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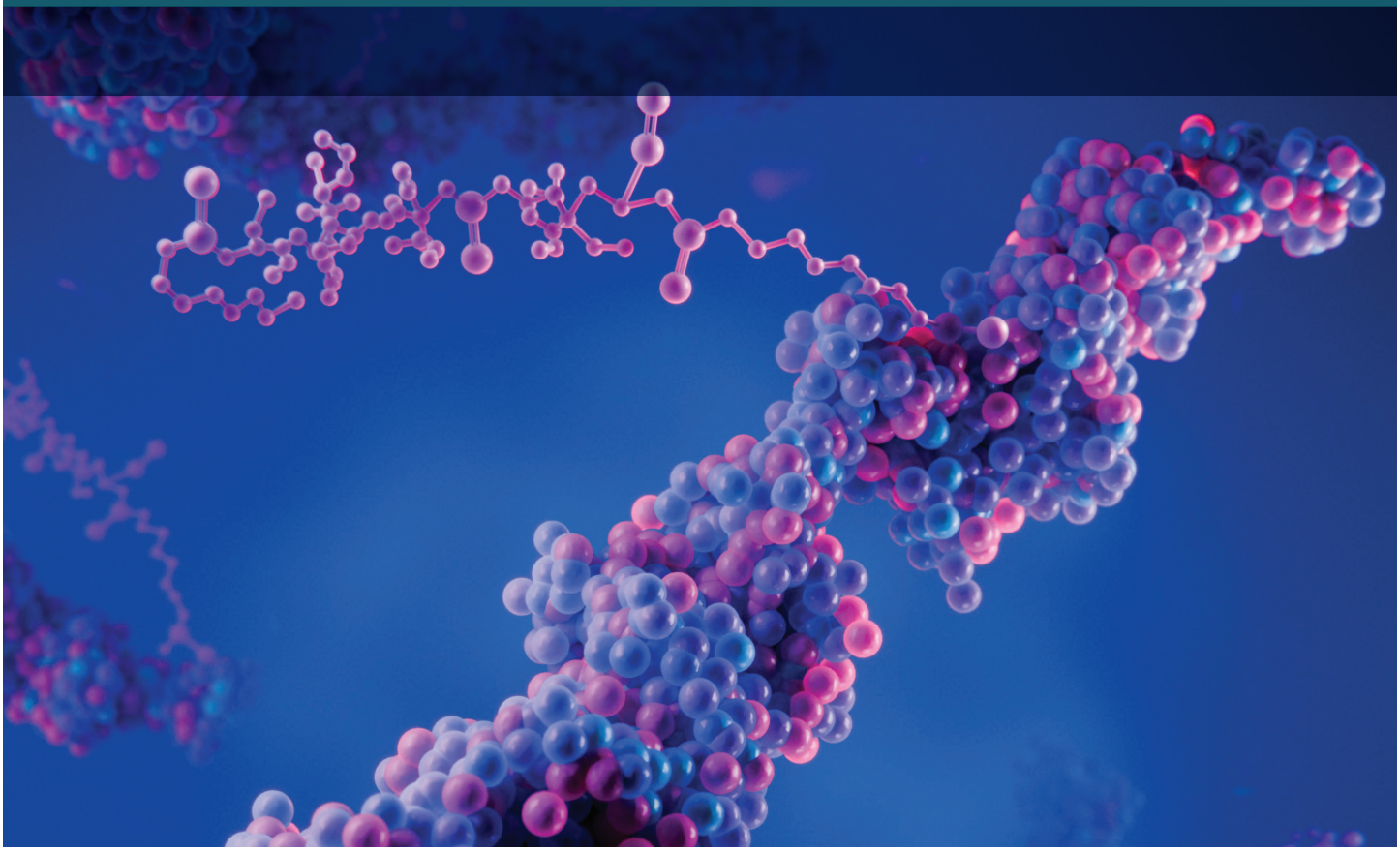
Complete Analytical Workflows for GLP-1 Receptor Agonists

Applications for peptide characterization, purification, and bioanalysis



Contents

Introduction

Peptides occupy a unique space between small molecules and biologics, offering distinct and tunable pharmacokinetic (PK) and pharmacodynamic (PD) profiles for therapeutic applications. They generally have a lower risk of triggering immune reactions compared to biologics such as monoclonal antibodies, are less expensive to produce, and penetrate tissues more effectively due to their smaller size. Although most peptides do not easily cross cell membranes, advances in engineering have improved intracellular targeting and cellular uptake. Compared to small molecules, peptides often demonstrate higher specificity and selectivity, reducing unwanted side effects. Because peptides degrade into naturally occurring amino acids, they are less likely to accumulate in tissues or cause long-term toxicity. Their size and flexibility allow them to modulate large protein surfaces and protein-protein interactions (PPIs).

Manufacturing peptides is more straightforward and cost-effective than producing proteins or antibodies, allowing for high purity and consistent quality. Additionally, peptides are often more stable in storage and may not require refrigeration, cutting down on logistical challenges and costs.

Recent innovations in drug design, including methods to extend peptide half-life (like acylation, PEGylation, or fusion to larger proteins), alongside advances in delivery platforms, have significantly accelerated the development of next-generation peptide therapeutics. These improvements address common issues of rapid breakdown and clearance, which traditionally required frequent injections. New peptide-based therapies have also expanded into areas like targeted delivery and vaccines.

Despite these advances, the increasing complexity of peptide drugs—due to varied molecular structures, conjugation strategies, and delivery formats—poses challenges for quality control.

Peptides work through diverse mechanisms—acting as receptor agonists or antagonists, enzyme inhibitors, immune modulators, or disrupting intracellular signaling. They are used to treat a broad range of conditions, including metabolic and cardiovascular diseases, cancer, and infectious diseases.

Therapeutic peptides are a fast-growing segment of the pharmaceutical market, with over 110 approved products globally as of May 2024, and are expanding into new therapeutic areas. The most esablished market remains metabolic diseases, particularly type 2 diabetes and obesity. Peptides drugs like insulin and glucagon-like peptide-1 receptor agonists (GLP-1 RA) have revolutionized treatments. GLP-RAs help regulate glucose levels, promote weight loss, and reduce cardiovascular risk. Among them, Ozempic (semaglutide), is notable for its convenient once-weekly dosing. The pipeline for GLP-1 RAs is evolving rapidly, with new multi-target candidates like tirzepatide, which combines GLP-1 and GIP receptor activation and has shown superior results in trials. Combination therapies that pair GLP-1 RAs with basal insulin are also gaining traction, offering more comprehensive management of diabetes. Figure 1 presents the classification of FDA-approved peptide drugs and diagnostics, along with their respective clinical indications, approved between 1940 and October 2024. The data was compiled from the publicly available PepTherDia database, with additional information on insulin-based drugs sourced from other resources.

Return to Contents Section

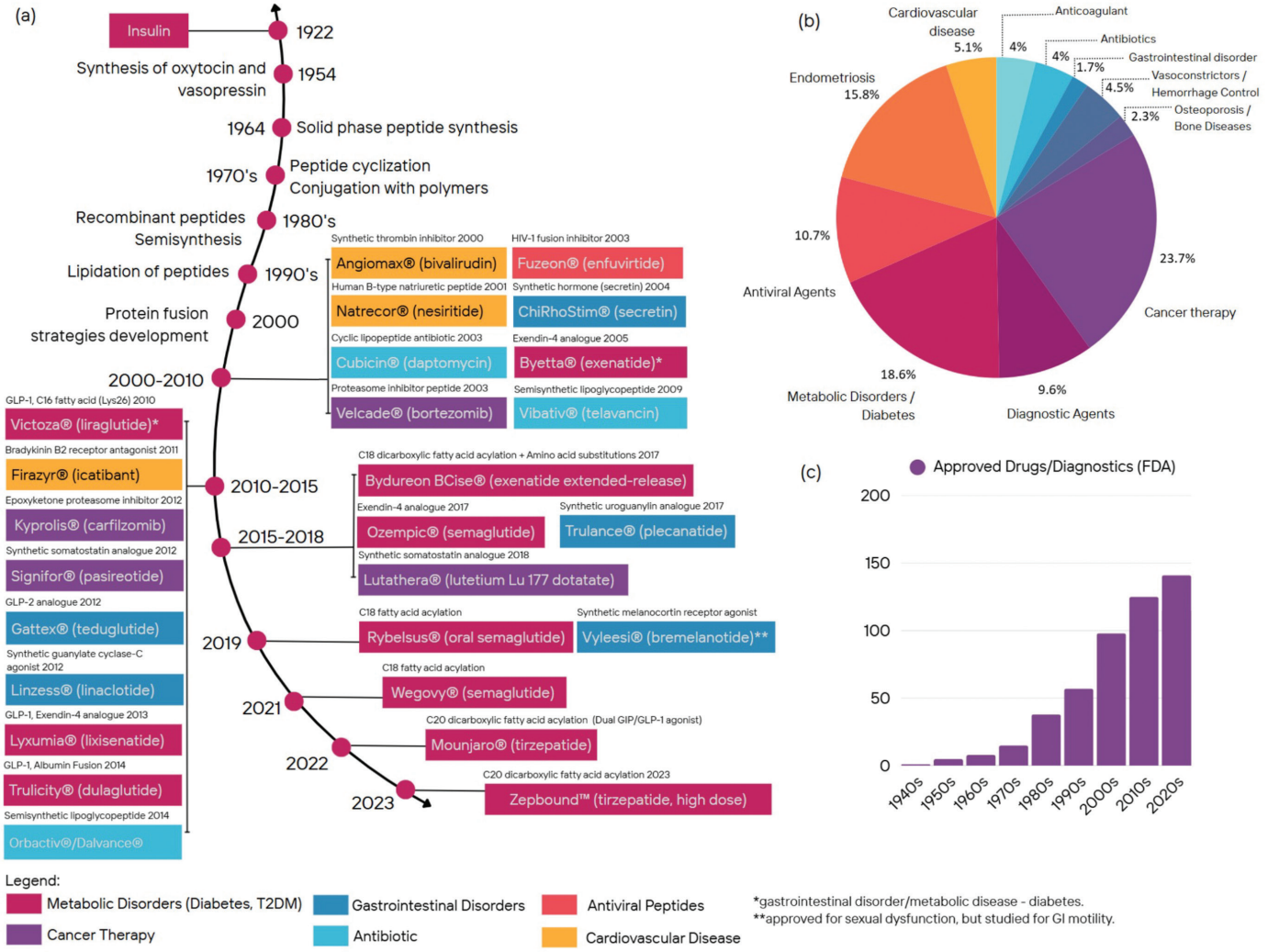


Figure 1. (a) Timeline of relevant milestones in the development of therapeutic peptides (only selected classes of FDA-approved drugs from 2000 onwards), (b) Classification of the FDA-approved molecules (from 1940 until October 2024) and their respective clinical use, (c) Distribution of peptide-based drugs and in-vivo diagnostics approval times from 2000 to 2024, compiled from the freely available database PepTherDia [7], with insulin-based drug data sourced from [8].

Analysis of peptide therapeutics

As the complexity and diversity of peptide-based products continue to grow, the importance of robust analytical capabilities becomes increasingly evident. Sensitive and selective methods are essential for determining and monitoring critical quality attributes (CQAs), such as identity, content, purity, and impurities, to ensure the quality, safety, and efficacy of these therapeutics (Table 1). The challenge extends to the bioanalysis of these modalities, including PK and PD assessments in biological matrices. Liquid chromatography (LC) and mass spectrometry (MS) play a central role in supporting the above measurements.

Return to Contents Section

Table 1. Therapeutic peptide attributes studied by LC and MS.

Applications	Goal	Approach
Identity	Ensuring that the peptide has the correct structure which is, primarily, defined by the amino acid sequence and intended modifications (lipidation, amidation, PEGylation, etc.)	- Comparison of retention time to reference standard by LC/UV - Molecular mass determination by LC/MS - Sequence determination by LC/MS/MS - Amino acid analysis by LC/FLD
Purity and impurity	Assessing the proportion of the desired peptide relative to impurities (purity) and detecting, identifying and quantifying process- (solvents, reagents, metals, host-cell materials, etc.) and product-related impurities (impurity)	- Purity and product-related impurities: LC/UV, LC/MS, LC/MS/MS, 2D-LC - Process-related impurities: LC/UV, LC/ELSD, LC/RI, LC/MS, LC/MS/MS, GC/FID, GC/MS, ICPMS
Content	Determination of the absolute quantity of the therapeutic peptide	- LC/UV, LC/MS, LC/MS/MS using external or internal calibration - Amino acid analysis by LC/FLD
Bioanalysis (PK/PD)	Quantification of therapeutic peptides in biological matrices (blood, plasma, serum, urine, …)	- 1D or 2D-LC/MS/MS with tailored sample preparation and absolute quantification using isotopically labeled synthetic peptides or analogue peptides

This compendium brings together practical insights and application notes focused on analytical challenges and method development for GLP-1 receptor agonists and related peptides. It is designed to support scientists involved in ensuring the quality and reliability of these innovative therapeutics as they continue to transform patient care.

With content adapted from contributions by RIC Group.

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Return to Contents Section

Identity, Purity, and Impurity Assessment

Introduction

The determination of the identity, purity, and impurity profile of therapeutic peptides is essential to guarantee safety, efficacy, and regulatory compliance. Peptides are inherently more complex than small molecules, resulting in a broader spectrum of product-related impurities that arise during production (synthesis, purification, and formulation) or storage (Table 1). The impurities often share similar physicochemical properties with the therapeutic peptide, posing significant challenges not only for their sensitive detection but also for achieving sufficient selectivity to distinguish the impurities from the main peptide.

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Return to Contents Section

Table 1. Common product-related impurities and degradation products.

Parameters	Value
Amino acid deletion	Missing one or more amino acids at the termini or within the sequence (synthesis-related)
Amino acid insertion	Addition of one or more amino acids during synthesis
Protecting group	Incomplete removal of protecting group during synthesis
Diastereomers	L-/D-amino acid, *cis*-trans isomerism of Pro
Oxidation	Oxidation of Met, Trp, His, Cys
Reduction	Reduction of S-S bridges
Deamidation	Asn to Asp or Gln to Glu conversion
Isomerization	Asp to isoAsp
Cyclization	Formation of diketopiperazine, pyroglutamate, succinimide
Other side- and end-chain impurities	Reduction of S-S bridges
Oligomers	Covalent oligomers connected via disulfide bridges or amino acid cross-links (due to His, Trp or Tyr oxidation or Cys β-elimination) or non-covalent oligomers associated via hydrophobic or electrostatic interactions

Reversed-phase liquid chromatography (RPLC) is the gold standard in peptide analysis for its resolving power, robustness, and MS compatibility, capable of detecting most impurities in Table 1. To address slow diffusion of large peptides, sub-3 µm fully or superficially porous silica columns are used, with C18 phases most common. Pore sizes of 120–300 Å allow access to the stationary phase; column lengths of 50–250 mm balance throughput and resolution. For MS or lower solvent use, 2.1–3 mm IDs are preferred.

Ion-pairing RPLC (IP-RPLC), often with trifluoroacetic acid (TFA), mitigates non-specific interactions but can suppress electrospray ionization (ESI). Charged surface particles enable the use of milder acids (e.g., formic acid) to improve MS sensitivity. Alternatives include difluoroacetic acid (DFA), heptafluorobutyric acid (HFBA), and, for LC/UV only, non-volatile acids such as phosphoric or methanesulfonic acid. Ammonium formate/acetate are also used. Peptide elution is achieved with increasing organic solvent (methanol/acetonitrile) at 25–60°C, balancing mass transfer gains with peptide stability.

Complete resolution of all impurities may require orthogonal methods such as IP-RPLC, size-exclusion chromatography (SEC), ion-exchange (IEC), hydrophilic interaction (HILIC), chiral, or mixed-mode chromatography. SEC, widely used for oligomer/monomer analysis, employs sub-3 µm, ~130 Å particles in 150–300 mm columns with neutral phosphate buffers or acidic alternatives. Additives (e.g., NaCl, Arg, acetonitrile), hydrophilic coatings, or deactivated steel prevent non-specific interactions. SEC combined with MALS can determine molar mass.

Two-dimensional liquid chromatography (2D-LC), coupled with orthogonal modes, is employed to resolve co-eluting species or achieve MS-friendly conditions. This is accomplished using heart-cutting or high-resolution sampling, sometimes with active solvent modulation (ASM) to ensure compatibility between the 1D and 2D separations.

Mass spectrometry (MS) enables quantitation and structural characterization of impurities. ESI in positive mode produces multiply charged ions detected by LRMS (e.g., single quad) or high-resolution mass spectrometry (HRMS) (e.g., TOF). HRMS supports accurate mass and structural resolution; spectral deconvolution gives molecular weights. Tandem MS (Q-TOF, QQQ) allows sequencing; CID, ECD, and ion-mobility MS (IM-MS) help locate modifications, distinguish isobars (Leu/Ile, Asp/isoAsp), study disulfide bonds, and separate closely related peptides.

Specialized methods (e.g., ICP-MS/OES for metals, GC-FID/MS for residual solvents, RPLC-MS/MS for host-cell proteins, HILIC/mixed-mode with ELSD for excipients) are used for certain process-related impurities.

Return to Contents Section

Application Note

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Molecular Weight Confirmation of a Peptide Using MS Spectral Deconvolution for OpenLab CDS and the Agilent InfinityLab LC/MSD XT System

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Abstract

Intact mass analysis of proteins and peptides by liquid chromatography/mass spectrometry (LC/MS) is a valuable tool in biopharmaceutical research. This application note shows the development of a molecular weight confirmation method for a peptide sample using the MS Spectral Deconvolution feature of Agilent OpenLab CDS version 2.8.

Data were acquired on an Agilent 1290 Infinity II LC coupled to the Agilent InfinityLab LC/MSD XT using the latest version of OpenLab CDS. A recombinant peptide sample was analyzed, and its intact molecular weight, as well as an oxidized species molecular weight, were confirmed by deconvolution of the mass spectra.

Return to Contents Section

Introduction

Intact mass by LC/MS is a common analytical workflow used to quickly confirm a biomolecule's molecular weight. Large biomolecules such as proteins, peptides, and oligonucleotides have multiple ionizable functional groups, and during electrospray ionization take on multiple charge states, leading to a complicated mass spectrum. This characteristic spectrum, often referred to as a charge envelope, can be used to back-calculate the molecular mass of the originating species by a process called deconvolution.

Although it is common to use high-resolution accurate mass detection and spectral deconvolution for intact mass determination, unit mass instruments may also be used when mass accuracy is secondary to robustness. For molecular weight confirmation workflows, unit mass instruments are easier to operate and present a more cost-effective solution than high resolution instruments[1,2].

This application note shows how OpenLab CDS can be used with an InfinityLab LC/MSD XT system to automate the molecular weight confirmation of a recombinant 42 amino acid polypeptide, gastric inhibitory peptide-1 (GIP-1). Spectral deconvolution was used to quickly confirm the molecular weight of the peptide. Additionally, an oxidized species of GIP-1 was identified and confirmed using the deconvolution feature. This demonstrates the utility of the LC/MSD XT and the MS Spectral Deconvolution feature to increase confidence in assigning a specific modification to an event such as oxidation without the need for high resolution mass spectrometry.

Experimental

Instrumentation

A 1290 Infinity II LC and LC/MSD XT were used to analyze the peptide samples. The complete instrument setup consisted of the following modules:

- Agilent 1290 Infinity II flexible pump (G7104A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II DAD (G7117B)
- InfinityLab LC/MSD XT (G6135C) with electrospray ionization (ESI) source (G1948B)

LC method parameters applied for the analysis are shown in Table 1, and MS parameters shown in Table 2.

Table 1. Agilent 1290 Infinity II LC method parameters.

Parameter	Value	
Column	ZORBAX RRHT StableBond 2.1 x 50 mm, 1.8 µm. 80 Å, 80 °C	
Mobile Phase A	0.1 % formic acid in water	
Mobile Phase B	0.1 % formic acid in acetonitrile	
Gradient	Time (min)	%B
	0	5
	5	40
	5.5	5
Flow Rate	0.5 mL/min	
Injection Volume	5 µL, standard wash	
Detection UV	285 nm, 4 nm bandwidth, ref. 360 nm	
Acquisition Rate	2.5 Hz	

Table 2. Agilent InfinityLab LC/MSD XT parameters.

Parameter	Value
Ion Source	ESI
Polarity	Positive
Drying Gas Temperature	350 °C
Gas Flow	10 L/min
Nebulizer Pressure	35 psi
Capillary Voltage Positive	4,000 V
Scan Range (m/z)	400-2,000
Scan Time (ms)	936
Fragmentor Voltage	200 V
Gain	3
Threshold	0
Storage Mode	Profile

Return to Contents Section

Software

OpenLab CDS version 2.8 with MS Spectral Deconvolution capabilities was used to operate the instrument and perform the analysis. The algorithm used in OpenLab CDS for spectral deconvolution is optimized for simplifying spectra of multiply charged molecules obtained from unit mass instrumentation. The deconvolution parameters needed only slight adjustments for the target analyte, as it produced a good-quality spectrum with highly abundant peaks. Table 3 summarizes the basic settings chosen for the target analyte. A detailed overview over the capabilities of MS Spectral Deconvolution is available in the application note[3] describing the feature.

Table 3. MS Spectral Deconvolution settings.

Automatic Deconvolution	
Run Automatic Deconvolution	Checked
All Peaks	Selected
TIC top (n) Peaks	3
Basic Settings	
Use m/z Range	Unselected
Low/High Molecular Weight	1,000—8,000
Maximum Charge	10
Minimum Peaks in Set	3
Advanced Settings	
MW Agreement (0.01%)	5 (0.05%)
Absolute Noise Threshold	1,000
Relative Abundance Threshold (%)	10%
MW Algorithm	Curve Fit
MW Algorithm Threshold	40%
Envelope Threshold (%)	50%

Chemicals

Acetonitrile, formic acid, hydrogen peroxide, and LC/MS grade water were purchased from VWR (Bruchsal, Germany). Recombinant human GIP-1 was purchased from GenScript (Piscataway, NJ, USA). Unless stated otherwise, samples were prepared from lyophilized GIP-1 to a concentration of 1 mg/mL stock solution by diluting with de-ionized water, aliquoted to 50 µL in sample tubes, and kept frozen at -20 °C until use. To force oxidation of GIP-1, a 50 µL aliquot was diluted to 50 µg/mL using a 2% hydrogen peroxide solution and incubated for five minutes at room temperature.

Results and discussion

LC/MS analysis of a stored aliquot of GIP-1 showed two peaks in the total ion current (TIC) chromatogram (Figure 1a). The mass spectra of the peaks at 3.8 and 4.0 minutes displayed distinctive charge envelope patterns common to large molecules (Figure 1b). The deconvolution algorithm reported an apparent mass of 4998.7 and 4982.7 Da, respectively, for the main components under the two peaks (Figure 1c). The larger peak at 4.0 minutes was therefore identified as the unmodified peptide GIP-1 with a theoretical mass of 4983.6 Da. The delta mass of the main component in the earlier eluting peak amounts to 16 Da, which suggested an oxidized species of GIP-1.

To confirm the identity of the impurity peak as an oxidation product, a second aliquot was treated with 2% hydrogen peroxide to force oxidation. Additionally, a fresh standard was prepared from lyophilized GIP-1 and analyzed immediately without cold storage to rule out the storage as a source for the observed impurity. The peroxide-treated sample displayed a single peak at 3.8 minutes (Figure 2a) and a deconvoluted mass spectrum that matched the one for the suspected oxidized impurity shown in Figure 1b. The freshly prepared GIP-1 sample showed two peaks as before (Figure 2a), but with a different ratio of the peak areas. Consequently, it was concluded that the impurity observed in the GIP-1 samples was an oxidation product that was naturally present in the samples. Although a possible relationship to storage conditions could not be ruled out, it was not investigated further.

With the observed components identified, the method was then extended to include an automatic PDF report, as shown in Figure 3. The report template presents the deconvolution results per peak and summarizes calculated masses for components and their relative abundance in the spectrum, providing a clear mass readout per peak. This increases the confidence of correct identification of the species without relying on retention time alone, or requiring manual calculations based on known charge states.

Return to Contents Section

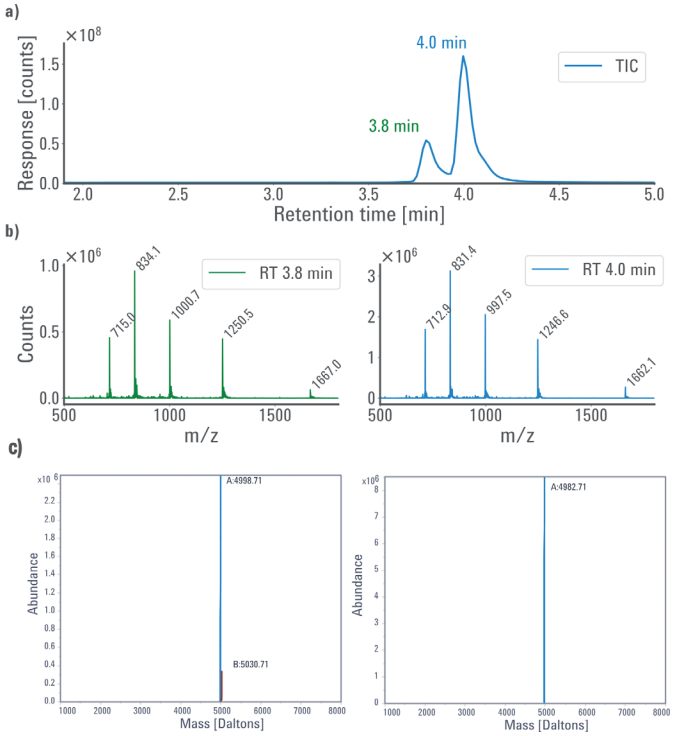


Figure 1. A) Total ion current chromatogram of GIP-1 frozen aliquot (100 µg/mL in water). B) Extracted mass spectra for peaks at 3.8 and 4.0 minutes, respectively. C) Deconvoluted mass spectra for the two identified peaks.

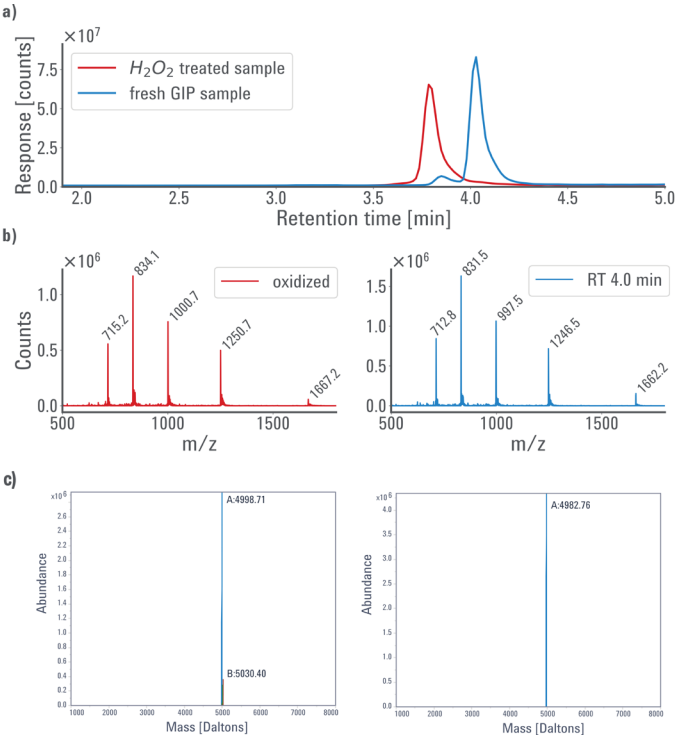


Figure 2. A) Overlaid TIC chromatograms of a GIP-1 sample prepared fresh from lyophilized standard and a hydrogen peroxide treated aliquot (50 µg/mL each). B) Extracted mass spectra for the respective peaks in A). C) Deconvoluted mass spectra for the peroxide-treated sample, and in comparison, the one for GIP-1 prepared fresh.

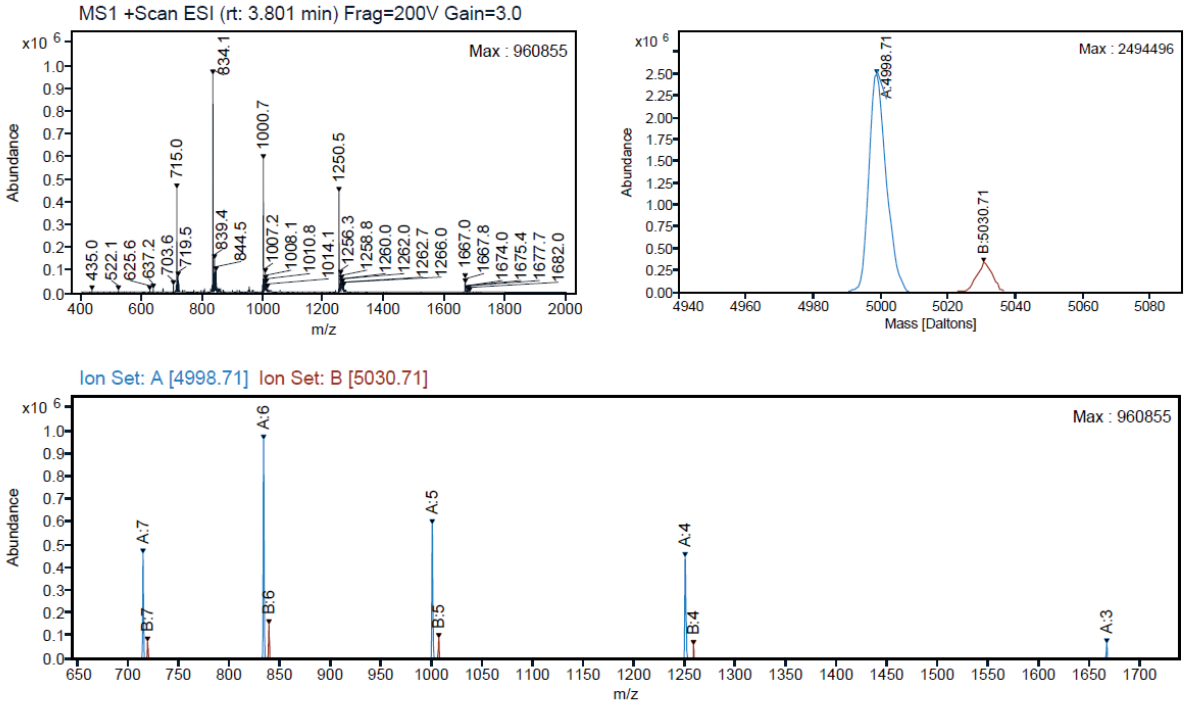
Return to Contents Section

Single Injection Report

Deconvolution of peak at RT: 3.801

Signal: MS1 +TIC SCAN ESI Frag=200V Gain=3.0

Spectrum: MS1 +Scan ESI (rt: 3.801 min) Frag=200V Gain=3.0



Component	Mass	Absolute Abundance	Relative Abundance (%)	Relative Quantitation (%)
A	4998.71	2494496	100.00	88.19
B	5030.71	333957	13.39	11.81

Figure 3. Excerpt from an automatic report showing the deconvolution results for the peak of the oxidized GIP-1 species. The report gives a detailed view of the mass spectrum, the deconvolution result, the ions considered in deconvolution, and a table with estimates of the relative abundance of the two components.

