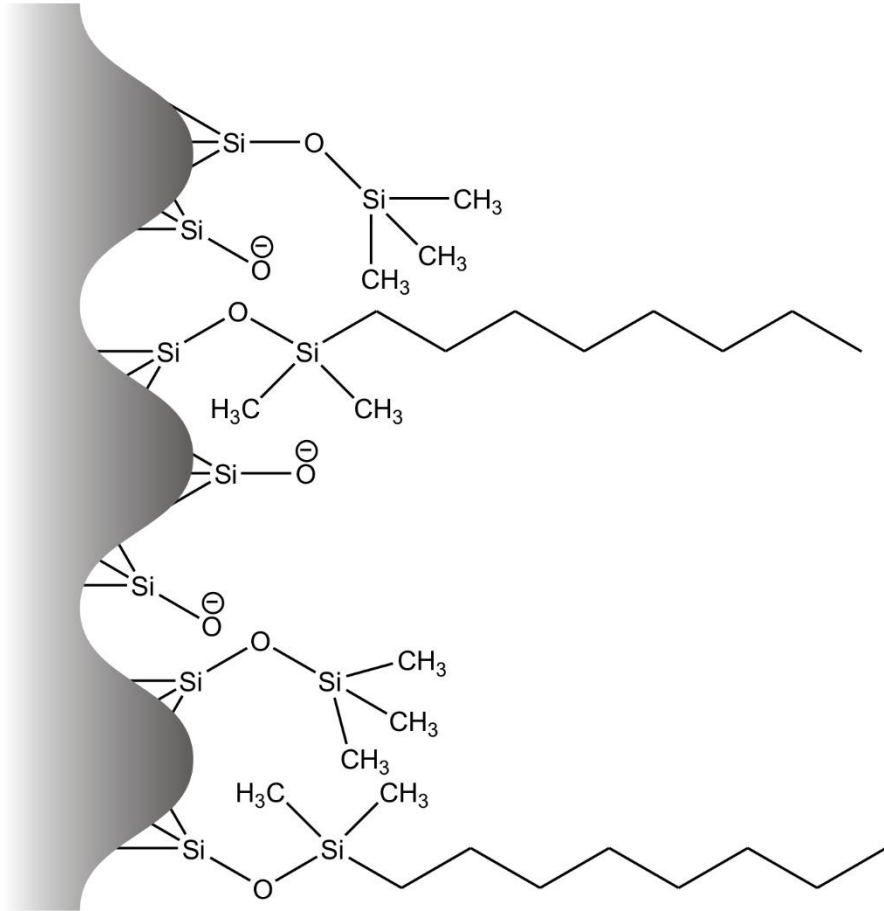


Alkyl bonded, endcapped silica

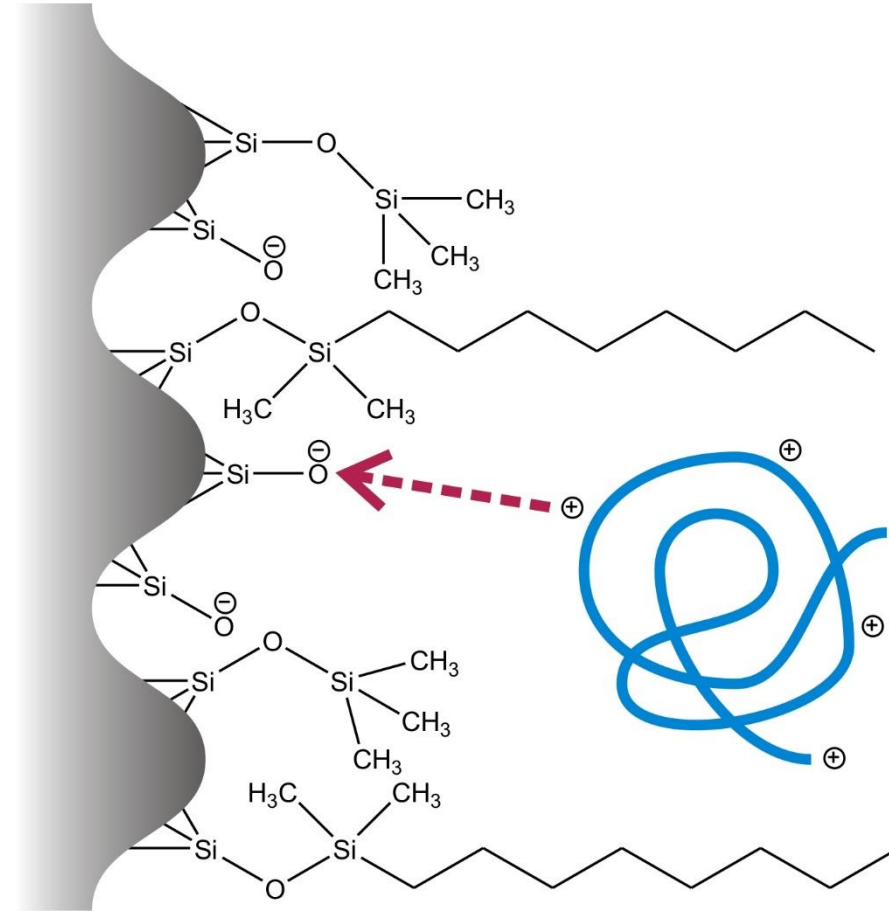


Reversed-Phase Chromatography of Peptides

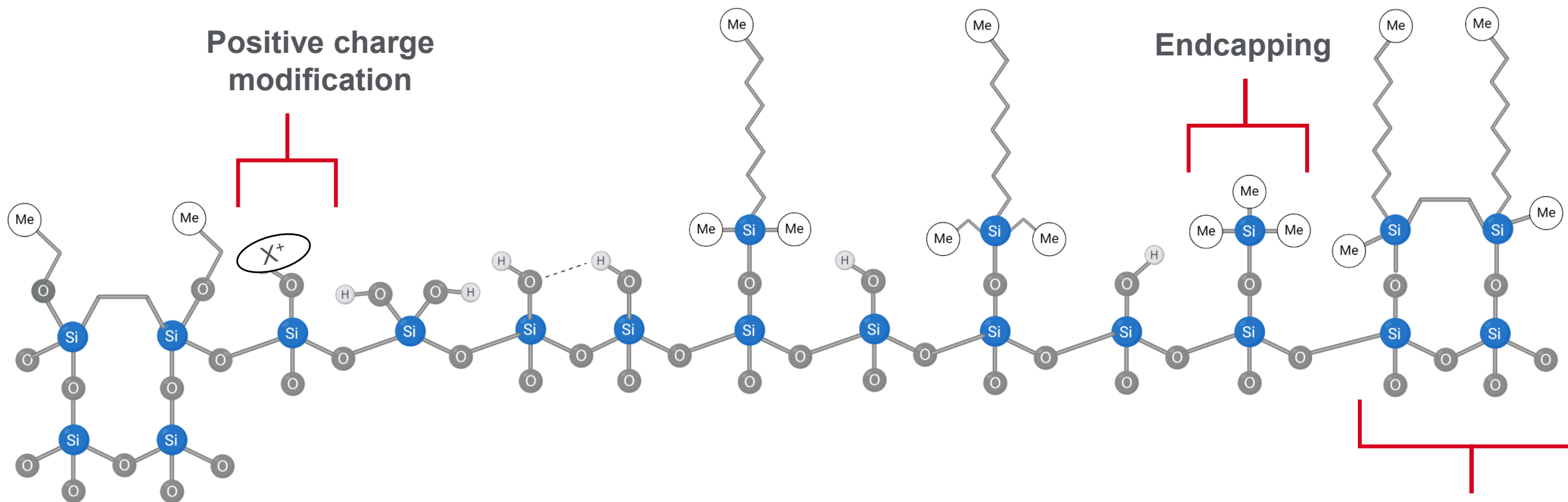
Deprotonated silanols (pH > 4)



Undesirable ionic interactions

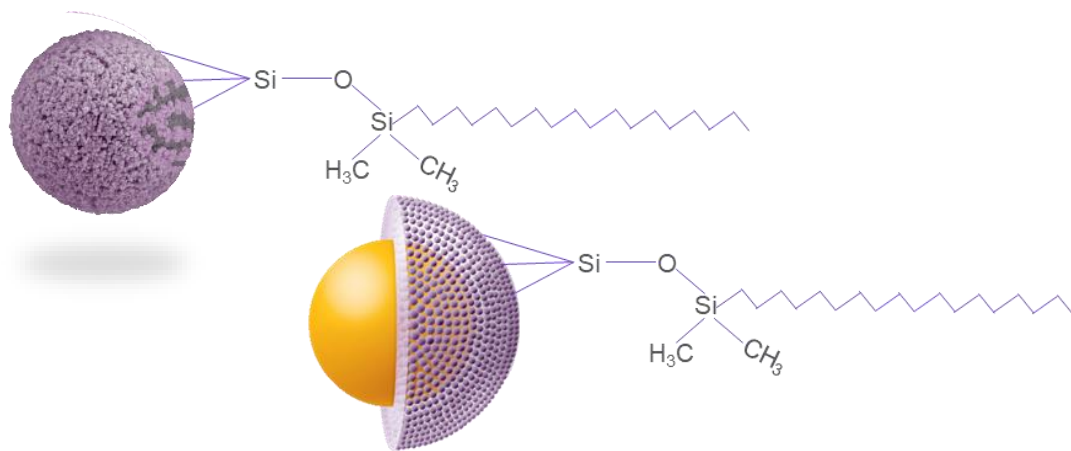


Endcapping and Novel Chemistries Can Block Silanols



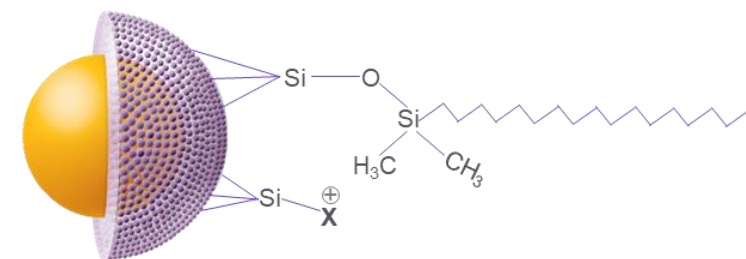
- Modification of the particle chemistry blocks silanols
- Charged surface modification most effectively blocks silanol interactions, improving peak shape with weaker acid modifiers like formic acid

Charged Surface Chemistry Versus Traditional C18



Altura ZORBAX Eclipse Plus C18 (1.8 μm) AdvanceBio Peptide Mapping (2.7 μm)

- Traditional endcapped C18 chemistry, with very similar selectivity to each other
- Good retention of small, hydrophilic peptides
- Solid all-round option

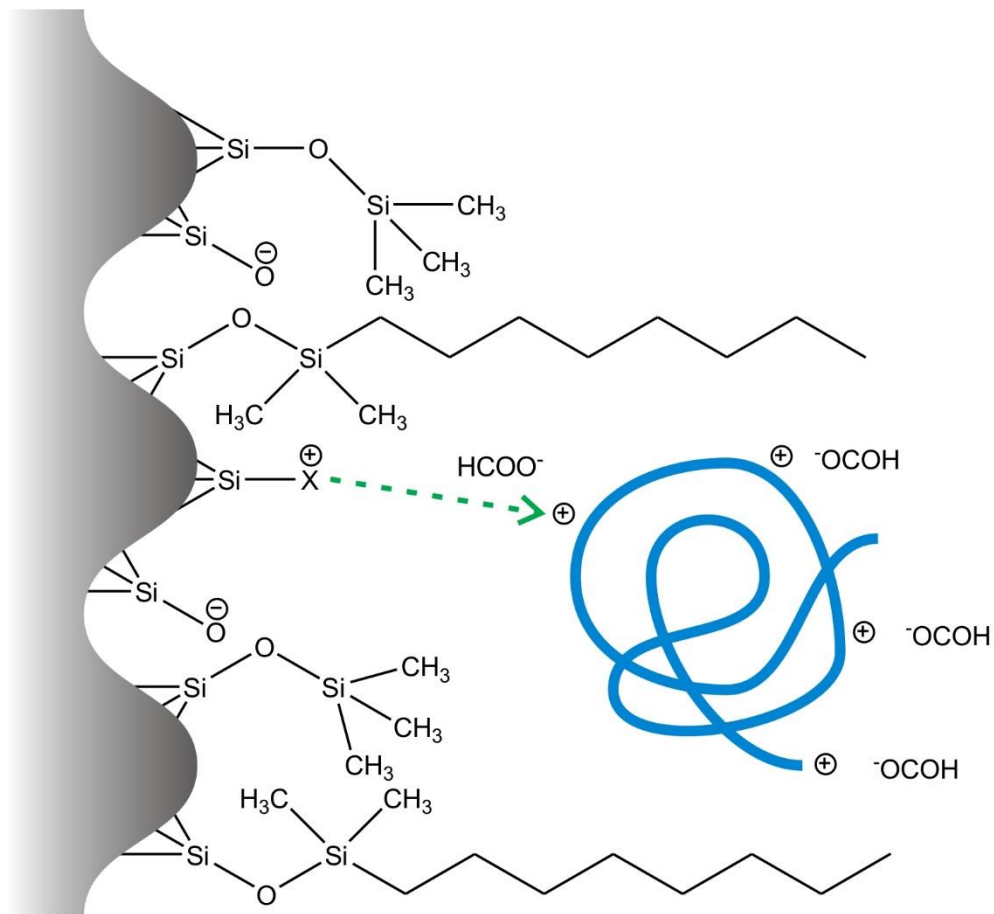


Altura Peptide Plus (AdvanceBio Peptide Plus in SS)

- Charged surface C18 chemistry
- Alternate selectivity, especially for deamidated peptides
- Excellent peak shape with formic acid mobile phase and under high loading conditions
 - Helps identify low-abundance peptides, such as from host cell proteins or impurities

Charged-Surface Hybrids

Peptide Plus charge effect



Application Note
Biopharma

Agilent
Trusted Answers

Analysis of a Synthetic Peptide and Its Impurities

Using mass spectrometry compatible mobile phase with an Agilent AdvanceBio Peptide Plus column

Authors
Andrew Coffey and
Veronica Qin
Agilent Technologies, Inc.

Abstract
Conventionally, chromatographic peptide separation with UV detection is performed using C18 reversed-phase HPLC columns and mobile phase containing trifluoroacetic acid (TFA) as ion pair reagent. However, although TFA improves resolution, it can suppress the mass spectrometry (MS) signal. Trifluoroacetic acid (TFA) is a preferred ion pair reagent for MS detection but can be replaced with many traditional C18 columns. This application note demonstrates the use of the Agilent AdvanceBio Peptide Plus column to separate synthetic peptides with MS-compatible FA as a mobile phase modifier.

Application Note
Biologics

Agilent
Trusted Answers

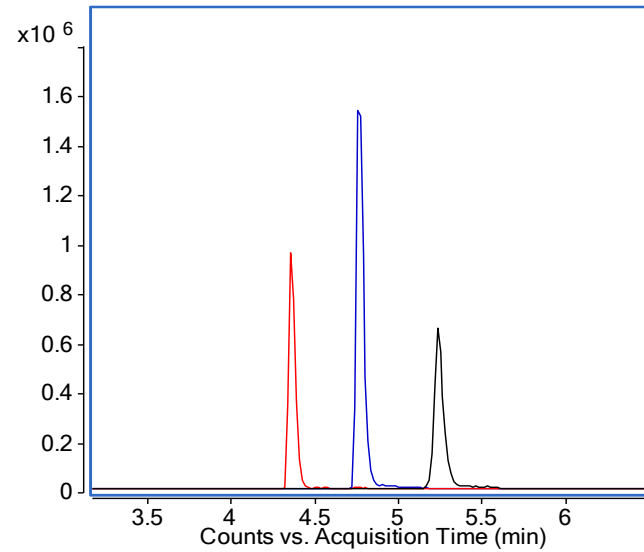
Separation of Deamidated Peptides with an Agilent AdvanceBio Peptide Plus Column

Authors
Oscar Potter and Veronica Qin
Agilent Technologies, Inc.

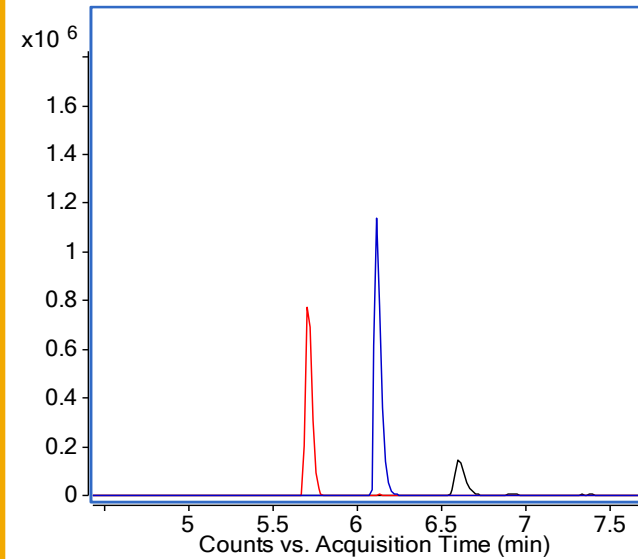
Abstract
Deamidations of glutamine and asparagine are amongst the most common degradations affecting proteins. However, analysis of deamidation by mass spectrometry is challenging due to the small mass shift of less than 1 Da versus the unmodified form. Site-specific deamidation is often determined by protease digestion followed by LC/MS analysis, but even this approach can fail when the unmodified and deamidated forms are not chromatographically resolved. Fortunately, a charged surface C18 column dramatically improves the resolution of deamidated peptides from their unmodified variants. Furthermore, mobile phase optimization can provide additional control over the resolution of these analytes.

Phosphopeptide: Power of an Inert Flow Path

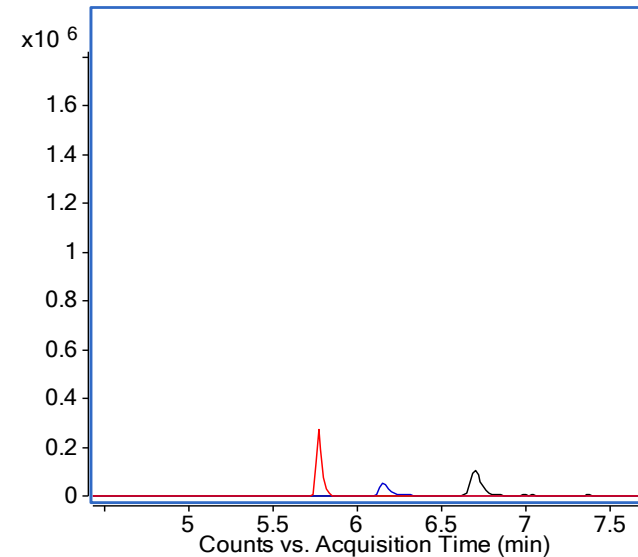
Bio LC
Altura Ultra Inert column



Standard LC
Altura Ultra Inert column



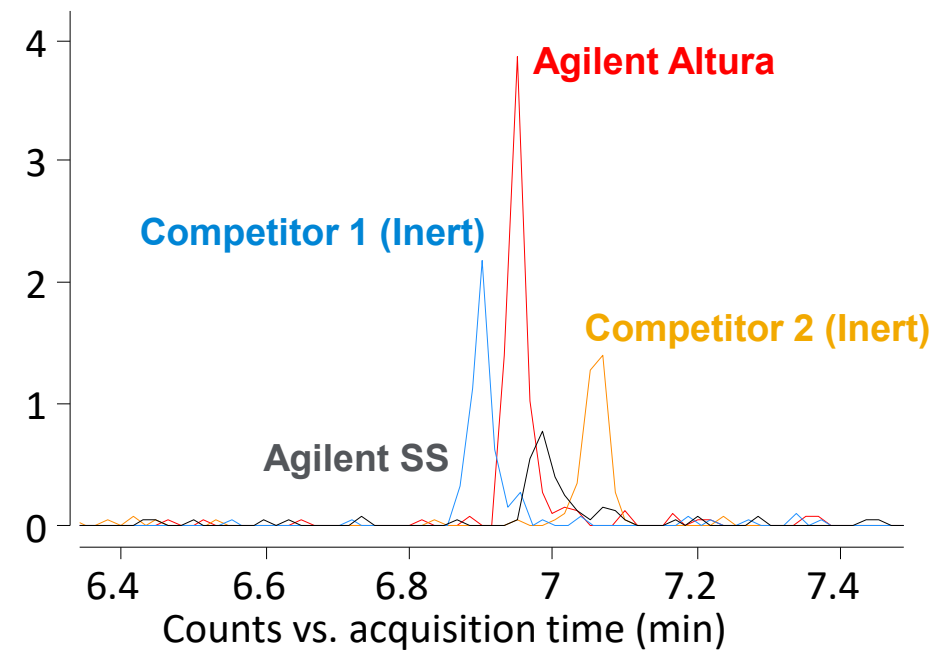
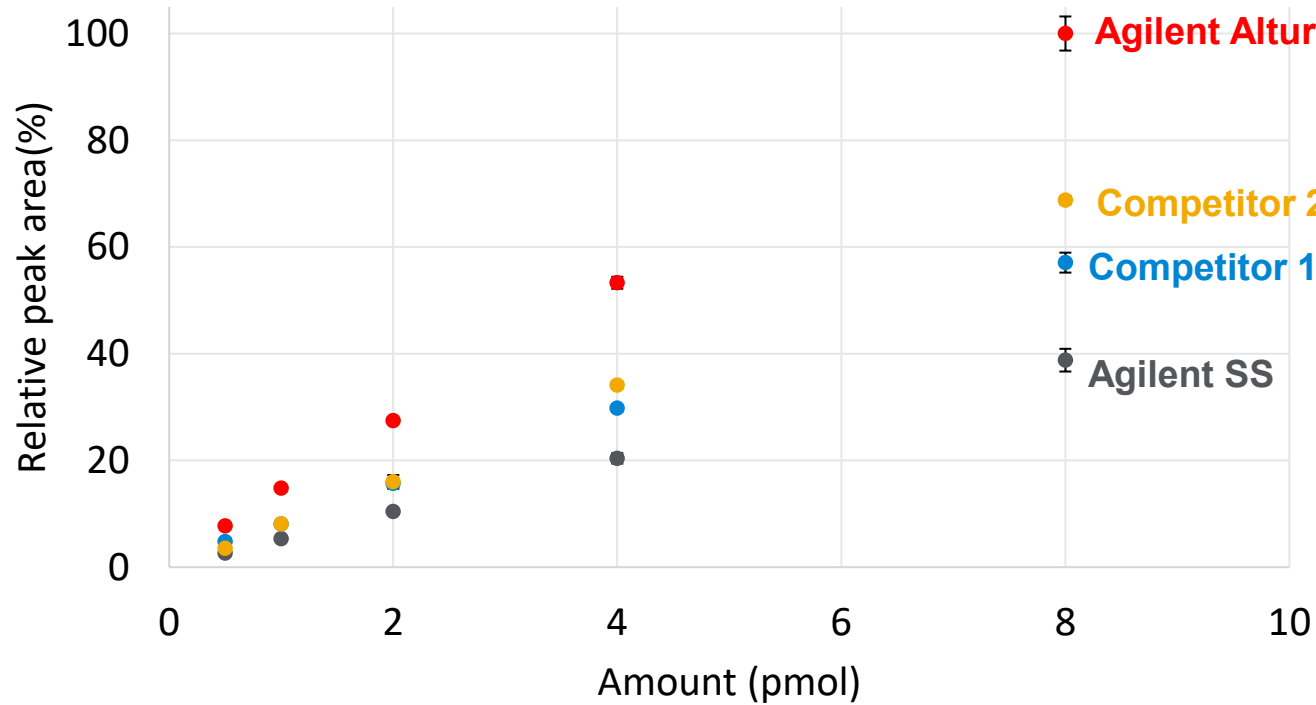
Standard LC
SS column



Up to 25x increase
in peak area

■ SFVLNPTNIGM(**pS**)KSSQGHVTK
■ FQ(**pS**)EEQQQTEDELQDK
■ TRDIYETD(**pY**)YRK

Altura Enables Highly Sensitive Peptide Analysis



0.5 pmol peptide
FQ(**pS**)EEQQQTDELQDK

Peptide Mapping Separation: Column Selection

Column choice depends on:

- Instrument capabilities and requirements
 - UV versus MS detection or both
 - Pressure capabilities
- Sample
 - Hydrophilic versus hydrophobic peptides
 - Larger polypeptides present
- Mobile-phase requirements
 - High pH required for sample
 - TFA versus formic acid

Peptide Mapping Separation: Column Selection

Choose your column carefully for best results

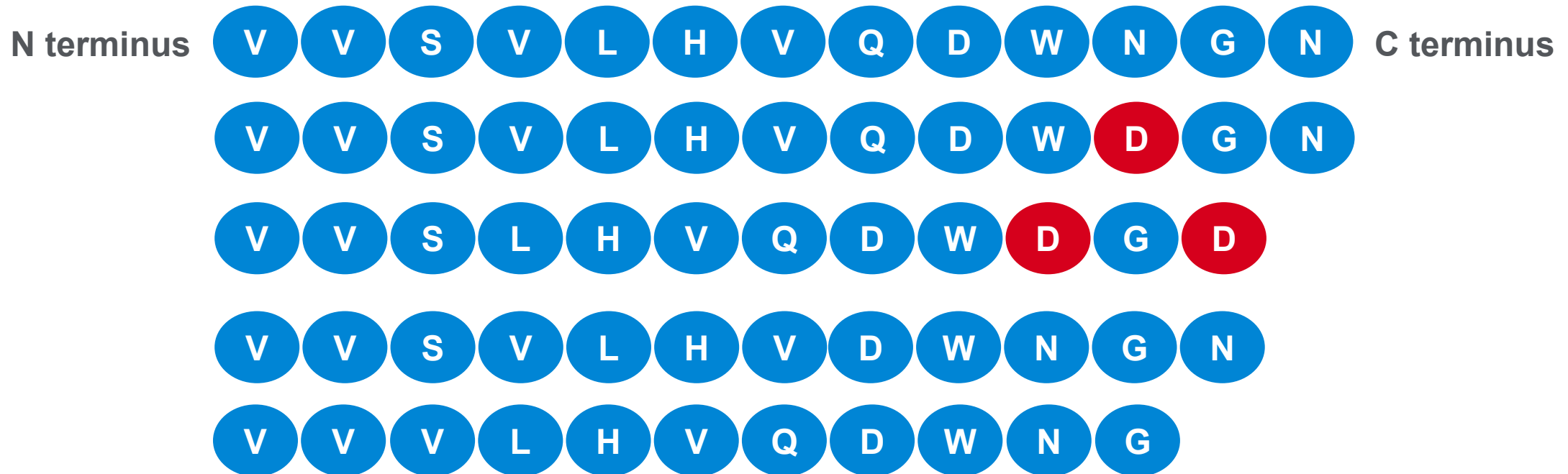
- Usually, longer columns are better—good to start with 150 mm
- Longer columns enable higher resolution for more complex peptide maps
- Use 2.1 mm id for mass spec sensitivity
- Smaller pore sizes—100 to 150 Å—are ideal for peptides

Agilent Biomolecule Columns Portfolio

Protein Therapeutics										Oligonucleotides		Capsid Therapeutics			
Titer Determination	Aggregate Analysis	Intact Purity & PTM Analysis		Peptide Mapping & PTM Analysis	Charge Variant Analysis	Glycan Analysis	Surfactant Analysis	Amino Acid / Cell Culture Media Analysis		Purification & Impurity Analysis		Aggregation	Empty/Full	Capsid Identity	Surfactant Analysis
Affinity	Size Exclusion	Reversed Phase >150 Å	Hydrophobic Interaction	Reversed Phase <150 Å	Ion Exchange	Hydrophilic Interaction	Reversed Phase >150 Å	Reversed Phase <150 Å	Hydrophilic Interaction	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase	Reversed Phase >150 Å
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S 1,000 Å 5 µm	AdvanceBio HIC	Altura Eclipse Plus C18	Bio mAb / Bio IEX 5 µm	AdvanceBio Amide HILIC	AdvanceBio Surfactant Profiling	AdvanceBio Amino Acid Analysis	Altura Poroshell HILIC-Z	Altura Oligo HPH-C18	*PL-SAX	AdvanceBio SEC 2.7 µm 500 & 1,000 Å	Bio SAX	ZORBAX RRHD 300 Å, 1.8 µm	AdvanceBio Surfactant Profiling
Bio-Monolith Protein A	AdvanceBio SEC	PLRP-S*		AdvanceBio EC-C18	Bio mAb (WCX)	AdvanceBio Glycan Mapping		ZORBAX Eclipse AAA 3.5 µm		*AdvanceBio Oligonucleotide	Bio SAX	Bio SEC-5 2,000 Å	Bio-Monolith (QA, DEAE, SO3)	Altura Eclipse Plus C18	
Bio-Monolith Protein G	*Bio SEC-5	AdvanceBio RP mAb 450 Å		AdvanceBio Peptide Mapping	AdvanceBio EC-C18						*PLRP-S	*Bio SAX		AdvanceBio Peptide Mapping	
	*Bio SEC-3	ZORBAX RRHD 300 Å, 1.8 µm		AdvanceBio Peptide Plus	*PL SCX, SAX			Biomolecule Sample Preparation							
	PROTEEMA	*ZORBAX 300SB 3.5, 5, and 7 µm		ZORBAX RRHD 300 Å, 1.8 µm	Bio-Monolith (QA, DEAE, SO3)			Desalting & Buffer Exchange	Released N-Glycan						
		Poroshell 300 5 µm						AdvanceBio Spin	AdvanceBio Gly-X						
								AdvanceBio Desalting-RP	AdvanceBio Sialic Acid						
												Column hardware			
												*Scalable to prep	Stainless steel (SS)	Solid PEEK or PEEK-lined SS bioinert	Ultra Inert column hardware

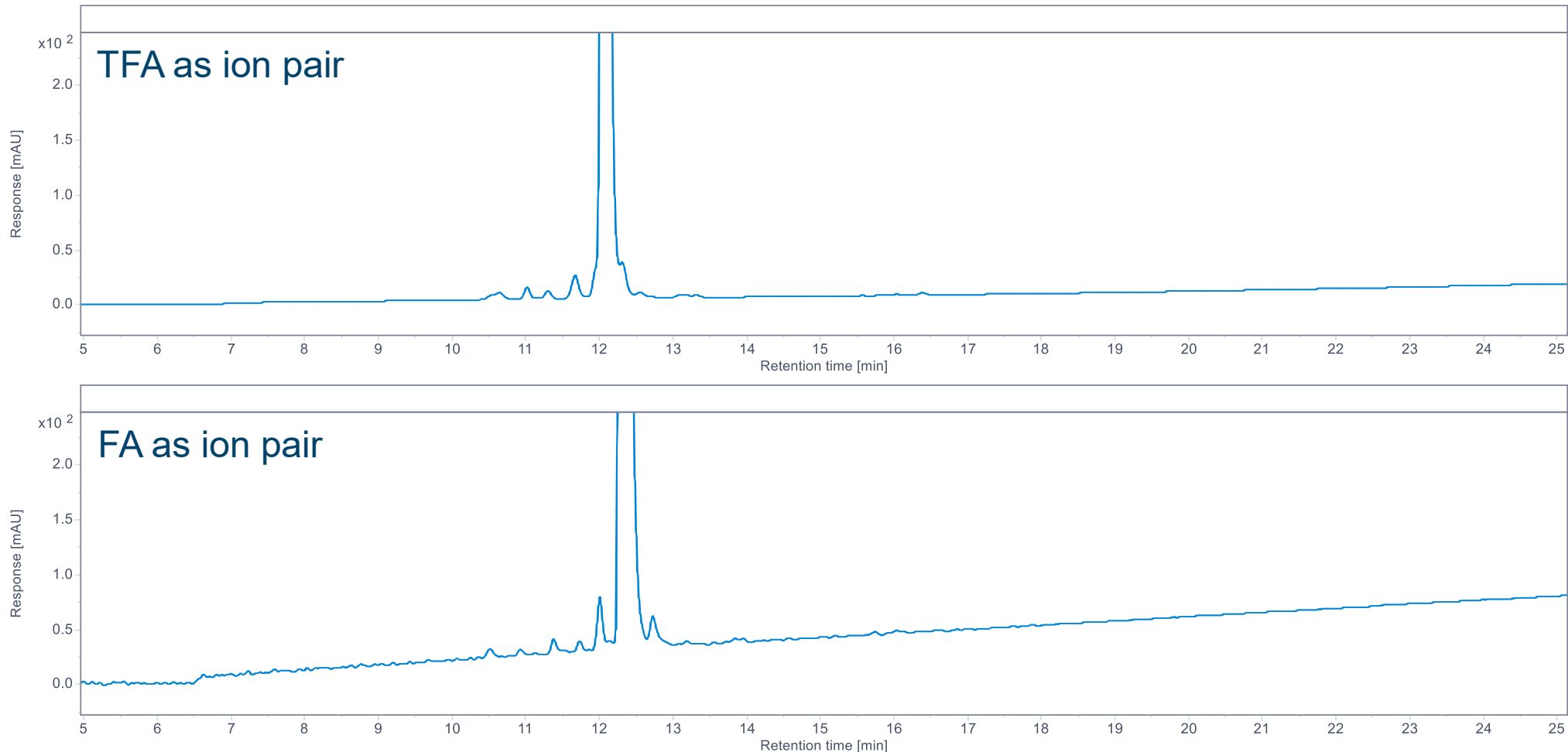
Reversed-Phase Impurity Analysis

- Detection, identification, or quantitation of production or process-related impurities
 - Amino acid addition, insertion, deletion, or truncation
 - Deamidation, oxidation, stereo-isomerization
- Reversed-phase separates based on differences in hydrophobicity



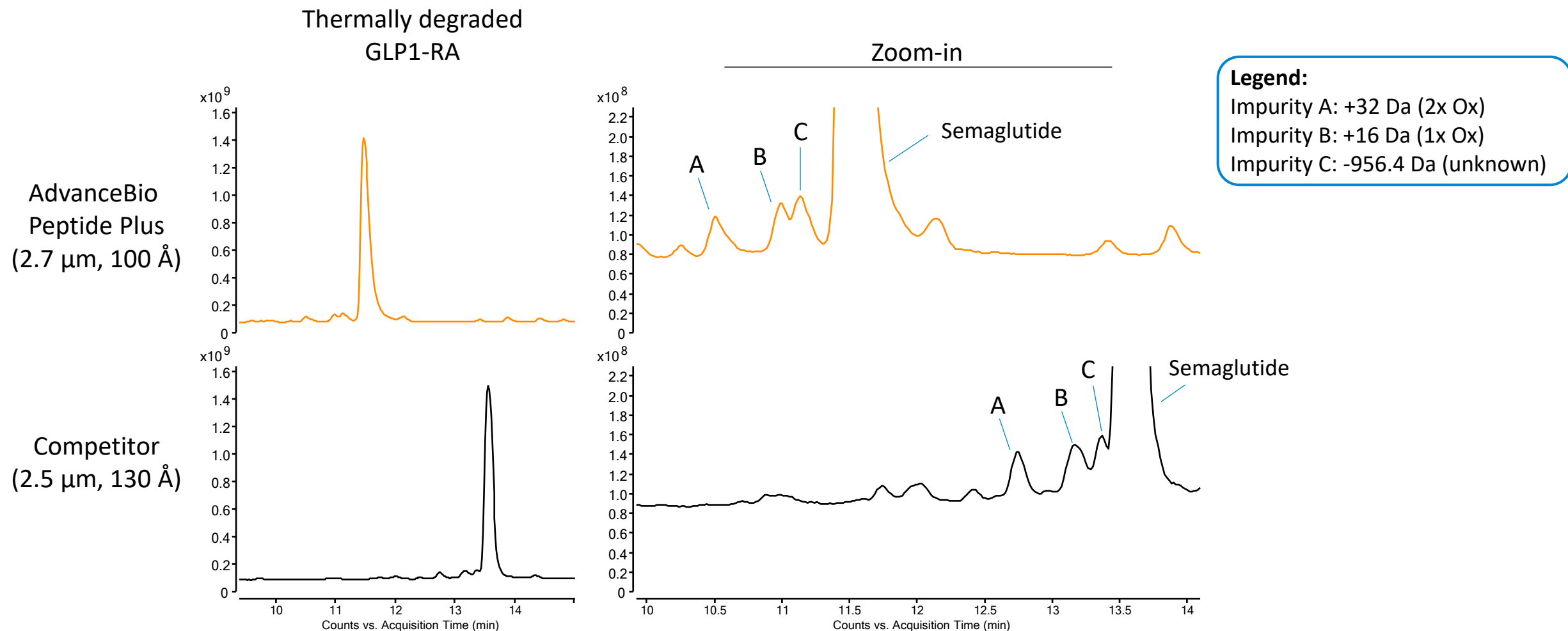
Heat-Treated Semaglutide with AdvanceBio Peptide Plus

Impurities are well resolved with formic acid as ion pair



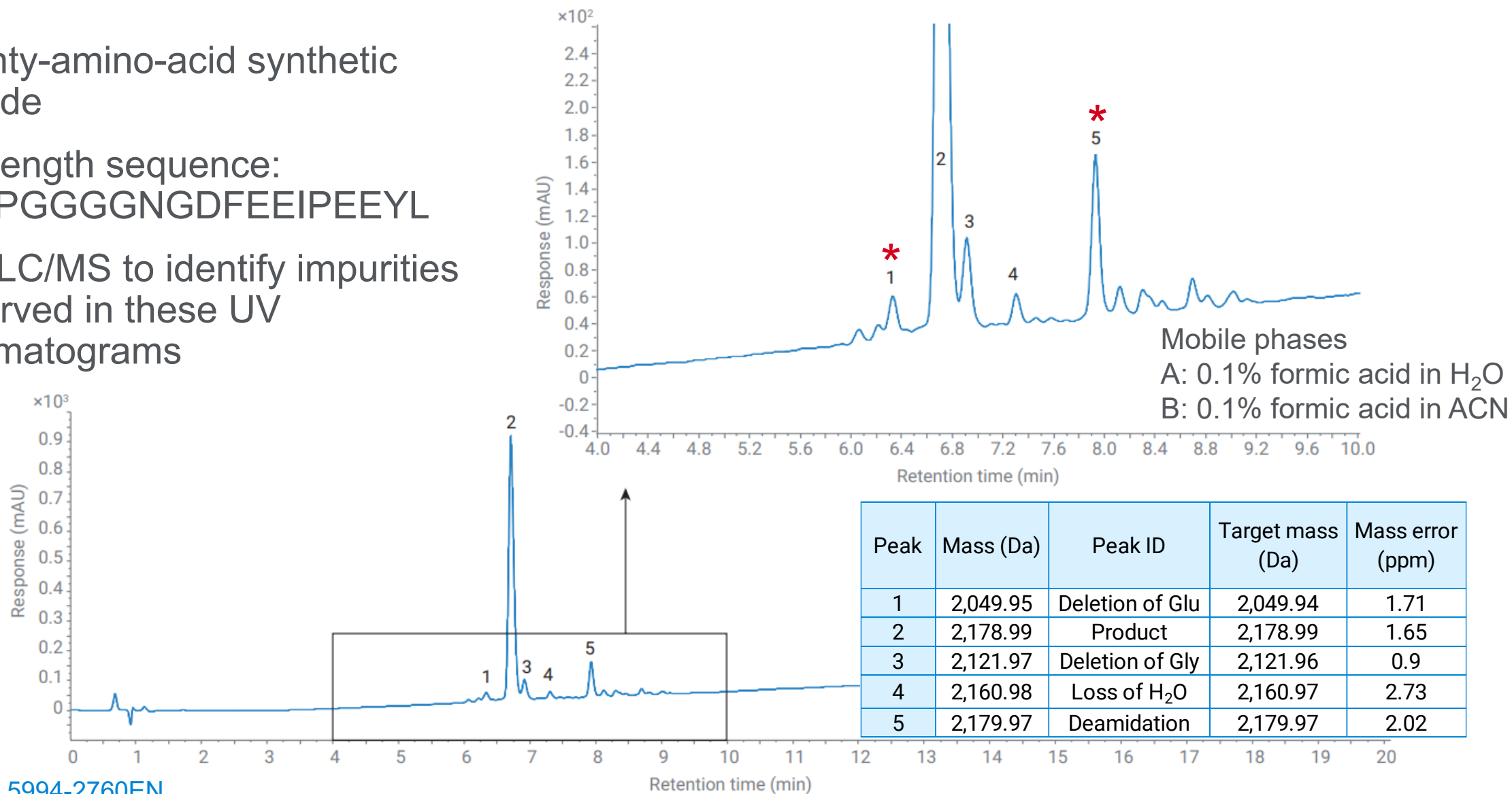
Application note: [5994-7419EN](#)