Return to Contents Section

Conclusion

The combination of the Agilent 1290 Infinity II LC system with the Agilent InfinityLab LC/MSD XT and Agilent OpenLab CDS version 2.8 with MS Spectral Deconvolution is a powerful tool to perform peptide analysis on a rugged platform. The automatic deconvolution enables mass confirmation for a target peptide, as well as its impurities, improving confidence in assigning the correct species. The deconvolution process can be run interactively through the CDS interface during method development, with various options to control the output. The fully automated workflow, where a report can be generated without user intervention, makes it easy to transition to higher throughput analyses. This versatility makes OpenLab CDS an attractive option for both research and routine quality control laboratories where efficiency, accuracy, and time-to-result depends on the instrumentation and associated software.

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3. Unit Mass Spectral Deconvolution for Molecular Weight Confirmation of Large Molecules. Agilent Technologies technical note, publication number 5994-6928EN, **2023**.

Application Note

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An In-Depth Analysis of Semaglutide, a Glucagon-Like Peptide-1 Receptor Agonist

Comparative IP-RP analytical outcomes using different column chemistries

Authors

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Abstract

Peptide biotherapeutics represent a class of pharmaceuticals that hold significant importance in modern medicine due to their unique properties and diverse therapeutic applications. Peptides are short chains of amino acids, typically comprising fewer than 50 residues, and they play crucial roles in various physiological processes within the human body. With advancements in biotechnology and pharmaceutical research, the development and use of peptide-based therapeutics have surged, offering novel treatment options for a wide range of medical conditions. This application note presents some of the challenges when analyzing a glucagon-like peptide-1 (GLP-1) receptor agonist, semaglutide acetate, comparing different gradient conditions, temperatures, and column chemistries. Furthermore, sequence identification was achieved by LC/MS analysis using an Agilent AdvanceBio Peptide Plus column.

Return to Contents Section

Introduction

GLP-1 receptor agonists (a group of peptide compounds) have gained importance for early-stage therapy of type II diabetes and obesity. Yet, ensuring optimal purity for these peptides presents a significant hurdle. Semaglutide (MW: 4,113.58 Da) is one of the main GLP-1 agonists commercially available today and possesses a fatty acid side chain modification (Figure 1). As with all medicinal substances, the existence of impurities arising from the manufacturing process or during storage holds the potential to compromise its safety, effectiveness, and overall quality. Crude peptides are normally analyzed by HPLC using reversed-phase columns with gradient elution using aqueous acetonitrile (ACN) (typically containing 0.1% trifluoroacetic acid (TFA) as the ion-pair reagent). TFA anions form an ion pair with positively charged peptides, increasing their hydrophobicity and therefore their retention time. However, to identify the impurity peaks in an LC/MS method, formic acid (FA) is the preferred mobile phase modifier, as the weaker acid causes less ion suppression. However, FA is less effective at suppressing nonspecific interactions, and creates a less hydrophobic (and therefore less retentive) ion pair with the peptide. Consequently, resolution when using FA as an ion-pair reagent can be compromised. This application note demonstrates how choosing different column chemistries can greatly enhance the selectivity for certain impurities, increasing the confidence in the ability to detect and quantify modifications present in the sample. Furthermore, the 250 mm long columns used for this work (PLRP-S 100 Å and 300 Å, Polaris and Pursuit) were chosen specifically because they are also available in preparative dimensions.

The characterization of semaglutide was confirmed using the Agilent 6545XT AdvanceBio LC/Q-TOF with an Agilent AdvanceBio Peptide Plus column (2.1 × 150 mm).

Experimental

Reagents and chemicals

All reagents were HPLC-grade or higher.

Analytical equipment

An Agilent 1290 Infinity II LC system consisted of the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117C) with a 10 mm Max-Light cartridge cell (G7117-60020)

Method parameters are listed in Table 1.

LC/MS equipment

A 1290 Infinity II LC system was coupled to the 6545XT AdvanceBio LC/Q-TOF.

Software and data processing

- Agilent OpenLab software suite, version 2.6
- Agilent MassHunter data analysis software, version B.09
- Agilent MassHunter BioConfirm software, version 10.00

Figure 1. Structure of semaglutide showing differences to a native GLP-1 fragment sequence.

Return to Contents Section

Table 1. LC/UV method conditions.

Agilent 1290 Infinity II Analytical LC Conditions	
Parameter	**Value**
Column	(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
Mobile Phase	Eluent A1: 0.1% TFA in Water Eluent B1: 0.1% TFA in ACN Eluent A2: 0.1% FA in Water Eluent B2: 0.1% FA in ACN
Flow Rate	1.0 mL/min
Column Temperature	25 °C
Injection Volume	10 µL
Detection	UV, 220 nm
Total Run Time	30 minutes

Table 3. LC/MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System			
Parameter	**Value**		
Source	Dual AJS		
Polarity	Positive		
Gas Temperature	325 °C		
Gas Flow	13 mL/min		
Nebulizer	35 psi		
Sheath Gas Temperature	275 °C		
Sheath Gas Flow	12 L/min		
Capillary Voltage	4,000 V		
Nozzle Voltage	500 V		
Fragmentor	175 V		
Skimmer	65 V		
Acquisition Mode	2.5 Hz		
Mass Range	100 to 2,100 *m/z*		
Acquisition Rate	5 spectra/s		
Agilent 1290 Infinity II LC System			
Column	Agilent AdvanceBio Peptide Plus 2.1 × 150 mm		
Thermostat	7 °C		
Solvent A	FA 0.1% in water		
Solvent B	FA 0.1% in acetonitrile		
Gradient	Time (min) 0 5 50 51 56	%A 70% 70% 45% 70% 70%	%B 30% 30% 55% 30% 30%
Column Temperature	25 °C		
Flow Rate	0.21 mL/min		
Injection Volume	0.2 µL		

Table 2. LC/UV gradient optimization.

Gradient	%B	Time (min)
1	25 to 55	0 to 30
2	30 to 55	0 to 30
3	35 to 55	0 to 30
4	30 to 60	0 to 30
5	35 to 60	0 to 30
6	40 to 60	0 to 30

Sample preparation

Semaglutide acetate was purchased from Cayman Chemical and dissolved to 1.0 mg/mL in mobile phase A containing 0.1% TFA. Thermal degradation was performed by heating to 85 °C for 60 minutes.

Results and discussion

Agilent offers a variety of reversed-phase columns and media designed to simplify your synthetic peptide analytical workflows.

To determine the effect of different stationary phases on selectivity for the analysis of semaglutide reference material and semaglutide that had been thermally degraded (see conditions above), several different reversed-phase products were screened.

These included columns with fully porous and superficially porous particles, columns with different pore sizes, and columns with different bonding modifications (Table 4).

Three different ACN gradients were evaluated using TFA as the ion-pair reagent in order to determine which gradient would be most suitable for testing all seven column chemistries.

Duplication injections of semaglutide reference material and semaglutide that had been thermally degraded were made on each column in turn, followed by a blank gradient to check for signs of carryover (a potential hazard with peptides that are modified with fatty acid side chain groups).

Return to Contents Section

Table 4. Column characteristics.

Product Name	Pore Size (Å)	Particle Size (µm)	Particle Type	Bonding Chemistry	Dimensions (mm)
AdvanceBio Peptide Mapping	120	2.7	Superficially porous	Endcapped C18	4.6 × 150
AdvanceBio Peptide Plus	120	2.7	Superficially porous	Endcapped C18 with charged surface modification	4.6 × 150
PLRP-S	100	8	Fully porous	Polystyrene/divinylbenzene	4.6 × 250
PLRP-S	300	8	Fully porous	Polystyrene/divinylbenzene	4.6 × 250
Polaris Amide C18	180	5	Fully porous	Amide modified C18	4.6 × 250
Polaris C18-A	180	5	Fully porous	C18 Type A	4.6 × 250
Pursuit C18	200	5	Fully porous	Endcapped C18	4.6 × 250

Figure 2 shows the chromatograms from one injection of semaglutide reference material on each of the seven columns overlaid using the gradient described in Table 2. It must be taken into consideration that the two columns packed with superficially porous materials were 150 mm long, compared to the other columns which were all 250 mm long. However, the peak shapes for all columns were sharp and relatively symmetrical. The two polystyrene/divinylbenzene columns gave slightly broader peaks, as would be expected given the larger particle size. The AdvanceBio Peptide Plus column also has a lower bonding density as a result of the presence of the charge surface modification, which accounts for the shorter retention time. The Polaris C18-A and Pursuit C18 columns gave the greatest retention time, which was likely due to higher surface area.

The same approach was taken to evaluate each column using different ACN gradients with FA as the ion-pair reagent. FA is a weaker acid than TFA and also considerably less hydrophobic. The optimum gradient was therefore different compared to the TFA separations. Figure 3 shows chromatograms from each of the columns tested, and there are noticeable changes. Firstly, the peak shape is inferior on several columns as a result of interactions between basic side chain residues of the peptide and residual acidic silanols on the surface of the silica stationary phase, leading to broader peaks and increased tailing. However, the AdvanceBio Peptide Plus, Polaris Amide C18, and Polaris C18-A all gave good peak shapes. The key difference with these materials is the presence of residual positive charge in the bonding chemistry, which helps prevent undesirable secondary interactions that result in increased tailing on other columns.

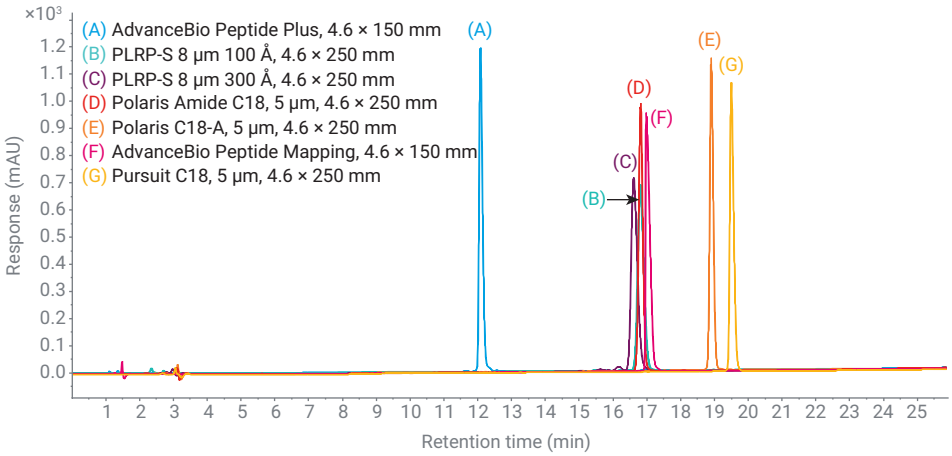

Figure 2. Comparison of analytical LC/UV chromatograms of semaglutide under 0.1% TFA conditions using gradient 5 (Table 2).

Return to Contents Section

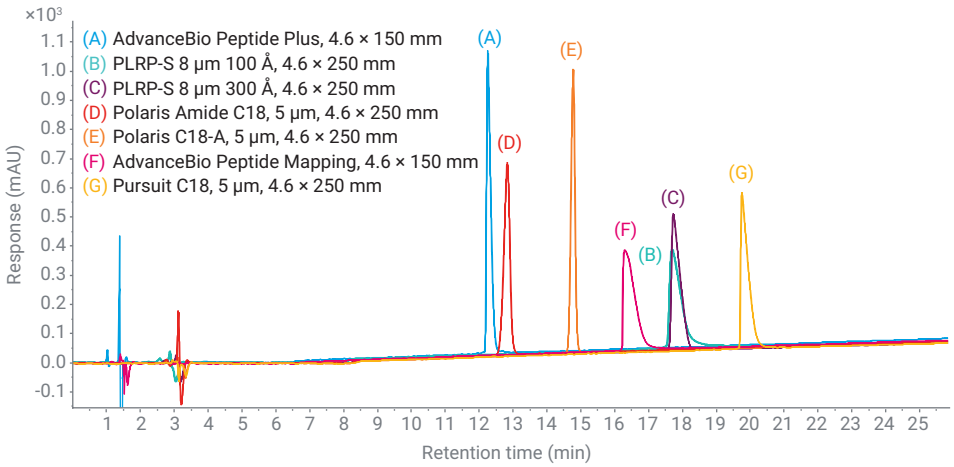

Figure 3. Comparison of analytical LC/UV chromatograms of semaglutide under 0.1% FA conditions using gradient 2 (Table 2).

Figure 4 compares the heat-treated, degraded, semaglutide sample run on the AdvanceBio Peptide Plus column with TFA and FA as ion-pair reagents. It is clear that the level of resolution remains excellent in FA. Figure 5 shows the same comparison using the Polaris Amide C18 column. Although the performance in FA is not compromised too much, there has been some loss in resolution compared to TFA.

For this reason, the AdvanceBio Peptide Plus column was chosen for LC/MS analysis using formic acid as the ion-pair reagent.

A further consideration when choosing an appropriate column for peptide analysis and purification is the pore size of the stationary phase. Although peptides are generally very small, with longer sequences or sequences that are modified, it may be beneficial to consider a wider pore size column. Larger pores do not restrict the mass transfer in and out of the pores as much as smaller pore sized columns, which can then lead to sharper peaks. This is best illustrated in Figure 6, which shows the same degraded sample analyzed on the PLRP-S 100 Å column versus PLRP-S 300 Å column. The resolution was clearly improved on the wider pore 300 Å column.

Return to Contents Section

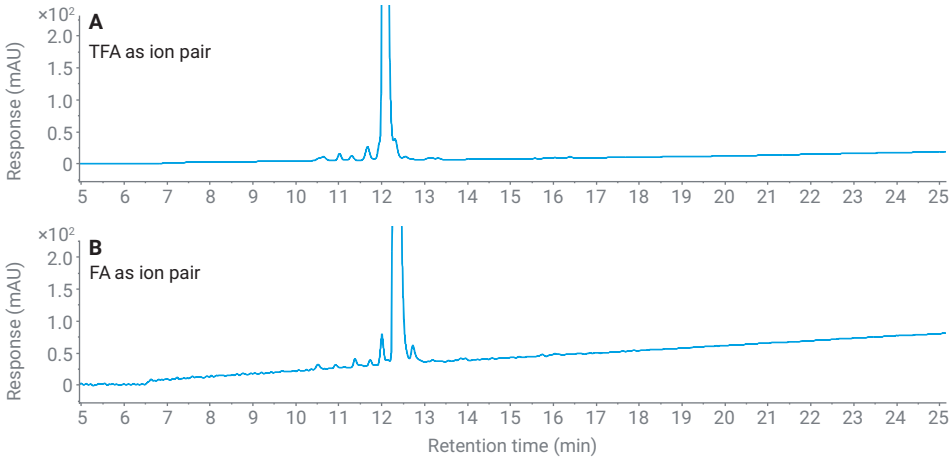

Figure 4. Comparison of heat-treated semaglutide LC/UV chromatograms showing the effect of ion-pair reagents TFA (A) using gradient 5 and FA (B) using gradient 2 with the Agilent AdvanceBio Peptide Plus column.

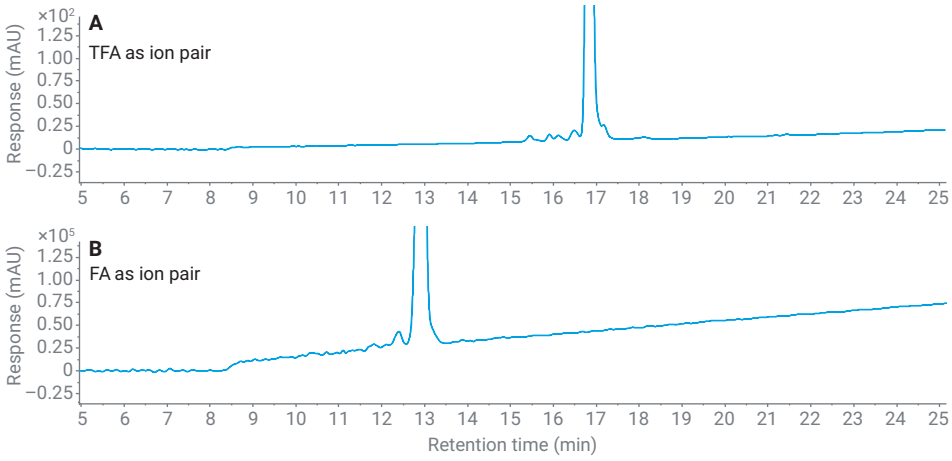

Figure 5. LC/UV chromatogram comparison of heat-treated semaglutide between TFA (A) using gradient 5 and FA (B) using gradient 2 with the Agilent Polaris Amide C18 column.

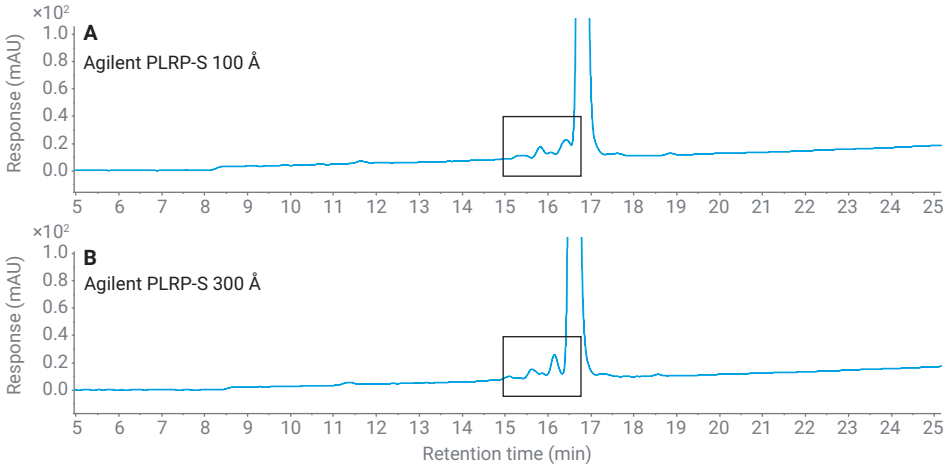

Figure 6. LC/UV chromatogram comparison of heat-treated semaglutide using Agilent PLRP-S columns, 100 Å (A) and 300 Å (B).

Return to Contents Section

Finally, Figure 7 shows the LC/MS data for the analysis of the semaglutide reference material using the AdvanceBio Peptide Plus column. The analysis is made more complicated by the presence of the fatty acid side chain modification, however, the data clearly confirms the identity of the molecule with the expected $[M + 3H]^{3+}$ at *m/z* 1,372.05, $[M + 4H]^{4+}$ at *m/z* 1,029.29, $[M + 5H]^{5+}$ at *m/z* 823.63, and $[M + 6H]^{6+}$ at *m/z* 686.53 (Figure 8) corresponding to the full-length amino acids of semaglutide of 4,113.58 Da.

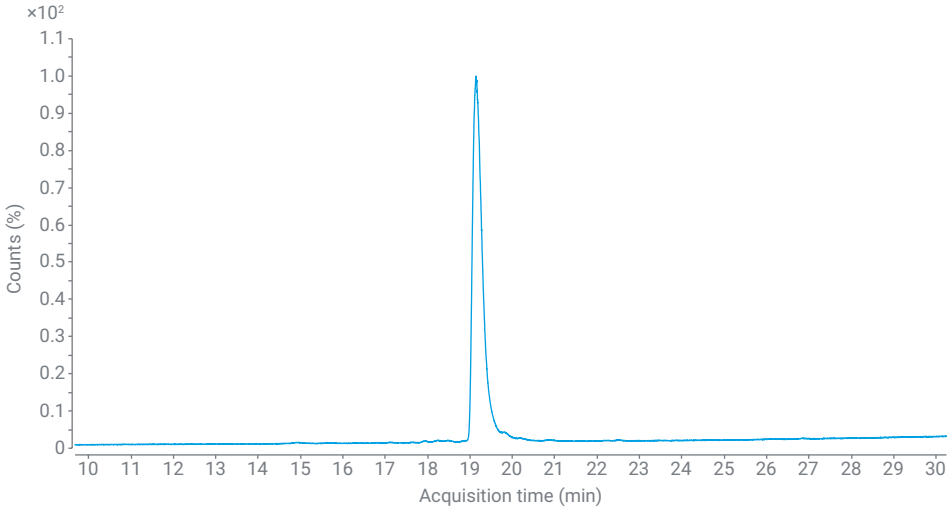

Figure 7. Total ion chromatogram (TIC) result of semaglutide acetate analyzed by LC/MS on an Agilent AdvanceBio Peptide Plus column (for method conditions, see Table 3).

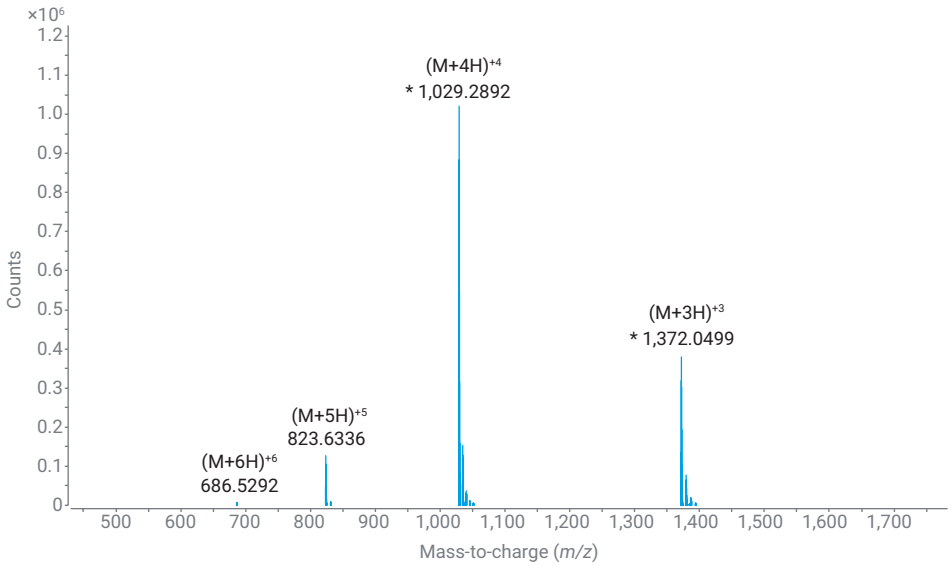

Figure 8. Mass spectrum result of semaglutide acetate analyzed by LC/MS on an Agilent AdvanceBio Peptide Plus column (for method conditions, see Table 3).

Return to Contents Section

Conclusion

This application note demonstrates that it is always critically important to select the right stationary phase chemistry. Moreover, it is crucial in designing an optimal gradient, keeping in mind the differences between trifluoroacetic acid and formic acid in order to achieve maximum resolution and identification. All Agilent columns used for this work have demonstrated that the analysis of semaglutide can be easily performed and impurities well separated when using TFA as ion-pair reagent. The Agilent AdvanceBio Peptide Plus column stands out by having greater selectivity in both TFA and (most importantly) under FA conditions when compared to the C18 and PLRP-S polymeric stationary phase columns. Finally, LC/MS analysis and sequence confirmation was successfully achieved under formic acid using the Agilent AdvanceBio Peptide Plus column on an Agilent 6545XT AdvanceBio LC/Q-TOF.

Application Note

Purity Analysis

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Quantification of Glucagon-Like Peptide-1 Agonist Tirzepatide Using an Agilent 6495D Triple Quadrupole LC/MS System

Suitable for Agilent
1290 Infinity III LC

Authors

Suresh Babu C.V.
Agilent Technologies, Inc.

Abstract

Synthetic peptide-related impurities generated during manufacturing and storage may affect the safety and efficacy of therapeutic peptides. Glucagon-like peptide-1 (GLP-1) receptor agonists represent the most promising class of synthetic peptide therapeutics. Tirzepatide, a GLP-1 agonist, regulates blood sugar levels and lowers body weight. This application note demonstrates the use of the Agilent 6495D triple quadrupole LC/MS system to measure unmodified and mono-oxidized tirzepatide. With the multiple reaction monitoring (MRM)-based method, the limit of quantitation (LOQ) for tirzepatide is 0.025 ng/mL.

Return to Contents Section

Introduction

Peptide-based therapeutics have gained great attention in the research and development of biomedicines. However, peptides can undergo various chemical modifications during formulation, manufacturing, and storage. These changes may impact the efficacy and safety of therapeutic peptides. Therefore, sensitive analytical methods must be used to characterize and quantify these peptide impurities during the biotherapeutic development life cycle. Liquid chromatography-mass spectrometry (LC/MS) is one of the major tools for the characterization and quantification of peptides and their post-translational modifications (PTMs).

Glucagon-like peptide-1 (GLP-1) plays an important role in metabolic regulation and helps in both insulin secretion and the promotion of weight loss.[1] GLP-1 analogs such as tirzepatide are synthetic versions of GLP-1. Tirzepatide binds to both the glucose-dependent insulinotropic peptide (GIP) and the GLP-1 receptors, acting as a dual agonist. Tirzepatide is an acylated peptide composed of 39 amino acids with an incorporated C_20 fatty-diacid moiety. Furthermore, this analog achieves a longer half-life compared to native GLP-1. Tirzepatide is approved by the U.S. Food and Drug Administration (FDA) for the treatment of type 2 diabetes mellitus (T2DM).[2]

During the drug development process, monitoring drug stability is crucial to ensure the right dosage for achieving optimal therapeutic levels. Due to greater accuracy and precision, a wide dynamic range, and short method development time, LC/MS has become a popular method to quantitate biomolecules.

This application note demonstrates the quantitative analysis of unmodified/native and oxidized tirzepatide using an Agilent 1290 Infinity II bio LC system and a 6495D triple quadrupole LC/MS system. Calibration curves were generated to determine the LOQ, and the results showed excellent sensitivity for both peptide forms.

Table 1. Liquid chromatography parameters.

Parameter	Value
Instrument	Agilent 1290 Infinity II bio LC system
Column	Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, 2.7 µm, 120 Å
Sample Thermostat	10 °C
Mobile Phase A	0.1% DFA
Mobile Phase B	0.1% DFA in ACN
Gradient	Time (min) %A %B 0.00 70 30 1.00 70 30 4.00 30 70 5.00 10 90 7.50 5 95 8.00 70 30
Stop Time	8 min
Column Temperature	40 °C
Flow Rate	0.4 mL/min

Experimental

Reagents and chemicals

Tirzepatide was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Difluoroacetic acid (DFA), dimethyl sulfoxide (DMSO), and 30% (v:v) hydrogen peroxide (H_2O_2) were procured from Sigma-Aldrich (St. Louis, MO, USA). LC/MS-grade acetonitrile (ACN) and methanol were obtained from Fisher Scientific (Waltham, MA, USA). Ultrapure water was collected from an in-house Millipore Sigma Milli-Q system (Billerica, MA, USA).

Instumentation

Agilent 1290 Infinity II bio LC system including:

- Agilent 1290 Infinity II bio high-speed pump (G7132A)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6495D triple quadrupole LC/MS system (G6495D)

Software and data processing

- Agilent MassHunter Workstation acquisition LC/TQ software (12.1)
- Agilent MassHunter Workstation quantitative analysis software (12.1)

Sample preparation

Tirzepatide peptide was dissolved in methanol to a concentration of 1.0 mg/mL. Calibration curve samples were prepared by serial dilution using 30% ACN and 2% DFA. Concentrations of the prepared calibration curve samples ranged from 0.025 to 250 ng/mL. Quality control (QC) samples were prepared at 0.75, 7.5, and 75 ng/mL. For oxidative stress, stock solutions were diluted to 0.5 mg/mL in 30% ACN and incubated with the oxidizing agent H_2O_2 (2% v:v) overnight at room temperature.

LC/MS analysis

Data acquisition was performed using a 1290 Infinity II bio LC system connected to a 6495D triple quadrupole LC/MS system with an Agilent Jet Stream source. The LC separation was performed on an Agilent AdvanceBio Peptide Mapping column (part number 653750-902) using an 8-minute gradient. Tables 1 and 2 list the LC and MS parameters.

Return to Contents Section

Results and discussion

Optimizing the sample preparation, LC, and MS conditions is crucial for enhancing the sensitivity and reproducibility of tirzepatide quantitation. Different solvents, including methanol, ACN, and DMSO, were evaluated for their ability to dissolve the sample and maintain the stability of the MS signal. Of these solvents, methanol was found to be the most effective diluent. Unmodified and oxidized tirzepatide forms were well separated with LC using the Agilent AdvanceBio Peptide Mapping column. The MS conditions were optimized using the Method Optimizer feature in the MassHunter Workstation acquisition software.

In this study, tirzepatide was treated with 2% H_2O_2 to generate the oxidized peptide product.[3] The oxidized peptide represents the potential degradation product that could form in conditions present during storage. Table 3 shows the details for unmodified/native and mono-oxidized tirzepatide.

Positive electrospray ionization of both unmodified/native and mono-oxidized tirzepatide produces molecular ion peaks with multiple charge states (3+, 4+, and 5+). The most intense precursor ion signals for unmodified/native and mono-oxidized tirzepatide are observed at *m/z* 1,204.4 and 1,208.1, respectively, from the $[M + 5H]^{5+}$ ions. Several MRM transitions were observed, and the *m/z* 396.3 y4 fragment from the 5+ precursor ions was selected as the quantifier (Table 4).

Table 2. MS data acquisition parameters.

Parameter	Value
Instrument	Agilent 6495D LC/TQ system
Ion mode	Positive, Agilent Jet Stream
Gas Temperature	290 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	360 °C
Sheath Gas Flow	12 L/min
Nebulizer Gas	35 psi
Capillary Voltage	4,400 V
Nozzle Voltage	0 V
High Pressure Radio Frequency	150 V
Low Pressure Radio Frequency	80 V

Table 3. Unmodified/native tirzepatide and mono-oxidized tirzepatide.

GLP-1 Peptide	Formula	Molecular Weight (Da)	Sequence
Tirzepatide	$C_{225}H_{348}N_{48}O_{68}$	4,813.55	Y-{Aib}-EGTFTSDYSIXLDKIAQ-{C20 diacid-gamma-Glu-(AEEA)2-Lys}-AFVQ**W**LIAGGPSSGAPPPS
Mono-Oxidized tirzepatide	$C_{225}H_{348}N_{48}O_{69}$	4,829.55	

Tryptophan (W) oxidation is highlighted in bold

Table 4. Observed MRM transitions for unmodified/native tirzepatide and mono-oxidized tirzepatide.

Compound Name	Charge State	Precursor Ion (*m/z*)	MS1 Res	Frag (V)	Product Ion (*m/z*)	MS2 Res	CE (V)
Tirzepatide	5+	963.7	Wide	166	396.3	Unit	22
	4+	1,204.4	Wide	166	396.3	Unit	23
	4+	1,204.4	Wide	166	795.2	Unit	35
	4+	1,204.4	Wide	166	909.5	Unit	35
	3+	1,605.5	Wide	166	396.2	Unit	28
Mono-Oxidized tirzepatide	5+	966.5	Wide	166	396.2	Unit	23
	4+	1,208.1	Wide	166	396.3	Unit	23
	4+	1,208.1	Wide	166	795.8	Unit	23
	4+	1,208.1	Wide	166	910.1	Unit	23
	3+	1,610.5	Wide	166	369.3	Unit	23

Res = resolution, Frag = fragmentation voltage, CE = collision energy

Return to Contents Section

Figure 1 shows the MRM chromatograms for unmodified/native tirzepatide and mono-oxidized tirzepatide. These MRMs verify the peptide identity and demonstrate the use of MRM to monitor PTMs such as oxidation. As expected, the oxidized form elutes early (at 3.9 minutes) due to reduced hydrophobicity compared to the native peptide, which elutes later (at 4.0 minutes). The m/z 1,208.1 & 396.3 MRM transition shows another peak at 4.3 minutes, corresponding to an unknown signal that was also present in the unmodified/native tirzepatide sample.

The calibration curve and quality control samples were subjected to quantitative analysis using MassHunter Workstation quantitative analysis software. Figure 2 shows the quantitative performance of the unmodified/native tirzepatide and the mono-oxidized tirzepatide. Excellent standard curve fitting is observed for a dynamic range spanning four orders of magnitude (0.025 to 250 ng/mL) for native tirzepatide and three orders of magnitude (0.25 to 250 ng/mL) for the mono-oxidized tirzepatide.

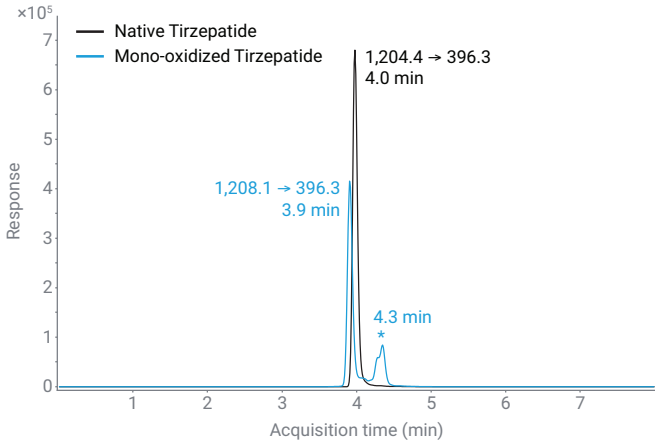

Figure 1. MRM chromatograms of native tirzepatide (standard, black) and mono-oxidized tirzepatide (2% H_2O_2-treated, blue). The peak for the native/unmodified peptide transition (*m/z* 1,204.4 & 396.3) occurs at 4.0 minutes, and the peak for the mono-oxidized peptide transition (*m/z* 1,208.1 & 396.3) occurs at 3.9 minutes. An asterisk (*) marks the unknown peak.

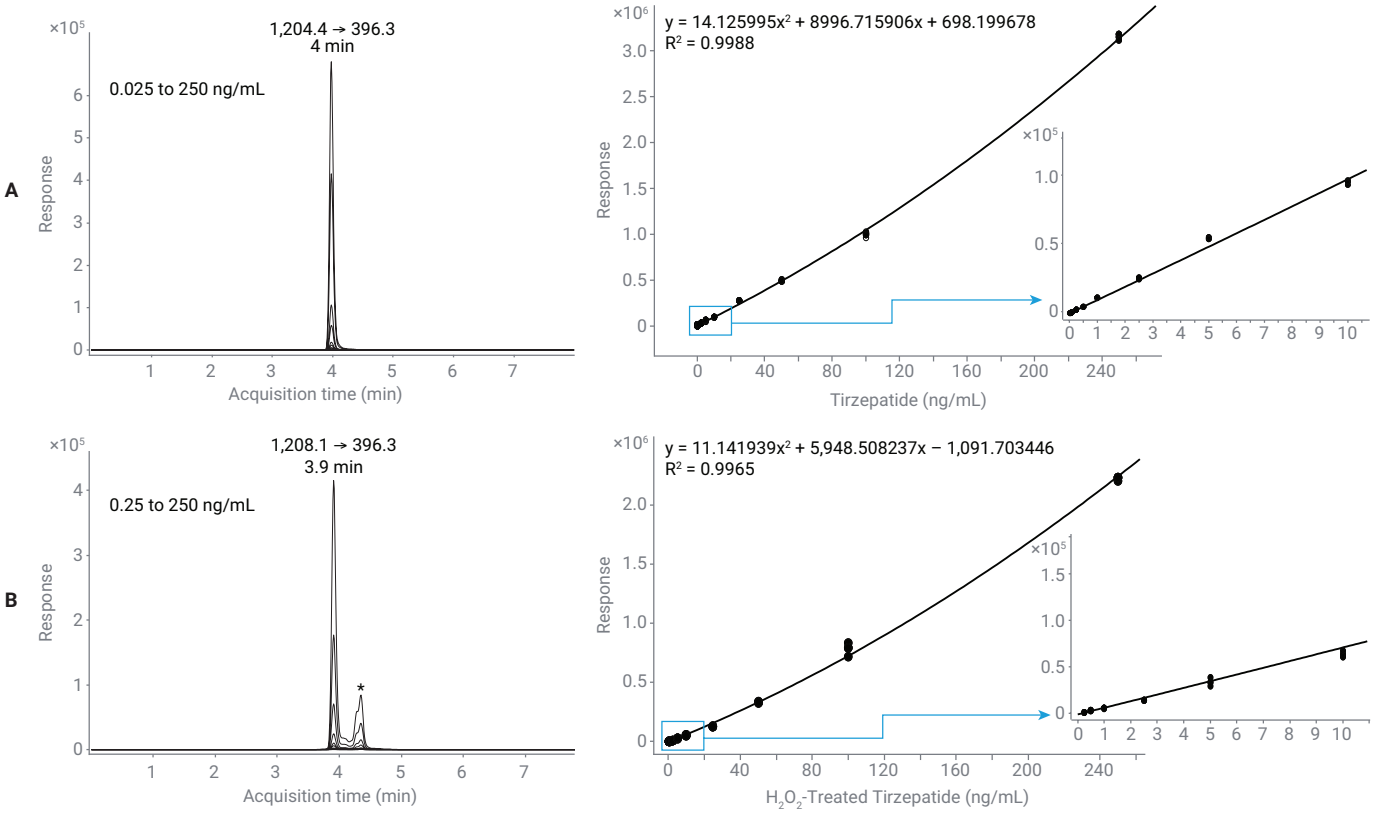

Figure 2. Quantitative performance of (A) unmodified/native and (B) tryptophan mono-oxidized tirzepatide (n = 4). On the left, overlays of MRM chromatograms for a range of concentrations are shown. An asterisk (*) marks the unknown peak. On the right, the standard curves for each form of tirzepatide are shown, including insets showing the details of the curve at lower concentration levels.

Return to Contents Section

The correlation coefficients (R2) were 0.997 and 0.996 for the native and mono-oxidized forms, respectively. Precision and accuracy were excellent at all levels, with percent relative standard deviation (%RSD) < 6% and accuracy ranging from 81 to 115% (Table 5).

Figure 3 shows MRM chromatograms for blank samples and the lowest concentration levels. This method achieves an LOQ of 0.025 ng/mL for unmodified/native tirzepatide and 0.25 ng/mL for mono-oxidized tirzepatide (Figure 3). The method performance was tested with three QC samples at low, middle, and high concentration levels (Table 6). The QC sample precision (percent coefficient of variation, %CV, n = 4) for both native and mono-oxidized tirzepatide rangeed from 1.01 to 9.55%. At LOQ levels, the %CV for native tirzepatide (9.55%) and mono-oxidized tirzepatide (2.43%) were within the acceptance criteria recommended by the U.S. FDA.[4]

Table 5. Precision (area %RSD) and accuracy for the standard curve analysis of unmodified/native (standard) tirzepatide and tryptophan mono-oxidized (2% H_2O_2-treated) tirzepatide (n = 4).

Samples	Tirzepatide Standard		2% H_2O_2-Treated Tirzepatide	
Concentration (ng/mL)	Area %RSD	Accuracy (%)	Area %RSD	Accuracy (%)
0.025	2.52	81.37	–	–
0.05	4.28	82.1	–	–
0.1	5.20	81.8	–	–
0.25	4.98	113.7	5.72	117.92
0.5	4.01	101.1	5.97	108.07
1.0	2.06	118.2	5.18	103.05
2.5	3.00	106.3	2.54	90.67
5	1.15	112.7	11.41	97.4
10	1.41	97.6	4.52	89.8
25	0.51	112.9	2.23	82.32
50	1.29	100.2	1.73	99.6
100	1.11	95.6	2.29	107.15
250	0.94	100.4	0.52	99.27

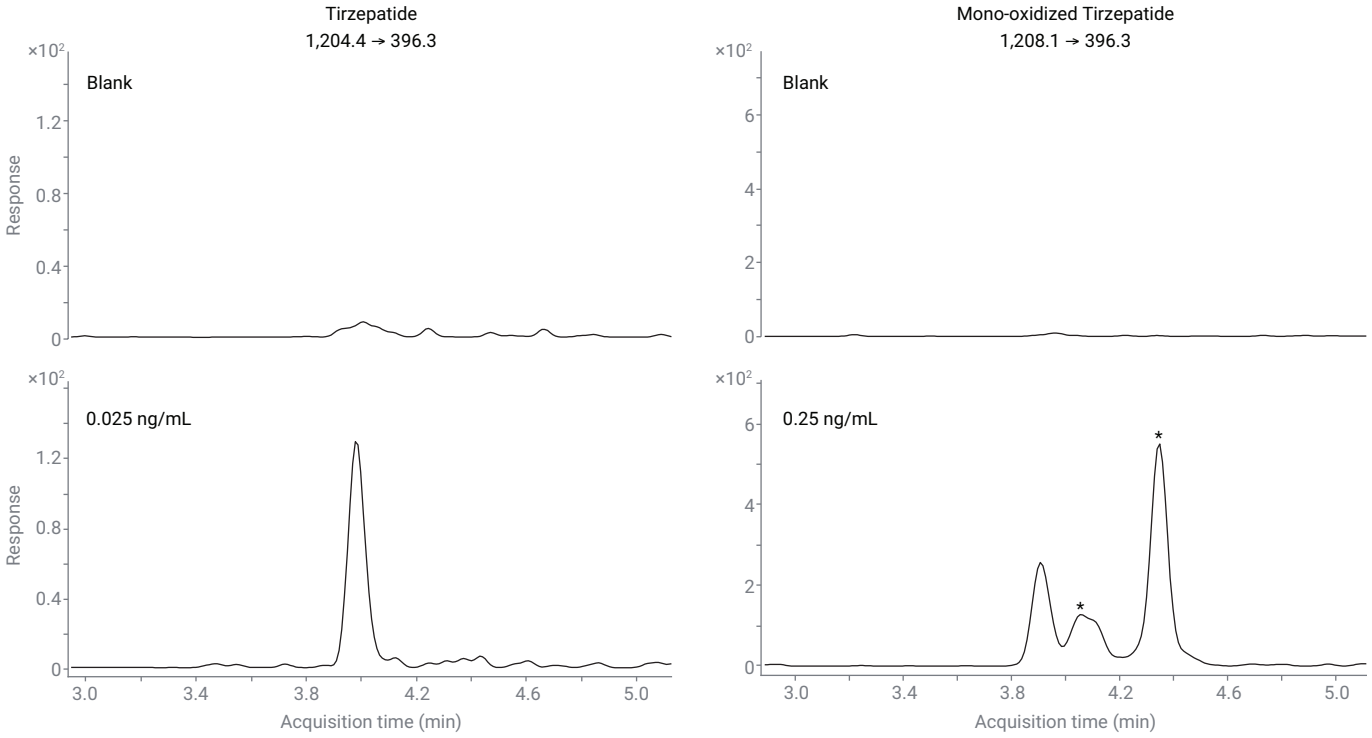

Figure 3. Extracted ion chromatograms of blank samples and samples at the lowest concentration levels. Each unknown peak is marked by an asterisk (*).

Return to Contents Section

Table 6. Precision (%CV) and accuracy results for QC samples (n = 4).

Tirzepatide Standard (ng/mL)	0.025 (LOQ)	0.75 (Low)	7.5 (Mid)	75 (High)
Mean (ng/mL)	0.022	0.82	8.80	82.55
CV (%)	9.55	2.48	1.21	1.01
Accuracy (%)	81.37	100.07	117.3	110.1
2% H_2O_2-Treated Tirzepatide (ng/mL)	**0.25 (LOQ)**	**0.75 (Low)**	**7.5 (Mid)**	**75 (High)**
Mean (ng/mL)	0.29	0.77	8.27	85
CV (%)	2.43	1.82	8.97	3.34
Accuracy (%)	117.92	114.67	109.5	103.05

Conclusion

In this study, a rapid multiple reaction monitoring (MRM)-based method for the highly sensitive quantitative analysis of both native and oxidized tirzepatide was developed using an Agilent 6495D triple quadrupole LC/MS system coupled with an Agilent 1290 Infinity II bio LC system. The calibration curve demonstrated a wide dynamic range, spanning four orders of magnitude for native tirzepatide and three orders of magnitude for mono-oxidized tirzepatide. These results show that this method provides reliable quantitation accuracy and precision.

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Application Note

Impurity Analysis

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Confirmation of Peptide-Related Impurity Intact Mass Using Agilent 1290 Infinity II Bio 2D-LC and InfinityLab LC/MSD XT

Author

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Agilent Technologies, Inc.

Abstract

This application note presents the analysis of peptide-related impurities for semaglutide using an Agilent 1290 Infinity II bio 2D-LC system and an Agilent InfinityLab LC/MSD XT system. Peptide-related impurities are challenging to separate from peptide-active pharmaceutical ingredients due to their structural diversity and relatively large molecular weights. To address this challenge, an Agilent AdvanceBio Peptide Plus column, which is stable under high-concentration acidic conditions, was used to achieve good separation of semaglutide impurities. A solvent system compatible with mass spectrometry (MS) was used with the 1290 Infinity II bio 2D-LC and InfinityLab LC/MSD XT, enabling the acquisition of MS spectra. The deconvolution feature in Agilent OpenLab CDS software, version 2.8, facilitated the easy determination of molecular weights for impurity peaks analyzed by two-dimensional liquid chromatography (2D-LC).

Return to Contents Section

Introduction

The Federal Drug Administration (FDA) and European Medicines Agency (EMA) have published guidelines for the Abbreviated New Drug Application (ANDA) of highly purified synthetic peptides.[1,2] With the expiration of patents for several GLP-1 receptor agonists, numerous pharmaceutical companies are developing generic drug products. As emphasized in the FDA guidelines, peptide-related impurities must be strictly controlled, and any new peptide-related impurity identified at levels higher than 0.5% must be evaluated for immunogenicity. This is critical, as demonstrated by the clinical trial discontinuation of taspoglutide due to reported anaphylaxis[3], and similar reactions observed with exenatide administration.[4]

Taspoglutide, despite having an amino acid sequence nearly identical to semaglutide and native GLP-1, exhibited significantly different side effects regarding immune response.[5] Impurities generated during the synthetic process, such as diastereoisomers, protected sequences, amino acid insertions or deletions, side chain reactions, oxidation, and reduction, can present substantial challenges.[6] Minor sequence differences make separation through reversed-phase HPLC conditions difficult.[7] Thus, HPLC method optimization, tailored to each peptide drug product's impurity profile, is necessary to enhance specificity. Acidic modifiers with ion-pairing properties can improve separation, with high concentrations of trifluoroacetic acid (TFA) aiding in the resolution of coeluting impurities from the main peak.[8] However, TFA adversely affects peptide-related impurity detection by MS due to ion suppression.[9]

2D-LC proves to be a valuable tool to decrease TFA effects while increasing resolution. The 1290 Infinity II bio 2D-LC system, developed with biocompatible materials, is highly suitable for biomolecule analysis. Using 2D-LC, conventional HPLC methods can be converted for MS-friendly conditions, and resolution can further be increased by applying different column chemistries in first (1D) and second (2D) dimensions.

This application note describes the analysis of semaglutide impurities arising from a forced degradation assay using the AdvanceBio Peptide Plus column under high-TFA conditions with the 1290 Infinity II bio 2D-LC. Formic acid (FA) was used in 2D before MS detection with the LC/MSD XT. Also, peaks coeluting in 1D were successfully separated in 2D. The mass spectra acquired from the LC/MSD XT were used to determine molecular weights using the deconvolution feature in OpenLab CDS 2.8.

Experimental

Instumentation

The following instrumentation was used in this application note:

- Agilent 1290 Infinity II bio flexible pump (part number G7131A)
- Agilent 1290 Infinity II bio high-speed pump (part number G7132A)
- Agilent 1290 Infinity II bio multisampler (part number G7137A) with sample thermostat
- Agilent 1290 Infinity II multicolumn thermostat (part number G7116B) with InfinityLab Quick-Connect heat exchanger 1290 bio standard flow (part number G7116-60071)
- Two Agilent 1290 Infinity II diode array detectors (DAD HS) (part number G7117B) with InfinityLab Max-Light cartridge cell LSS, 10 mm (part number G7117-60020) and bio-inert Max-Light cartridge cell, 60 mm (part number G5615-60017)
- Agilent 1290 Infinity valve drive (part number G1170A) with InfinityLab bio 2D-LC ASM valve (part number G5643B)
- Two Agilent 1290 Infinity valve drives (part number G1170A) with InfinityLab multiple heart-cutting (MHC) valve and biocompatible 40 µL loops
- Agilent InfinityLab LC/MSD XT (part number G6135C)

Reagents

TFA, FA, and hydrochloric acid (HCl) were purchased from Sigma-Aldrich; sodium hydroxide (NaOH) was purchased from Merck, and acetonitrile (ACN) was purchased from B&J.

Samples

Semaglutide (molecular weight: 4,113.58 g/mol) was donated by a local customer.

Semaglutide was dissolved in 30% ACN to prepare a concentration of 1 mg/mL, and was heated at 60 °C for two days. Separately, 100 µL of 1 M HCl was added to 1 mL of the semaglutide solution, heated at 60 °C for two days, then neutralized with 100 µL of 1 M NaOH.

Columns

The following columns were used in this application note:

- First dimension (1D): Agilent AdvanceBio Peptide Plus, 2.1 × 250 mm, 2.7 µm (part number 693775-949)
- Second dimension (2D): Agilent InfinityLab Poroshell 120 CS-C18, 2.1 × 100 mm, 2.7 µm (part number 695775-942)

Return to Contents Section

Methods

The ^{1}D and ^{2}D parameters are displayed in Tables 1 and 2, respectively; LC/MSD XT data acquisition parameters are displayed in Table 3; and MS Spectral Deconvolution settings are displayed in Table 4.

Table 1. ^{1}D parameters.

Parameters	Value
Column	Agilent InfinityLab Poroshell 120 CS-C18, 100 Å, 2.1 × 100 mm, 2.7 µm
Flow	0.6 mL/min (Idle flow: 0.05 mL/min)
Column Temperature	60 °C
Mobile Phase	A) 0.1% FA in water B) 0.1% FA in ACN
2D-LC Operation Mode	Time-based heart-cut MHC
Gradient	Time (min) %A %B 0 75 25 10 60 40 ^{2}D run time: 10 min ^{2}D equilibration: 3 min Cycle time: 13 min
ASM Setting	Factor: 3 Flush factor: 2.5
Sample Loop	40 µL
Detector	UV 280 nm (DAD HS with bio-inert Max-Light cartridge cell, 60 mm)

Table 2. ^{2}D parameters.

Parameters	Value
Ion Source	ESI (+)
Source Parameters	Gas temperature: 325 °C Gas flow: 11 L/min Nebulizer: 45 psi Capillary voltage: 4,500 V
Acquisition	Scan range: *m/z* 500 to 2,500 Fragmentor: 150 V Scan time: 1,950 ms (0.5 Hz) Gain: 1 Storage: Profile

Software

Agilent OpenLab CDS software, version 2.8, was used for spectral deconvolution.

Table 3. Agilent InfinityLab LC/MSD XT data acquisition parameters.

Parameters	Value
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 250 mm, 2.7 µm
Flow	0.4 mL/min
Column Temperature	60 °C
Injection Volume	20 µL
Mobile Phase	A) 0.4% TFA in water B) 0.4% TFA in ACN
Gradient	Time (min) %A %B 0 70 30 0.5 70 30 65 45 55 70 10 90 75 10 90 75.1 70 30 80 70 30
Detector	UV 280 nm (DAD HS with Max-Light cartridge cell LSS 10 mm + aperture)

Table 4. MS spectral deconvolution settings.

Parameter	Value
Basic Settings	
Use *m/z* Range	Unselected
Low/High Molecular Weight	2,500 to 8,000
Maximum Charge	10
Minimum Peaks in Set	3
Advanced Settings	
MW Agreement (0.01%)	10
Relative Abundance Threshold	50%
MW Algorithm	Curve Fit
MW Algorithm Threshold	40%
Envelope Threshold	50%

Return to Contents Section

Results and discussion

Developing the first-dimension LC conditions for forced degradation analysis of impurities from semaglutide

To achieve high-resolution separation of Semaglutide-derived impurities, an HPLC method using the AdvanceBio Peptide Plus column with 0.4% TFA as an acidic modifier was used. A 1 mg/mL semaglutide solution was subjected to heat and acidic conditions then analyzed using LC/UV conditions, as outlined in Table 1. As shown in Figure 1, various impurity peaks were identified in samples heated at 60 °C for two days and treated with 0.1 M HCl at 60 °C for two days. A shallow gradient and long analysis time are required to separate various impurity peaks derived from the peptide. From the results of Figure 1, it was confirmed that the retention time of semaglutide remained consistent even though the mobile phase ratio was changing at a low rate of 0.39% per minute.

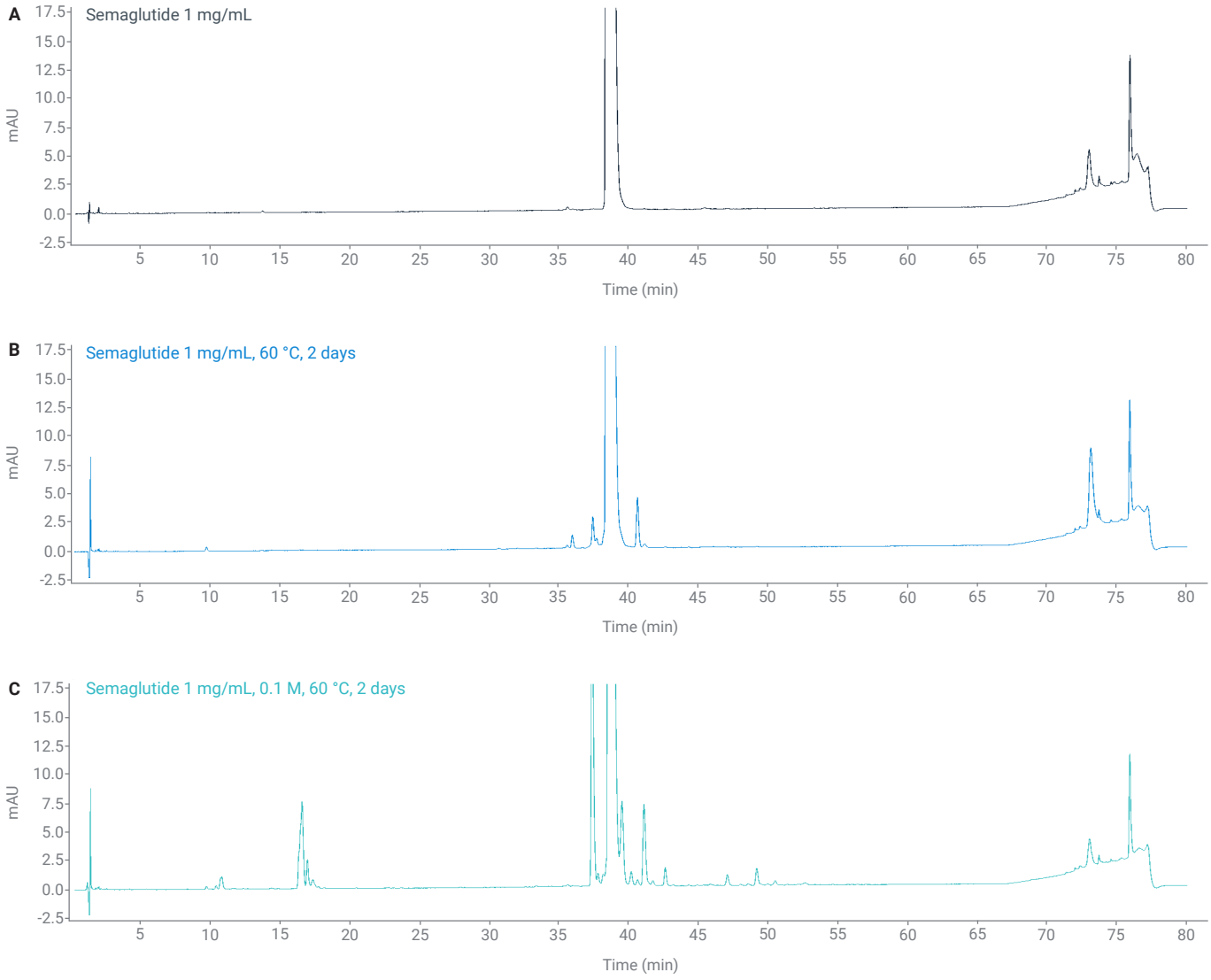

Figure 1. UV chromatogram of a 1 mg/mL semaglutide solution (A), heated at 60 °C for two days (B), and a semaglutide solution heated in 0.1 M HCl conditions for two days (C).

Return to Contents Section

Analysis of impurities from thermal degradation by 2D-LC/MS

Six characteristic peaks were identified that formed during two days of heating at 60 °C, as shown in Figure 1, and were selected for further analysis (Figure 2) using the 2D-LC conditions described in Table 2. The 2D conditions used FA as the acidic modifier for MS compatibility, and the InfinityLab Poroshell 120 CS-C18 column to reduce cycle time for analyzing each heart-cut. The 2D total ion chromatogram (TIC) for each cut was obtained under the LC/MSD XT conditions listed in Table 3, and the TICs are shown in Figure 3. Cut number 3 was separated into two peaks at 7.3 and 8.5 minutes under 2D conditions, while cut numbers 5 and 6 displayed reversed retention order (Figure 3).

The MS raw spectra for each peak are presented in Figure 4, demonstrating that even for cuts with low UV chromatogram heights in ^{1}D (cut numbers 1 and 6), interpretable MS spectra were obtained. The raw spectra were processed using the deconvolution feature of OpenLab CDS 2.8 with the parameters listed in Table 4, resulting in the deconvoluted mass spectra shown in Figure 4.

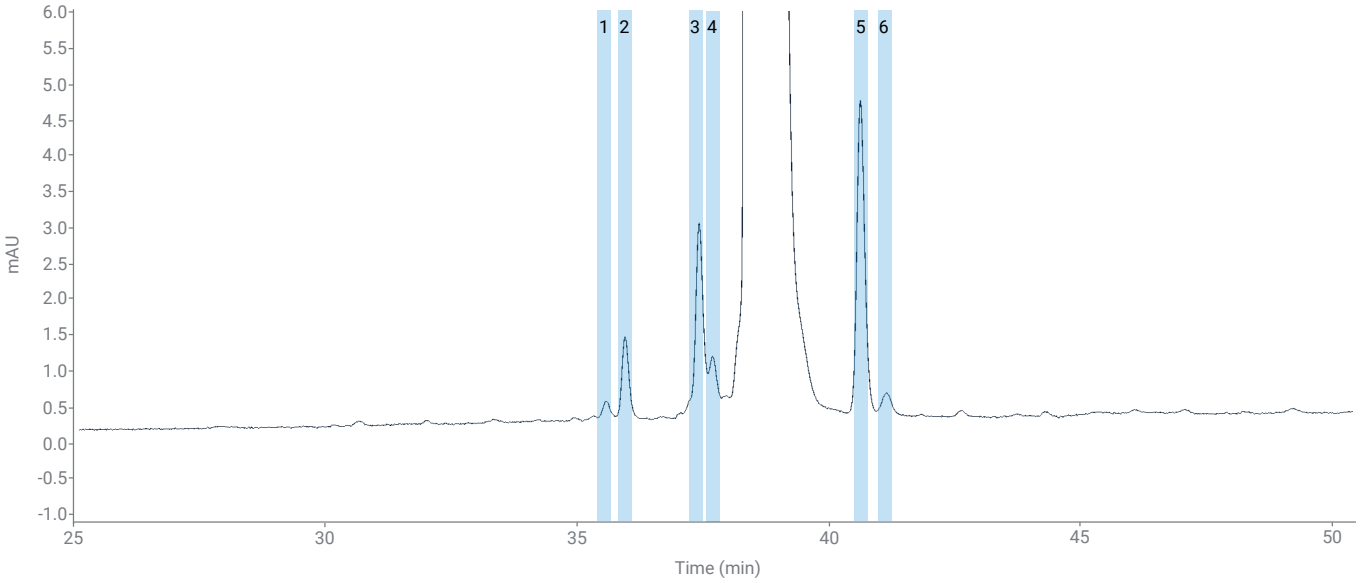

Figure 2. UV chromatogram of a 1 mg/mL semaglutide solution heated at 60 °C for two days, with indication of the position of the ^{2}D cut.

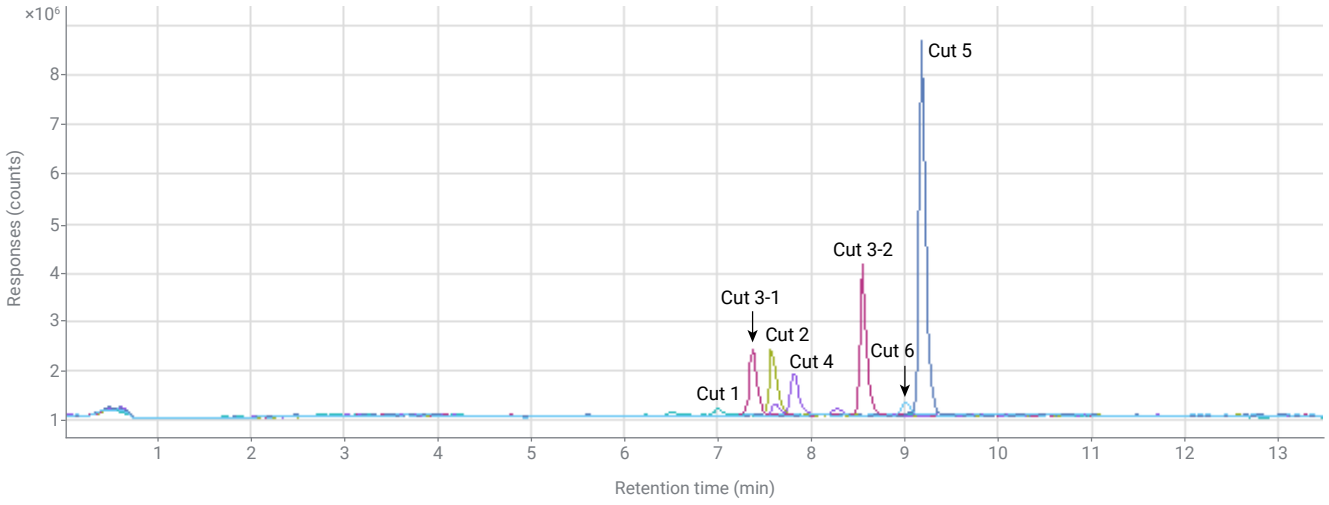

Figure 3. ^{2}D TIC illustrating the analysis of impurities caused by thermal degradation using an Agilent InfinityLab Poroshell CS-C18 column with 0.1% FA as the mobile phase additive.