AdvanceBio Peptide Plus Versus Competitor Column



Synthetic Peptide Impurity Profiling of Bivalrudin

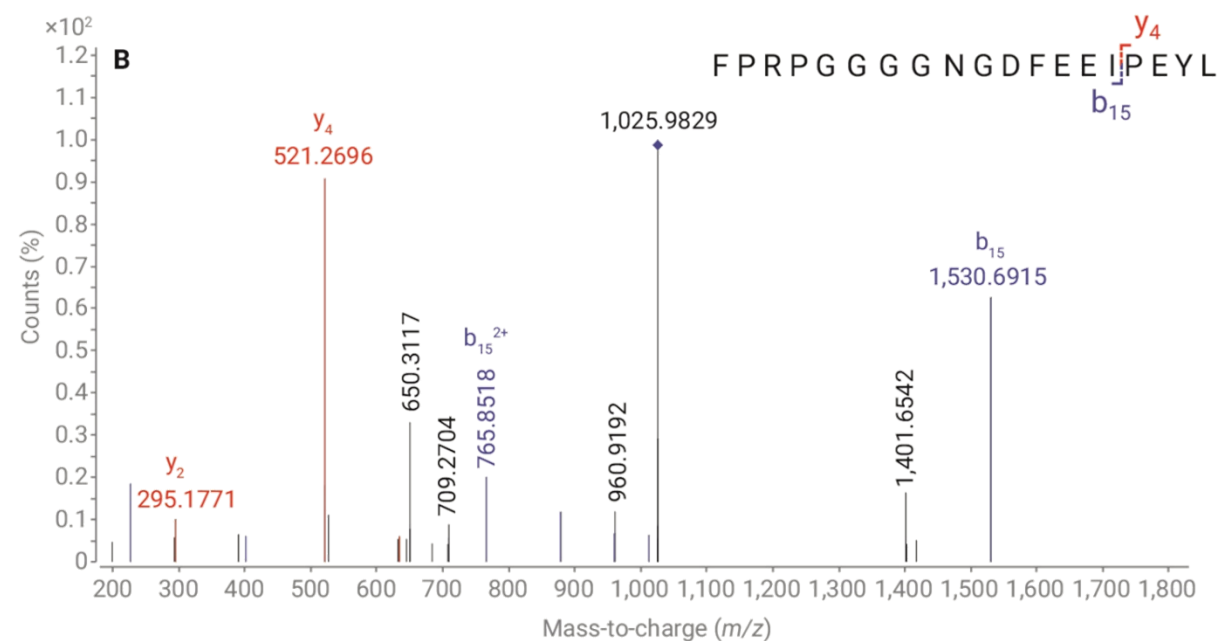
- Twenty-amino-acid synthetic peptide
- Full-length sequence:
FPRPGGGGNGDFEEIPEEYL
- Use LC/MS to identify impurities observed in these UV chromatograms



Application note: [5994-2760EN](#)

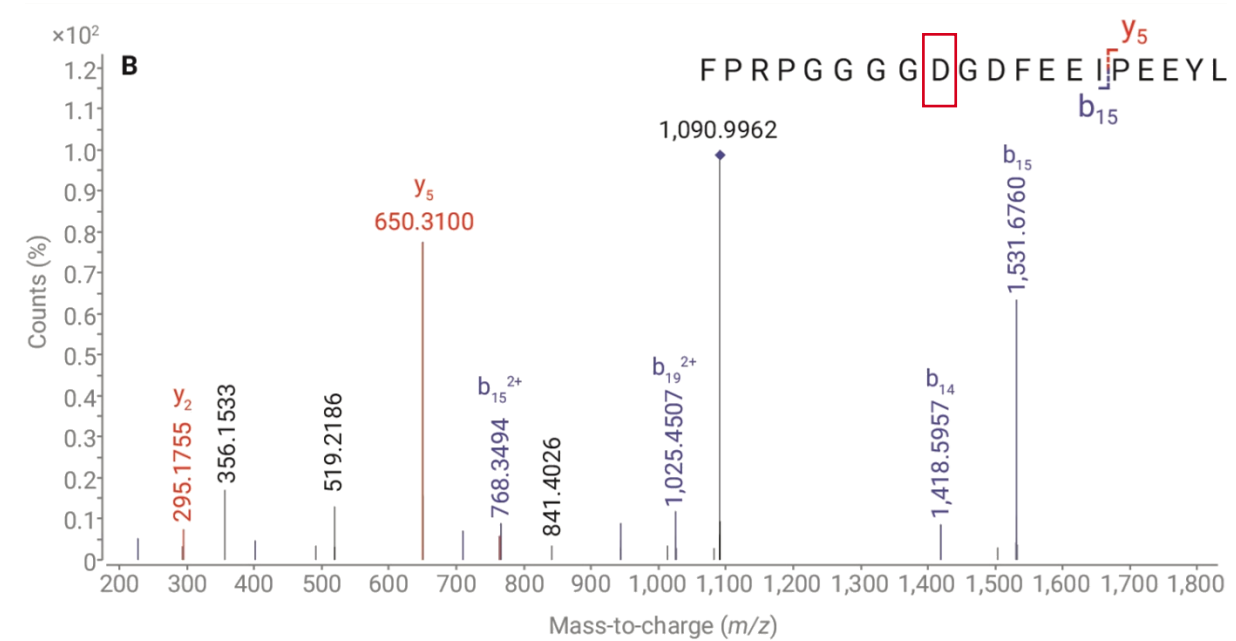
MS/MS Identifies Impurity Peaks

Full length sequence:
FPRPGGGG**N**GDFEEI**P**EEYL



MS/MS spectrum of Peak 1 reveals the location of missing glutamic acid residue

Peak	Mass (Da)	Peak ID	Target mass (Da)	Mass error (ppm)
1	2,049.95	Deletion of Glu	2,049.94	1.71
2	2,178.99	Product	2,178.99	1.65



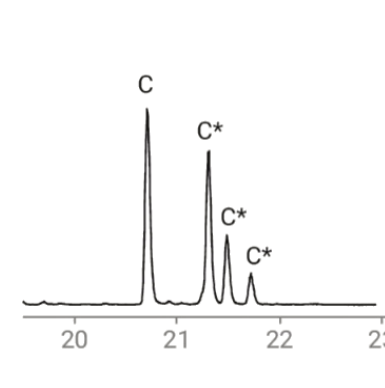
MS/MS spectrum of Peak 5 confirms deamidation of the Asn at residue 9 to an Asp

Peak	Mass (Da)	Peak ID	Target mass (Da)	Mass error (ppm)
2	2,178.99	Product	2,178.99	1.65
5	2,179.97	Deamidation	2,179.97	2.02

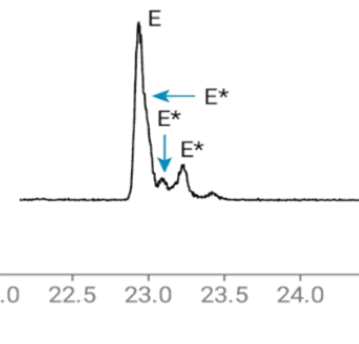
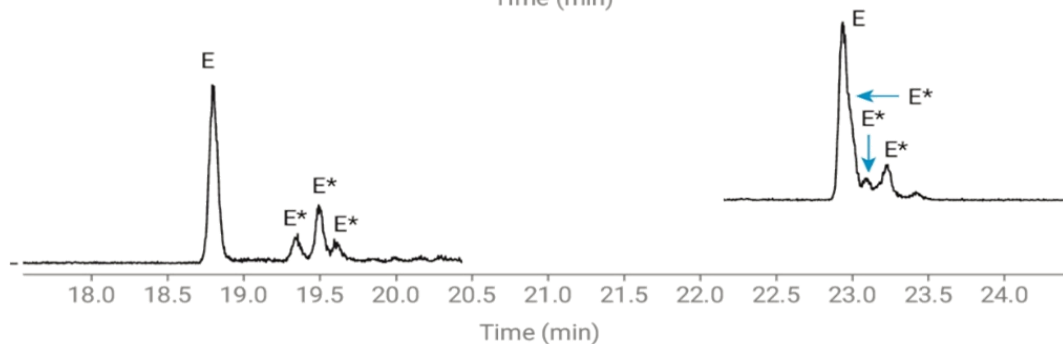
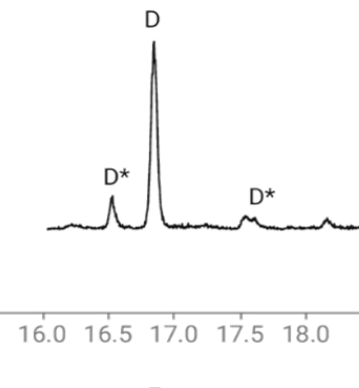
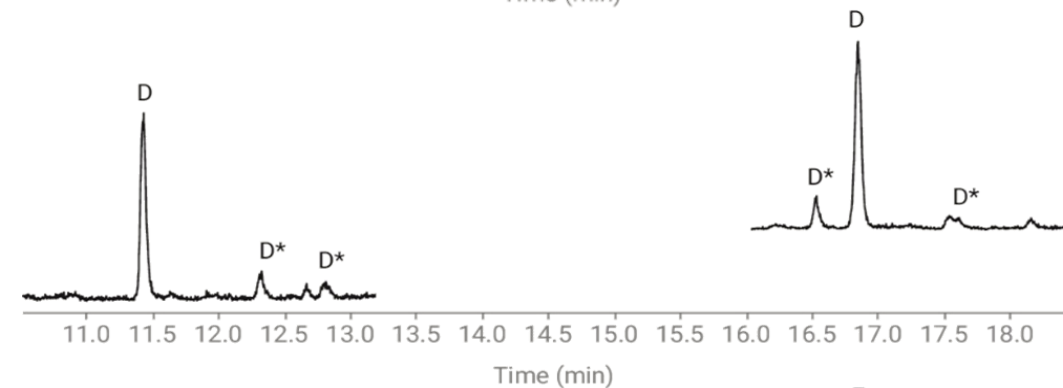
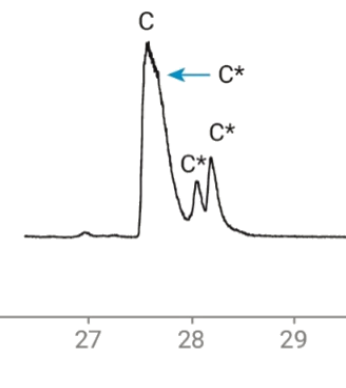
Separation of Deamidated Variants

AdvanceBio Peptide Plus has enhanced selectivity for deamidated variants.

AdvanceBio Peptide Plus



AdvanceBio Peptide Mapping



Application note: [5994-2971EN](#)

Synthetic Peptide Analysis Reversed-Phase Workflow

MW or sequence confirmation, impurity analysis



Therapeutic peptide samples,
including GLP-1



Altura Peptide Plus columns
with Ultra Inert technology
2.1 × 150 mm

InfinityLab bio solvent inlet
filter



Propylene InfinityLab bio
solvent bottle



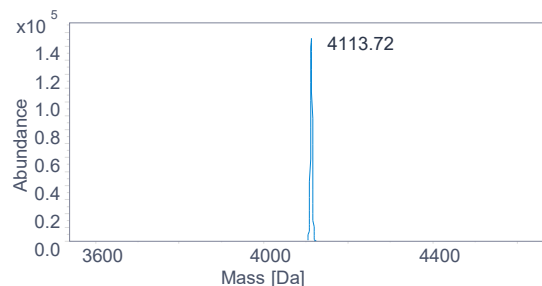
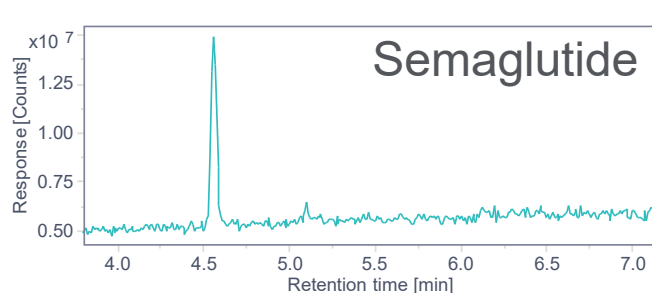
1260 Infinity III LC(bio-inert) or
1290 Infinity III LC (biocompatible)
with UV or MS detection



BIO

Molecular Weight Confirmation of GLP-1 Agonists

Deconvoluted spectra

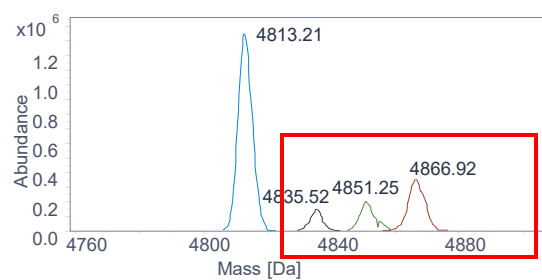
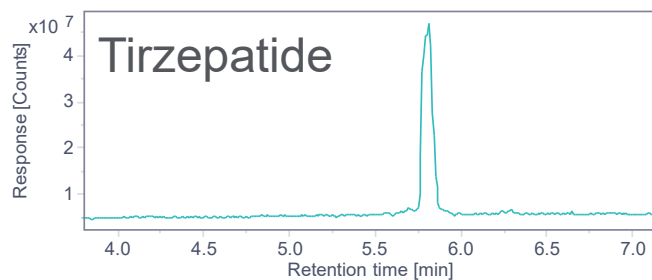
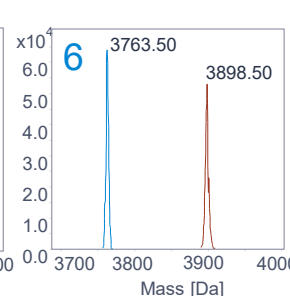
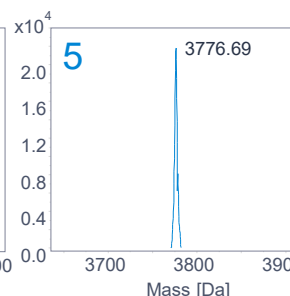
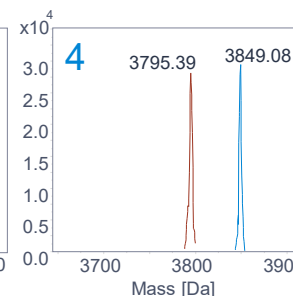
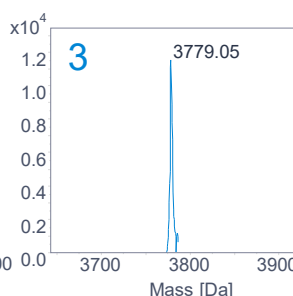
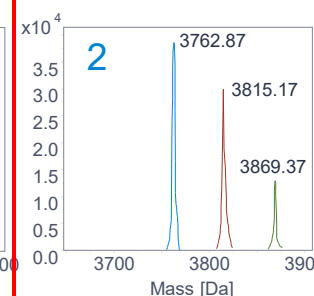
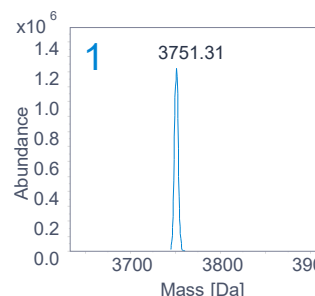
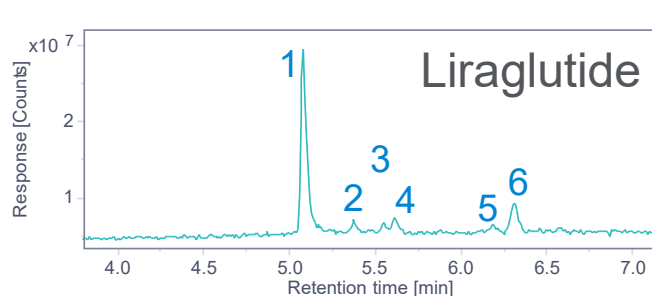