Return to Contents Section

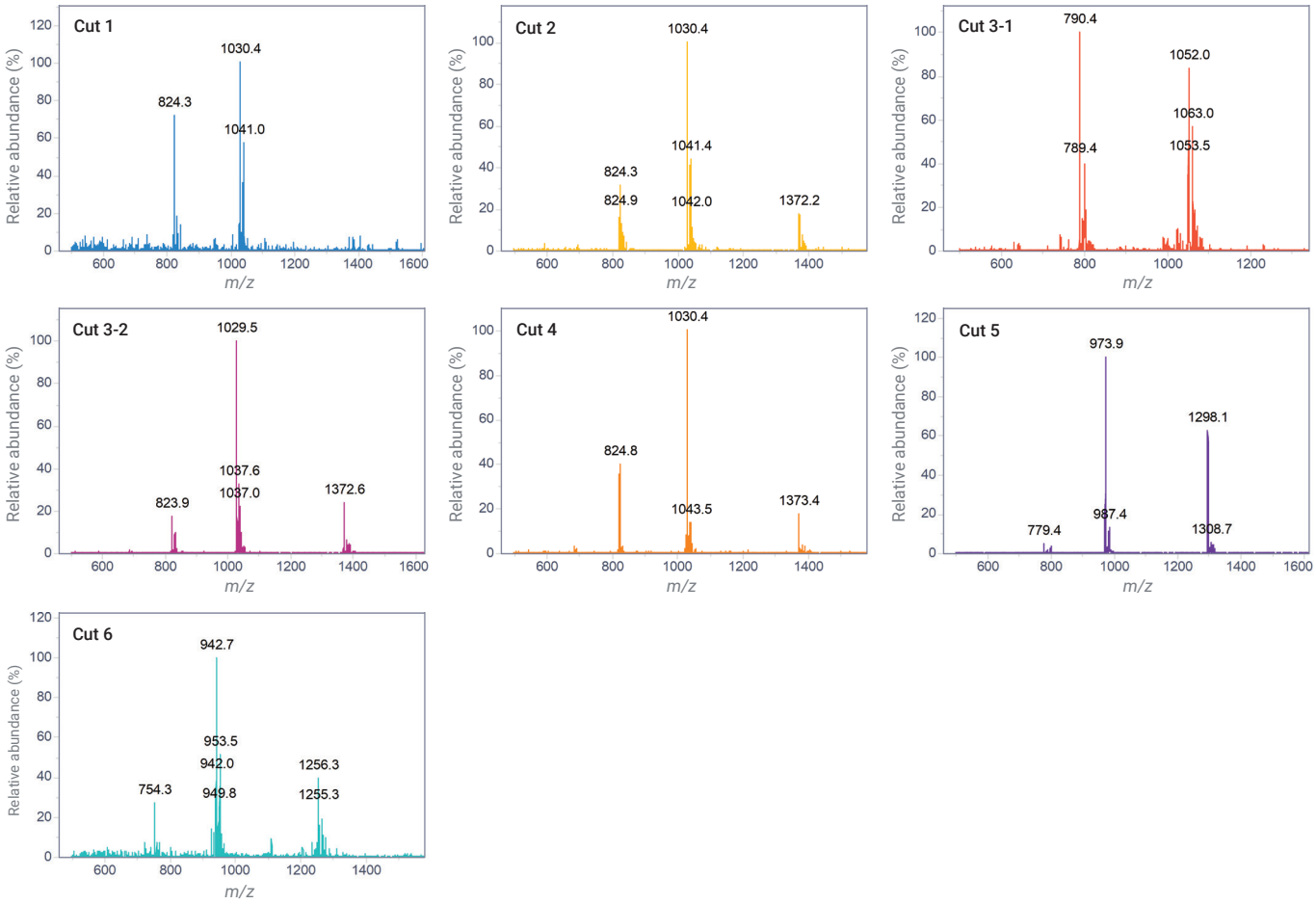

Figure 4. MS spectra of thermal degradation impurities of Semaglutide.

Return to Contents Section

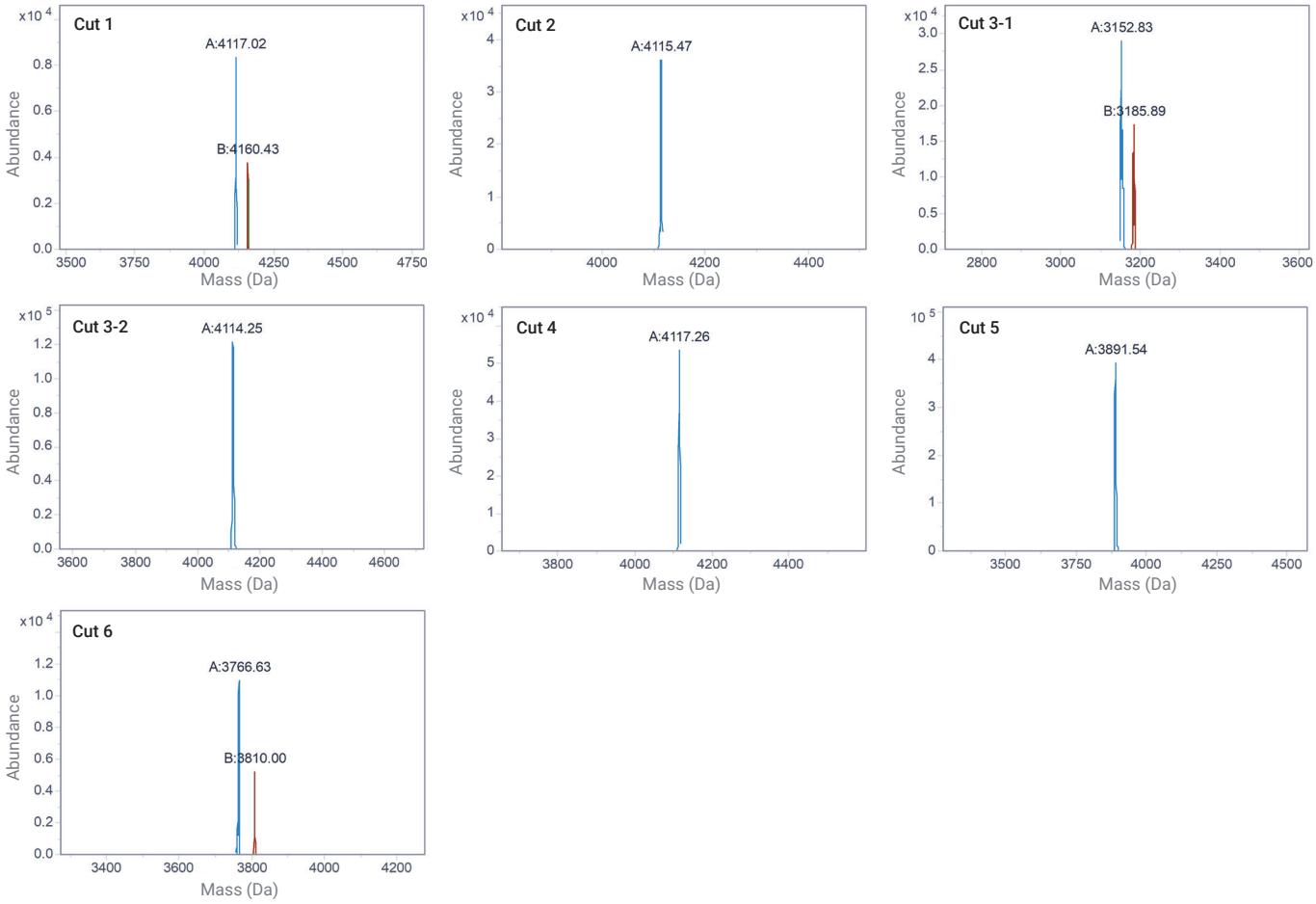

Figure 5. Deconvoluted mass spectra of thermal degradation impurities of Semaglutide.

Return to Contents Section

Analysis of impurities from acidic degradation by 2D-LC/MS

The degradation impurities peaks of semaglutide generated under 0.1 M HCl conditions, as identified in Figure 1, were subjected to heart-cutting and analyzed using the same ^{2}D conditions as in Figure 6. Cut number 3 from Figure 2 and cut number 5 from Figure 6 exhibited the same retention time in the ^{1}D chromatogram. Corresponding deconvoluted mass spectra for cut numbers 3-1 and 5 were found to be 3,152.83 and 3,153.60 Da, respectively, showing similar results (Figure 9).

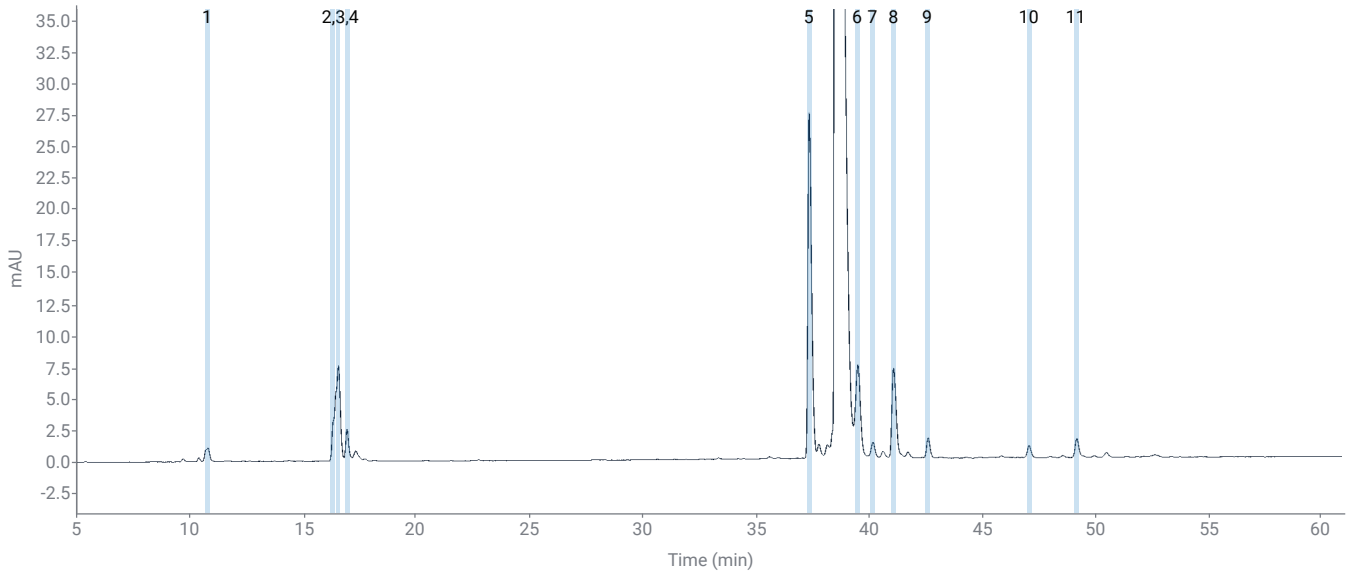

Figure 6. UV chromatogram of a 1 mg/mL semaglutide solution heated at 60 °C for two days under 0.1 M HCl conditions, along with the location of the ^{2}D cut.

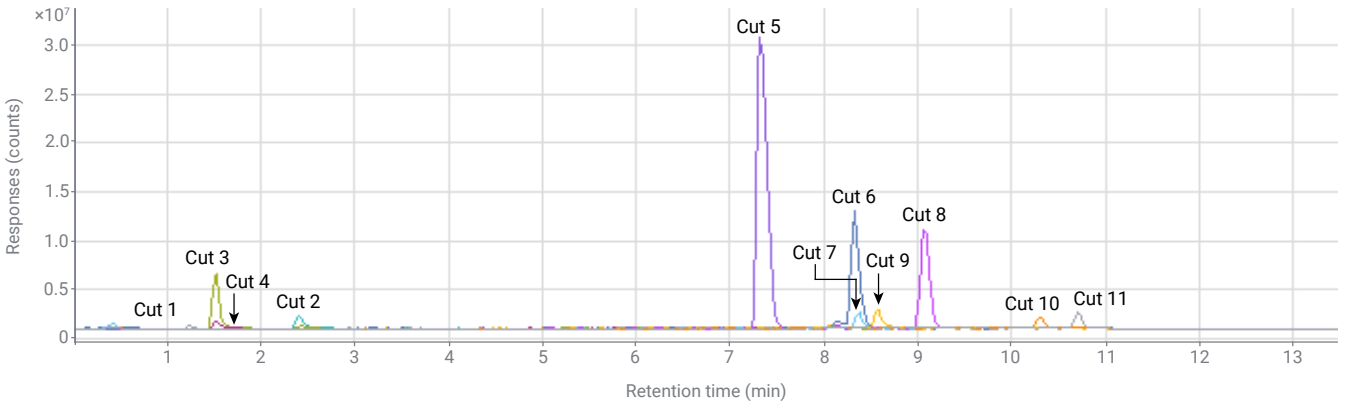

Figure 7. ^{2}D TIC illustrating the analysis result of acid degradation impurities using an Agilent InfinityLab Poroshell 120 CS-C18 column with 0.1% FA as the mobile phase.

Return to Contents Section

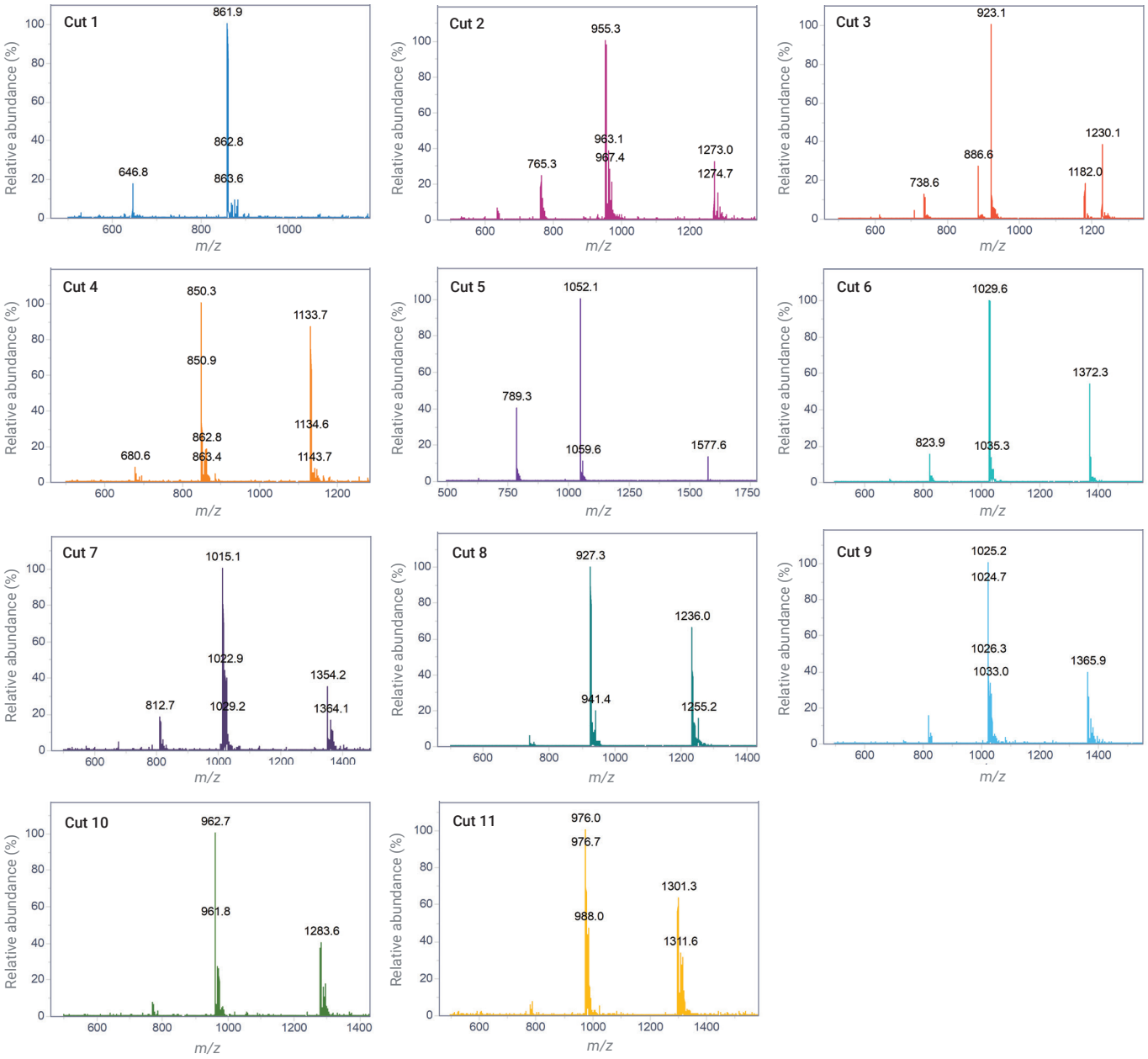

Figure 8. MS spectra of acid degradation impurities of Semaglutide.

Return to Contents Section

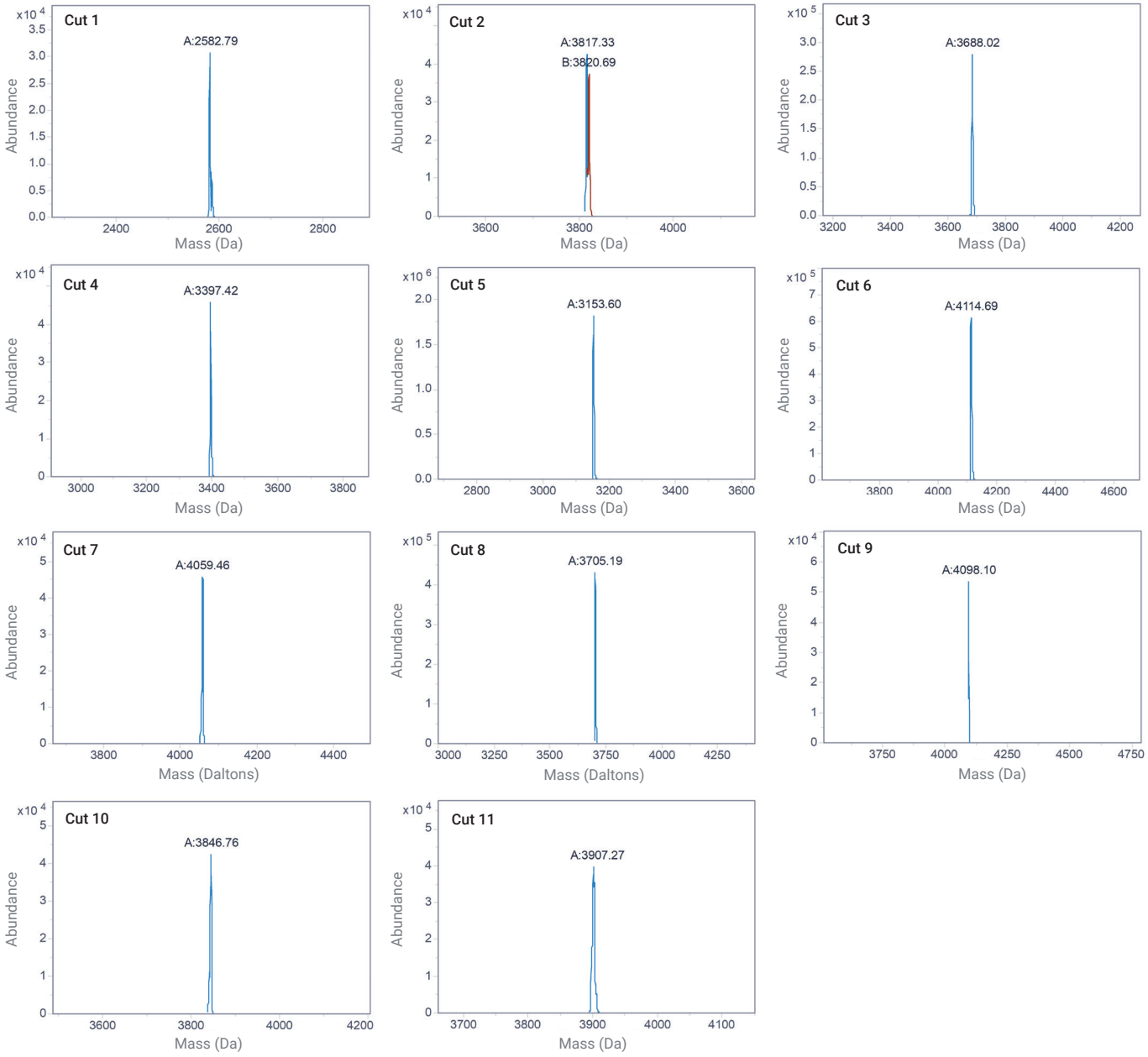

Figure 9. Deconvoluted mass spectra of acid degradation impurities of Semaglutide.

Return to Contents Section

The degradation impurities peaks of semaglutide generated under 0.1 M HCl conditions, as identified in Figure 1, were subjected to heart-cutting and analyzed using the same ^{2}D conditions as in Figure 6. Cut number 3 from Figure 2 and cut number 5 from Figure 6 exhibited the same retention time in the ^{1}D chromatogram. Corresponding deconvoluted mass spectra for cut numbers 3-1 and 5 were found to be 3,152.83 and 3,153.60 Da, respectively, showing similar results (Figure 9).

Table 5. Deconvolution results for major impurities of semaglutide obtained through 2D-LC/MS analysis.

Condition	Cut Number	^{1}D Retention Time (min)	^{2}D Retention Time (min)	Component	Mass (Da)	Mass Difference (Da)*
Heat	1	35.44	7.000	A	4,117.02	3
				B	4,160.43	47
	2	35.82	7.582	A	4,115.47	2
	3-1	37.27	7.381	A	3,152.83	−961
				B	3,185.89	−928
	3-2	37.27	8.555	A	4,114.25	1
	4	37.60	7.821	A	4,117.26	4
	5	40.45	9.188	A	3,891.54	−222
	6	41.02	9.018	A	3,766.63	−347
				B	3,810.00	−304
Acid	1	10.75	1.216	A	2,582.79	−1,531
	2	16.31	2.404	A	3,817.33	−296
				B	3,820.69	−293
	3	16.54	1.514	A	3,688.02	−426
	4	16.93	1.514	A	3,379.42	−734
	5	37.35	7.326	A	3,153.60	−960
	6	39.50	8.328	A	4,114.69	1
	7	40.16	8.368	A	4,059.46	−54
	8	41.07	9.073	A	3,705.19	−408
	9	42.60	8.573	A	4,098.10	−15
	10	47.06	10.295	A	3,846.76	−267
	11	49.15	10.709	A	3,907.27	−206

* The mass difference was calculated in comparison to the molecular weight of semaglutide (4,113.53 Da).

Return to Contents Section

Conclusion

Generally, difficult-to-separate peptide-related impurities can benefit from improved resolution through optimizing the concentration of acidic modifiers and fine-tuning the column performance. However, confirming the molecular weight of impurities can be challenging due to mobile phases not being suitable for MS analysis. In this application note, a stability indicating test for semaglutide demonstrated efficient transition to LC conditions compatible with MS analysis using the Agilent InfinityLab multiple heart-cutting valve feature of an Agilent 1290 Infinity II bio 2D-LC. An Agilent InfinityLab LC/MSD XT exhibited the sensitivity to confirm MS spectra, even when analyzing impurities at low concentrations in the second dimension, and the deconvolution feature of Agilent OpenLab CDS software, version 2.8, facilitated easy confirmation of accurate molecular weights. Based on these results, it is anticipated that this method will provide foundational data for the management of peptide-related impurities not only for formulation compatibility but also for stability studies.

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Application Note

Impurity Analysis

Agilent

Trusted Answers

Characterization of Forced Degradation Impurities of Glucagon-Like Peptide-1 Agonists by LC/Q-TOF Mass Spectrometry

Author

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Agilent Technologies, Inc.

Abstract

Peptide biotherapeutics have gained increased attention due to their many therapeutic uses. This application note presents a liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF MS) method to study the forced chemical degradation of glucagon-like peptide-1 (GLP-1) agonists. The results obtained are helpful for the GLP-1 agonists characterization during development.

Return to Contents Section

Introduction

GLP-1 agonists are an emerging class of biotherapeutics. These synthetic peptides mimic the GLP-1 hormone to trigger the action through GLP receptors. GLP-1 agonists help to lower blood sugar levels and can lead to weight loss.[1] Currently, various GLP-1 agonists are available in the market for medication.

During the biotherapeutics development and manufacturing process, studies are required to assess the active pharmaceutical ingredient (API) impurity profiles. The presence of impurities can play an important role in the efficacy and safety aspects of biotherapeutics. The impurities associated with the product constitute a significant risk associated with adverse immunological reactions.[2] These impurities may arise from various sources throughout the product life cycle, including production processes, degradation, storage conditions, and even during delivery. It is crucial to identify and quantify these impurities to ensure that the therapeutic proteins perform as intended without causing adverse effects.

Forced degradation or stress testing studies are used to understand the chemical and physical changes of biopharmaceuticals. According to International Conference on Harmonisation (ICH) Q5E, manufacturers should evaluate the stability data through stress conditions to provide potential product-related impurities.[3] Therefore, it is essential to have analytical methods that can detect and identify the degradation products. Liquid chromatography/mass spectrometry (LC/MS) is well suited for effectively identifying and characterizing synthetic peptides.

This study used an LC/MS method to identify and characterize GLP-1 agonist impurity products under oxidative and pH stress conditions. The LC/Q-TOF MS identifies mass changes of GLP-1 agonists with oxidative and deaminated conditions. The results demonstrate the suitability of the LC/MS method for determining the GLP-1 agonist impurities and may be applied to routine quality control analysis.

Experimental

Reagents and chemicals

Semaglutide, liraglutide, and tirzepatide peptides were purchased from MedChemExpress (Monmouth Junction, NJ, USA) and stored according to the manufacturer's instructions. Difluoroacetic acid (DFA), Trizma base, tris HCl, and 30% (v:v) hydrogen peroxide (H_2O_2) were procured from Sigma (St. Louis, MO, USA); LC/MS grade acetonitrile (ACN) was obtained from Fisher (Waltham, MA, USA). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Billerica, MA, USA).

Analytical equipment

Agilent 1290 Infinity II bio LC system including:

- Agilent 1290 Infinity II bio high-speed pump (G7120A)
- Agilent 1290 Infinity II bio multisampler (G7137A)
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)

Software and data processing

- Agilent MassHunter data acquisition software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1
- Agilent MassHunter Qualitative Analysis version 12.0.

Sample preparation

Liraglutide, semaglutide, and tirzepatide peptides were dissolved to 1.0 mg/mL in 30% ACN. For oxidative stress, stock solutions were diluted to 0.5 mg/mL and incubated at different concentrations of the oxidizing agent H_2O_2 (0.05%, 0.5%, 1%, 2% v:v) overnight at room temperature. For a deamidation test, diluted samples were kept at a temperature of 37 °C in a tris buffer pH 8.9 for 3, 6, 12, and 24 days.

LC/MS analysis

The LC separation was performed on an Agilent AdvanceBio Peptide Mapping column (part number 653750-902) using a 30-minute gradient (Table 1). The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF (Table 2) and data analysis was performed in MassHunter BioConfirm version 12.1 and MassHunter Qualitative Analysis version 12.0.

Return to Contents Section

Results and discussion

To characterize the synthetic peptides, the workflow comprises the 1290 Infinity II bio LC, AdvanceBio peptide mapping column, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm version 12.1 (Figure 1). The biocompatibility of the instrument configuration is a critical factor in the analysis of synthetic peptides. Instruments that are compatible with biological materials ensure that the analysis of peptides is accurate and free from interference caused by the instrument itself. This biocompatibility is particularly important when identifying impurities that may affect the quality and efficacy of peptide-based drugs.

To assess the forced chemical degradation products, the GLP-1 agonist was subjected to H_2O_2 and high pH treatment. The treatment conditions were tested at different time points and concentrations of chemical agents. Methionine and tryptophan are the most common oxidation residues in protein and peptide pharmaceuticals. The three GLP-1 agonists employed in this study contain one tryptophan residue in each peptide sequence. Typically, under laboratory conditions, H_2O_2 is used to assess oxidative degradation species. Figure 2 depicts the LC/MS monitoring of GLP-1 agonist profiles following overnight H2O2 treatment. The total ion chromatograms (TICs) of GLP-1 agonists can reveal significant insights into their oxidative profiles under stress conditions. Initial experiments were conducted with different time points (data not shown), and overnight incubation resulted in several oxidation forms of the peptides. The observation of multiple peaks with increasing H_2O_2 concentration indicates the formation of various oxidized forms of peptides, which can be effectively separated using the AdvanceBio peptide mapping column. Table 3 shows the theoretical and experimental monoisotopic masses determined by resolved isotope deconvolution.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II Bio LC System			
Parameter	**Value**		
Column	Agilent AdvanceBio peptide mapping, 2.1 × 150 mm, 2.7 µm, 120 Å		
Sample Thermostat	10 °C		
Mobile Phase A	0.1% DFA in water		
Mobile Phase B	0.1% DFA in ACN		
Gradient	Time (min)	%A	%B
	0.00	80	20
	1.00	80	20
	20.00	40	60
	25.00	10	90
	25.10	80	20
	30.00	80	20
Stop Time	30 min		
Column Temperature	40 °C		
Flow Rate	0.4 mL/min		

Table 2. MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Parameter	**Value**
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Nebulizer	35 psi
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
Oct RF Vpp	750 V
Reference Mass	922.009798
Acquisition Mode	Data were acquired in extended dynamic range (2 GHz)
MS Mass Range	100 to 1,700 *m/z*
Acquisition Rate	2 spectra/s

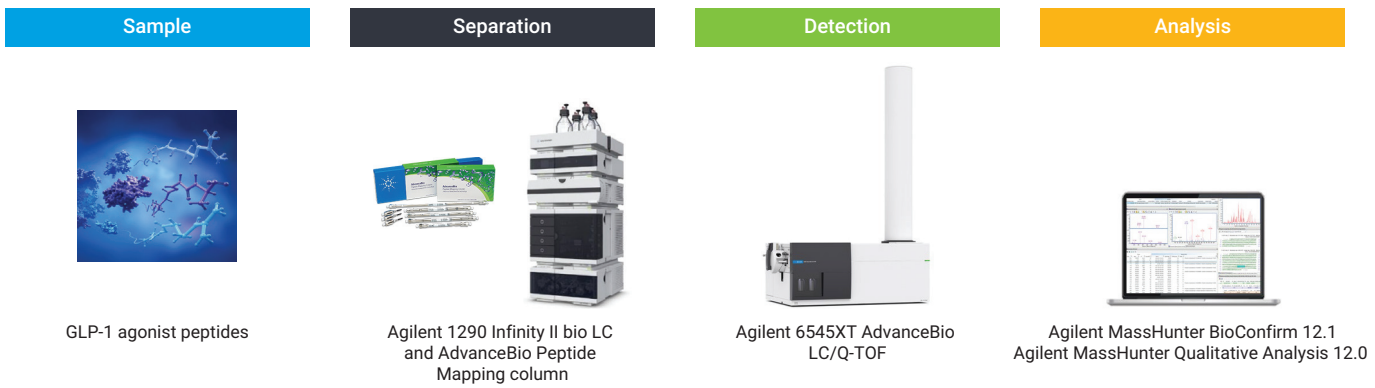

Figure 1. Synthetic peptide characterization workflow configuration.

Return to Contents Section

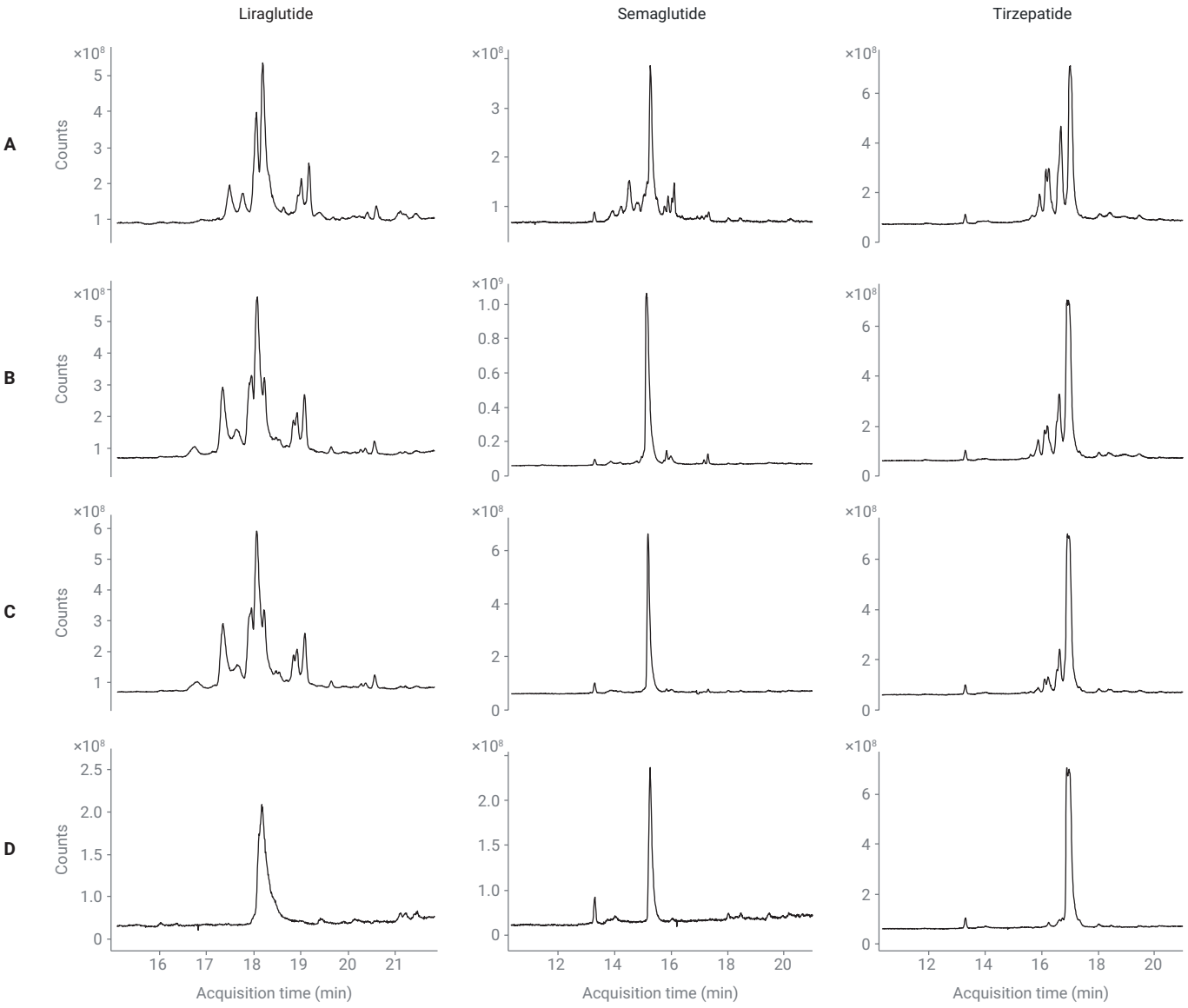

Figure 2. LC/MS monitoring of GLP-1 agonist TIC profiles following H_2O_2 stress. (A) 2% H_2O_2, (B) 1% H_2O_2, (C) 0.5% H_2O_2, and (D) 0.05% H_2O_2.

Table 3. GLP-1 agonist sequence and mass accuracy (monoisotopic mass).

GLP-1 Agonist	Sequence	Theoretical (Da)	Observed (Da)	Mass Accuracy (ppm)
Liraglutide	HAEGTFTSDVSSYLEGQAA-{Lys-N6-[N-(1-oxohexadecyl)-L-g-glutamyl]}-EFIA**W**LVRGRG	3,748.94	3,748.97	8.0
Semaglutide	H-{Aib}-EGTFTSDVSSYLEGQAA-{C18 diacid-γ-Glu-(AEEA)2-Lys}-EFIA**W**LVRGRG	4,111.11	4,111.12	2.43
Tirzepatide	Y-{Aib}-EGTFTSDYSIXLDKIAQ-{C20 diacid-gamma-Glu-(AEEA)2-Lys}-AFVQ**W**LIAGGPSSGAPPPS	4,810.52	4,810.53	2.07

Tryptophan (W) oxidation is highlighted in bold

Return to Contents Section

Multiply charged spectra corresponding to different oxidation forms were observed under 2% H_2O_2 treatment (Figure 3). Quadruple charge ($[M + 4H]^{4+}$) is the most abundant charge state in all three GLP-1 agonist samples. The charge states were corresponding to the expected $[M + 3H]^{3+}$, $[M + 4H]^{4+}$, $[M + 5H]^{5+}$, and $[M + 6H]^{6+}$ charge states of oxidized and non-oxidized peptide forms.

The mass spectrum was deconvoluted using a resolved isotope deconvolution algorithm in MassHunter Qualitative Analysis version 12.0 software. It converts the multiply-charged spectrum to the zero-charge mass spectrum. Figure 3 shows the results obtained from the deconvolution. The deconvolved mass confirms the multiple oxidation forms of the GLP-1 agonist. A mass shift of +16 Da, +32 Da, and +48 Da corresponds to mono, di, and tri oxidation products, respectively.

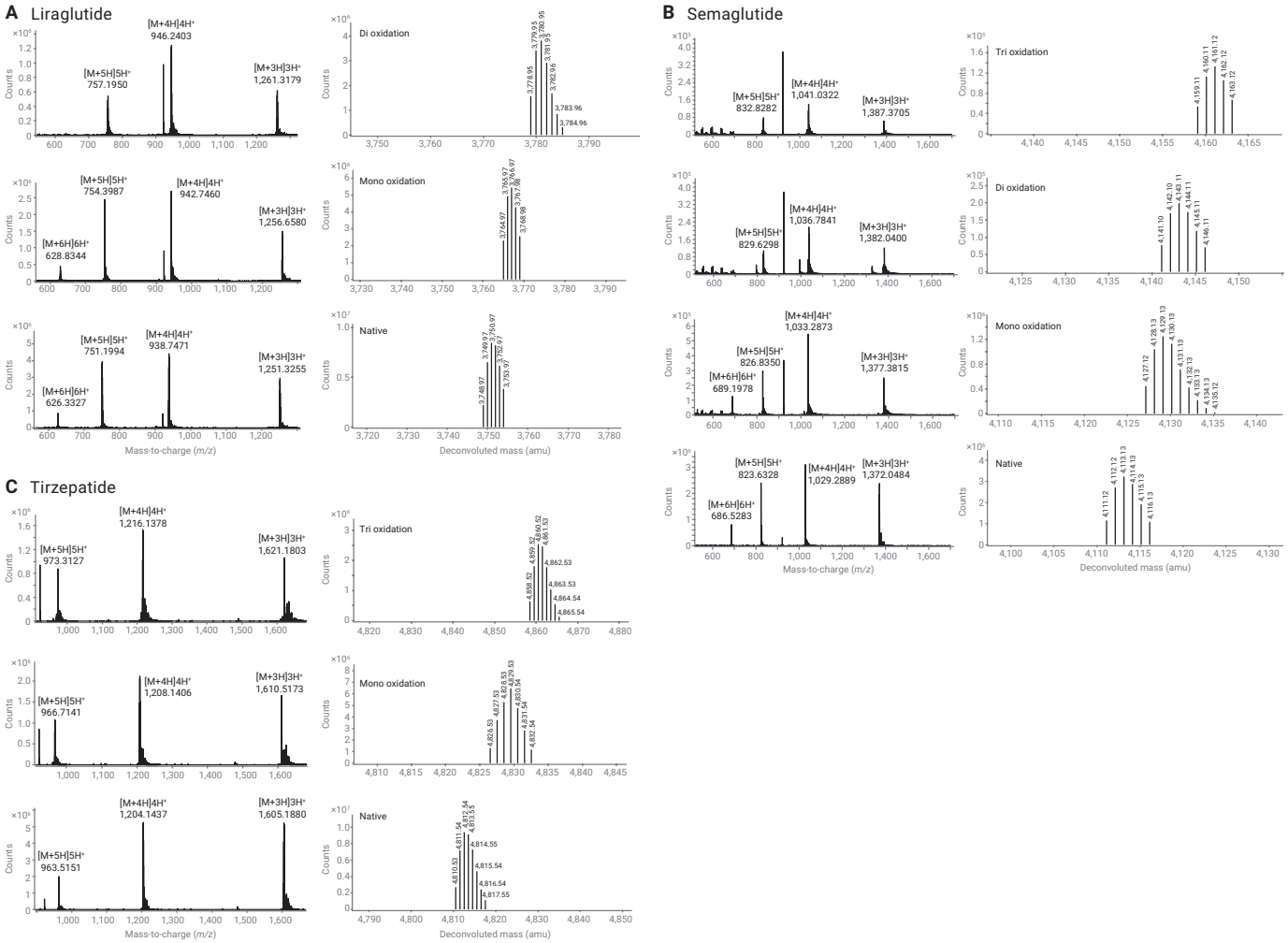

Figure 3. The charge state envelopes and resolved isotope deconvoluted spectra of oxidized and native GLP-1 agonists: (A) liraglutide, (B) semaglutide, and (C) tirzepatide.

Return to Contents Section

Tryptophan is most susceptible to oxidation and can produce isomeric peptide oxidation products.[4] Based on deconvolved mass, different peptide oxidation products are assigned and shown in Figure 4. Partial separation of singly, doubly, and triply oxidized isomeric peptides was achieved by reversed-phase chromatography.

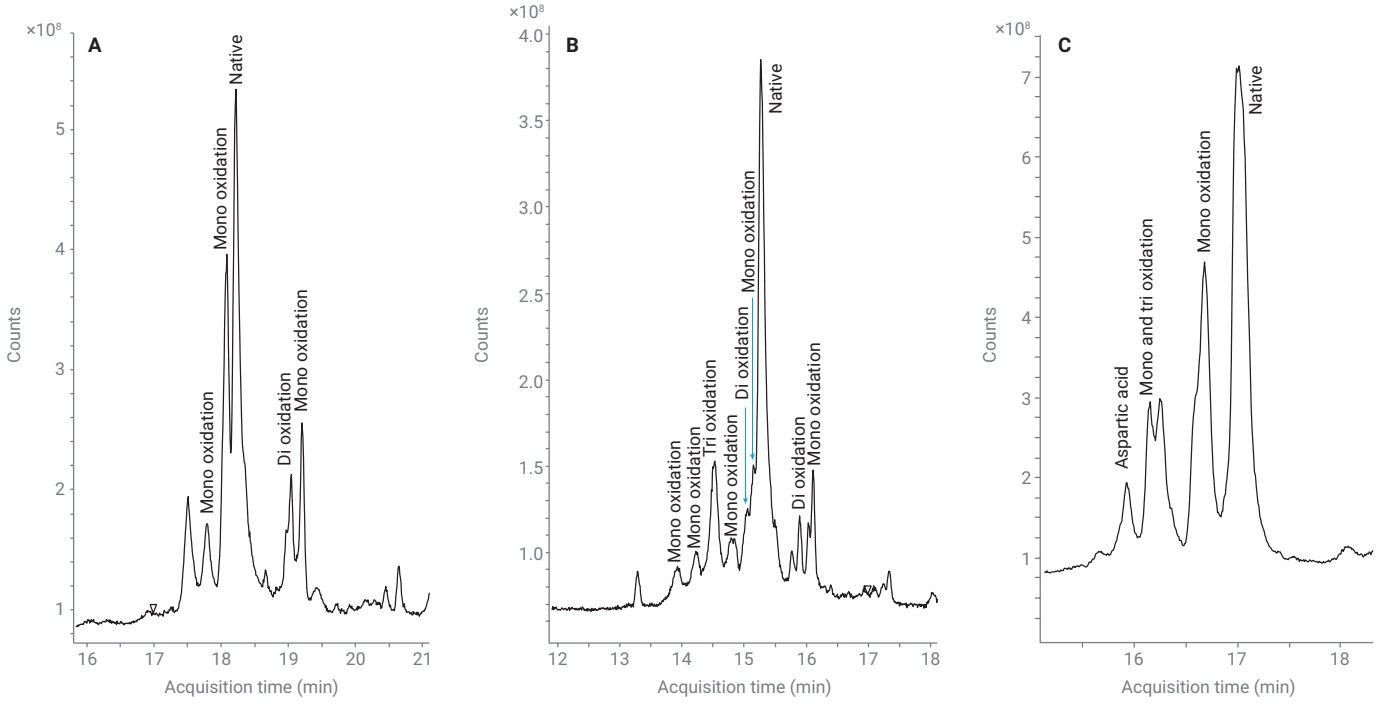

Figure 4. TIC profiles of oxidation of GLP-1 agonists: (A) liraglutide, (B) semaglutide, and (C) tirzepatide.

Return to Contents Section

Figure 5 shows the representative MS/MS spectrum for both unmodified and oxidized liraglutide. Inspection of the y ion shows an increase of *m/z* ~8 in the oxidized MS/MS spectra, suggesting oxidation of the tryptophan residue. Progression of the oxidation reaction was monitored using extracted ion chromatograms (EICs) for different identified oxidized species (Figure 6). The analysis of the forced oxidation samples showed that the mono-oxidation state is highly abundant in all three molecules.

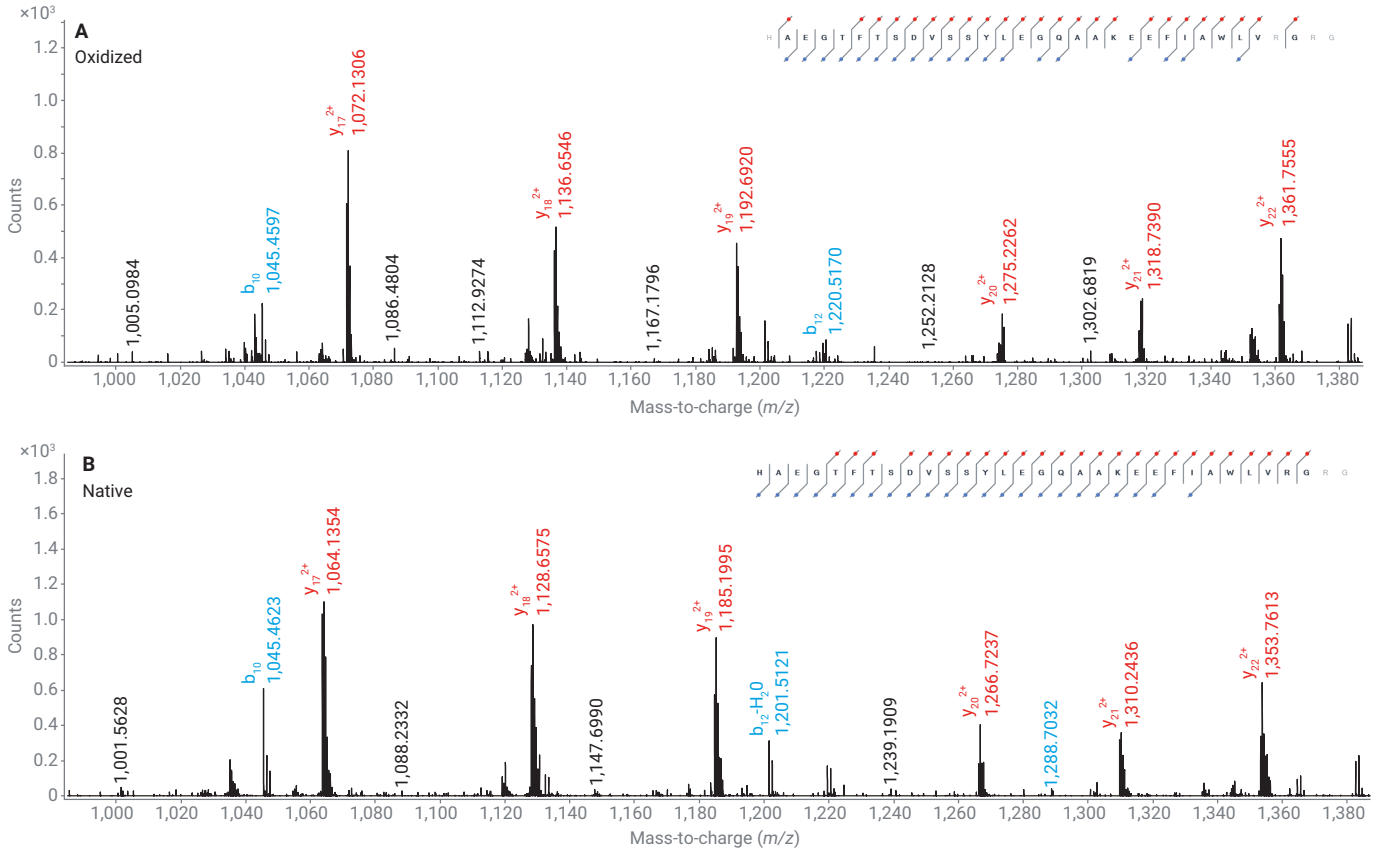

Figure 5. MS/MS spectra of unmodified and oxidized liraglutide.

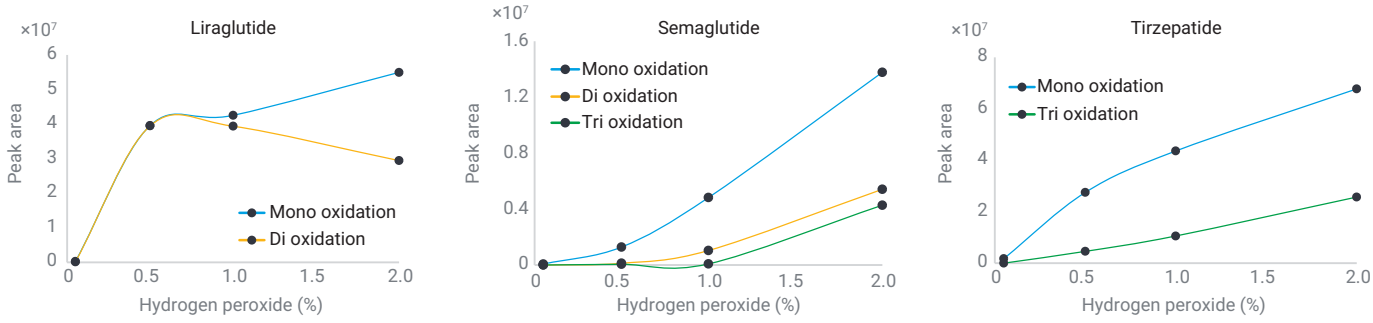

Figure 6. Oxidation responses of GLP-1 agonists. EIC peak area of quadruple charged ions.

Return to Contents Section

The intact level analysis of forced degradation conditions for 24 days (at high pH) indicates no formation of deamidation species (Figure 7). It is noteworthy that all three peptides contain only glutamine as a possible deamidation site, which happens to have a much slower rate of deamidation than asparagine.

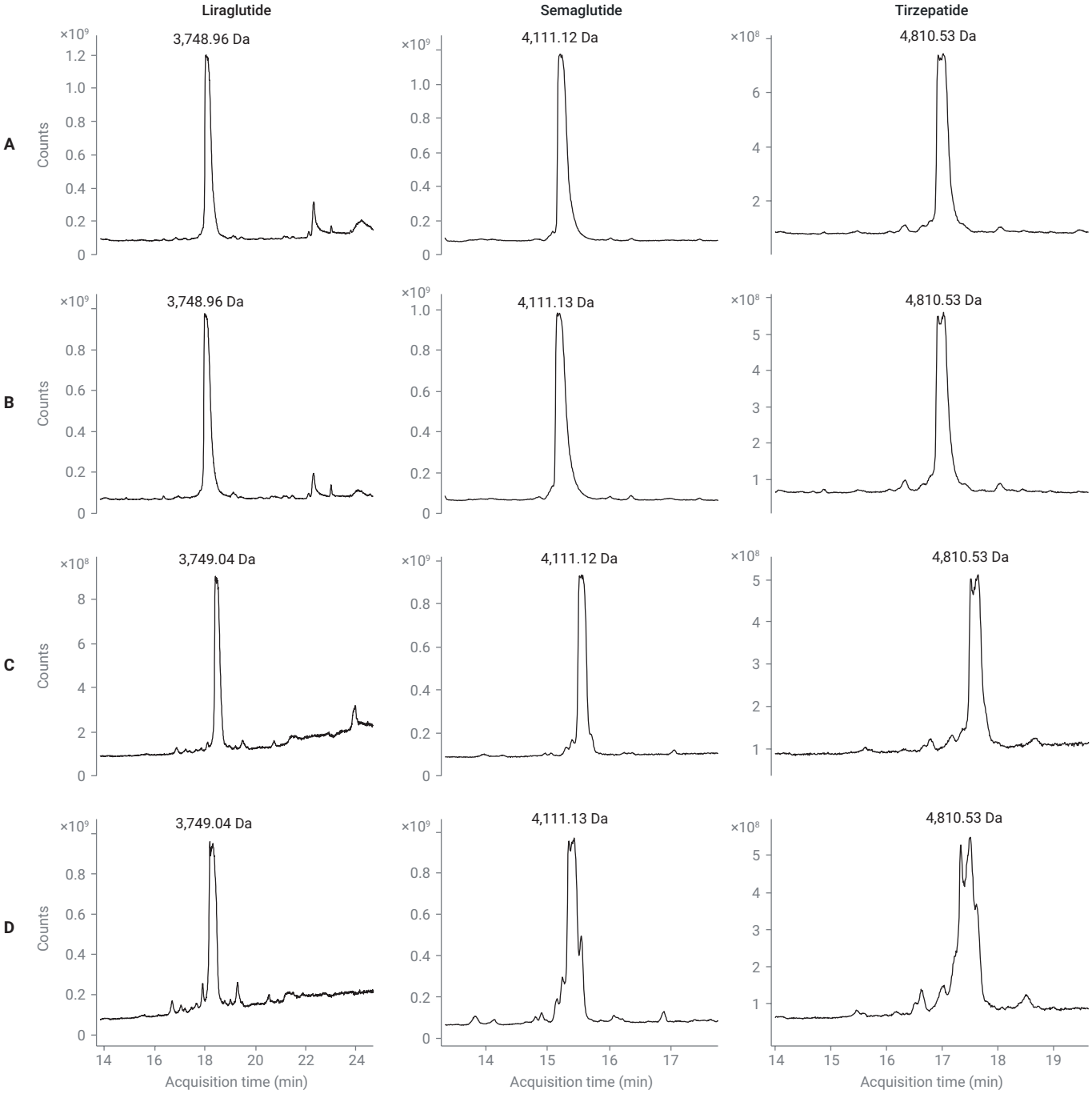

Figure 7. LC/MS monitoring of GLP-1 agonists at pH 8.9. TIC profiles show at (A) 3 days, (B) 6 days, (C) 12 days, and (D) 24 days. Monoisotopic masses are presented.

Return to Contents Section

Conclusion

This application note demonstrates the analysis of GLP-1 agonist forced chemical degradation products using an Agilent LC/Q-TOF system. Precise characterization of forced chemical degradation of GLP-1 was achieved with high-efficiency chromatographic separation and high-quality MS spectra. The Agilent MassHunter BioConfirm software is easy to use and provides an integrated environment for the analysis of synthetic peptide. The developed LC/MS method demonstrates the applicability of the method in peptide drug research and manufacturing.

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Return to Contents Section

Application Note

Sequence Confirmation

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LC/MS Based Characterization Workflow of GLP-1 Therapeutic Peptide Liraglutide and Its Impurities

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Abstract

This application note demonstrates the power of the Agilent 1290 Infinity II LC system coupled with the Agilent 6545XT LC/Q-TOF for identification and characterization of therapeutic peptides. This application is exemplified for the GLP-1 agonist liraglutide and its impurities. The therapeutic peptide workflow within Agilent MassHunter BioConfirm software allows for accurate mass-based identification, sequence conformation, and identification of impurities, with extra or missing amino acid impurities that can arise during solid phase synthesis. These impurities may pose a potential safety risk and eventually may impact the efficacy of the product.

Introduction

Therapeutic peptides such as GLP-1 agonists have become a rapidly increasing sector of the pharmaceuticals market and are among the best-selling pharmaceutical products. Current growth projections indicate that the importance of therapeutic peptides will increase over the next decade.[1]

However, according to the Food and Drug Administration (FDA) guidance, synthetic peptide substances are required to exhibit impurity levels equal to or lower than those well identified as the reference listed drug (RLD). The confirmation of the impurity profile mandates proving the safety of impurities exceeding 0.5% of the drug substance.[2]

The 1290 Infinity II LC system combined with the AdvanceBio Peptide Plus column and the 6545XT AdvanceBio LC/Q-TOF is an optimized workflow to determine sequence confirmation of the drug substance and to identify low-level impurities simultaneously. The data analysis by MassHunter BioConfirm software is straightforward and simplifies detection and identification of the impurities. This application note describes a workflow for LC/MS analysis of liraglutide and its impurities, using reversed-phase chromatography combined with Q-TOF MS/MS detection.

Liraglutide is a full agonist of the GLP-1 receptor and shares 97% of its amino acid sequence identity with human GLP-1. Liraglutide was created by substituting arginine for lysine at position 34 in the GLP-1 peptide and adding a palmitic acid chain with a glutamic acid spacer on the lysine residue at position 26 to improve the pharmacokinetic effects.[3]

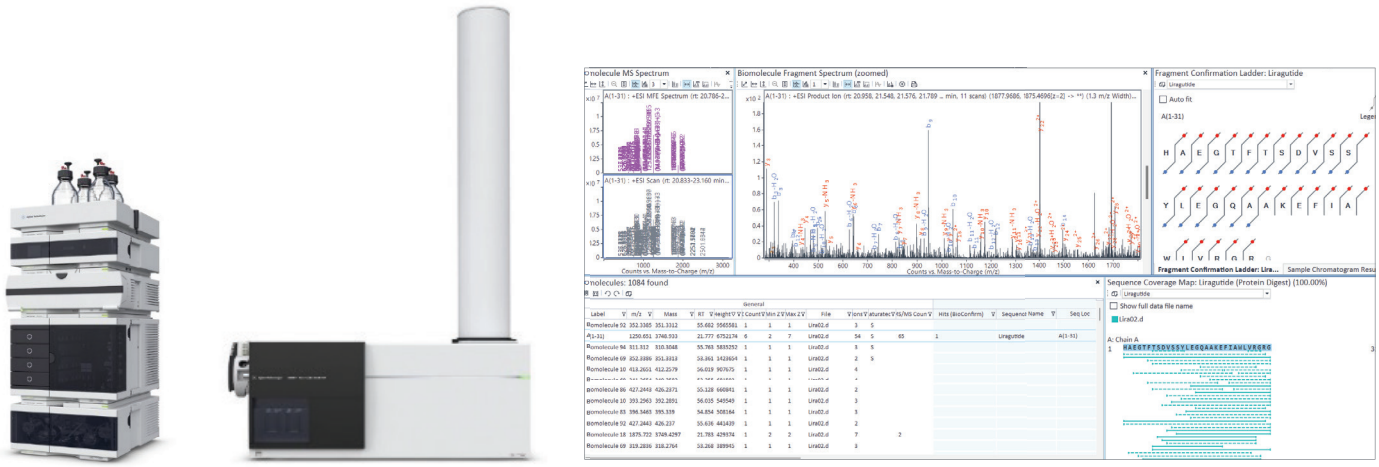


Figure 1. Agilent 1290 Infinity II LC coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF and Agilent MassHunter BioConfirm software.

Figure 2. Structure of liraglutide.

Return to Contents Section

Experimental

Instrument

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B) with Agilent InfinityLab sample thermostat (G4761A, option #101)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent Dual Jet Stream ESI source

Software

- Agilent MassHunter Acquisition software version 11.1
- Agilent MassHunter BioConfirm software version 12.1

Samples

Liraglutide (approximately 2 mg/mL) was dissolved in water to 1 mL of water:methanol (70:30).

Columns

Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, 2.7 µm (part number 695775-949)

Chemicals and reagents

Ultra-grade LC/MS acetonitrile was purchased from Biosolve (MS grade, Biosolve, Dieuze, France) and trifluoroacetic acid from Merck was used (Merck KGaA, Darmstadt, Germany). Water was purified using a Milli-Q-IQ 7003 purification system (Merck KGaA, Darmstadt, Germany).

Instrument configuration

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System			
Parameter	**Value**		
Column	Agilent AdvanceBio peptide mapping, 2.1 × 150 mm, 2.7 µm, 120 Å		
Sample Thermostat	10 °C		
Mobile Phase A	0.1% DFA in water		
Mobile Phase B	0.1% DFA in ACN		
Gradient	Time (min)	%A	%B
	0.00	80	20
	1.00	80	20
	20.00	40	60
	25.00	10	90
	25.10	80	20
	30.00	80	20
Stop Time	30 min		
Column Temperature	40 °C		
Flow Rate	0.4 mL/min		

Table 2. MS parameters.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	270 °C
Drying Gas Flow	11 L/min
Nebulizer	35 psig
Sheath Gas Temperature	375 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	500 V
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 300 to 3,200
Acquisition Rate	2 Spectra/second
Auto MS/MS Range	*m/z* 50 to 3,200
Min MS/MS Acquisition Rate	3 Spectra/second
Isolation Width	Narrow (~ *m/z* 1.3)
Precursors/Cycle	Top 3
Collision Energy	3.8 × (*m/z*)/100 + 2 for charge 2 5.3 × (*m/z*)/100 − 3 for charge 3 5 × (*m/z*)/100 − 2 for charge > 3
Threshold for MS/MS	2,000 counts and 0.001%
Dynamic Exclusion On	1 Repeat, then exclude for 0.2 minutes
Precursor Abundance-Based Scan Speed	Yes
Target	25,000 Counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Purity	100% Stringency, 30% cutoff
Isotope Model	Peptides
Sort Precursors	By abundance

Return to Contents Section

Results and discussion

The LC/Q-TOF MS methodology for therapeutic peptide mass confirmation and impurity characterization uses the BioConfirm peptide mapping workflow. The matching rules of intact mass, missing and modified amino acids perfectly confirm the main peak and impurities.

Peptide characterization using the BioConfirm workflow

The LC/MS approach for a therapeutic peptide workflow uses the chromatographic separation, in-spectrum dynamic range, isotopic fidelity, and accurate mass for identification and impurity profiling.

The chemically modified silica with a hybrid charged C18 surface provides alternate selectivity over traditional C18 columns, resulting in improved separation of liraglutide impurities. The total ion chromatogram (TIC) (Figure 4) shows that a few minor peaks eluting before and after the main peak are also observed. However, the possibility for additional impurities coeluting with the main peak cannot be excluded.

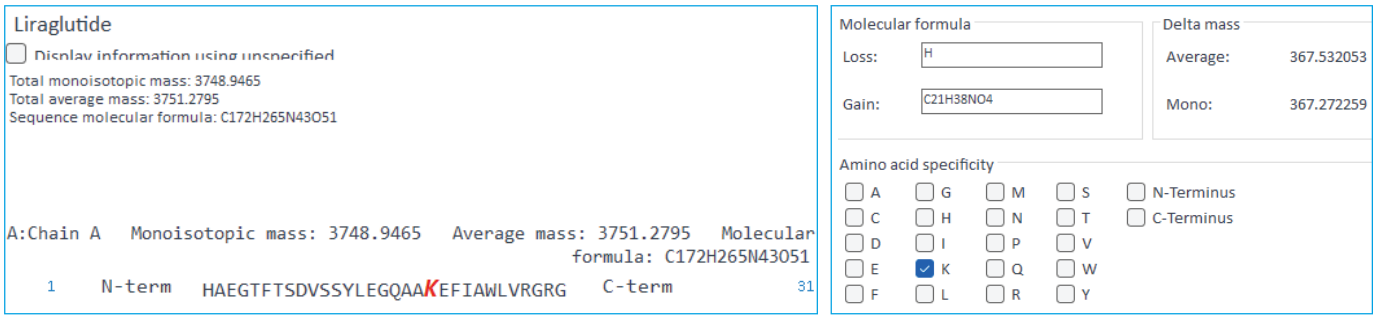


Figure 3. Setting up of chemical modification at lysine 25 (K25) sequence of liraglutide in Agilent MassHunter BioConfirm Sequence Manager. Additional impurities were included in the BioConfirm Sequence Manager as modifications.