Molecular masses of GLP-1 agonists

Semaglutide: 4113.6 Da

Liraglutide: 3851.3 Da

Tirzepatide: 4813.5 Da

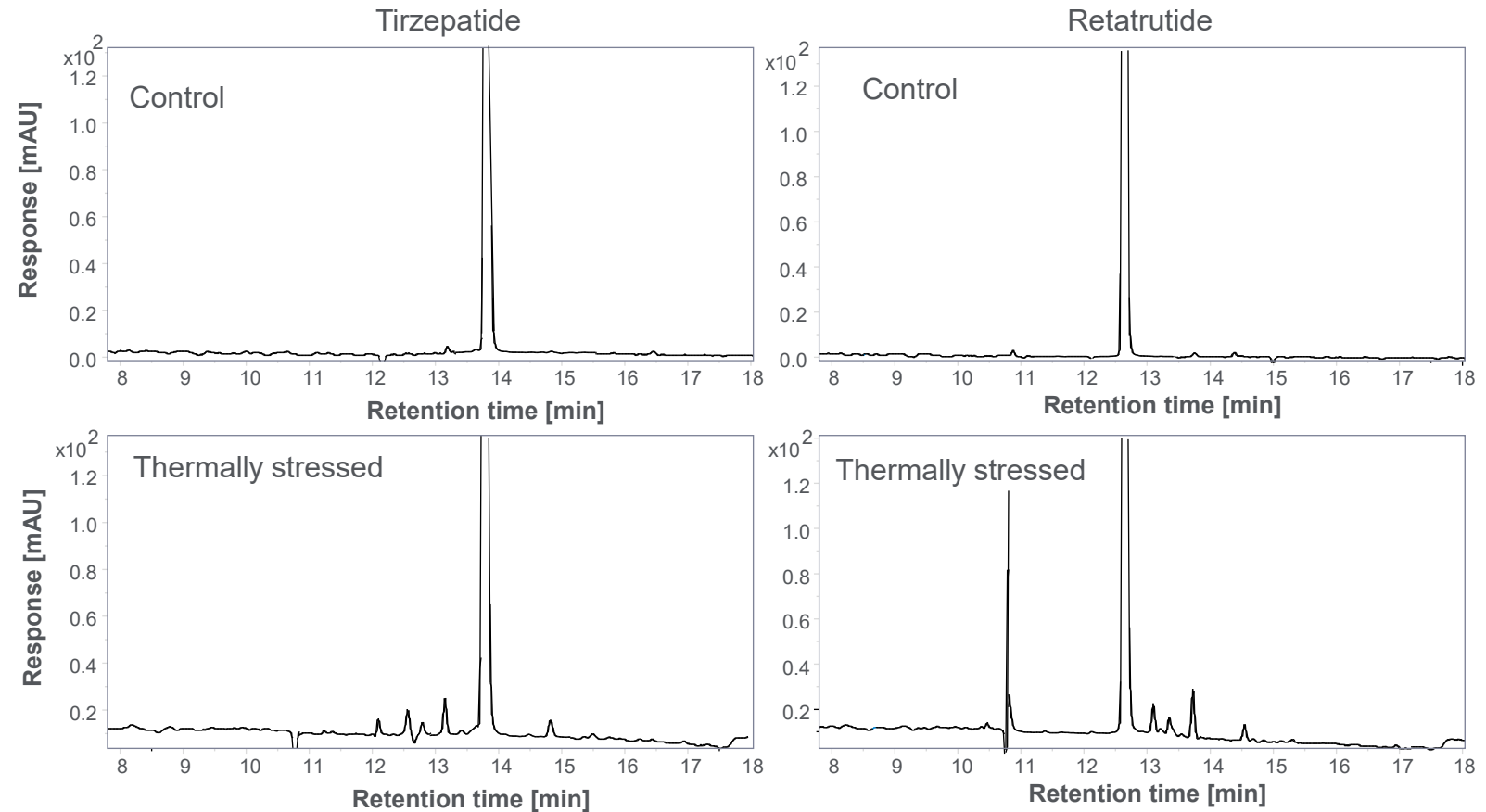


Impurities present in drug substances

Deconvolution of mass spectra helps to detect coeluting impurities

Impurity Analysis of Heat-Stressed GLP-1

Samples stressed at 85 °C for 3 hours to induce impurities



Peptide Sequence Confirmation Workflow



Protein digest or peptide



Altura ZORBAX Eclipse Plus C18
columns with Ultra Inert technology
2.1 × 150 mm

InfinityLab bio solvent inlet
filter



Propylene InfinityLab bio
solvent bottle



1260 Infinity III LC (bio-inert) or
1290 Infinity III LC (biocompatible)
with UV or MS detection



BIO

Peptide Mapping Altura Column Selection

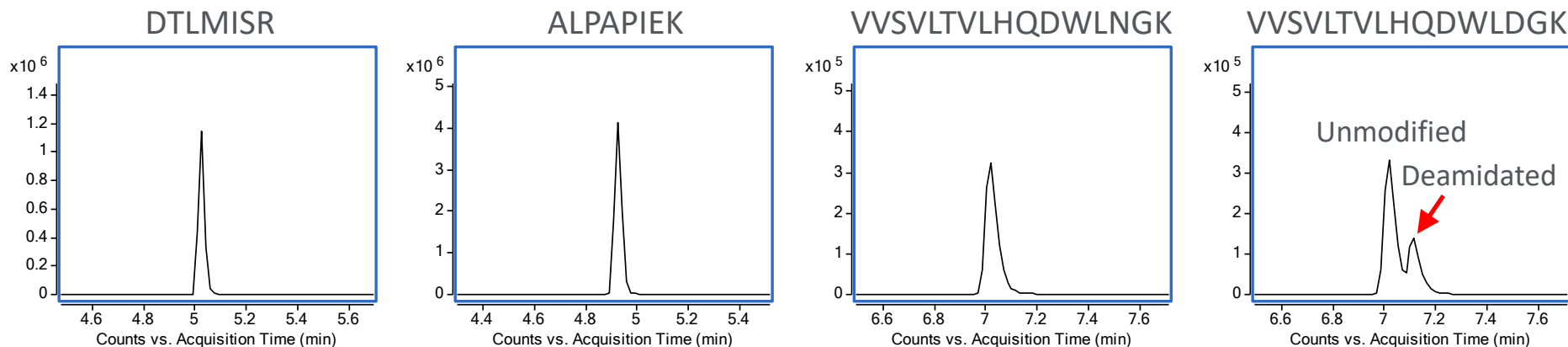
- Altura ZORBAX Eclipse Plus C18
 - Totally porous
 - 1.8 μm particle
 - Traditional endcapped C18 chemistry
 - Good all-round retention of diverse peptides



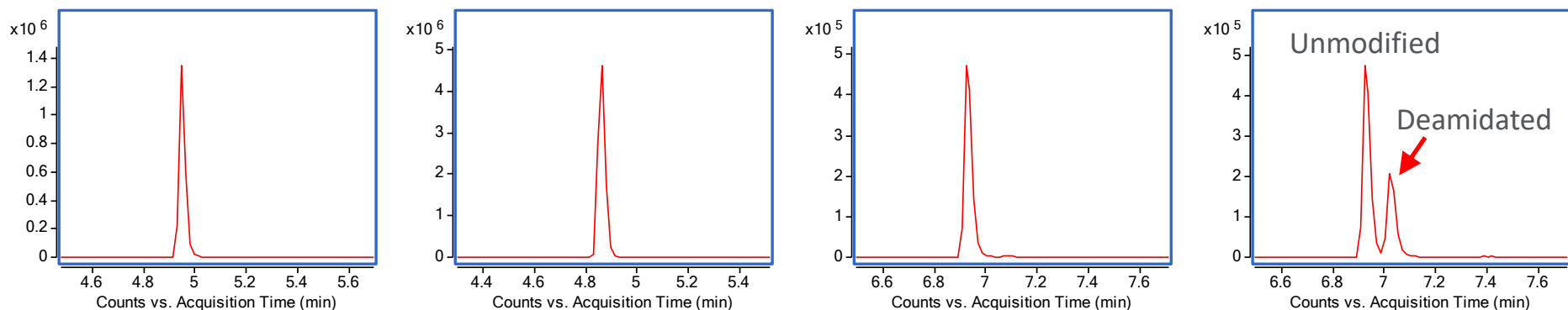
Agilent 1290 Infinity II Bio LC System		
Parameter	Value	
Mobile Phase A	Water with 0.1% formic acid	
Mobile Phase B	Acetonitrile with 0.1% formic acid	
Column Temperature	60 °C	
Flow Rate	0.4 mL/min	
Sampler Temperature	8 °C	
Gradient 1 for Synthetic Peptides	Time (min)	%B
	0.00	2
	1.00	2
	7.00	38
	7.10	80
	9.00	80
	9.10	2
	14.0	2
Gradient 2 for Herceptin Tryptic Digest	Time (min)	%B
	0	2
	2	2
	42	42
	43	80
	47	80
	48	2
	55	2

PTM and Degradation Monitoring Improved by Ultra Inert Technology

ZORBAX Eclipse Plus C18



Altura ZORBAX Eclipse Plus C18



Even after extensive condition of the stainless-steel column, the Altura ZORBAX Eclipse Plus C18 column has significantly better resolution of the deamidated variant.

Agilent's Inert Sample Path Solution



1260 and 1290 Infinity III Bio LC



Altura HPLC columns with
Ultra Inert technology



MP35N capillaries &
Quick Connect fittings

Polypropylene InfinityLab
bio solvent bottle
p/n [9301-6028](#)



InfinityLab bio solvent
inlet filter
p/n [5320-0070](#)



Vials

First Wave Altura Ultra Inert Portfolio

Peptide therapeutics

Impurity analysis with
Altura Peptide Plus

Peptide mapping and
PTM monitoring with
**Altura ZORBAX Eclipse
Plus C18**

Protein therapeutics



Oligo therapeutics

Impurity analysis with
Altura Oligo HPH-C18

Monitor amino acids and other
polar metabolites or excipients
with **Altura Poroshell HILIC-Z**

Cell culture and formulations

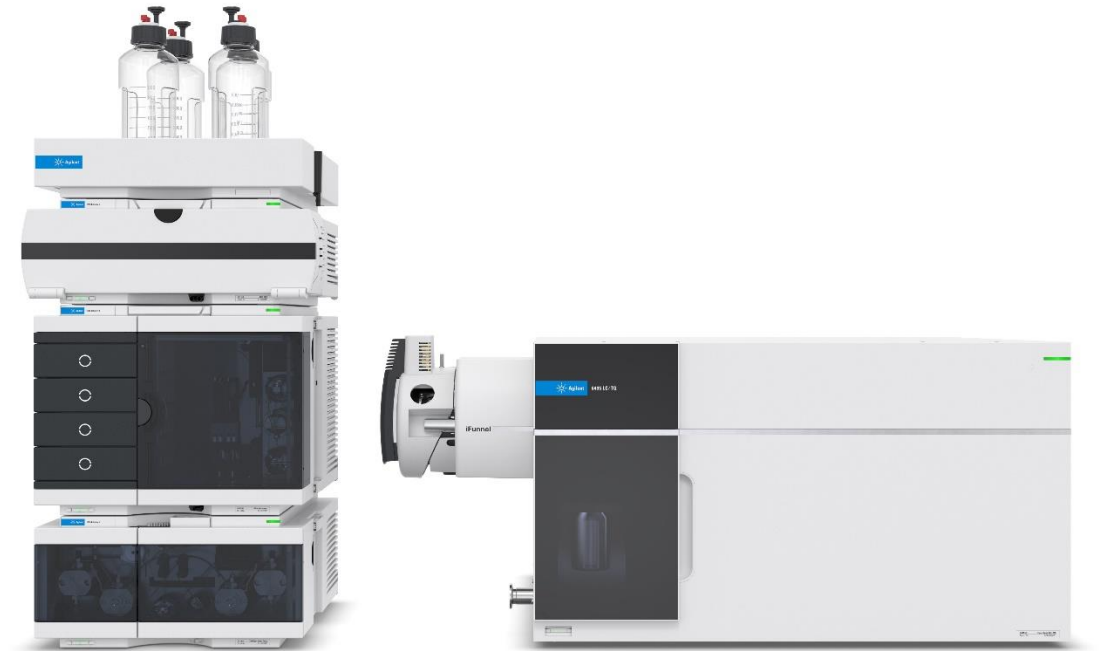
Agilent Biomolecule Columns Portfolio

Protein Therapeutics										Oligonucleotides		Capsid Therapeutics			
Titer Determination	Aggregate Analysis	Intact Purity & PTM Analysis		Peptide Mapping & PTM Analysis	Charge Variant Analysis	Glycan Analysis	Surfactant Analysis	Amino Acid / Cell Culture Media Analysis		Purification & Impurity Analysis		Aggregation	Empty/Full	Capsid Identity	Surfactant Analysis
Affinity	Size Exclusion	Reversed Phase >150 Å	Hydrophobic Interaction	Reversed Phase <150 Å	Ion Exchange	Hydrophilic Interaction	Reversed Phase >150 Å	Reversed Phase <150 Å	Hydrophilic Interaction	Reversed Phase	Ion Exchange	Size Exclusion	Anion Exchange	Reversed Phase	Reversed Phase >150 Å
Bio-Monolith Protein A	AdvanceBio SEC 1.9 µm	PLRP-S 1,000 Å 5 µm	AdvanceBio HIC	Altura Eclipse Plus C18	Bio mAb / Bio IEX 5 µm	AdvanceBio Amide HILIC	AdvanceBio Surfactant Profiling	AdvanceBio Amino Acid Analysis	Altura Poroshell HILIC-Z	Altura Oligo HPH-C18	*PL-SAX	AdvanceBio SEC 2.7 µm 500 and 1,000 Å	Bio SAX	ZORBAX RRHD 300 Å, 1.8 µm	AdvanceBio Surfactant Profiling
Bio-Monolith Protein A	AdvanceBio SEC	PLRP-S*		AdvanceBio EC-C18	Bio mAb (WCX)	AdvanceBio Glycan Mapping		ZORBAX Eclipse AAA 3.5 µm		*AdvanceBio Oligonucleotide	Bio SAX	Bio SEC-5 2,000 Å	Bio-Monolith (QA, DEAE, SO3)	Altura Eclipse Plus C18	
Bio-Monolith Protein G	*Bio SEC-5	AdvanceBio RP mAb 450 Å		AdvanceBio Peptide Mapping	AdvanceBio EC-C18					*PLRP-S	*Bio SAX			AdvanceBio Peptide Mapping	
	*Bio SEC-3	ZORBAX RRHD 300 Å, 1.8 µm		AdvanceBio Peptide Plus	*PL SCX, SAX			Biomolecule Sample Preparation							
	PROTEEMA	*ZORBAX 300SB 3.5, 5, and 7 µm		ZORBAX RRHD 300 Å, 1.8 µm	Bio-Monolith (QA, DEAE, SO3)			Desalting & Buffer Exchange	Released N-Glycan						
		Poroshell 300 5 µm						AdvanceBio Spin	AdvanceBio Gly-X						
								AdvanceBio Desalting-RP	AdvanceBio Sialic Acid						
												Column hardware			
												*Scalable to prep	Stainless steel (SS)	Solid PEEK or PEEK-lined SS bioinert	Ultra Inert column hardware

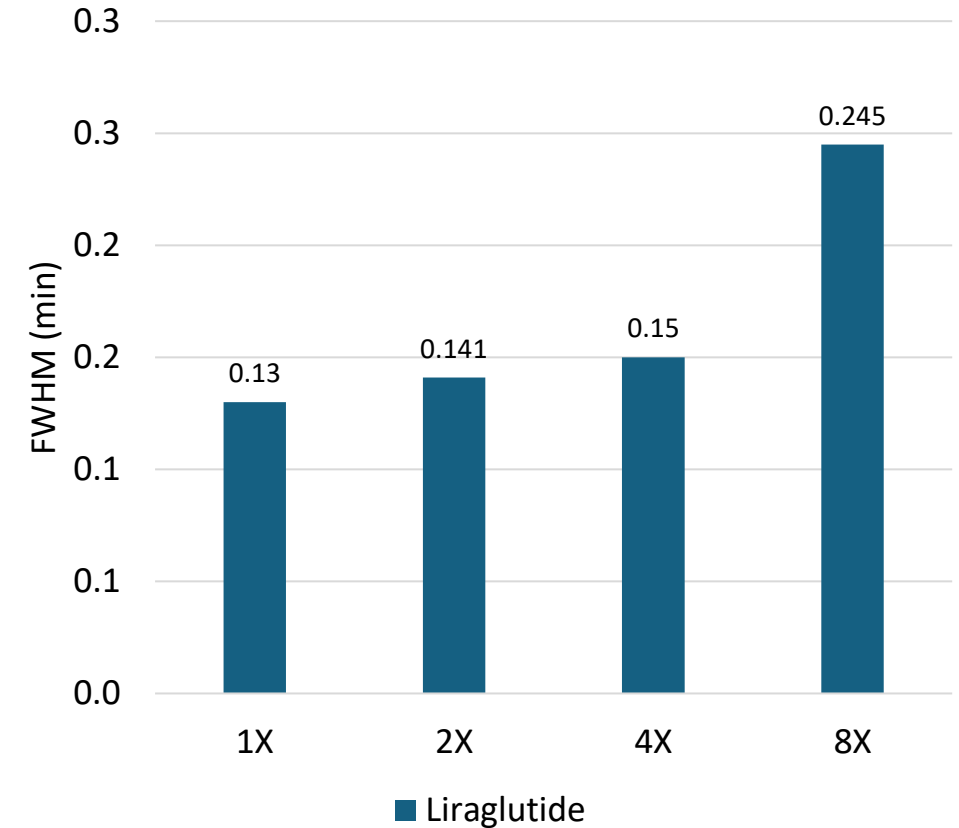
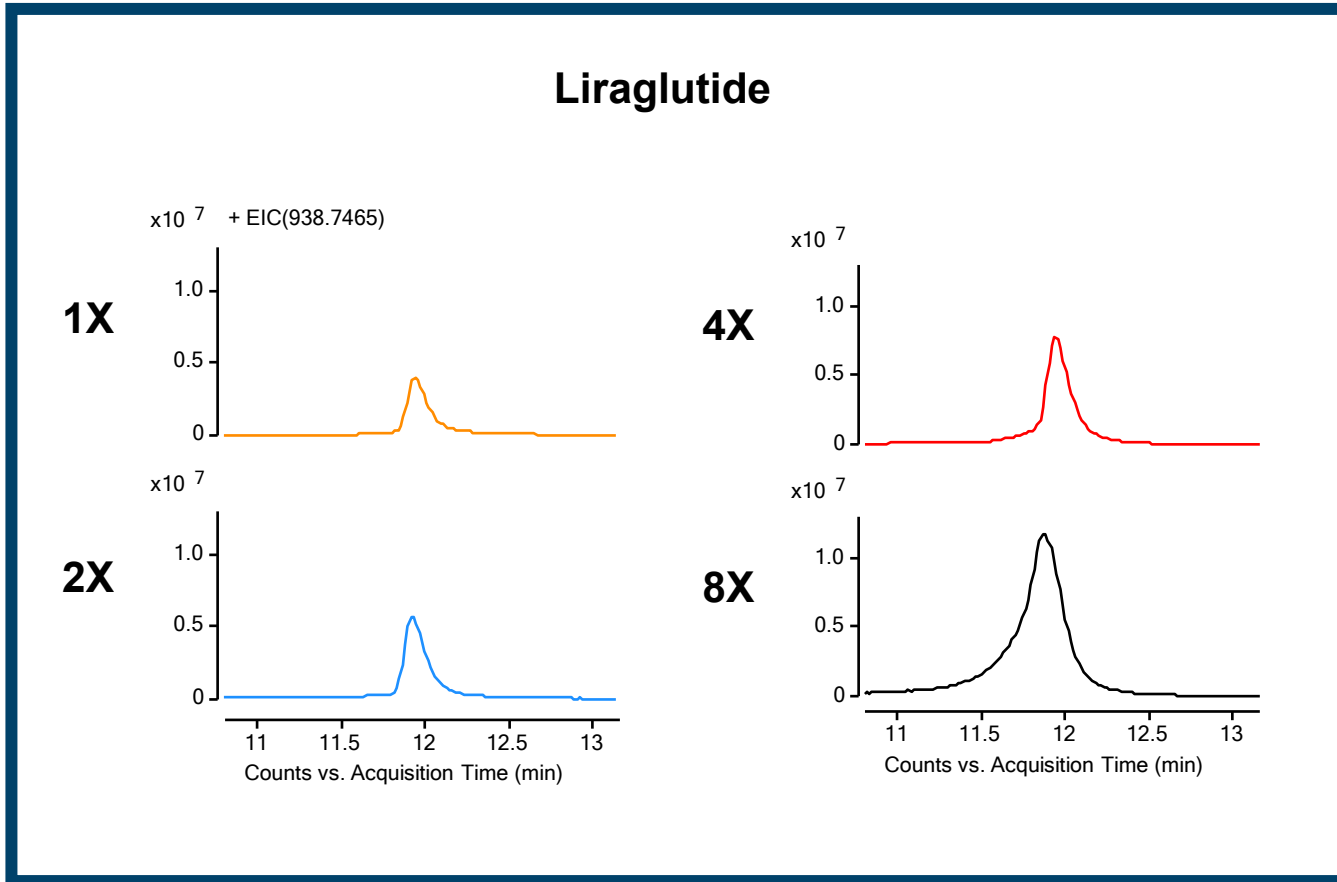
Method Development Considerations for Peptide Separation

Experimental parameters to optimize:

- Sample injection amount
- Column compartment temperature
- Mobile-phase modifiers
- Column chemistry and pore size
- Source optimization for MS
 - Gas temperature
 - Gas flow
 - Fragmentor voltage