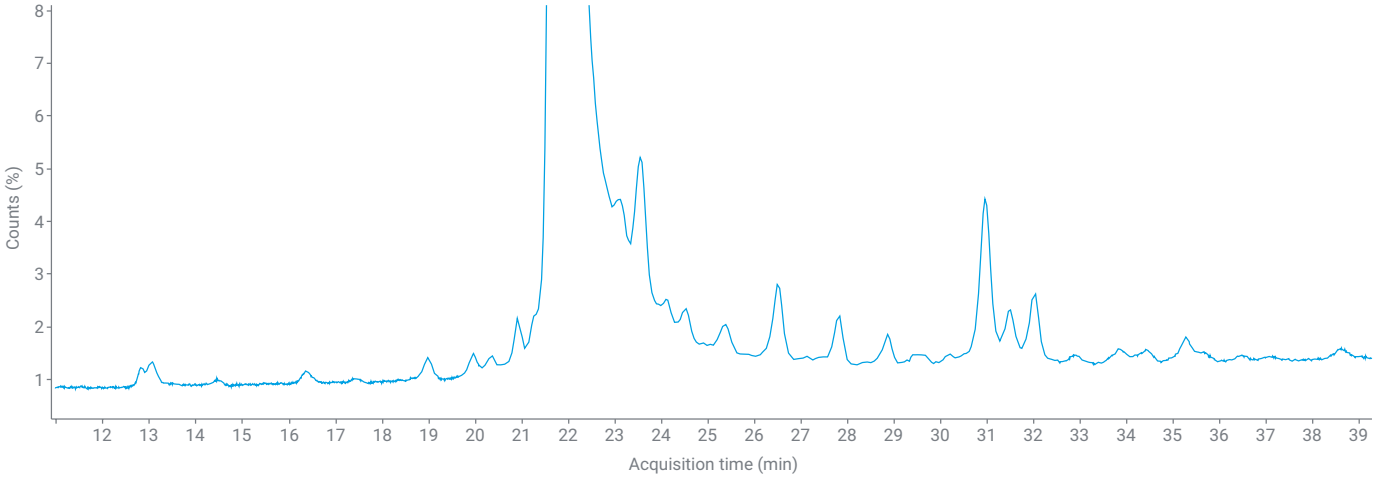
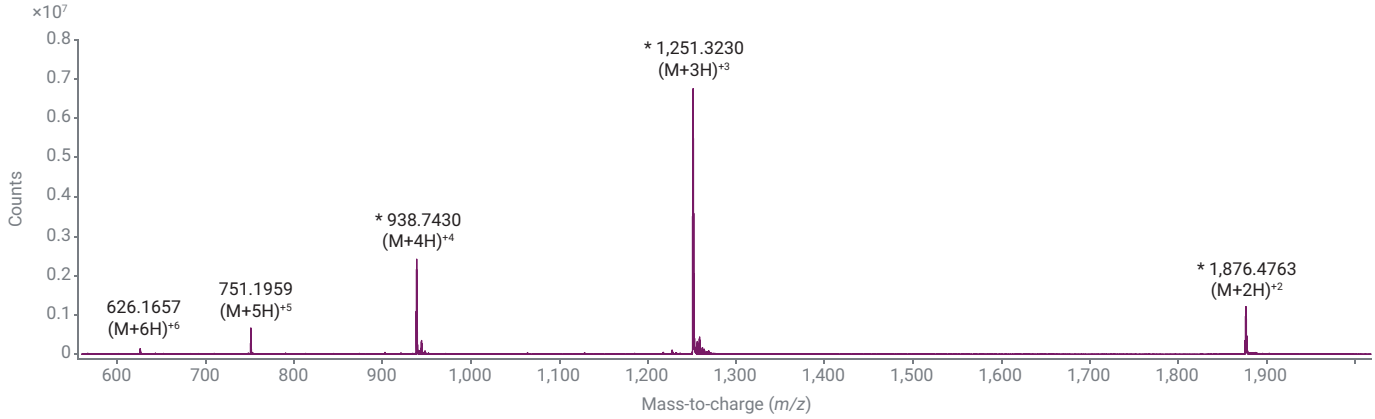


Figure 4. The zoomed TIC of a liraglutide sample and the separation of the main peak and impurities.

Return to Contents Section

Mass accuracy and charge states

The mass spectra of liraglutide and its related impurities were identified using BioConfirm software version 12.1. The mass of intact liraglutide and its impurities were matched with a respective sequence. Figure 5 shows the different charge states extracted with BioConfirm software resulted in mass accuracies (smaller than 5 ppm) matching well with the sequence of liraglutide. The isotopic fidelity of the raw spectra corresponding to the 3+ charge states of liraglutide is represented in Figure 6.



Best	Sequence Name	Seq Loc	Sequence	Fixed Mods	Variable Mods	Mass	Mass (Bio)	Diff (ppm
	Liragutide	A(1-31)	HAEGTFTSDVSSYLEGC	Palm-Glut 20		3748.933	3748.9465	-3.6

Z (Prec)	m/z (Prec)	Score (Bio, MS/MS)	CE
3	1250.6475	25	63.3
4	938.2405	18.14	44.6
2	1875.4693	14.78	75.1
5	750.7939	11.91	36.3
6	625.8294	6.62	29.9

Figure 5. The intact mass charge state distribution identified by Agilent MassHunter BioConfirm software for liraglutide.

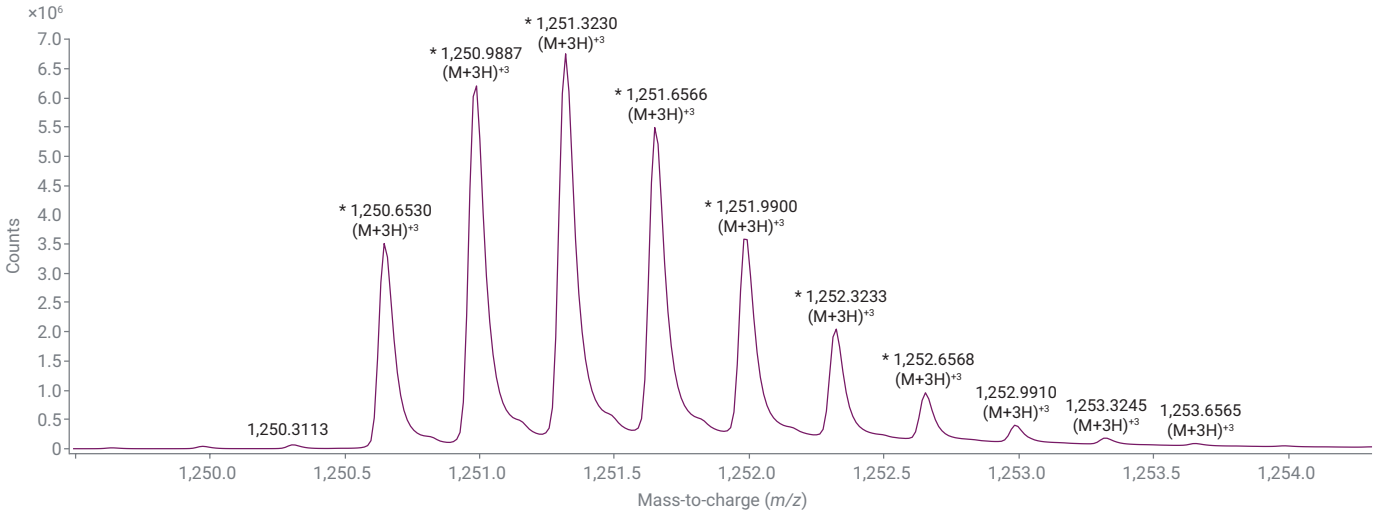


Figure 6. Isotopic fidelity of $[M+3H]^{+3}$ charge state of intact liraglutide.

Return to Contents Section

Figure 7 shows the MS/MS fragmentation maps for each of the liraglutide samples, with fragment locations with observed fragmentation of b and y ion series annotated in the spectrum and visualization on the MS/MS fragmentation pattern ladder for 3+ and 4+ charge states.

Figure 8 highlights differences in effective charge states for the impurity missing threonine (Des-T) due to deletion of the amino acid threonine from the main liraglutide sequence.

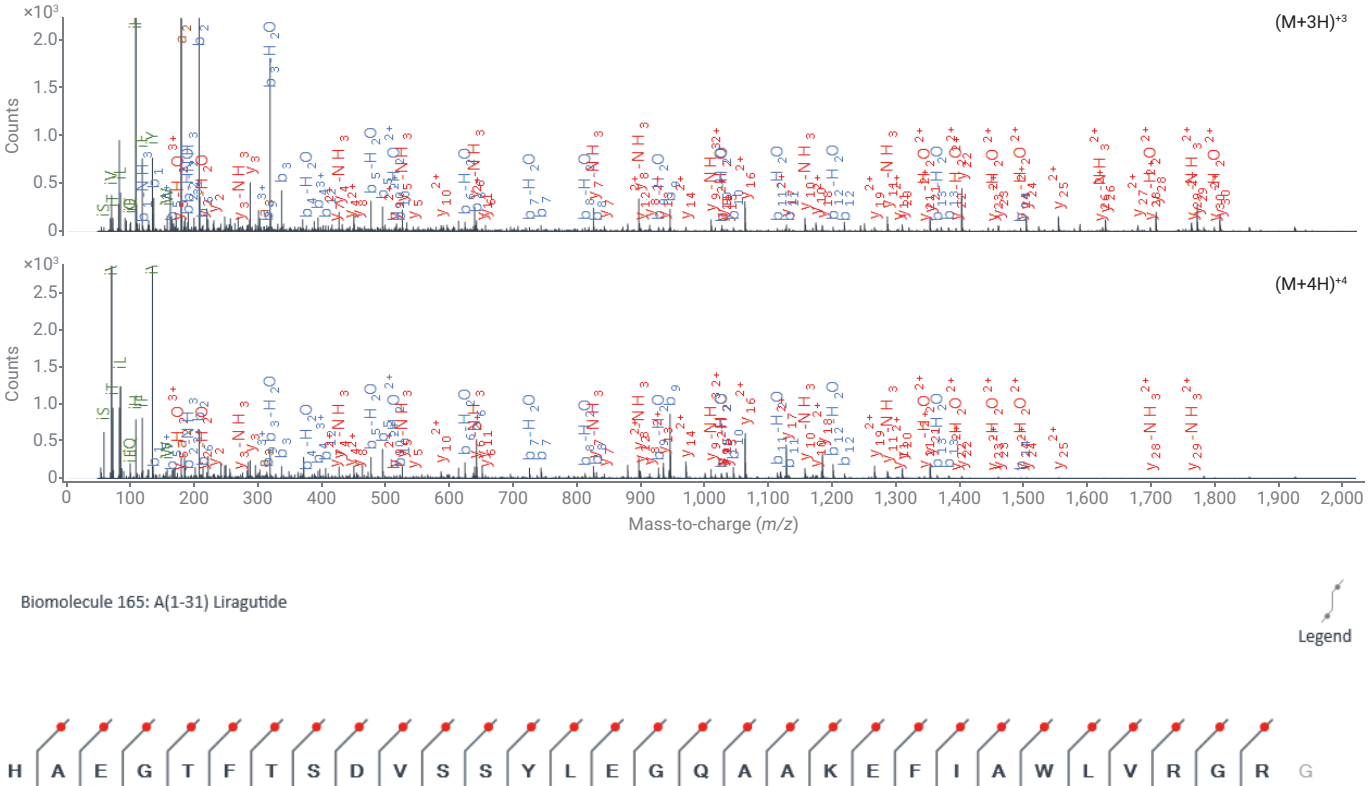


Figure 7. The MS/MS fragmentation pattern for the liraglutide peptide (z = 3, 4) with coverage of both b and y ion series. The fragmentation ladder annotates the identified b/y series (z = 3) for the sequence.

Return to Contents Section

An impurity of liraglutide with a missing amino acid alanine (missing H) was identified and confirmed by accurate mass and MS/MS fragmentation pattern, as illustrated in Figure 9. The impurity can be concluded from the H (1) impurity in liraglutide with a corresponding ion series of the y and b ions without histidine (H). MS/MS analysis in combination with accurate molecular weight determinations allows for both the amino acid composition and sequence confirmation.

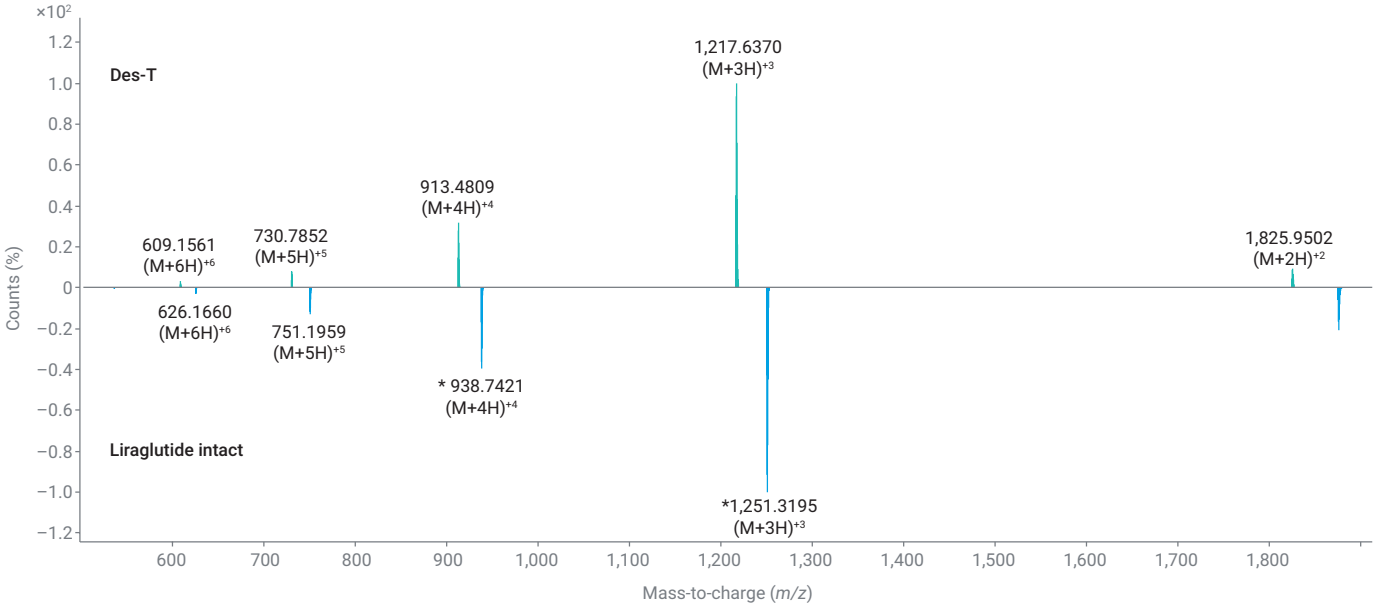


Figure 8. The different charge states of impurities compared by mirror plots of Agilent MassHunter BioConfirm software.

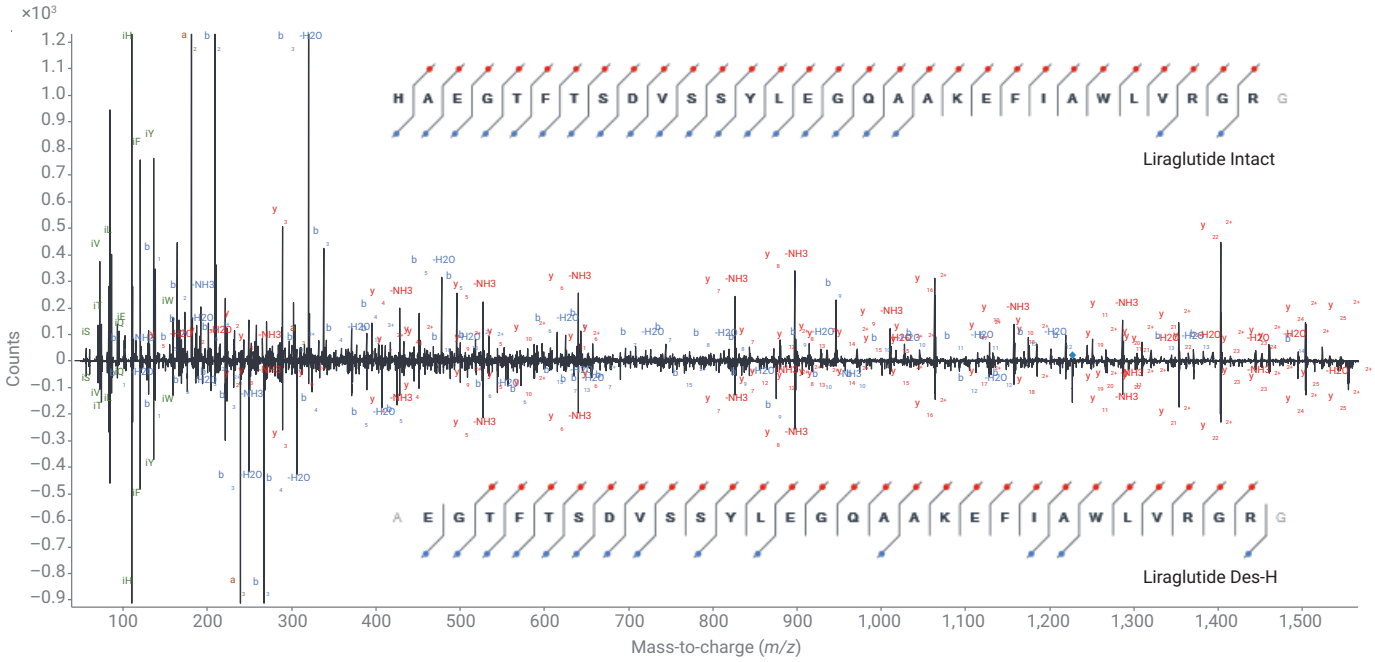


Figure 9. Missing H (1) impurity in liraglutide: Comparison of the y and b ions from the MS/MS spectrum for modified b1 ions shows an effective change over those ions in unmodified peptide, suggesting that histidine (H) is missing at position 1.

Return to Contents Section

Identification and quantification

The separation of the main peak and impurities were achieved by the chromatographic method. An overlay of extracted ion chromatograms (EIC) for different impurities separated chromatographically were illustrated in Figure 10. BioConfirm software allows seamless identification of intact liraglutide and its impurities while applying multiple sequences of predicted impurities in a single matching event.

All the identified impurities with sequence confirmation by BioConfirm software version 12.1 were summarized in Table 3. Figure 11 shows the relative quantification of the identified impurities with respect to intact liraglutide while extracting the data for the volume of each impurity.

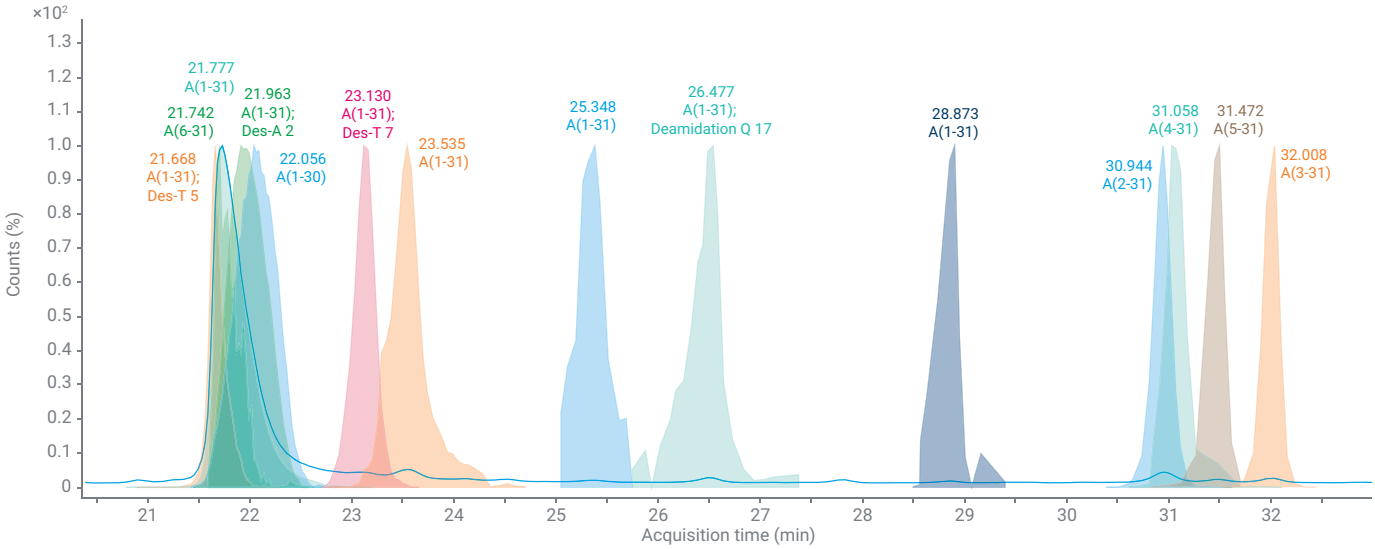


Figure 10. Overlaid chromatograms for EIC of different impurities separated at different retention times (RTs).

Return to Contents Section

Table 3. The list of impurities identified from the samples.

RT	Sequence	Sequence Location	Impurity	Mass	Difference (ppm)
21.8	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide main peak	3,748.9330	−3.6
23.5	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9287	−4.8
22.0	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Des-A 2	3,677.8926	−4.6
22.1	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR	(1-30)	Des-C-term-Gly	3,691.9037	−5.8
21.7	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Des-T 5	3,647.8852	−3.7
30.9	AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(2-31)	Des-^{1}His	3,611.8701	−4.8
23.1	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Des-T 7	3,647.8795	−5.3
32.0	EGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(3-31)	Des-^{1}His-^{2}Ala	3,540.8331	−4.9
22.8	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Des-A 2	3,677.8872	−6.0
21.8	TSDVSSYLEGQAAKEFIAWLVRGRG	(7-31)	Des-^{1}His-^{6}Phe	3,106.6610	−3.0
25.3	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Deamidation Q 17	3,749.9256	−1.3
21.8	DVSSYLEGQAAKEFIAWLVRGRG	(9-31)	Des-^{1}His-^{9}Asp	2,918.5784	−4.2
21.6	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR	(1-30)	1*Des-T	3,590.8588	−5.2
26.5	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Deamidation Q 17	3,749.9270	−0.9
31.0	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9209	−6.8
31.5	TFTSDVSSYLEGQAAKEFIAWLVRGRG	(5-31)	Des-^{1}His-^{4}Gly	3,354.7697	−5.0
24.7	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9255	−5.6
27.8	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9264	−5.4
21.7	FTSDVSSYLEGQAAKEFIAWLVRGRG	(6-31)	Des-^{1}His-^{5}Thr	3,253.7324	−1.9
25.3	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9237	−6.1
31.1	GTFTSDVSSYLEGQAAKEFIAWLVRGRG	(4-31)	Des-^{1}His-^{3}Glu	3,411.7887	−5.6
32.0	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9234	−6.2
26.5	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9263	−5.4
28.9	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	Liraglutide*	3,748.9262	−5.4
22.7	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	(1-31)	1*Deamidation Q	3,749.9361	1.5

* Isomeric impurities separated chromatographically

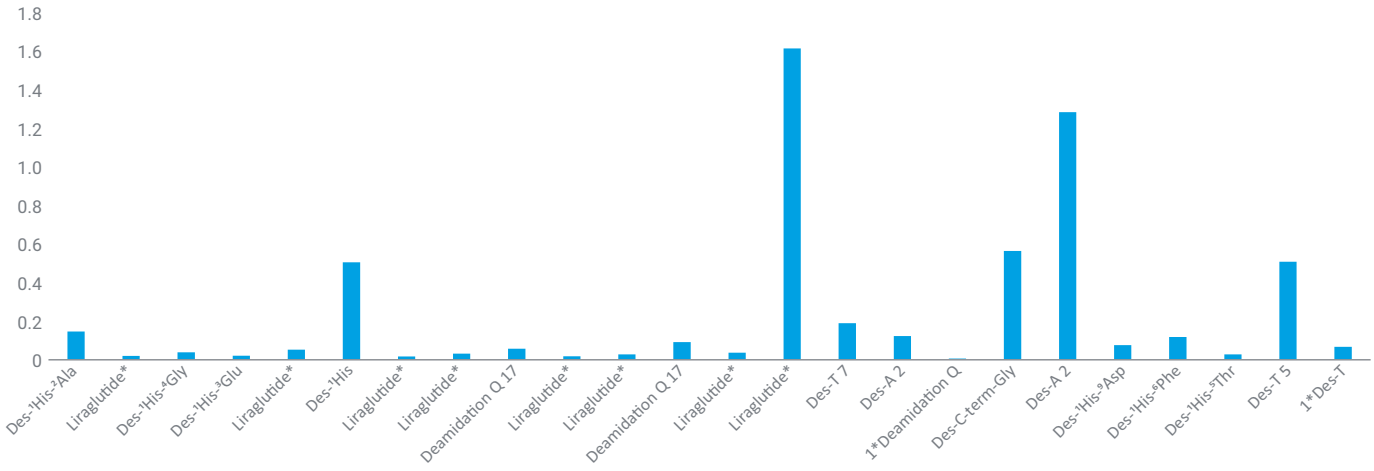


Figure 11. Relative percentage of identified impurities with respect to intact liraglutide. The isomeric impurities separated chromatographically are marked with an asterisk (*).

Return to Contents Section

Conclusion

A complete workflow was demonstrated to identify and characterize therapeutic peptides using LC/MS/MS with the Agilent 1290 Infinity II LC coupled with the Agilent AdvanceBio Peptide Plus column and an Agilent 6545XT Agilent AdvanceBio LC/Q-TOF. For data analysis, Agilent MassHunter version 12.1 software was used. The application of peptide mapping within one assay to achieve sequence confirmation and detection of low-abundant impurities was demonstrated. The separation of product-related impurities was achieved on an AdvanceBio Peptide Plus column, and the acquisition method using MS/MS on the 6545XT AdvanceBio LC/Q-TOF allowed for the identification of low-abundant impurities with accurate mass. Agilent MassHunter BioConfirm software version 12.1 can define sequences with chemical modification and provide automated matching of multiple sequences. A combination of these functions given in the 6545XT AdvanceBio LC/Q-TOF and MassHunter BioConfirm software enhances the workflow for therapeutic peptide mapping workflow significantly. This workflow meets data integrity standards with technical controls to securely acquire, process, report, and store data in laboratories that must follow the compliance guidelines of FDA 21 CFR Part 11, EU Annex 11, GAMP 5, ISO/IEC 17025, and EPA 40 CFR Part 160.

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Application Note

Sequence Confirmation

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Identification of Amino Acid Isomers Using Electron Capture Dissociation in the Agilent 6545XT AdvanceBio LC/Q-TOF System

Author

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Joseph Meeuwsen
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Abstract

Accurately determining the amino acid sequence is crucial for understanding a protein's structure and function. However, distinguishing between leucine (Leu) and isoleucine (Ile) poses a challenge, because they are positional isomers and cannot be differentiated using traditional collision-based fragmentation techniques.

Electron-based fragmentation offers a promising solution by producing side chain fragments that can be used to distinguish between Leu/Ile and other isobaric residues such as aspartate (Asp) and isoaspartate (isoAsp). This application note demonstrates the identification of isobaric residues in peptides and intact proteins using the Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an ExD cell for electron capture dissociation. Agilent ExDViewer software enables intuitive analysis of side chain fragmentation in Q-TOF datasets, enhancing the ability to discern between isobaric amino acids.

Introduction

Approximately one-sixth of the human proteome consists of the isobaric amino acids Leu or Ile.[1] Sequence variations involving these amino acids can significantly influence protein structure and function. For example, variations between Ile or Leu within the complimentary determining region of an antibody affect the strength of target binding.[2] Additionally, spontaneous isomerization of Asp to isoAsp is associated with protein aging and various diseases.[3–5] Therefore, reducing ambiguity in protein sequence analysis by accurately determining the identity of isobaric amino acids is crucial for understanding protein function and disease mechanisms.

Agilent's innovative solution for electron dissociation, the ExD cell, enables a more comprehensive and complementary characterization of protein sequences compared to using collision-induced dissociation (CID) alone. Radical z-type ions can undergo secondary fragmentation of amino acid side chains producing w-ions that can be used to distinguish between Leu and Ile.[1,6] The isomerization of Asp to isoAsp results in a carboxyl group shift, which can be detected as a 57 Da shift from the expected c and z-type ions.[4–6]

This application note describes a fast and highly efficient fragmentation of peptides and proteins using the 6545XT AdvanceBio LC/Q-TOF equipped with an ExD cell for electron capture dissociation. Isobaric amino acids were identified in MS/MS spectra from a synthetic peptide and using intact ubiquitin. While very few freely accessible tools exist for the identification of isobaric residues, ExDViewer makes annotation of side chain fragmentation in peptide and protein spectra simple and easily interpretable.[7] Together, the sensitivity and fragmentation capabilities of the 6545XT AdvanceBio LC/Q-TOF combined with ExDViewer for analysis provide an effective solution for the comprehensive characterization of protein sequence including the identification of isobaric amino acids.

Experimental

Chemical and standards

- Melittin/tune mix tuning standard formic acid, 99.0+%, Optima LC/MS Grade (part number A-117-50), Fisher Chemical
- Acetonitrile, LC/MS grade, 99.9%+, OmniSolv (part number AX0156-6), Supelco
- REALLYisoD synthetic peptide
- (part number 4144889), Bachem
- Bovine ubiquitin (part number U6253), Sigma
- Agilent Tuning Mix (G1969-85000)

Sample preparation

Lyophilized samples were stored at –20 °C before analysis. Before analysis, samples were reconstituted in 15% acetonitrile and 0.01% formic acid. The final concentrations of the samples were 1 µM for the REALLYisoD peptide and 10 µM for ubiquitin.

Instrumentation

- Agilent 6545XT AdvanceBio LC/Q-TOF
- Agilent ExD cell (G1997AA)

Software

- Agilent ExDControl software version, v 3.6
- Agilent MassHunter Acquisition software for LC/TOF and LC/Q-TOF, v. 11.0
- Agilent ExDViewer software, v 4.5.14

Mass spectrometry methods

All samples were directly infused using a 500 µL syringe at a rate of 20 µL/min. A New Era syringe pump (model number 300) was used for infusion. The samples were introduced using PEEK tubing connected with a finger-tight ferrule to the nebulizer inlet of the Agilent Dual Jet Stream (AJS) source. Mass spectrometry was performed using the 6545XT AdvanceBio LC/Q-TOF equipped with the ExD cell. A targeted acquisition method was set up in MassHunter Acquisition v11.0. Fragmentation results were analyzed using ExDViewer v4.5.14. Detailed instrument parameters for the 6545XT AdvanceBio LC/Q-TOF are listed in Table 1.

Table 1. Q-TOF LC/MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System	
Parameter	**Value**
Ion Source	Agilent Dual Jet Stream Electrospray ionization source
Polarity	Positive
Gas Temperature	325 °C
Drying Gas Flow	5 L/min
Nebulizer	20 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	4,000 V (ubiquitin), 3,200 V (peptide)
Nozzle Voltage	2,000 V
Fragmentor	175 V
Skimmer	45 V
Acquisition Rate	1 spectrum/sec
Isolation Window	Wide (*m/z* 9)
MS1 Spectra Range	*m/z* 100 to 3,200
MS2 Spectra Range	*m/z* 120 to 3,200 (ubiquitin) *m/z* 120 to 2,400 (peptide)

Table 2. A summary of the tune standards used to optimize the ExD cell lens voltages for transmission or ECD. The ExDControl autotune algorithm is installed with several built-in mass lists, including masses for tune mix, melittin, and ubiquitin. However, custom mass lists can also be created to optimize the intensities of a user-defined mass list.

ExD Cell Tuning Standards		
	MS1 Transmission	**MS2 ECD Fragmentation**
REALLYisoD (1.9 kDa)	Tune mix	Melittin
Ubiquitin (8 kDa)	Tune mix	Ubiquitin

ExD cell operation

The ExD cell is an add-on for the 6545XT AdvanceBio LC/Q-TOF systems that enables electron capture dissociation capabilities. The ExD cell is controlled using the ExDControl software, which is stand-alone software operated alongside MassHunter Acquisition to control ExD cell voltages and filament heating current. ExDControl features an autotune algorithm that automatically adjusts ExD cell voltages to optimize function for transmission or electron capture dissociation (ECD).

The following steps were used to set up the ExD cell isobar analysis. First, an appropriate filament heating current was established and allowed to warm up for 20 minutes. Next, the melittin/tune mix standard was infused from bottle B. An ExDControl autotune was performed on tune mix ions to adjust the ExD cell lens voltages to maximize MS1 transmission.

After optimizing transmission, the melittin 3+ precursor (*m/z* 949) was isolated. ExD cell voltages were optimized for fragmentation by performing an autotune on melittin fragment masses. A cell voltage profile optimized for ECD of melittin works effectively for most peptides, however, additional tuning standards may be needed for optimal ECD of proteins. For ubiquitin, an autotune was performed on a built-in mass list of ubiquitin ECD fragments to maximize the intensities of ubiquitin fragments (Table 2). After establishing MS1 transmission and MS2 ECD profiles in ExDControl, MassHunter was switched to the acquisition context, and targeted acquisition methods were created.

Return to Contents Section

Targeted analysis with ExDViewer

ExDViewer is a freely available software tool that enables confident analysis of all polypeptide fragment types in Q-TOF MS/MS data. ExDViewer provides a quick and intuitive method for analyzing complex fragmentation patterns from peptides or large, intact proteins. A key feature of the software is the support for annotating side chain fragmentation patterns and isoAsp ions.

The targeted deconvolution workflow is used to match fragmentation patterns against a known sequence. Target sequences are defined using the target editor where the sequence and minimum and maximum charge states are specified. After defining a target, .d files can be loaded directly into ExDViewer for targeted deconvolution. Figure 1 shows a screenshot of the input page of the target deconvolution workflow. Profile data featured in this document were processed using the restrictive matching presets for ExD fragmentation analysis with iterative multi-pass matching for overlapping ions.

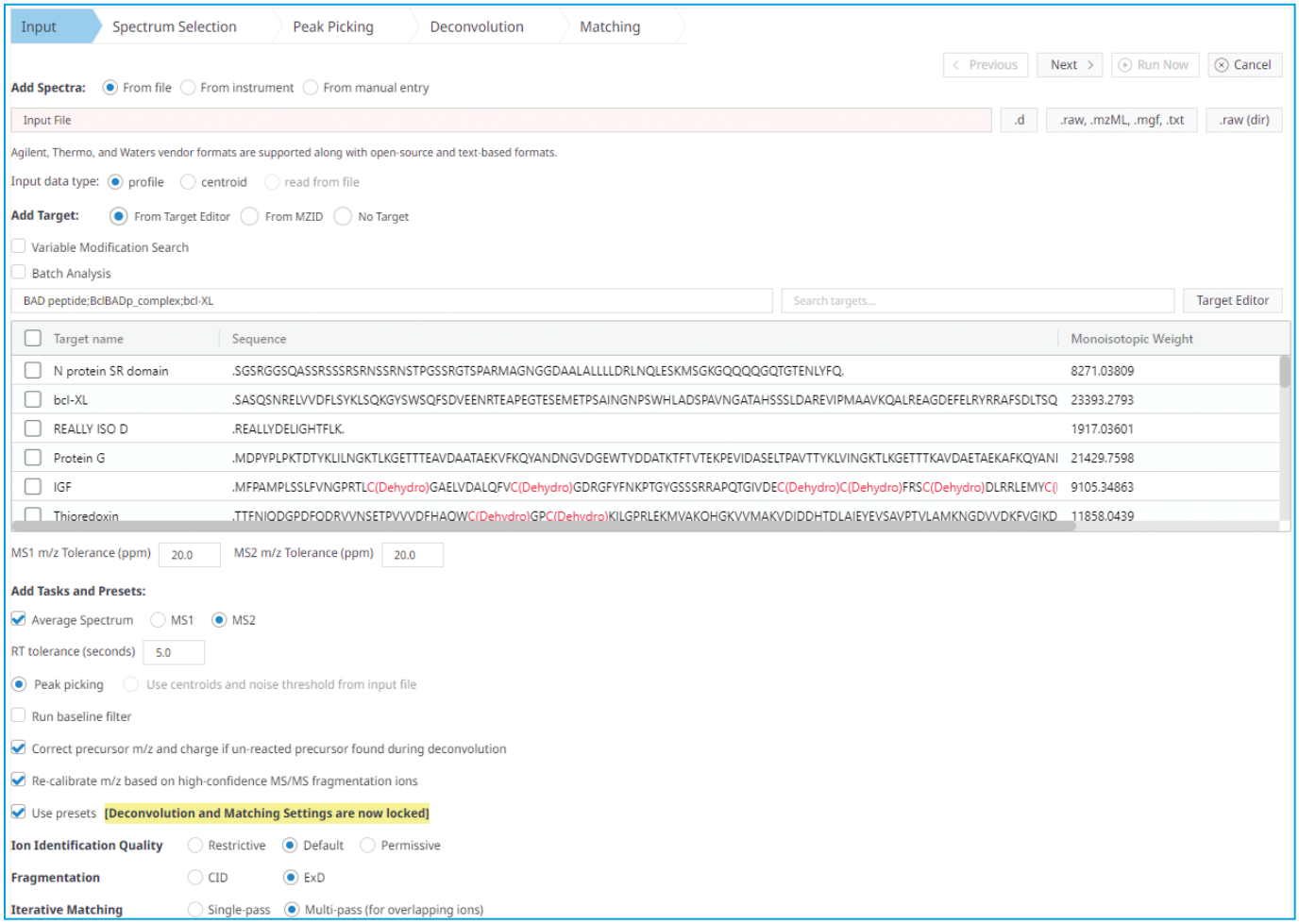

Figure 1. The ExDViewer targeted deconvolution input page. Preset match settings work well for a wide range of peptide and protein analytes.

Return to Contents Section

Results and discussion

Leu/Ile and Asp/isoAsp identification

Radical-driven side chain fragmentation leads to the formation of w-type ions, which enable Leu/Ile differentiation. Leu w-ions are formed by a radical loss of an isopropyl (z – 43 Da) group while the corresponding w-ion for Ile involves the loss of an ethyl radical (z – 29 Da).

In contrast, isoAsp formation is detected based on a structural change involving the peptide backbone, which results in the shift of 57 Da from the corresponding c or z ion for Asp (c + 57 Da, z – 57 Da).

Here, a synthetic peptide with the sequence REALLYisoDELIGHTFLK was used to demonstrate Leu/Ile and Asp/isoAsp identification using electron-based fragmentation (Figure 2).

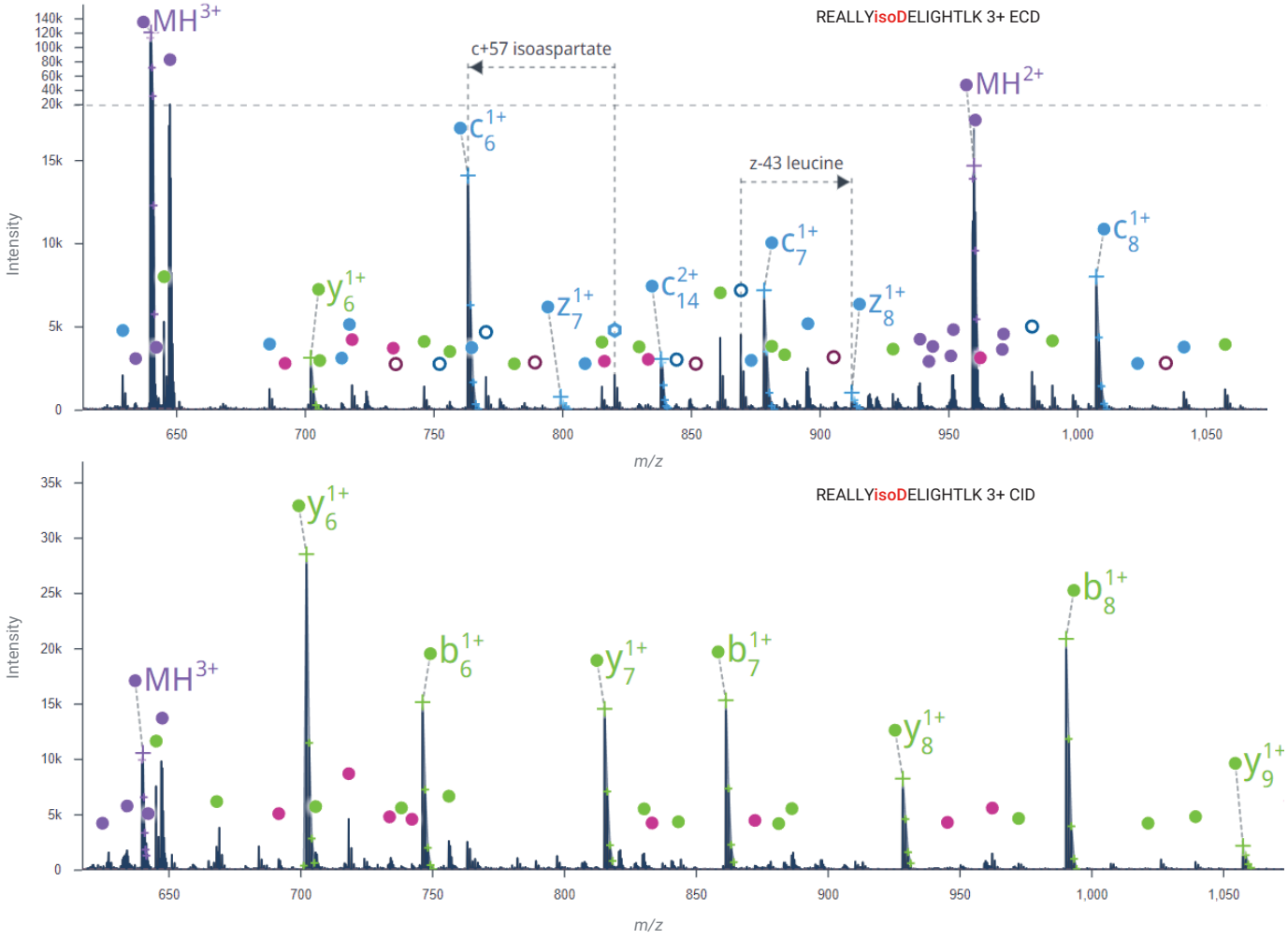

Figure 2. The top mass spectrum is an ECD fragmentation spectrum of the synthetic peptide REALLYisoDELIGHTFLK (1 µM). The Asp has been engineered to an isoAsp and is highlighted in red. Isobar fragment evidence is annotated with text in the spectrum. The bottom figure shows the CID mass spectrum for the same peptide. ECD type fragments are labeled in blue, CID type fragments are labeled in green, precursor ions are labeled in purple, and a-ions are in pink.

Return to Contents Section

REALLYisoDELIGHTFLK has five isobaric Leu/Ile residues and one engineered isoAsp, which yields 64+ possible isobaric sequences with identical theoretical CID spectra. Side chain fragmentation detected with electron fragmentation uniquely enables the identification of the exact peptide sequence with isobar specificity. Figure 2 compares the MS/MS spectrum of the 3+ REALLYisoDELIGHTFLK precursor using ECD or CID fragmentation. Using ExDViewer, diagnostic ions for Leu, Ile, and isoAsp are intuitively annotated in the spectrum.
The charge-reduced precursors are also annotated.

In Figure 3, the effect of collisional activation before ECD on the detection of diagnostic ions for Leu/Ile and isoAsp was investigated. A range of collision energies was applied to the 3+ REALLIYisoDELIGHTFLK precursor before electron capture. Overall, ion intensities went down with increasing collision energy. The 3+ precursor had a more gradual decline in intensity compared to the 4+ precursor which was more sensitive to collisional activation. These results suggested that using ECD alone was most effective for detecting diagnostic ions for this example peptide.

Top-down MS/MS isobar determination

In contrast to traditional peptide analysis, top-down mass spectrometry involves the sequencing of an entire protein without pre-enzymatic digestion. Avoiding enzymatic digestion reduces sample preparation steps, saving time while minimizing the risk of artifact introduction. Importantly, top-down analysis enables the characterization of unique proteoforms that are unable to be defined using a mixture of digested peptides. Here, top-down analysis was employed to characterize isobars in intact ubiquitin. 10 µM ubiquitin was directly infused and analyzed using a targeted acquisition method. The 11+ precursor was selected for isolation and subjected to ECD in the ExD cell. Data were collected at 1 spectrum/second for a total of 1 minute. Highly efficient fragmentation of the 11+ precursor resulted in 99% sequence coverage and the differentiation of 10/16 of isobaric Leu and Ile residues. Additionally, isoAsp c and z ions were identified that suggest the presence of isoAsp in amino acid positions 39 and 52. Figure 4 shows the sequence coverage map generated in ExDViewer, which enables the user to explore the fragment evidence supporting a target sequence interactively. Figure 5 highlights a *m/z* 350 wide slice of the ubiquitin fragmentation spectrum featuring abundant ECD ions and w-ions for Leu and Asp identification.

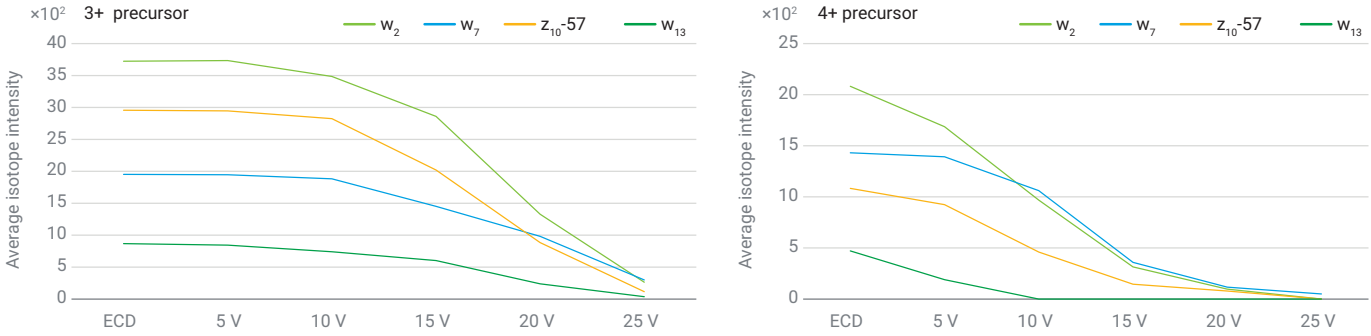

Figure 3. REALLYisoDELIGHTFLK isobar diagnostic ion intensities. Average ion intensities for w2, w7, z10 -57, and w13 were plotted as a function of collision energy.

Return to Contents Section

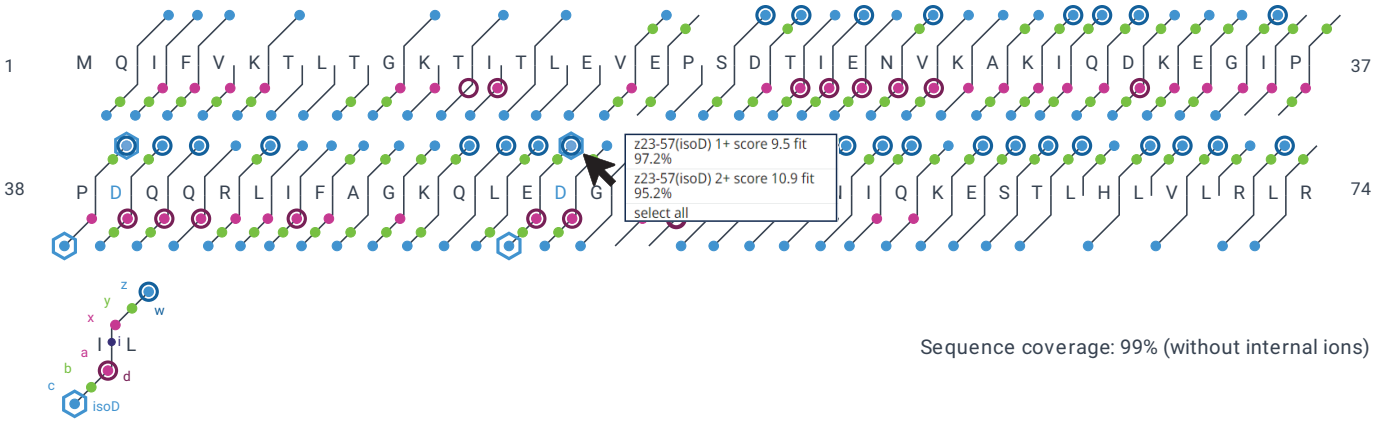

Figure 4. Sequence coverage map generated in ExDViewer. Each color dot represents a different type of ion. CID and ECD type backbone fragments ions are annotated along with side chain fragmentation and c/z isoAsp ions. Informative tool tips indicate the type of ion, charge states, and score for each fragment detected. Here, the tool tip is indicating that there were two charge states detected for the z23 -57 isoAsp ion.

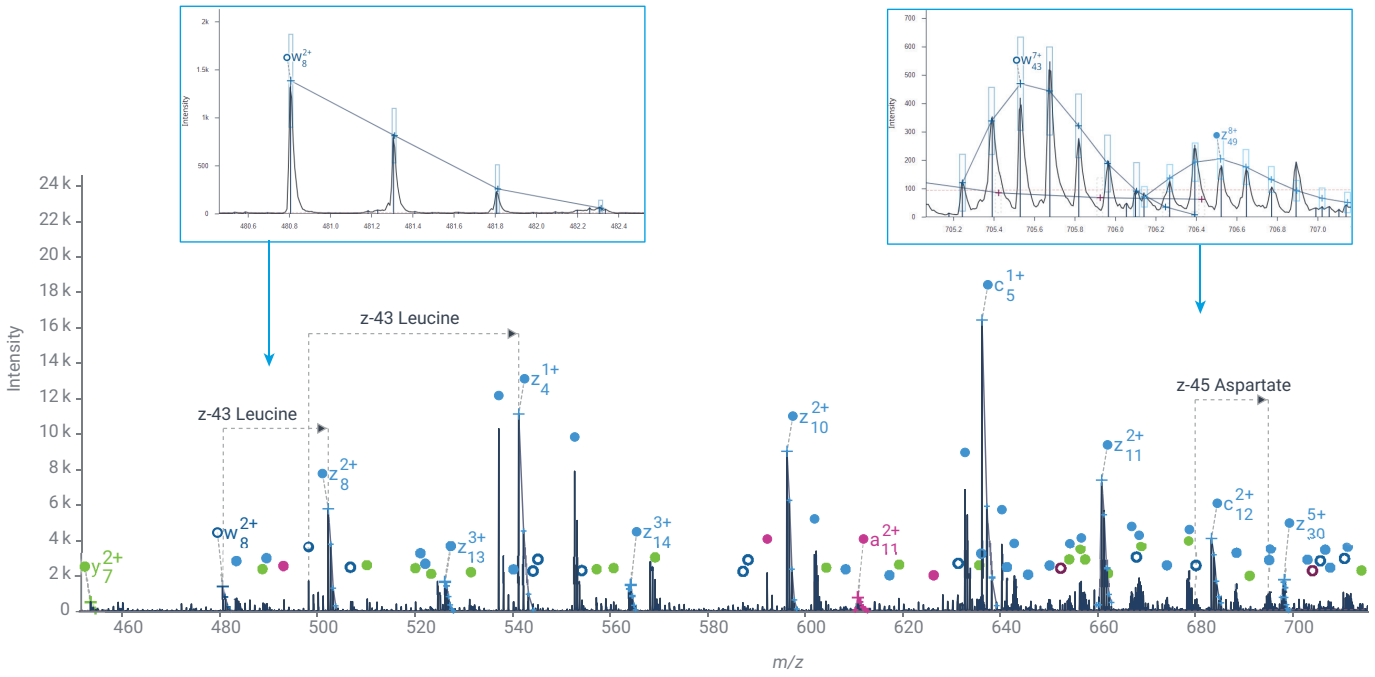

Figure 5. The ExD fragmentation spectrum of 11+ ubiquitin. Side chain fragment evidence identifying Leu and Asp is featured in the insets. Isobar evidence is automatically annotated with dashed lines and labels.

Return to Contents Section

Conclusion

This application note describes the analysis of isobaric amino acids using an Agilent 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent ExD cell and the Agilent ExDViewer software tool for analysis. Electron capture dissociation is a powerful fragmentation technique that provides complementary information to CID fragmentation. These methods can be applied to investigate peptide backbone and side chain fragmentation for a range of molecules, reducing ambiguity in protein sequence analysis. The sensitivity of the Q-TOF to detect ECD fragments combined with ExDViewer for fragment analysis provides an effective solution for targeted amino acid isobar identification.

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Return to Contents Section

Purification Solutions

Introduction

Therapeutic peptides are commonly manufactured via solid-phase (SPPS) or liquid-phase peptide synthesis (LPPS), recombinant expression in host organisms or combinations thereof. This process generates the crude peptide, a complex mixture containing the target molecule along with various synthesis-related impurities, including deletion or truncated sequences, modified peptides, residual reagents, or host-cell contaminants. These impurities must be effectively removed to ensure quality, safety, efficacy, and regulatory compliance of the final product.

Analytical reversed-phase liquid chromatography (RPLC) of the crude peptide is typically the first step in assessing the success of the synthesis. This analysis provides critical information about the identity, purity, and elution characteristics of the product and informs the design of the downstream purification strategy. Ion-paring RPLC (IP-RPLC), particularly using trifluoroacetic acid (TFA), remains the gold standard for both analytical and preparative purposes due to its ability to separate structurally similar compounds. Ultimately, the choice of purification mode depends on the peptide's physicochemical properties and impurity profile, which may require a combination of orthogonal techniques to achieve the desired purity and yield.

Scaling up from analytical to preparative LC presents several analytical challenges, including peptide solubility issues, non-specific adsorption to surfaces, poor recovery due to aggregation or hydrophobic interactions, and variability in retention behavior. Additionally, high resolving power, method robustness, and reproducibility are required to distinguish the product from highly similar impurities or degradation products. These factors require careful optimization of column chemistry, particle pore size, mobile phase composition, flow rate, and loading capacity to ensure scalability without compromising purity or yield.

Therapeutic peptides are produced via solid-phase (SPPS) or liquid-phase peptide synthesis (LPPS), recombinant expression, or hybrid approaches. These processes yield a crude peptide containing the target molecule alongside synthesis-related impurities such as truncated sequences, modified peptides, residual reagents, or host-cell contaminants, all of which must be removed to ensure quality, safety, efficacy, and regulatory compliance.

Analytical RPLC is typically the first step in evaluating synthesis success, providing critical data on identity, purity, and elution profile to guide downstream purification. IP-RPLC, especially with TFA, remains the gold standard for both analytical and preparative use due to its ability to resolve structurally similar species. Purification mode selection depends on the peptide's physicochemical properties and impurity profile, often requiring orthogonal techniques to achieve target purity and yield.

Scaling from analytical to preparative LC poses challenges such as solubility limitations, non-specific adsorption, aggregation, hydrophobic interactions, and retention variability. Achieving high resolution, robustness, and reproducibility requires optimization of column chemistry, pore size, mobile phase, flow rate, and loading capacity to enable efficient scale-up without compromising product quality. This section outlines strategies for effective method transfer from analytical to preparative LC to deliver high-purity peptides while minimizing development time and cost.

Return to Contents Section

Workflow Ordering Guide: Analysis and Purification of Synthetic Peptides by Liquid Chromatography

Synthetic peptide therapeutics have become increasingly important in drug development research because of their increased efficacy, specificity, and lower toxicity. Peptides play a critical role in a wide variety of biological pathways, from pathway signaling to enzyme regulation and cell communication.

Synthetic peptides are designed to replicate the function of naturally occurring peptides or proteins in the body that play important roles in these various biological functions. They can also be engineered to target new functions and can be further modified structurally to increase stability and bioavailability to improve the overall therapeutic efficacy.

Return to Contents Section

Techniques for purification of peptides

Peptides are short amino acid polymers ranging in sequence length from 2 to 50 amino acids. Many synthetic peptides are produced using solid-phase peptide synthesis (SPPS), which involves the step wise addition of amino acids to a peptide chain that is anchored to a polymeric solid support resin. The carboxyl group on one amino acid reacts with the amino group of another to form a peptide bond. The process of creating synthetic peptides through SPPS includes multiple deprotection, activation, and coupling steps, followed by the cleavage of the final sequence from the solid support. The crude final product contains impurities that can affect the safety and efficacy of the drug, and thus must be separated, characterized, and monitored by liquid chromatography.

Reversed-phase high-performance liquid chromatography (HPLC) coupled with UV detection is an established approach for this separation. Trifluoroacetic acid (TFA) is a common mobile phase additive and ion-pair reagent used for this analysis, creating a low pH environment that protonates the carboxyl groups of the amino acid sequence reducing secondary interactions and promoting better chromatographic separation and peak shapes, which is critical when looking to scale up your analytical methods for preparative purification. TFA can also form ion-pairs with positively charged functional groups in the peptide sequence, increasing the peptide's overall hydrophobic interaction with the non-polar reversed-phase chromatographic media. For confirmation and characterization of the purified fractions with mass spectrometry (MS) detection, TFA, however, can inhibit ionization efficiency and cause signal suppression. An alternative LC/MS method using formic acid is ideal for final product characterization.

Agilent reversed-phase columns for peptide purification

Agilent offers a variety of reversed-phase columns and media designed to simplify your synthetic peptide analytical workflows. Agilent PLRP-S reversed-phase columns contain rigid polystyrene/divinylbenzene (PS/DVB) particles that are available in a range of pore and particle sizes, making it an ideal particle chemistry for analytical peptide separations that can be easily scaled up to preparative purification. The polymeric particle is inherently hydrophobic and free from surface silanols and trace metals found in traditional silica-based particle supports that can cause unnecessary secondary interactions with charged functional groups in peptide sequences, leading to band broadening and peak tailing.

For confirmation analysis where MS characterization is needed, Agilent AdvanceBio Peptide Mapping and AdvanceBio Peptide Plus columns, with the high-resolution 2.7µm Agilent Poroshell particle support, are ideally suited for LC/MS separations where formic acid is used as an alternative mobile phase additive for TFA. The AdvanceBio Peptide Mapping column utilizes a C18 chemistry that has been especially designed to ensure suitability and robustness with peptide separations. For more challenging peptide separations, where secondary interactions with surface silanols cause peak broadening and tailing, the AdvanceBio Peptide Plus column chemistry was designed with an alternative C18 selectivity by incorporating a charged surface modification that helps reduce those unwanted secondary interactions for improved peak shape and resolution.

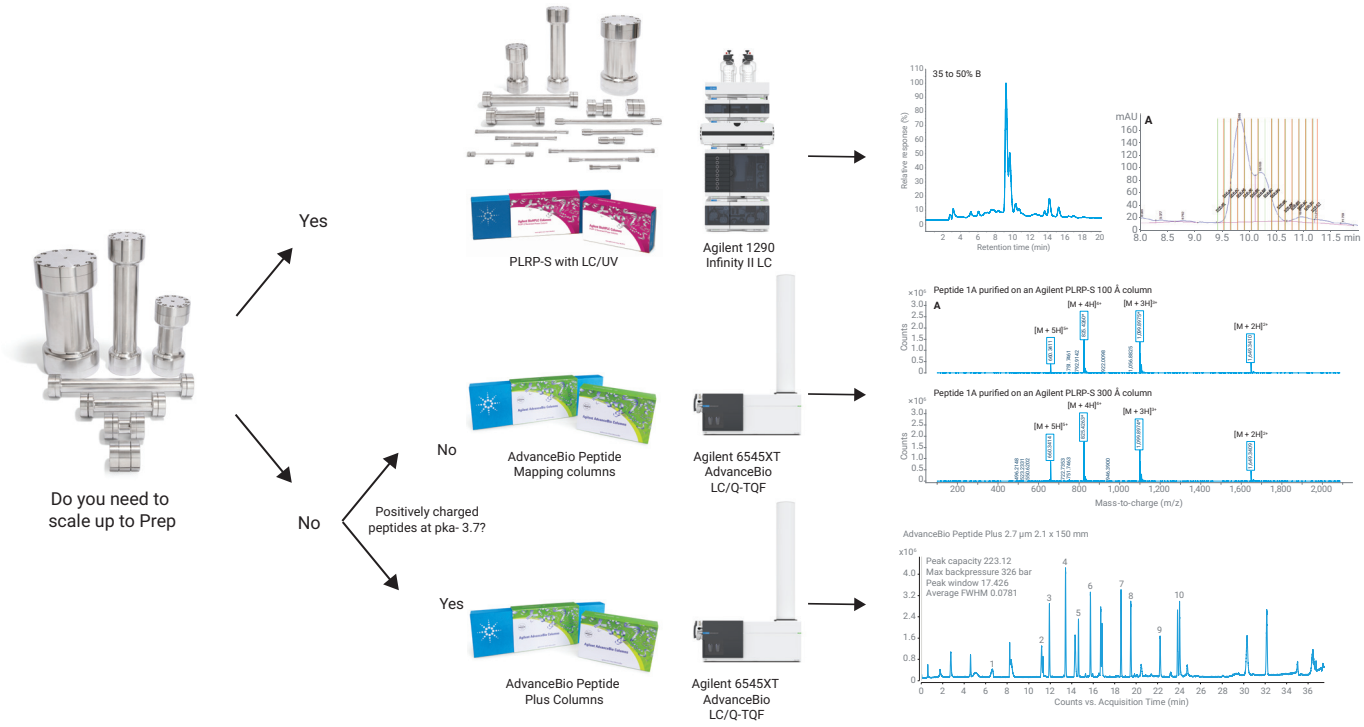

Figure 1. Choosing an analytical workflow for synthetic peptides depends on the scale of separation and need to characterize the sample with MS detection.

Return to Contents Section

Tips for optimal chromatographic separation and detection

Sample preparation

- Agilent StratoSpheres product line of high-performance and high-quality resins[1] represent a wide variety of polymer supports for the development and manufacture of synthetic peptides through solid support peptide synthesis pathways.
- Compatible with Boc and Fmoc chemistries, StratoSpheres SPPS resins provide superior synthesis performance for higher-purity peptides.
- Solid support resins allow for simplified cleanup of process related impurities and side products, simplifying the complexity of the crude sample (Figure 2).