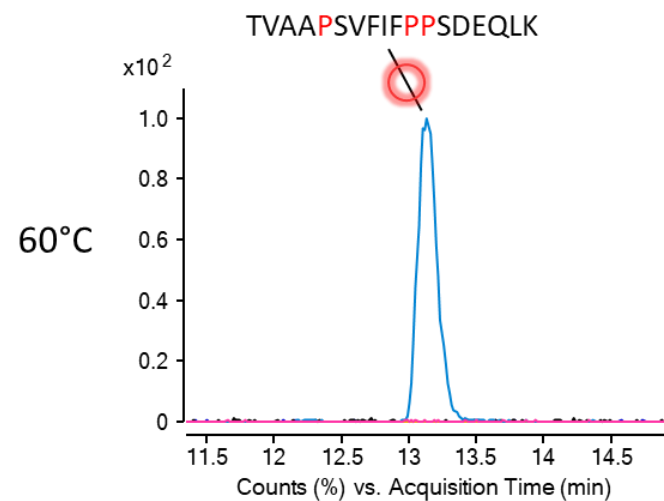
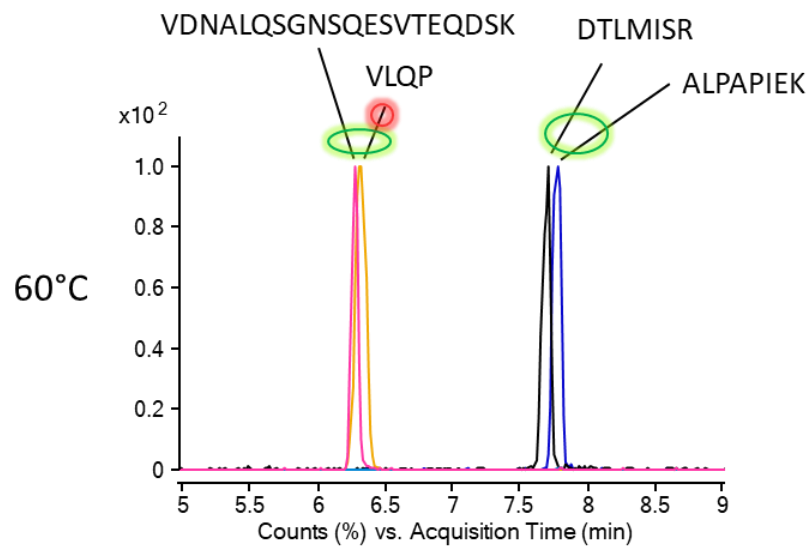
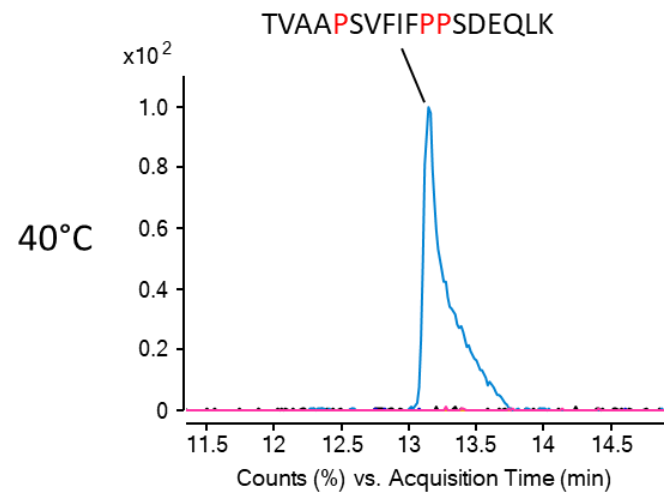
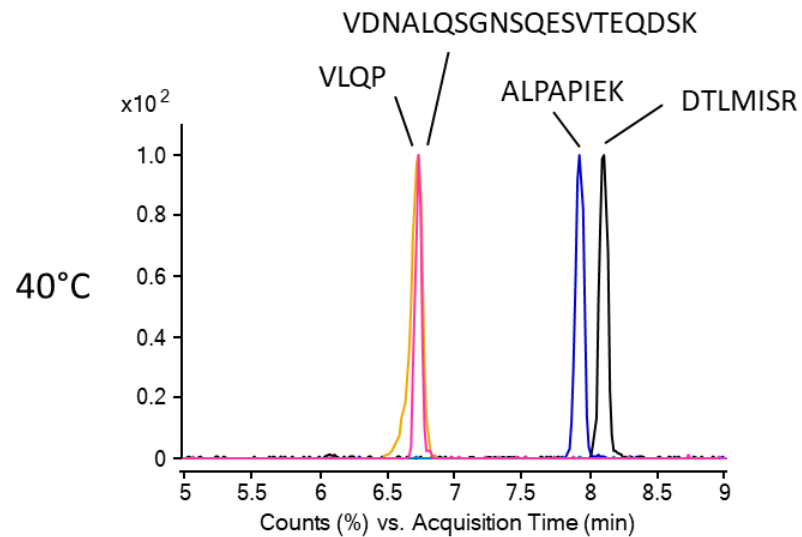
Optimal Chromatographic Peak Shape Based on Sample Load



Notes:

- Experiment was conducted at 40 °C
- As the amount of sample injected is increased, the FWHM increases with broader peak shapes

Changes to mAb Peptide Selectivity and Peak Shape with Column Temperature

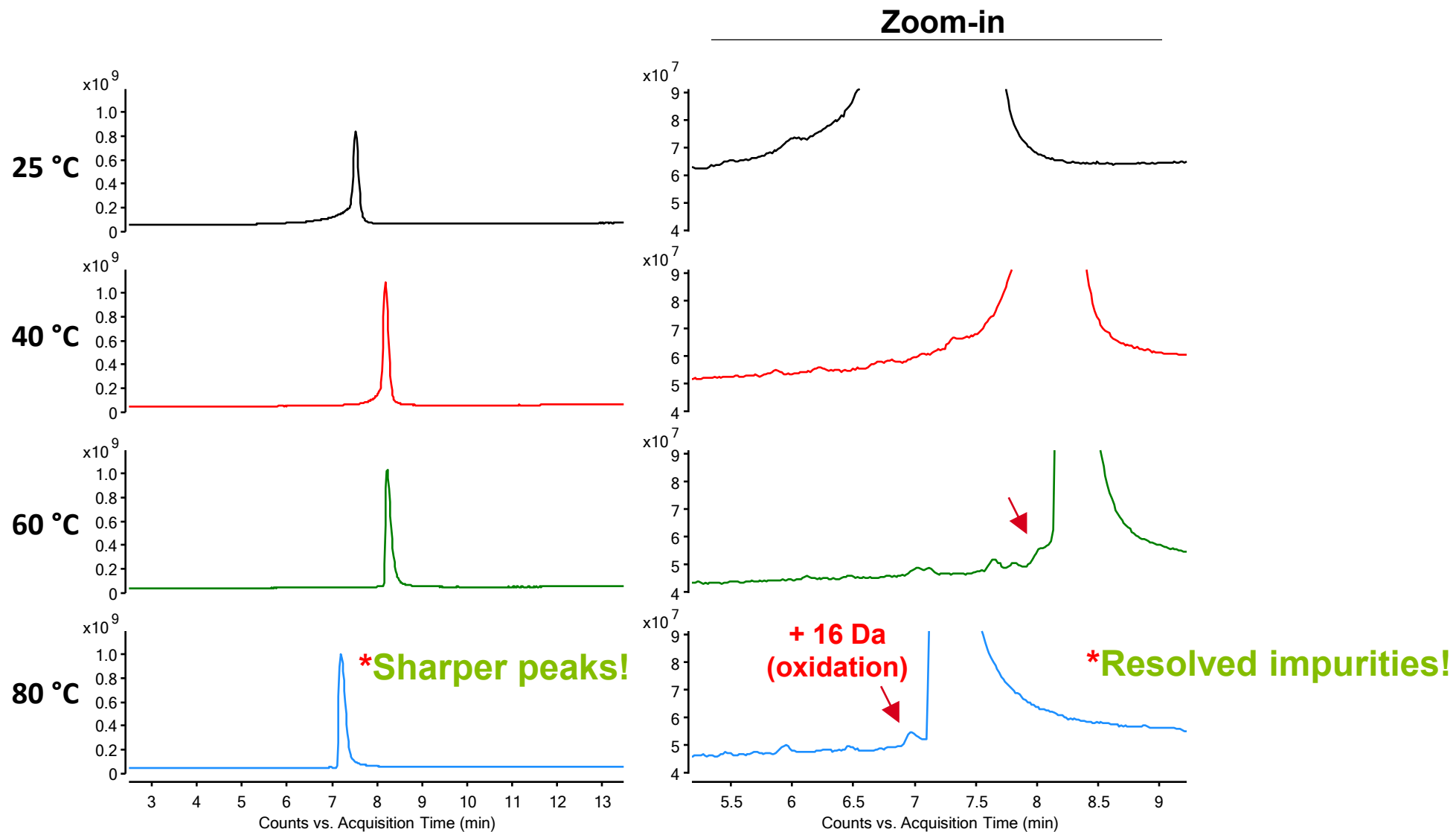


Legend:

○ Change in elution order

○ Improved peak shape

Increased Column Temperature Improves GLP1-RA Peak Shape and Impurity Separation



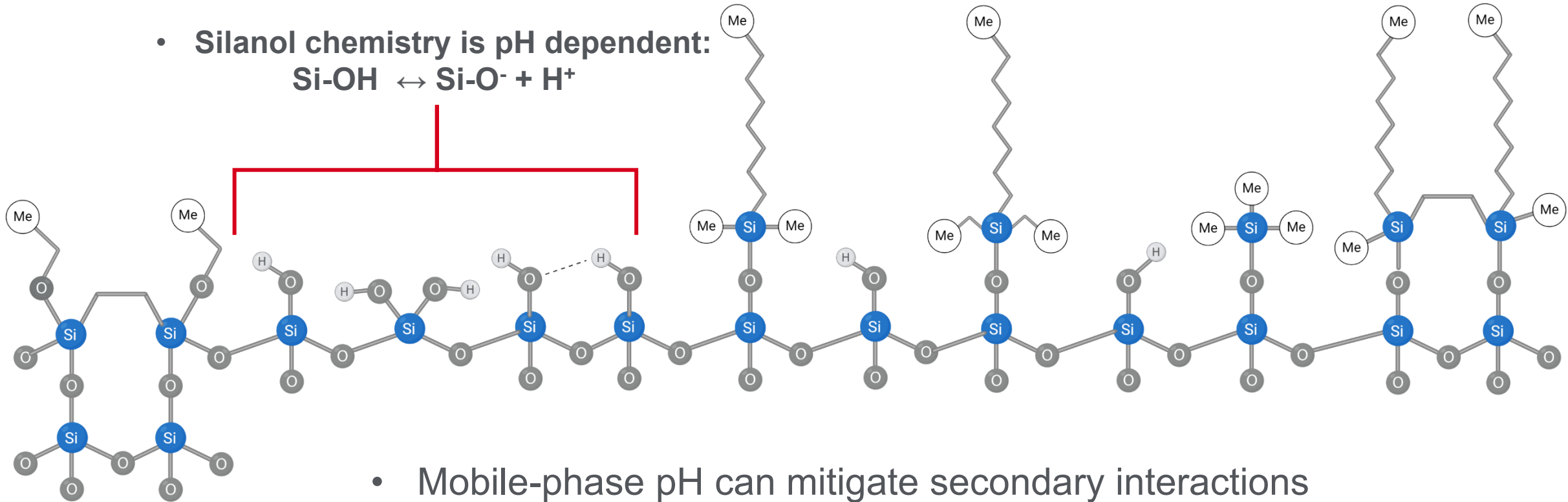
Mobile-Phase Selection

- In order of increasing eluotropic force and decreasing polarity: water, methanol, acetonitrile, isopropanol
- Selectivity differences and sample retention will vary significantly between mobile phases
- Ionic strength and pH of the aqueous portion of mobile phases are important in developing rugged methods
- It is very important to control pH to stabilize retention and selectivity
- Percentage of organic solvent: There is a pressure maximum and minimum for organic:aqueous mobile phases and it differs depending on the identity of the organic solvent used
- Use solvents that are compatible with the shipping solvent

Block Secondary Interactions by Changing pH

- Unprotected silanols lead to secondary interactions.

- Silanol chemistry is pH dependent:
 $\text{Si-OH} \leftrightarrow \text{Si-O}^- + \text{H}^+$

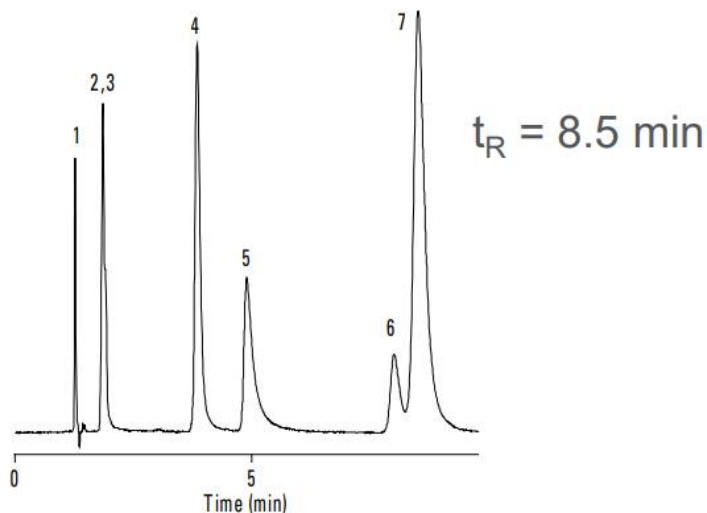


- Mobile-phase pH can mitigate secondary interactions (and change selectivity).
- Be sure to stay within the recommended pH range of the column.

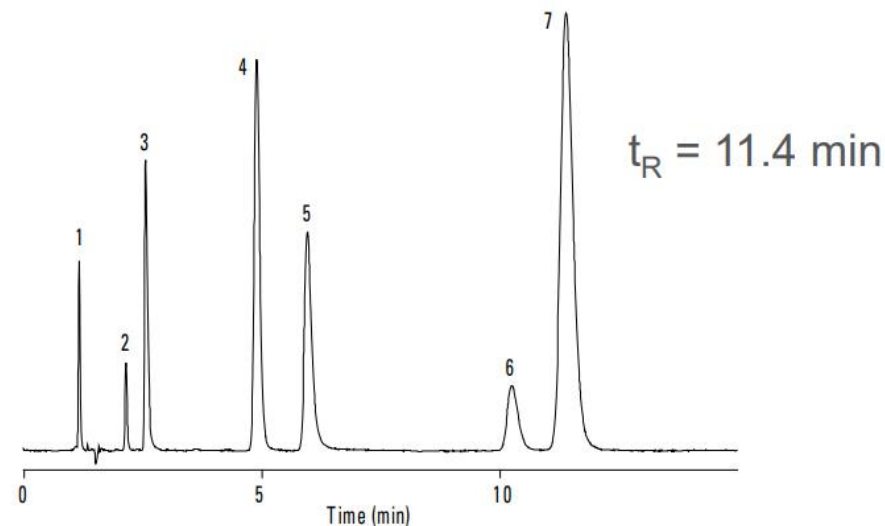
Peak Shape and Selectivity of Basic Compounds May Improve at High pH

pH range of ZORBAX Extend-C18 is 2–11.5

ZORBAX Extend-C18 at pH 7



ZORBAX Extend-C18 at pH 11



Mobile phase: 30% buffer: 70% MeOH; pH 7 buffer: 20 mM Na_2HPO_4 ; pH 11 buffer: 20 mM TEA

Flow rate: 1.0 mL/min; temperature: ambient; detection: UV 254 nm

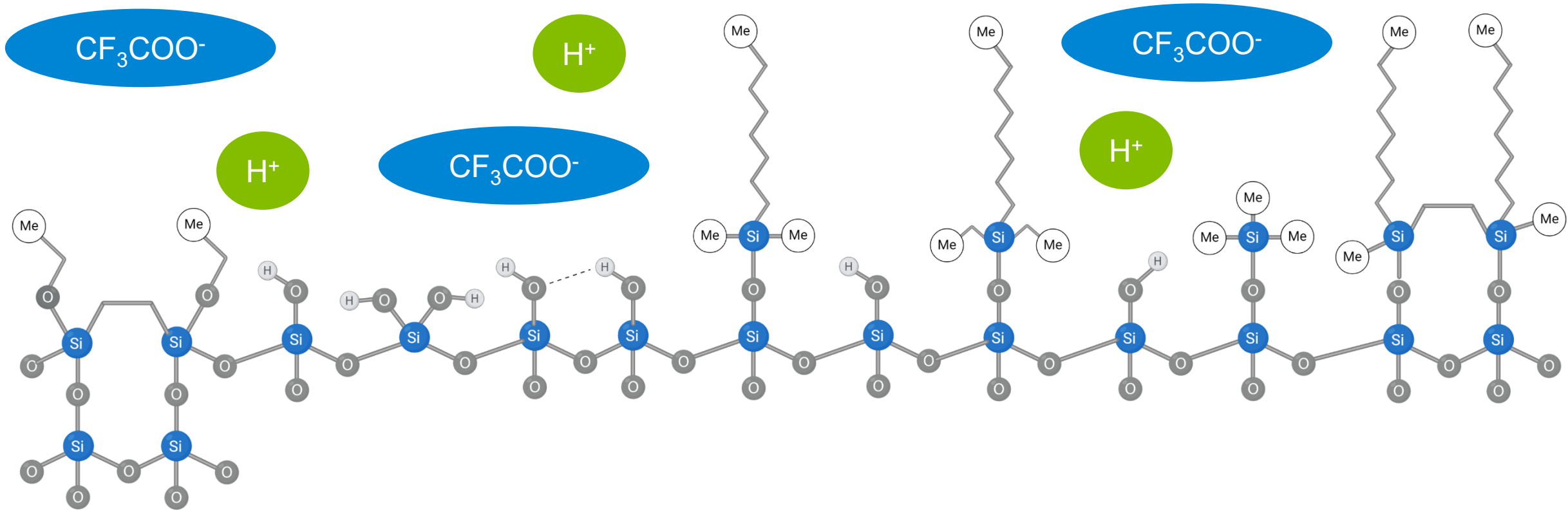
Sample: 1. Maleate 2. Scopolamine pKa 7.6 3. Pseudoephedrine pKa 9.8 4. Doxylamine pKa 9.2 5. Chlorpheniramine pKa 9.1

6. Triprolidine pKa 6.5 7. Diphenhydramine pKa 9.0

Column: ZORBAX Extend-C18, 4.6 x 150 mm, 5 μm

Retention and peak shape of basic compounds is improved at high pH on ZORBAX Extend-C18

Block Secondary Interactions with Mobile-Phase Additives

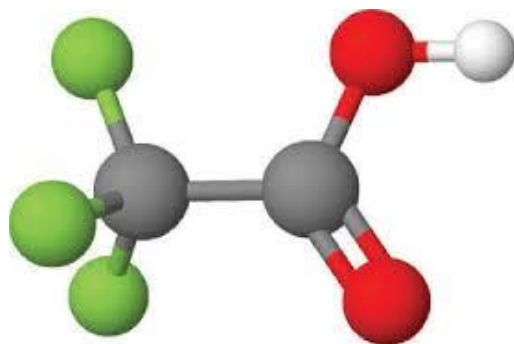


- Trifluoroacetic acid (TFA) and formic acid (FA) are the most common mobile-phase additives.

TFA Versus Formic Acid

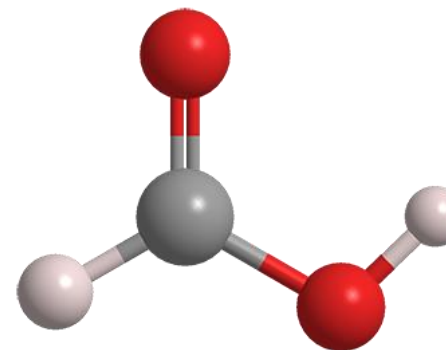
TFA

- Traditionally preferred for best chromatographic peak shape due to strong ion-pair capability
- Not ideal for MS detection
 - Lower volatility
 - Suppresses ionization
 - Limits MS sensitivity



Formic acid

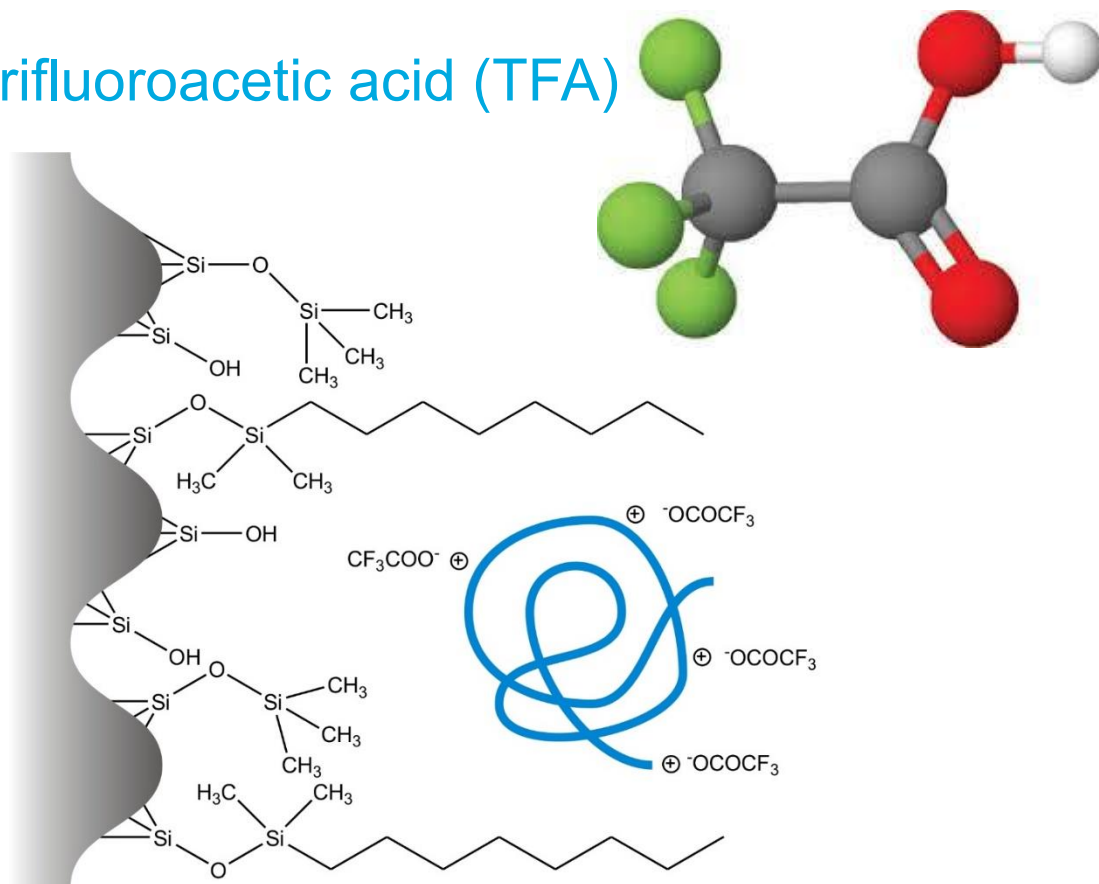
- Volatile ion-pair reagent, preserves MS sensitivity
- Less strong ion-pair reagent than TFA
 - Doesn't block secondary interactions with silica base particle as well
 - May see broader, more tailing peaks



How TFA and Formic Acid Work

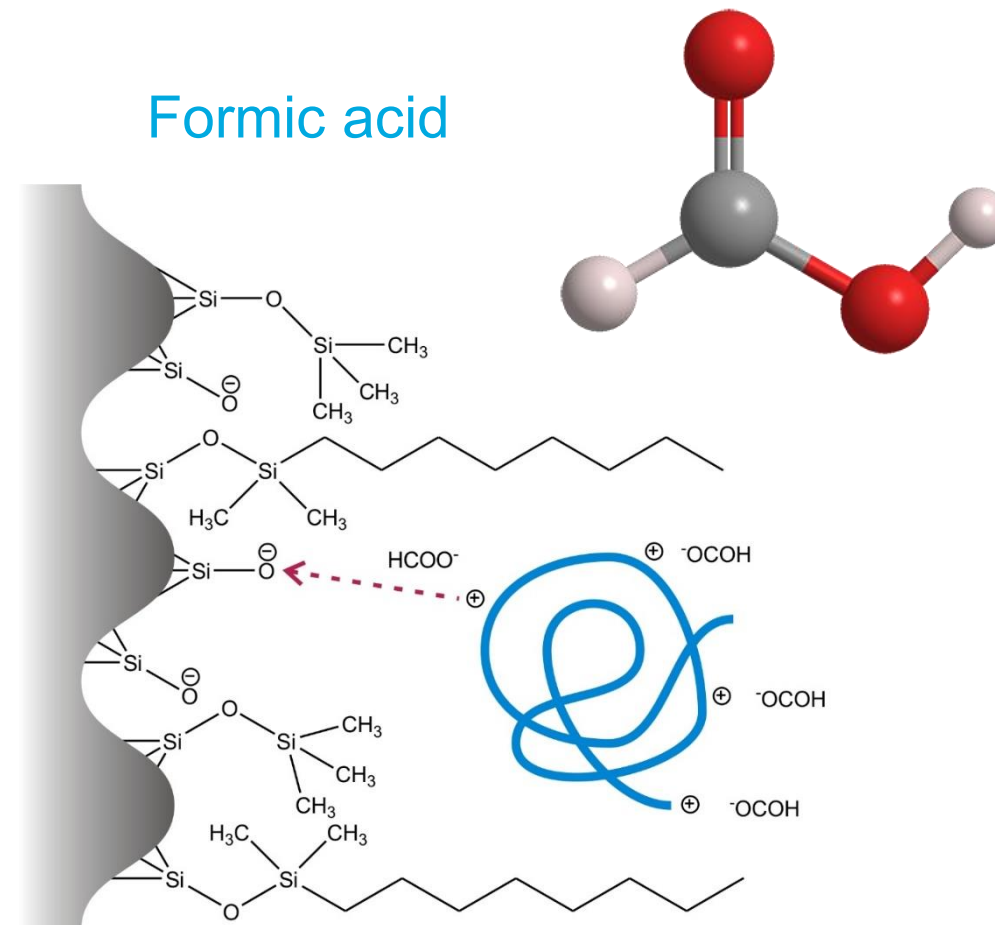
Mobile-phase modifiers

Trifluoroacetic acid (TFA)



TFA blocks silanol interaction, enabling good peak shape.

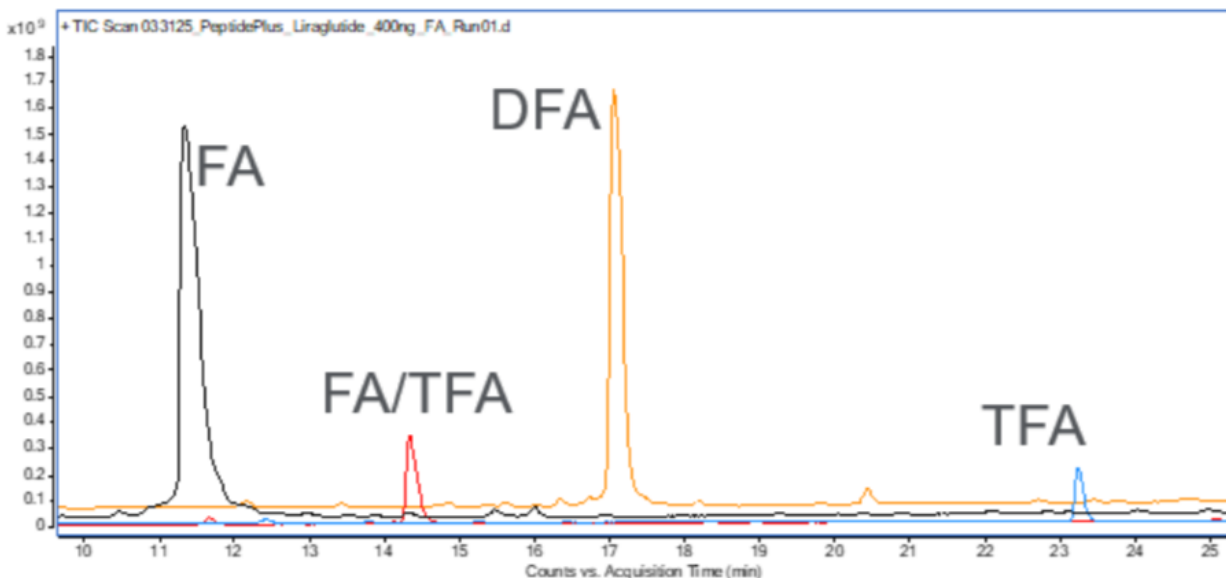
Formic acid



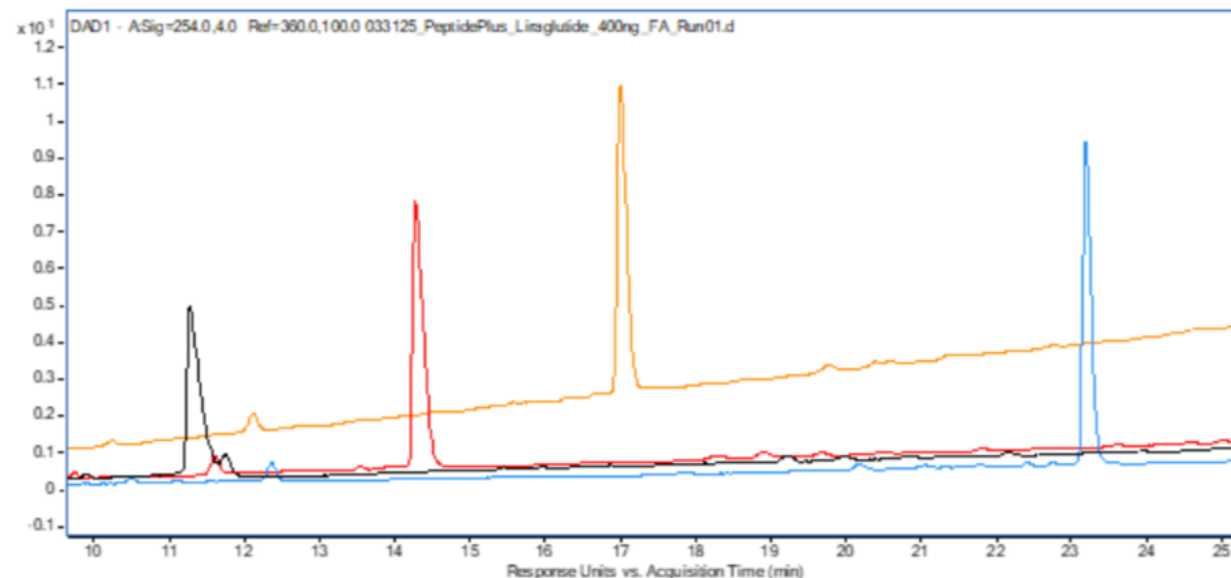
Formic acid weakens but doesn't block silanol interaction.

Trifluoroacetic acid Versus Formic acid Versus Difluoroacetic acid

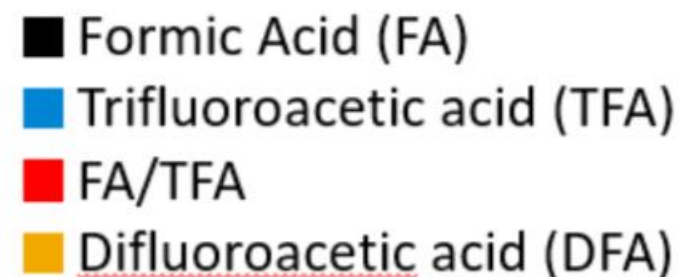
LC/MS



LC/UV

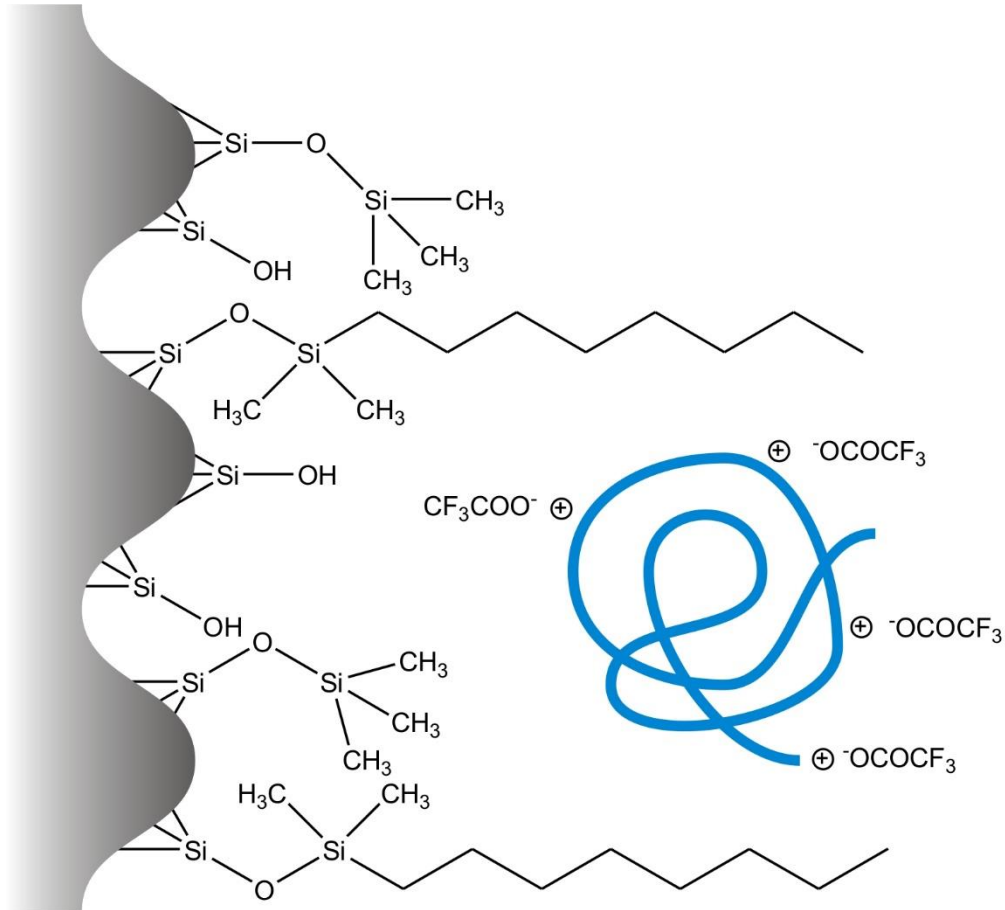


- Formic acid peaks tail more because FA ion pairs less effectively.
- TFA gives sharp, symmetrical peaks but poor MS signal.
- DFA is a nice compromise on peak shape but adds UV background at 254 nm.

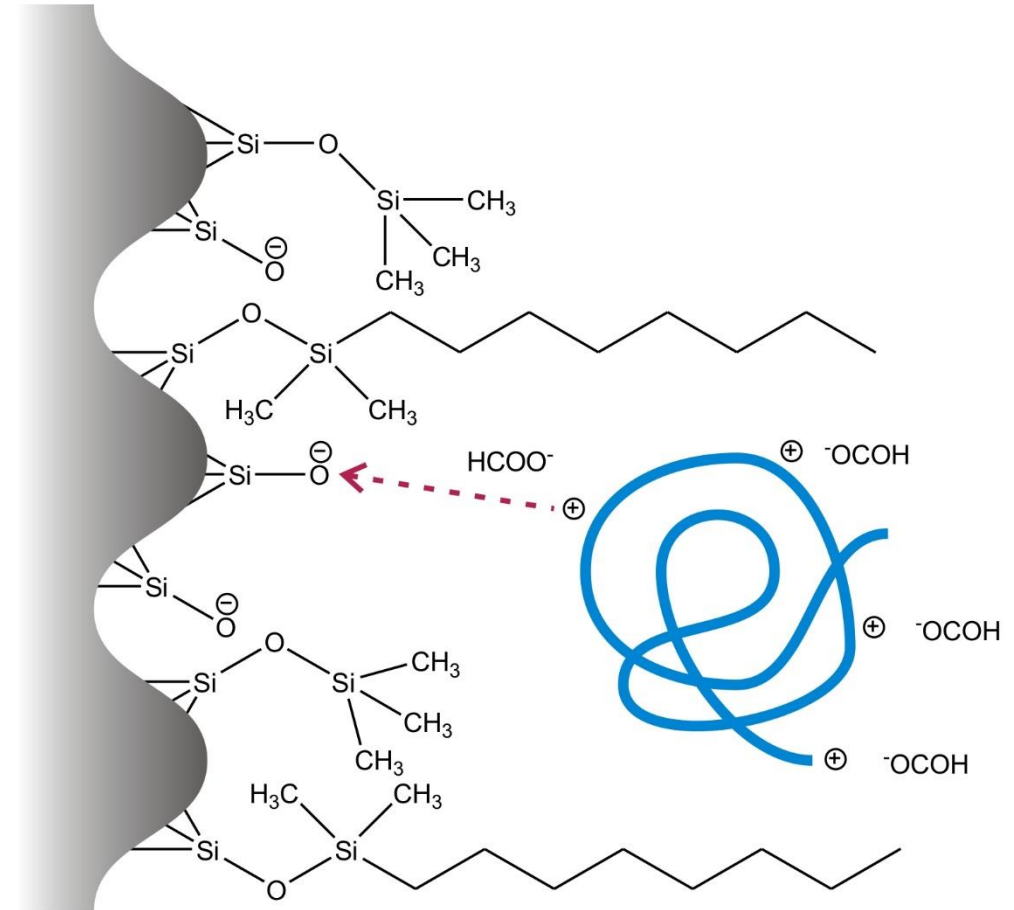


Ion-Pairing Reversed-Phase Chromatography of Peptides

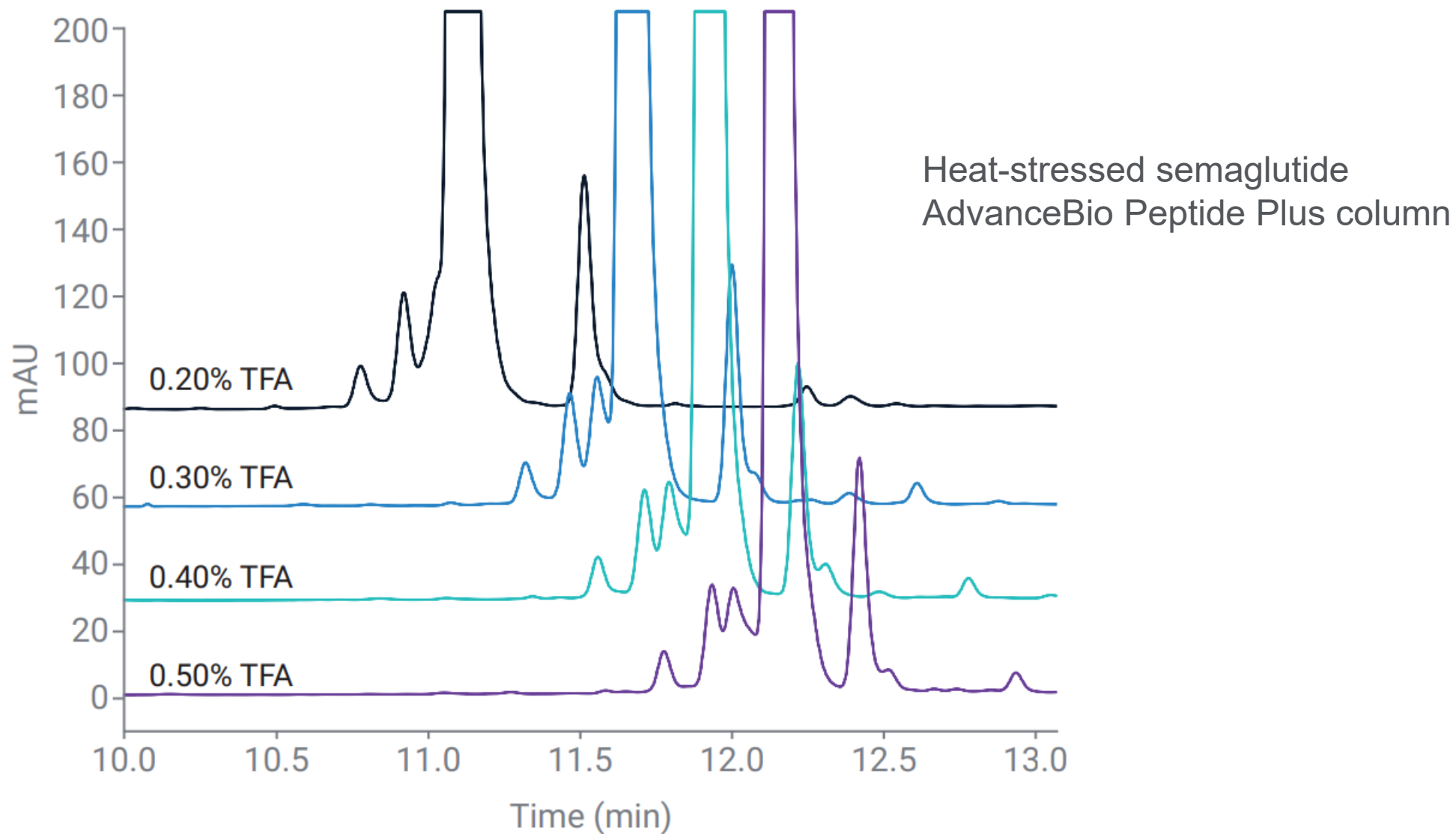
Trifluoroacetic acid (no interactions)



Formic acid (weak interactions)



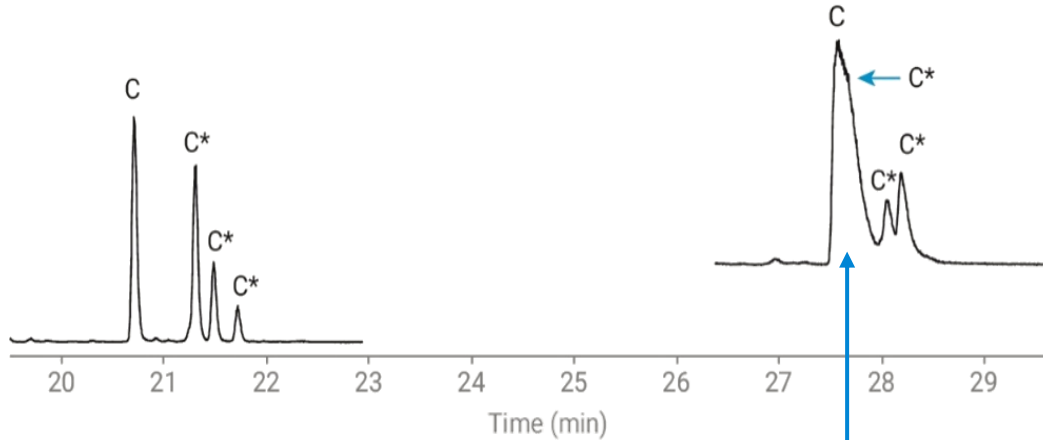
Modifying Additive Concentration May Improve Separation



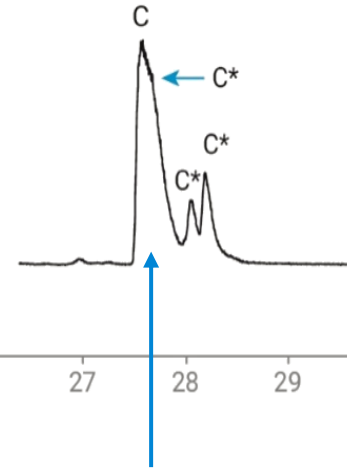
AdvanceBio Peptide Plus Versus AdvanceBio Peptide Mapping

0.1% formic acid mobile phase

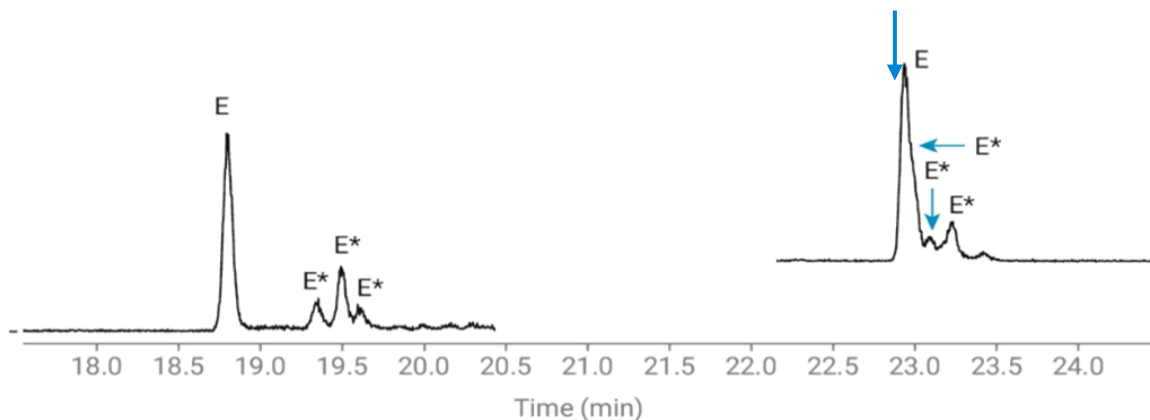
Peptide Plus



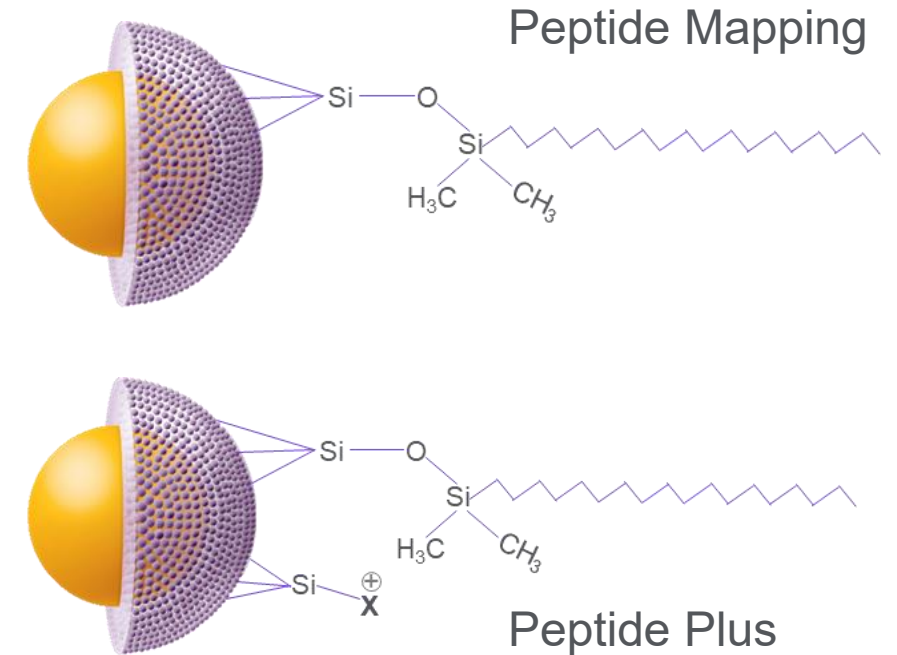
Peptide Mapping



One of the deamidated variants
coeluted with the unmodified form



Charged-surface chemistry better blocks secondary interactions with silica, resulting in good peak shape even with weak acid modifier.

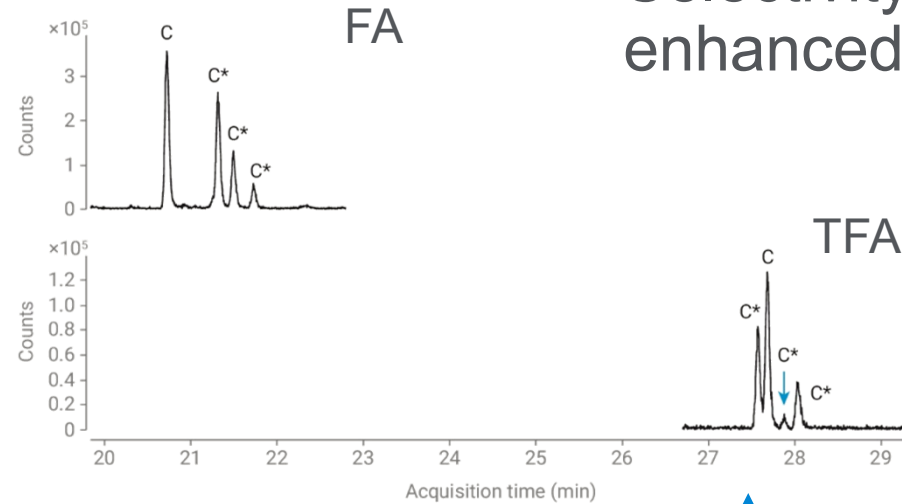


Acid Modifier Changes Selectivity

Peptide Plus

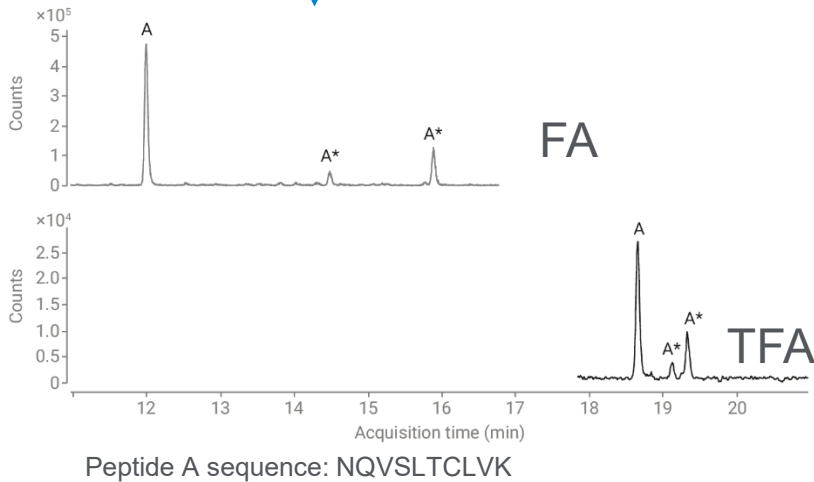
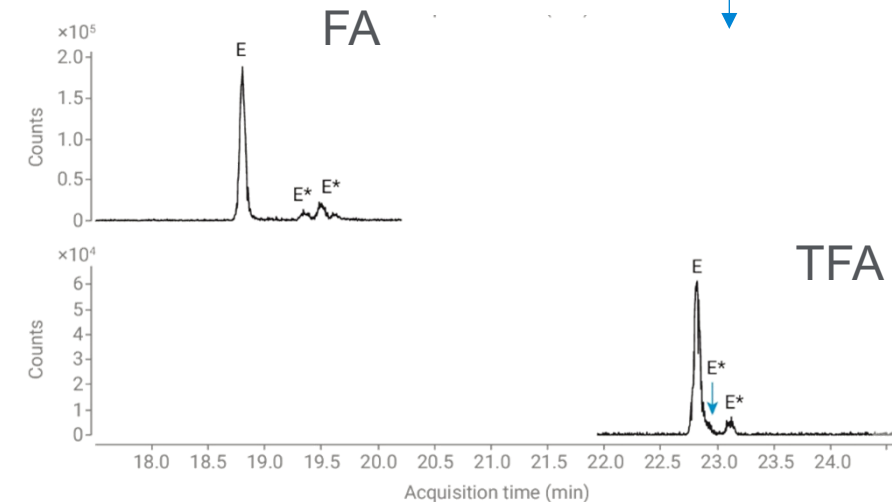
Selectivity for deamidated variants is enhanced with formic acid versus TFA

Peptide A shows more baseline between unmodified form and the variants

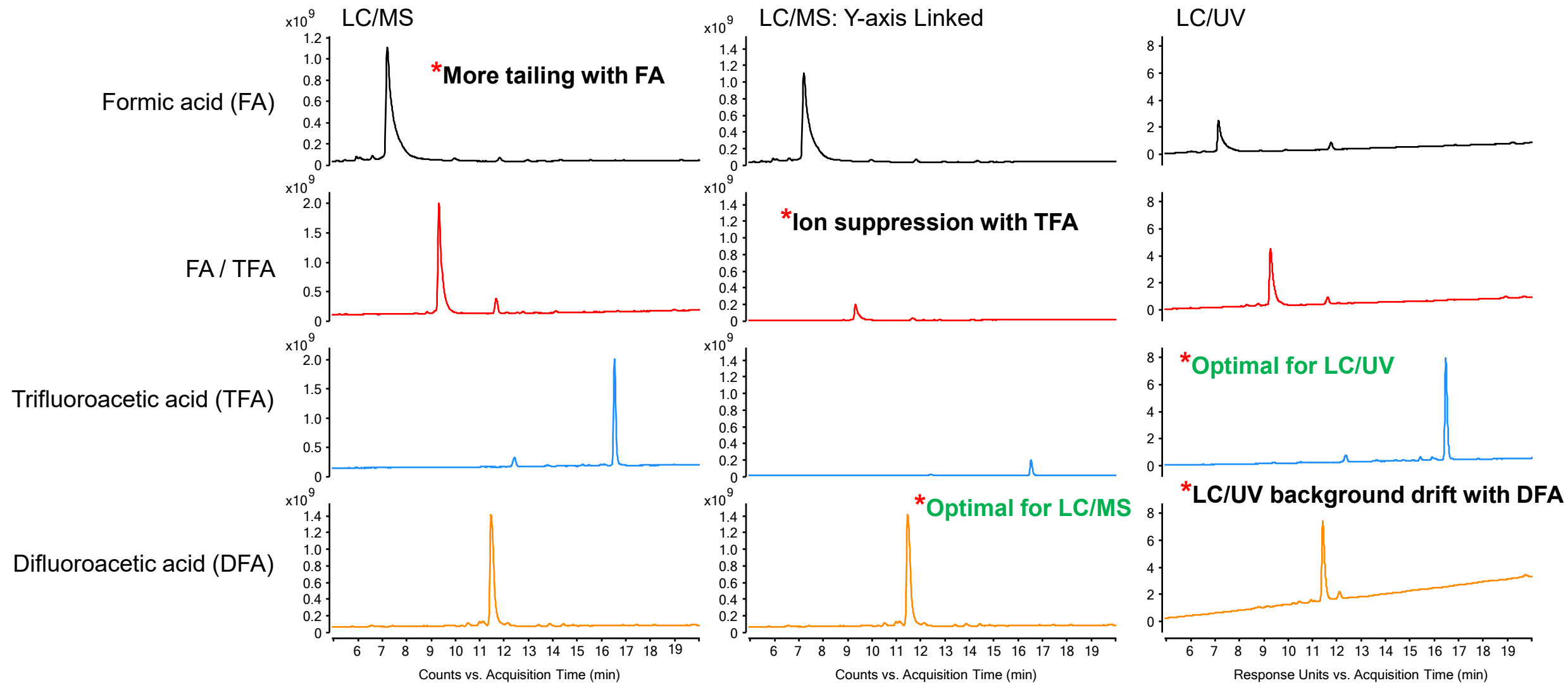


One of the deamidated variants eluted before the unmodified form

Resolution of one of the deamidated variants was lost

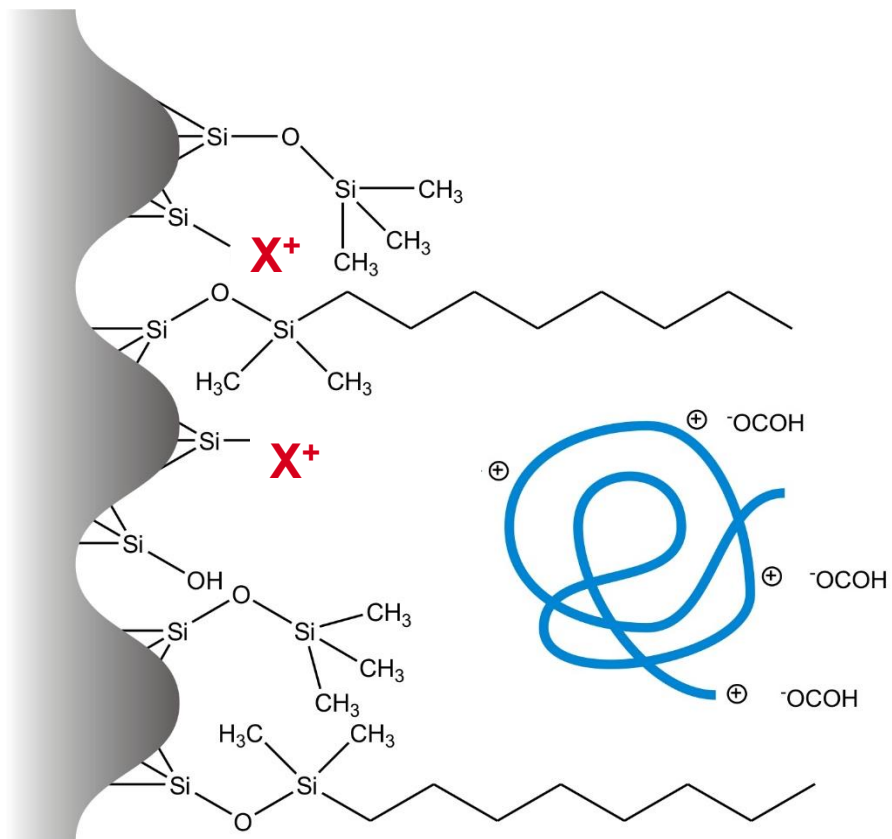


Mobile-Phase Modifiers' Impact on GLP1 Receptor Agonist Analysis

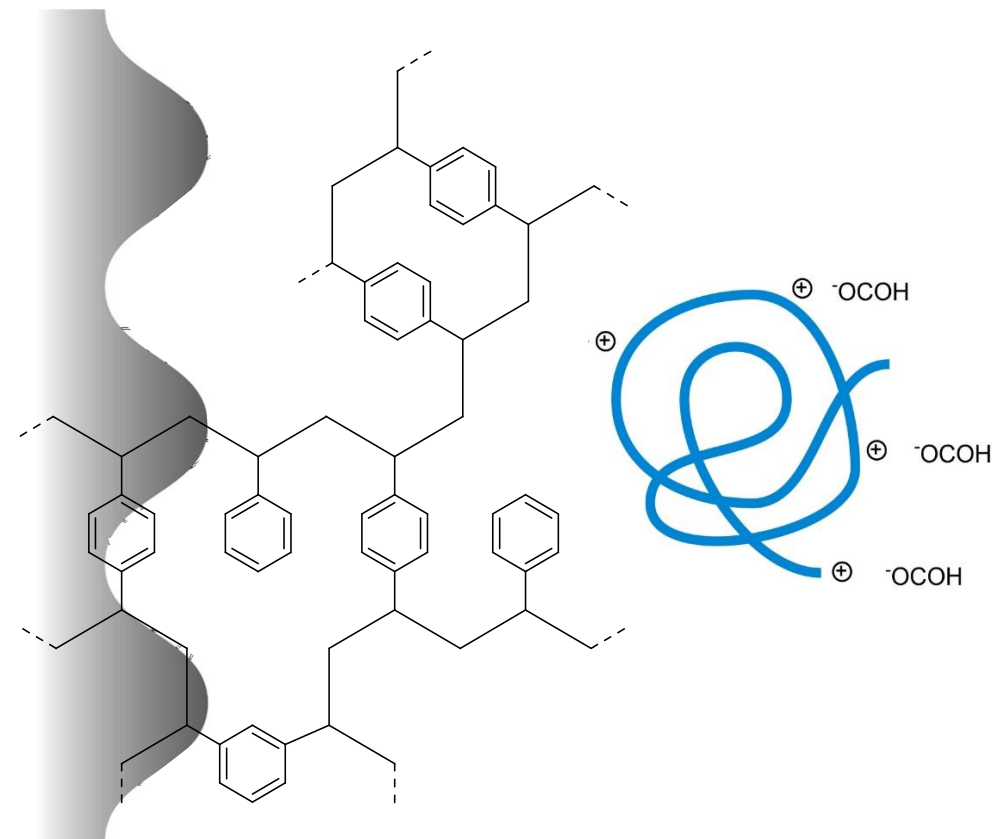


Stationary Phases with Good Formic Acid Performance

Charge-surface-modified C18 Chemistry



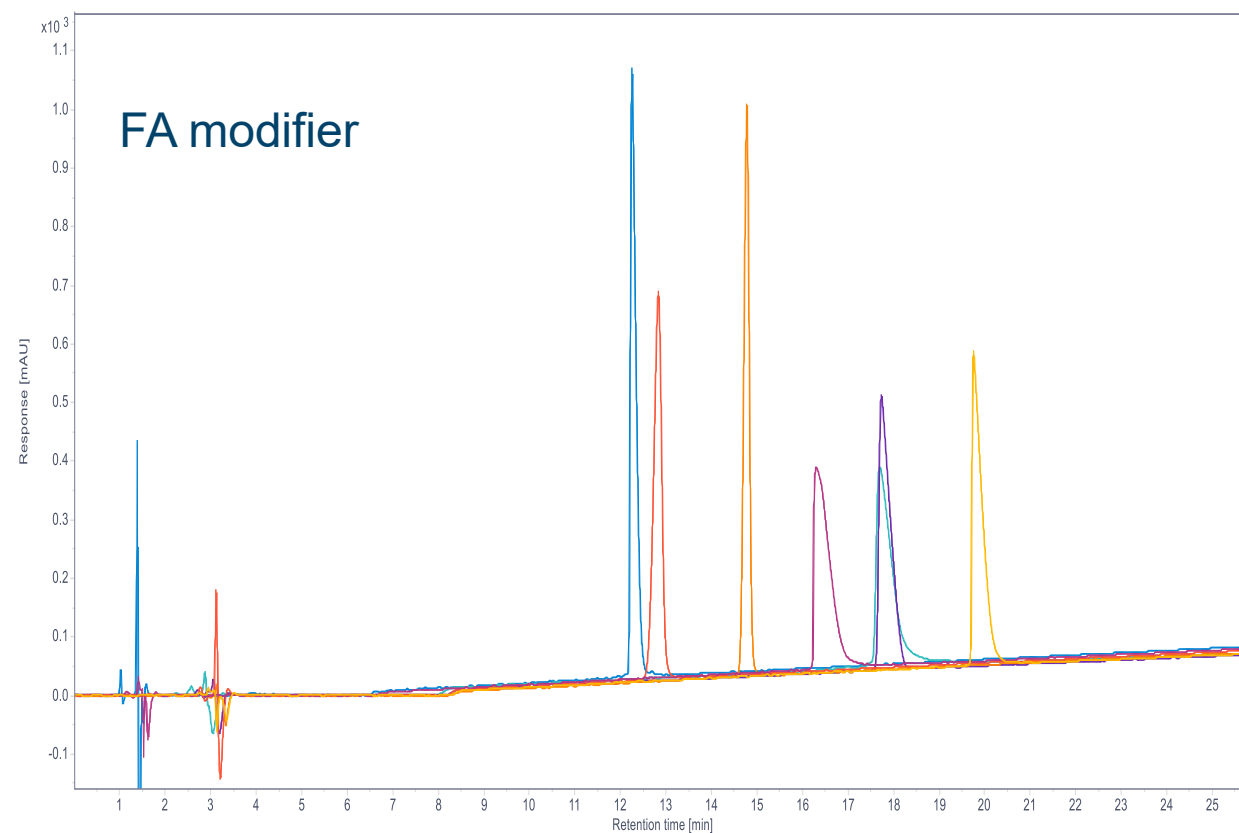
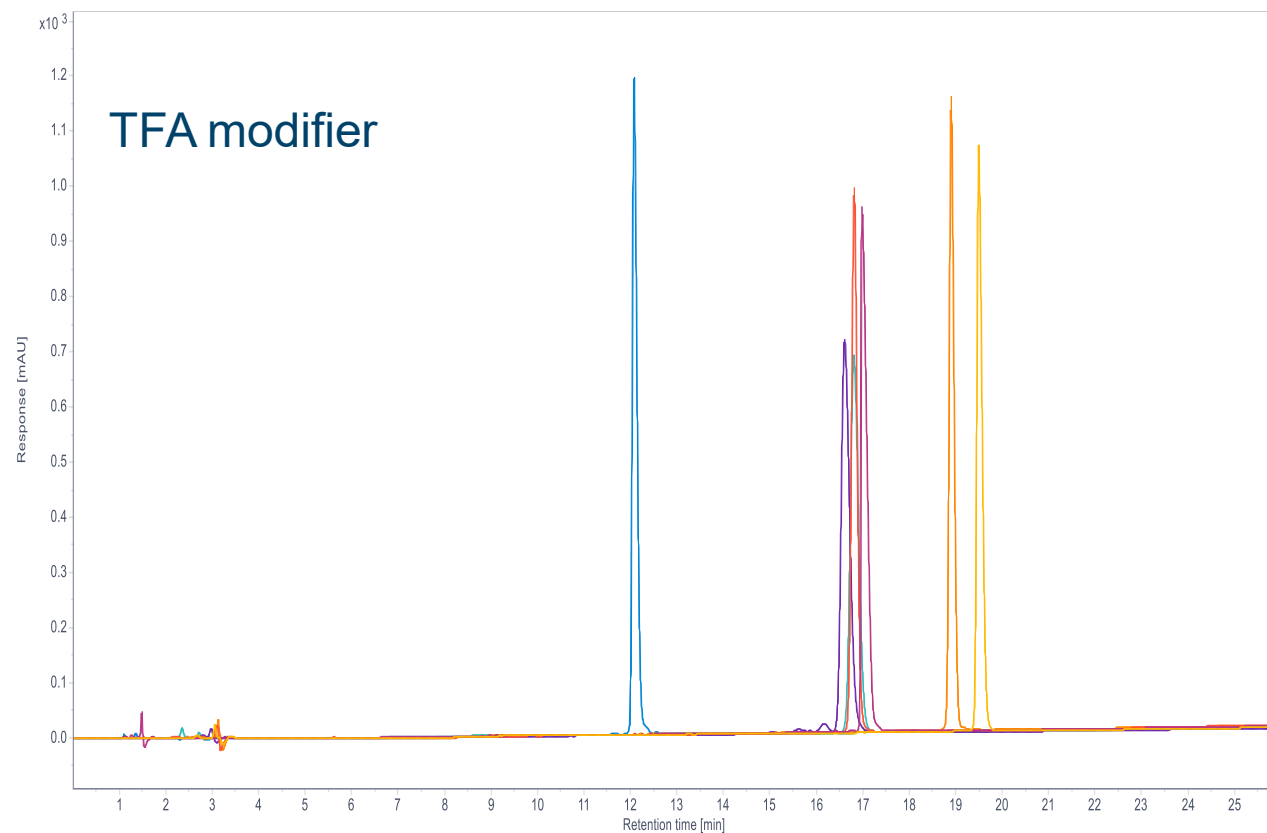
Polymeric stationary phase alternative



Comparison of Column Chemistries with Different Modifiers

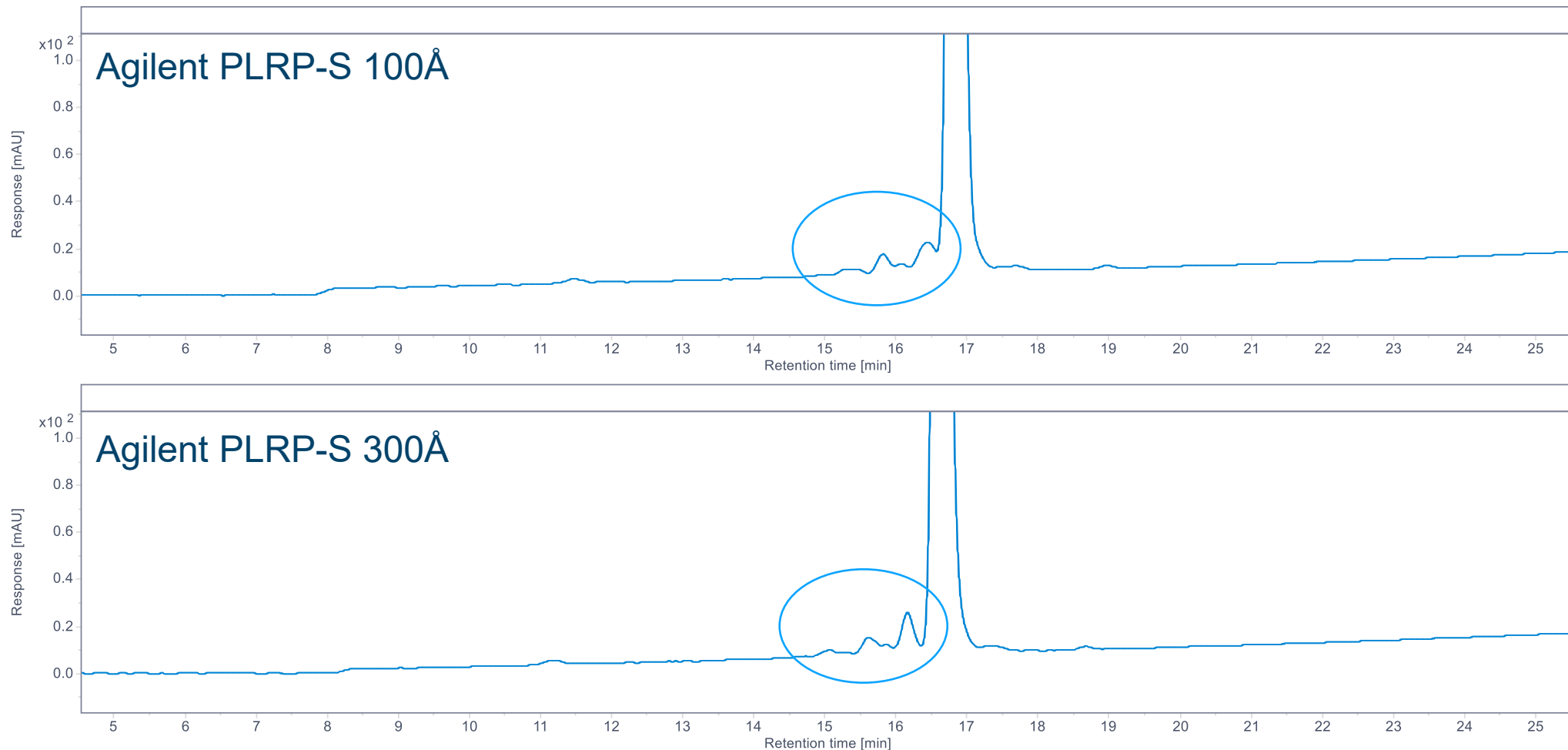
- (A) AdvanceBio Peptide Plus, 4.6 × 150 mm
- (B) PLRP-S 8 μm 100 Å, 4.6 × 250 mm
- (C) PLRP-S 8 μm 300 Å, 4.6 × 250 mm
- (D) Polaris Amide C18, 5 μm, 4.6 × 250 mm

- (E) Polaris C18-A, 5 μm, 4.6 × 250 mm
- (F) AdvanceBio Peptide Mapping, 4.6 × 150 mm
- (G) Pursuit C18, 5 μm, 4.6 × 250 mm



Heat-Treated Semaglutide with PLRP-S and Different Pore Sizes

The larger pore size allows mass transfer in and out of the pores and results in higher resolution.

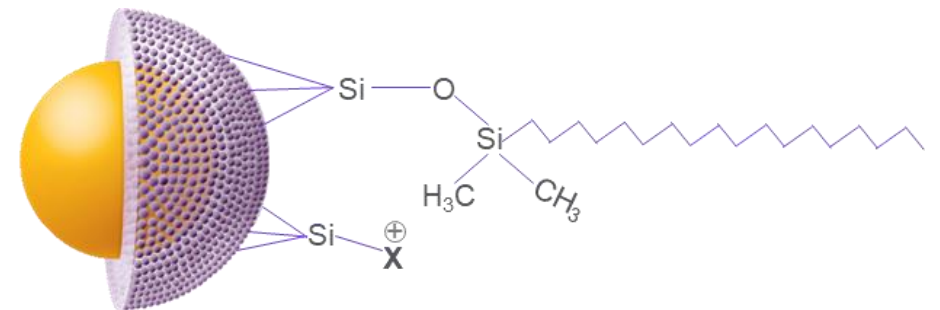


Application note: [5994-7419EN](#)

Reversed-Phase Recap

Method development tools to mitigate secondary interactions in reversed phase:

- Stationary-phase selection
 - Look for endcapping, or charged surface modification chemistry
- pH
 - Stay within pH range of your column or choose a column that tolerates higher pH
- Screen mobile-phase modifiers
 - TFA and formic acid most common, DFA also an option
 - Try varying concentration



Application Notes

2025 ASMS GLP-1 Posters

MP 229 Sensitive Quantitation of Glucagon-Like Peptide-1 (GLP-1) Analog Tirzepatide in Plasma Using Automated LC-MS/MS workflow

TP 622 Streamlined Analysis of Synthetic Peptides with Non-Standard Amino Acid Chemistry

TP 625 Characterization and Quantification of Glucagon-Like Peptide-1 Agonists and their Impurities Using Liquid Chromatography/Mass Spectrometry (LC/MS)

TP 629 Characterization of Synthetic Peptide Drug and Impurities Using High-Performance Liquid Chromatography (HPLC) and Liquid Chromatography/Mass Spectrometry (LC/MS)

TP 638 Advanced Characterization of Semaglutide and Its Impurities Using a Heart-Cutting 2D-LC/MS Workflow for Biopharmaceutical Analysis

TP 644 Monitoring Stability and Impurity Products of GLP-1 Agonists Using a Novel Single Quadrupole LC/MS

WP 603 Structural Characterization of Multiple GLP-1 Receptor Agonists Using Electron Capture Dissociation

ThP 595 Comprehensive Workflow for Automated Cleanup and Sensitive Quantitation of Glucagon-Like Peptide-1 (GLP-1) Analogs from Plasma

Impurity Analysis Workflow

Application notes:

- Semaglutide impurity profiling (1D): 5994-7419EN
- Liraglutide impurity resolution (1D): 5994-7415EN
- Efficient method optimization: 5994-7414EN
- Advanced 2D-LC/MS workflow: 5994-8288EN
- 2D-LC impurity identification in semaglutide (RP–RP): 5994-7654EN
- Peptide degradation impurity analysis (Q-TOF support): 5994-7794EN
- Peptide impurity characterization using 2D-LC: 5994-7727EN
- Peptide drug stability analysis: 5994-7500EN

Aggregate Analysis Workflow

- SEC-UV analysis of liraglutide (*in press*)
- In-depth analysis of semaglutide: 5994-7740EN

Amino Acid Analysis Workflow

- Amino acid analysis of GLP-1 analogs: 5994-7749EN

Peptide Sequence Confirmation/Peptide Quant Workflow

- Sequence and variant confirmation: 5994-7727EN
- Quantification of liraglutide and semaglutide: 5994-7415EN and 5994-7419EN
- Comprehensive characterization: 5994-7994EN
- Quantification of glucagon-like peptide-1 agonist tirzepatide: 5994-7992EN
- Quantification of therapeutic peptide exenatide: 5994-5224EN

Resources for Support

Technical support: www.agilent.com/chem/techsupport

Agilent product catalogs: www.agilent.com/en/promotions/catalog

- InfinityLab BioHPLC supplies catalog: ([5994-6123EN](#))

Resource page: www.agilent.com/chem/agilentresources

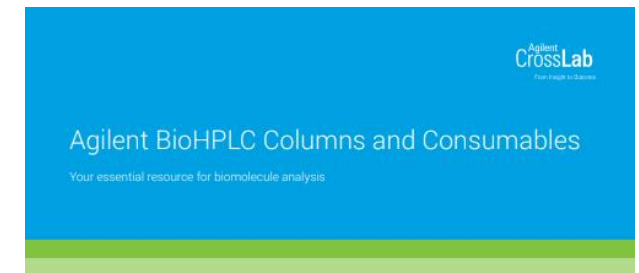
- Quick reference guides
- Catalogs, column user guides
- Online selection tools, how-to videos

Agilent University: <http://www.agilent.com/crosslab/university>

YouTube: [Agilent Channel](#)

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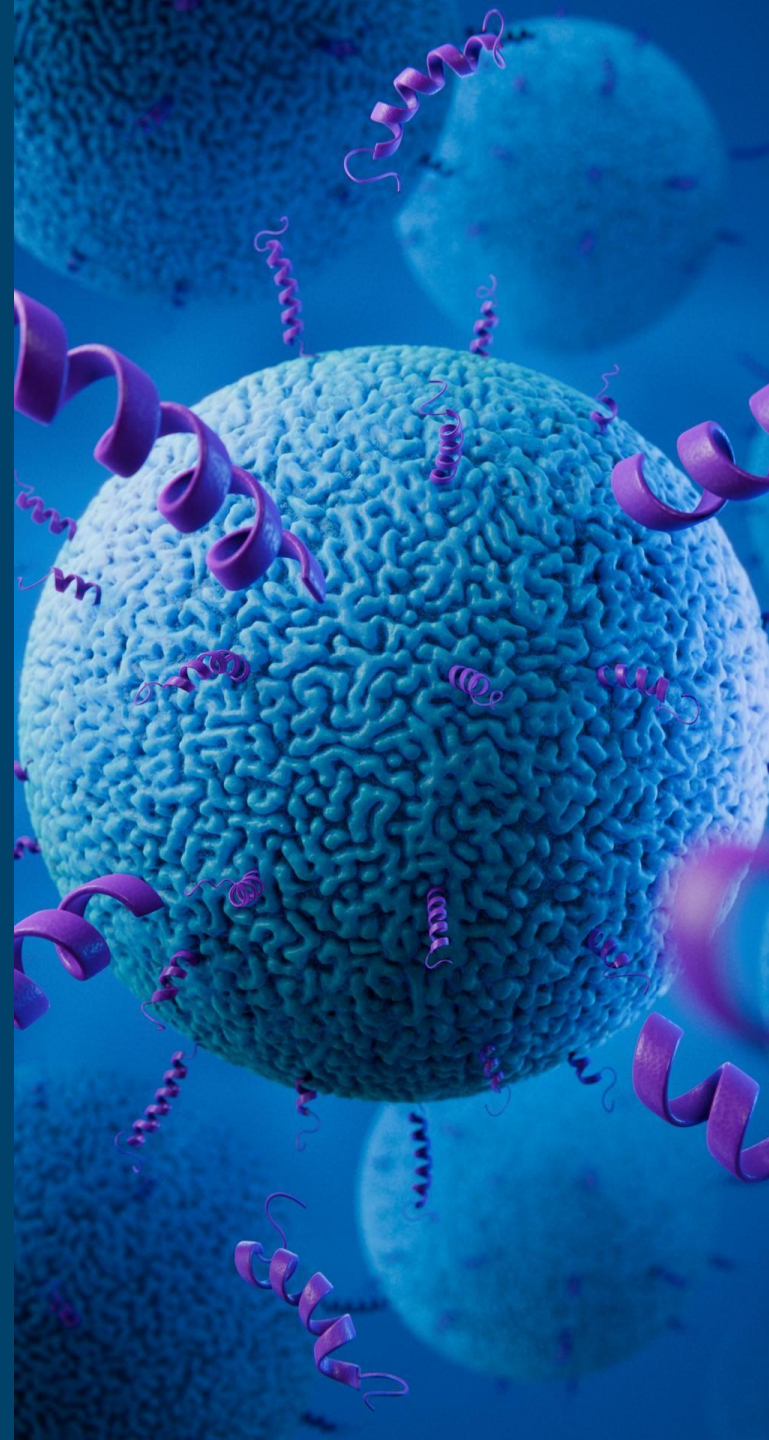
Web chat: product pages of Agilent.com



1-800-227-9770 option 3, option 3:

- Option 1: GC and GC/MS columns and supplies
gc-column-support@agilent.com
- Option 2: for LC and LC/MS columns and supplies
lc-column-support@agilent.com
- Option 3: for sample preparation, filtration, and QuEChERS
spp-support@agilent.com
- Option 4: for spectroscopy supplies
spectro-supplies-support@agilent.com
- Standards
chem-standards-support@agilent.com
- ProZyme products
pzi.info@agilent.com

Thank you!



Questions?