Chromatographic separation UV detection

- The choice of UV detection in your workflow allows for the use of TFA as a mobile phase additive. With a pKa ~ 0.23, TFA will lower the pH to protonate carboxyl functional groups on your peptide and residual silanols of the silica surface, to reduce unwanted secondary interactions that lead to peak broadening and tailing.
- The PLRP-S column portfolio offers flexibility of choice of particle and pore size. Evaluate different pore sizes (100 Å or 300 Å) depending on the sequence length of your synthetic peptide.
- Start your analytical method optimization[2] with an initial generic scouting gradient from 5% to 95% MPB to understand the elution profile of your target product and what impurities are still in your crude sample.
- A more focused gradient can be developed to shorten your overall analysis time and optimize the resolution between the full-length target product and any closely related impurities.
- Once an optimized separation method has been developed at the analytical scale, your method can be easily scaled up to larger inner diameter columns to allow for purification of larger sample sizes.
- It is critical to optimize your gradient on an analytical column dimension before scaling up to prep, as resolution will dictate the purity and sample size that can be purified at the prep scale.

Chromatographic separation: MS detection

- The choice of MS detection in your workflow limits the ability to use TFA as a mobile phase additive. TFA will affect the ionization efficiency of your sample and cause signal suppression, which can inhibit your ability to detect and characterize potential low-abundance impurities in your sample. Formic acid is a more compatible acidic modifier for MS detection, but with a higher pKa (~ 3.7), it can affect how your sample interacts with the stationary phase compared to TFA.
- AdvanceBio Peptide Mapping and AdvanceBio Peptide Plus column chemistries[3-4] on the 2.7 µm Poroshell particle support allow for high-resolution separations.
- Start your analytical method optimization with an initial generic scouting gradient from 5% to 95% MPB to understand the elution profile of your target product and what impurities are still in your crude sample.
- If the peptide has a high degree of positively charged functional groups in its sequence and the separation on the AdvanceBio Peptide Mapping column shows peak broadening and tailing, this may be due to interactions from the sample with residual surface silanols. Evaluate the AdvanceBio Peptide Plus column.
- After deciding on a column chemistry, a more focused gradient can be developed to shorten your overall analysis time and optimize the resolution between the full-length target product and any closely related impurities.

Mass spectrometry

- Do not use TFA or phosphate-containing buffers with MS detection!
- Divert the LC stream to waste outside of the retention time(s) of interest, especially during a high organic rinse at the end of the method and, if possible, as the void volume elutes.
- Use HPLC or higher-grade solvents.
- Establish a regular cleaning routine for the MS source.

Return to Contents Section

Sample method and chromatogram for each method

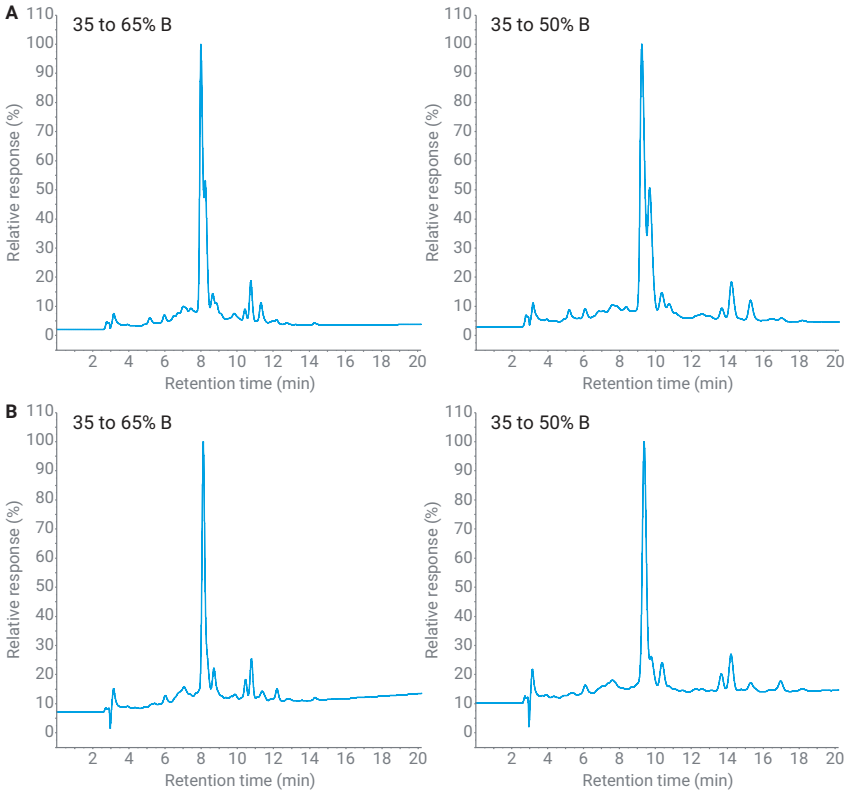

Figure 2. (A) Gradient optimization of peptide 1A on an Agilent PLRP-S 300 Å column. (B) Gradient optimization of peptide 1B on an Agilent PLRP-S 300 Å column.

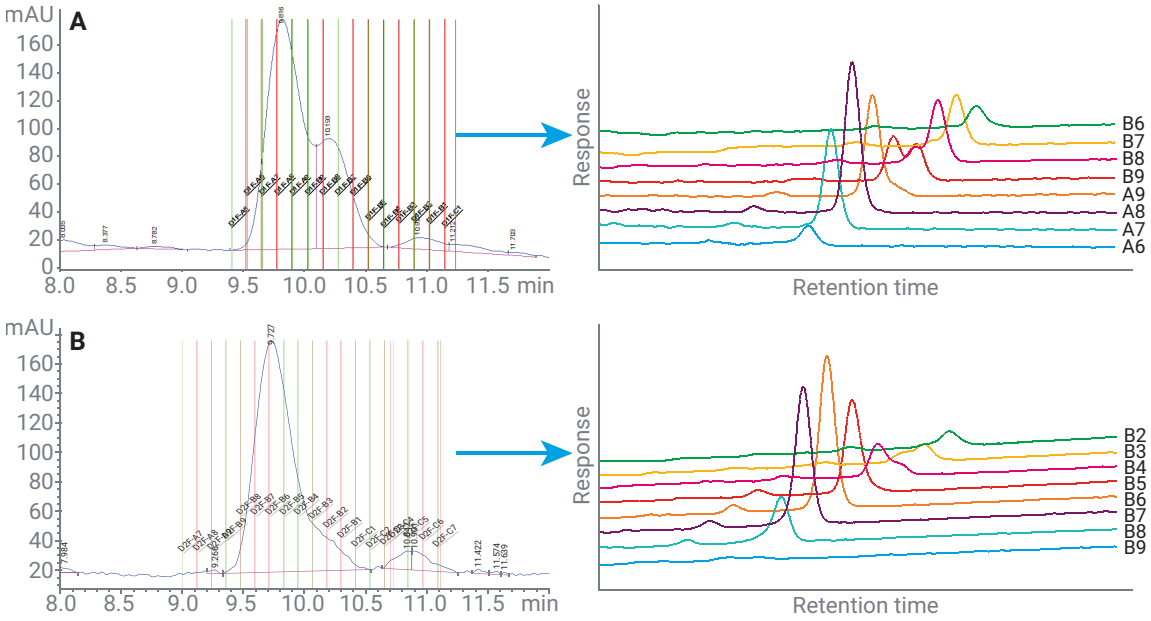

Figure 3. (A) Peptide 1A on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right). (B) Peptide 1B on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right).

Return to Contents Section

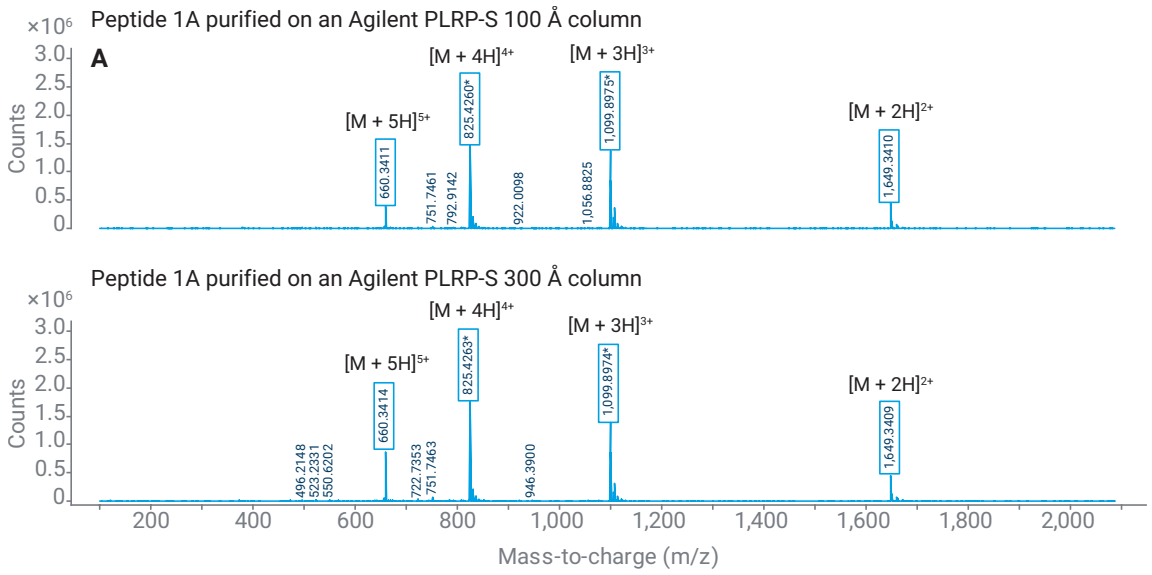

Figure 4. Mass spectral results of purified peptides analyzed by LC/MS on an Agilent AdvanceBio Peptide Mapping column (for method conditions see reference 2).

References

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3. Analysis of a Synthetic Peptide and its Impurities, 5994-2760EN
4. Agilent AdvanceBio Peptide Plus 2.7 µm Column for Peptide Characterization, 5994-3508EN

Easy selection and ordering information

To order items listed in the tables below, add items to your Favorite Products list by clicking on the MyList link in the header.

Your list will remain under Favorite Products for your use with future orders. If this is your first time using Favorite Products, you will be asked to enter your email address for account verification. If you have an existing Agilent account, you will be able to log in. If you do not have a registered Agilent account, you will need to register for one. This feature is valid only in regions that are e-commerce enabled.

Individual items can also be ordered from the Agilent online store by clicking on the part number hyperlinks or through your regular sales and distributor channels.

MyList 1: Solid-phase supports for peptide synthesis

Description	Part Number
Solid-Phase Peptide Synthesis	
AmphiSpheres 40RAM, 0.4 mmol/g, 75 to 150 µm, 100 g	PL3867-4764
PL-Rink Resin (1% DVB) 0.3 mmol/g 75 to 150 µm, 100 g	PL1467-4749

MyList 2: HPLC columns for peptide purification

Description	Part Number
PLRP-S Columns	
PLRP-S 100 Å, 8 µm, 2.1 × 150 mm	PL1512-3800
PLRP-S 100 Å, 8 µm, 2.1 × 250 mm	PL1512-5800
PLRP-S 100 Å, 8 µm, 25 × 150 mm	PL1212-3800
PLRP-S 300 Å, 8 µm, 2.1 × 150 mm	PL1912-3801
PLRP-S 300 Å, 8 µm, 2.1 × 250 mm	PL1912-5801
PLRP-S 300 Å, 8 µm, 4.6 × 150 mm	PL1512-3801
PLRP-S 300 Å, 8 µm, 4.6 × 250 mm	PL1512-5801
PLRP-S 300 Å, 8 µm, 25 × 150 mm	PL1212-3801
PLRP-S 300 Å, 8 µm, 50 × 150 mm	PL1712-3801
AdvanceBio Peptide Mapping	
AdvanceBio Peptide Mapping 120 Å, 2.7 µm, 2.1 × 100 mm	655750-902
AdvanceBio Peptide Mapping 120 Å, 2.7 µm, 2.1 × 150 mm	653750-902
AdvanceBio Peptide Plus Columns	
AdvanceBio Peptide Plus 100 Å, 2.7 µm, 2.1 × 150 mm	695775-949

Return to Contents Section

MyList 3: Supplies and solvents for HPLC purification of synthetic peptides

Description	Part Number
Ultralow Dispersion Kits‡	
Ultralow dispersion tubing kit for Agilent 1290 Infinity II LC	5067-5963
Ultralow dispersion tubing kit for Agilent 1290 Infinity II Bio LC	5004-0007
Sample Containment Supplies	
A-Line screw top vial, 2 mL, 12 × 32 mm (12 mm cap) amber, write-on spot, 100/pk	5190-9590
Screw cap, 12 mm, bonded, blue, PTFE/white silicone septa, 100/pk	5190-7021
Vial insert, 250 µL, 5.6 × 30 mm, deactivated glass with polymer feet, 100/pk	5181-8872
InfinityLab well plate 96/0.5 mL, 30/pk	5043-9310
InfinityLab well plate silicone closing mat for 96-well plates, 50/pk	5042-1389
Solvents and Additives	
InfinityLab Ultrapure LC/MS grade water, 1 L	5191-4498
InfinityLab Ultrapure LC/MS grade MeOH, 1 L	5191-4497
Formic acid, 99.5% purity, 5 mL	G2453-85060
Solvent Filtration Supplies*	
InfinityLab Solvent filtration assembly	5191-6776
InfinityLab solvent filtration flask, glass, 2 L	5191-6781
Filter membrane, Nylon 47 mm, pore size 0.2 µm, 100/pk	5191-4341
Filter membrane, regenerated cellulose 47 mm, pore size 0.2 µm, 100/pk	5191-4340
Solvent bottle glass filter, solvent inlet, 20 µm	5041-2168
Solvent Handling Supplies	
InfinityLab Stay Safe cap starter kit	5043-1222
InfinityLab solvent bottle, clear, 1 L	9301-6524
InfinityLab solvent bottle, amber, 1 L	9301-6526
Solvent bottle, clear, 2 L	9301-6342
Solvent bottle, amber, 2 L	9301-6341
InfinityLab Stay Safe purging bottle	5043-1339
InfinityLab waste can, GL45, 6 L with Stay Safe cap (charcoal filter 5043-1193 not included)	5043-1221
InfinityLab charcoal filter with time strip, 58 g (use with 5043-1221)	5043-1193

‡ If using an Agilent 1290 Infinity II Bio LC System, the ultralow dispersion tubing kit for Agilent 1290 Infinity II Bio LC is recommended.
* If using solvents other than those listed in this table, use the InfinityLab Solvent Filtration assembly prior to analysis.

Return to Contents Section

Application Note

Purification Solutions

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Optimizing Analysis and Purification of a Synthetic Peptide Using PLRP-S Columns

Reliable columns and media with the scale and pore size for successful purification of synthetic peptides

Author

Andrea Angelo P. Tripodi and
Andrew Coffey
Agilent Technologies, Inc.

Abstract

Reversed-phase ion-pair chromatography has become increasingly useful for the analysis and purification of synthetic peptides using mobile phases containing trifluoroacetic acid (TFA) as an ion-pair reagent. Scaling up from analytical to preparative high-performance liquid chromatography (HPLC) can be costly, time consuming, and often difficult to perform due to differences in chemistries, pH conditions, particle sizes, and column length. This application note describes the analysis of a synthetic peptide and how to scale up methods using an Agilent PLRP-S analytical HPLC column. The gradient and loading information are applied directly to a larger preparative column packed with an identical material.

Return to Contents Section

Introduction

Peptide therapeutics are gaining popularity because of developments in biotechnology and bioengineering, including cancer diagnosis and treatment, antibiotic drug development, and new vaccines. Most peptide drugs are produced using solid-phase peptide synthesis (SPPS). The synthesis is performed on a polymeric support or resin, which can easily be filtered from reactions. The synthetic route includes multiple deprotection, activation, and coupling steps. The final peptide sequence is separated from the resin using a cleavage cocktail containing scavengers and other components resulting in the final crude product that is ready for purification.

Crude peptides synthesized by solid-phase peptide synthesis are analyzed by HPLC using reversed-phase columns with gradient elution using aqueous acetonitrile (typically containing 0.1% trifluoroacetic acid (TFA) as the ion-pair reagent). Liquid chromatography/mass spectrometry (LC/MS) -based peptide analysis is normally used to confirm the structure of the target molecule. However, TFA is not ideal for LC/MS since it causes ion suppression, creating a weaker MS signal. The preferred ion-pair reagent for LC/MS methods is formic acid (FA), a weaker acid than TFA.

This work uses human glucagon-like peptide-1 (GLP-1) 7-36 amide, a single polypeptide chain containing 30 amino acids, which has a molecular mass of 3,297.7 Daltons (Da) (Figure 1).

This application note describes the ability to perform direct scale up from analytical PLRP-S 4.6 × 250 mm, 8 µm columns to larger scale preparative PLRP-S 21.2 × 250 mm, 8 µm columns. Two pore sizes appropriate for the separation of peptides, 100 and 300 Å, were investigated. Agilent PLRP-S is a rigid macroporous styrene divinylbenzene (PS-DVB) HPLC stationary phase with outstanding chemical and physical stability. PLRP-S HPLC media is inherently hydrophobic and does not require a bonded alkyl chain such as C8 or C18 to confer hydrophobicity. The characterization of the final product was confirmed using an Agilent 6545XT AdvanceBio liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF) with an orthogonal AdvanceBio Peptide Mapping column, 2.1 × 100 mm, 2.7 µm.

Sample preparation

Glucagon-like peptide GLP-17-36 amide was synthesized by CS Bio (Menlo Park, CA 94025, USA). The solid supports for the synthesis were provided by Agilent Technologies. The synthesis was achieved using standard side chain protection strategy and coupling conditions (fluorenylmethoxycarbonyl (Fmoc) chemistry).

Analytical equipment

An Agilent 1290 Infinity II LC system was composed of the following modules:

- Agilent 1290 Infinity II high-speed pump (G7120A)
- Agilent 1290 Infinity II multisampler with sample thermostat (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II diode array detector (G7117C) with a 10 mm InfinityLab Max-Light Cartridge Cell (G7117-60020)

Preparative equipment

An Agilent 1290 Infinity II preparative LC system was composed of the following modules:

- Agilent 1290 Infinity II preparative binary pump (G7161B)
- Agilent 1260 Infinity II fraction collector (G7157A)
- Agilent 1290 Infinity II preparative column compartment (G7163B)
- Agilent 1260 Infinity II diode array detector (G7165A)

LC/MS equipment

Agilent 1290 Infinity II LC system coupled to the 6545XT AdvanceBio LC/Q-TOF (G6549AA)

Software and data processing

- Agilent OpenLab software suite, version 2.6
- OpenLab ChemStation CDS, version C01.09
- Agilent MassHunter data workstation acquisition, v ersion B10.00
- Agilent MassHunter BioConfirm software, version 10.00

H-His-Ala-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Val-Ser-Ser-Tyr-Leu-Glu-Gly-Gln-Ala-Ala-Lys-Glu-Phe-Ile-Ala-Trp-Leu-Val-Lys-Gly-Arg-NH_2

Figure 1. The amino acid sequence of synthetic GLP-1 (7-36) amide.

Return to Contents Section

Columns

- **Analytical columns:** Agilent PLRP-S 100 Å, 4.6 × 250 mm, 8 µm (part number PL1512-5800); Agilent PLRP-S 300 Å, 4.6 × 250 mm, 8 µm (part number PL1512-5801)
- **Preparative columns:** Agilent PLRP-S 100 Å, 21.2 × 250 mm, 8 µm; Agilent PLRP-S 300 Å, 21.2 × 250 mm, 8 µm (custom dimension)
- **LC/MS column:** AdvanceBio Peptide Mapping 2.1 × 100 mm, 2.7 µm (part number 655750-902)

Solid support

- AmphiSpheres 40 RAM 0.4 mmol/g 75 to 150 µm (part number PL3867-4764)
- PL-Rink Resin (1% DVB) 0.3 mmol/g 75 to 150 µm (part number PL1467-4749)

Reagents and chemicals

All reagents were HPLC grade or higher.

Method conditions

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II Analytical LC System	
Parameter	**Value**
Column	Agilent PLRP-S, 4.6 × 250 mm, 8 µm
Thermostat	4 °C
Solvent A	0.1% TFA in water
Solvent B	0.1% TFA in acetonitrile
Gradient	Gradient 1: Time (min) %B 0 to 2 35 2 to 22 35 to 50 22 to 24 50 to 90 24 to 28 90 28 to 30 90 to 35 30 to 36 35 Gradient 2: Time (min) %B 0 to 2 35 2 to 22 35 to 65 22 to 24 65 to 90 24 to 28 90 28 to 30 90 to 35 30 to 36 35
Column Temperature	25 °C
Flow Rate	1.0 mL/min
Injection Volume	5.0 µL
Agilent 1290 Infinity II Preparative LC System	
Column	Agilent PLRP-S, 21.2 × 250 mm, 8 µm
Thermostat	4 °C
Solvent A	0.1% TFA in water
Solvent B	0.1% TFA in acetonitrile
Gradient	Time (min) %B 0 to 2 35 2 to 22 35 to 50 22 to 24 50 to 90 24 to 28 90 28 to 30 90 to 35 30 to 45 35
Column Temperature	Ambient
Flow Rate	21.2 mL/min
Injection Volume	100 µL
Fraction Collection	2.5 mL fractions; time based

Table 2. LC/MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Parameter	**Value**
Source	Dual AJS
Polarity	Positive
Gas Temperature	325 °C
Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Skimmer	65 V
Acquisition Mode	2.5 Hz
Mass Range	100 to 2,100 *m/z*
Acquisition Rate	5 spectra/s
Agilent 1290 Infinity II LC System	
Column	AdvanceBio Peptide Mapping, 2.1 × 100 mm, 2.7 µm
Thermostat	4 °C
Solvent A	0.1% Formic acid in water
Solvent B	0.1% Formic acid in acetonitrile
Gradient	Time (min) %B 0 to 2 3 2 to 23 3 to 47 23 to 25 47 to 50 25 to 26 50 to 97 26 to 27 97 to 3 27 to 30 3* *isocratic (postrun)
Column Temperature	55 °C
Flow Rate	0.3 mL/min
Injection Volume	20 µL

Return to Contents Section

Results and discussion

Two resins were used for the synthesis of the target GLP-1 7-36 amide peptide. The first, AmphiSpheres 40 RAM 0.4 mmol/g 75 to 150 µm, contains a polyethylene glycol chain to improve the performance of the resin for difficult peptides. The second, PL-Rink resin (1% DVB) 0.3 mmol/g 75 to 150 µm is lower loading and suitable for the synthesis of longer peptide chains.

The synthesis was performed under identical conditions and resulted in two crude peptides: peptide 1A (from the AmphiSpheres resin) and peptide 1B (from the PL-Rink resin).

Purification of peptides usually requires a pore size of 100 or 300 Å. This pore size maximizes the loading capacity while minimizing restricted access or exclusion of larger molecules and retaining the desired mass transfer to achieve best separation.

Analytical chromatography of the crude peptides is necessary as a starting point to confirm the presence of the desired molecule and understand the elution characteristics. Initially the elution profile of the two samples was unknown, so different gradient methods were screened (Figures 2A, 2B, 3A, and 3B), and the best (35 to 50 %B) was chosen for the preparative runs. For actual method conditions, see Table 1.

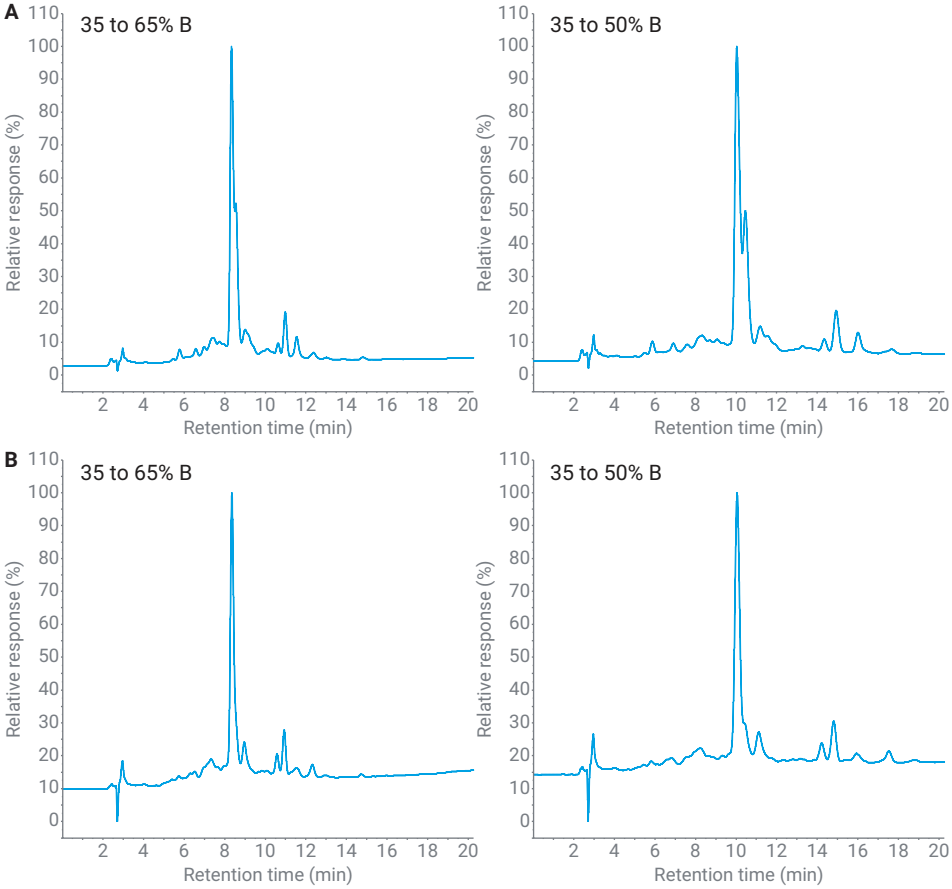

Figure 2. (A) Gradient optimization of peptide 1A on an Agilent PLRP-S 100 Å column. (B) Gradient optimization of peptide 1B on an Agilent PLRP-S 100 Å column.

Return to Contents Section

It is evident that peptide 1B (prepared using PL-Rink resin 0.3 mmol/g) resulted in higher crude purity than AmphiSpheres 40 RAM in this example (Table 3).

Table 3. Crude peptide purity.

	Peptide 1A	Peptide 1B
PLRP-S 100 Å	33.15%	43.19%
PLRP-S 300 Å	41.23%	46.53%

The preparative scale separation was performed by injecting 100 µL of crude peptide at a concentration of 1 mg/mL dissolved in mobile phase A (containing water with 0.1% TFA). A total amount of 1 mg was purified on both PLRP-S 100 Å and PLRP-S 300 Å columns with a 21.2 mm id scale up from 4.6 to 21.2 mm id column dimensions (Figures 4 and 6).

The fraction collector was set to collect the full-length product (FLP) using fixed 2.5 mL volume fractions over the time period that the main peak eluted. The product and any closely eluting impurities could easily be identified by reanalyzing the appropriate fractions on the analytical columns (Figures 5 and 7).

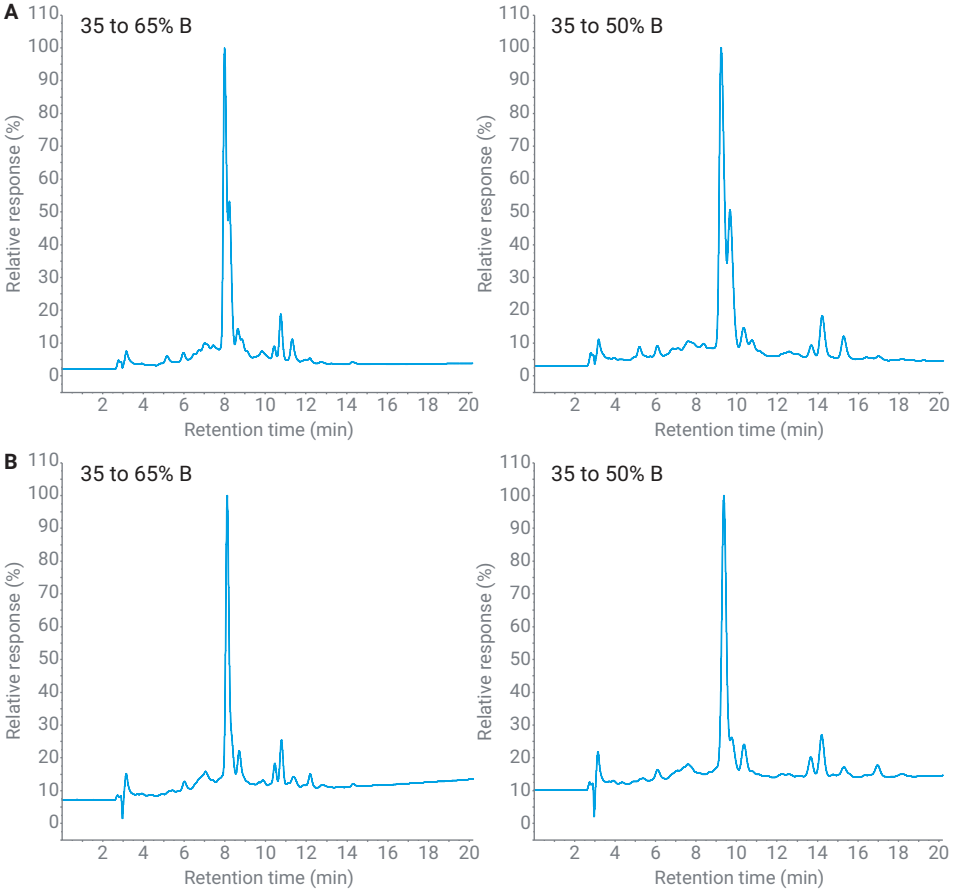

Figure 3. (A) Gradient optimization of peptide 1A on an Agilent PLRP-S 300 Å column. (B) Gradient optimization of peptide 1B on an Agilent PLRP-S 300 Å column.

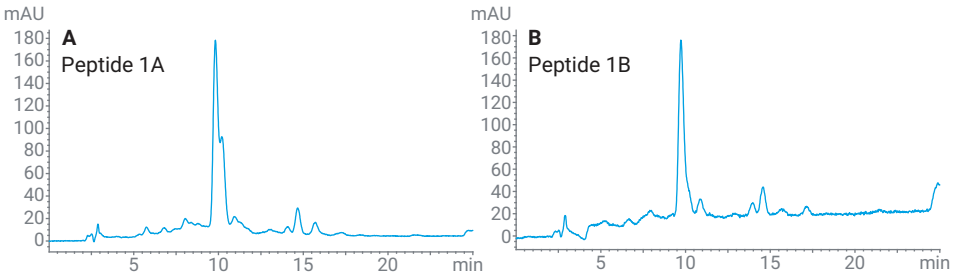

Figure 4. Preparative chromatograms of peptide 1A (A), and peptide 1B (B) on an Agilent PLRP-S 100 Å column.

Return to Contents Section

Overall purity level is calculated from the peak area percentage of each fraction (Table 4).

Table 4. Summary of purity and yield from combination of fractions.

Peptide 1A	Area% (Purity)	Overall Yield%
PLRP-S 100 Å (Fractions A6-B9)	89.28	85.59
PLRP-S 300 Å (Fractions B8-B5)	90.26	73.02
Peptide 1B	**Area% (Purity)**	**Overall Yield%**
PLRP-S 100 Å (Fractions B8-B4)	97.81	92.69
PLRP-S 300 Å (Fractions D7-D3)	90.55	90.93

LC/MS analysis of the main component purified was performed on an AdvanceBio Peptide Mapping column to confirm its identity.

Synthetic peptide samples can often contain a high number of different molecular weights, impurities, missing amino acids in the sequence, loss of water, sometimes the protecting groups from the synthesis can still be attached to our target molecule in case of unsuccessful cleavage from the solid support. It is therefore critical that analytical methods for synthetic peptides cover a wide range of potential impurities. The major component from the purest fraction gave the expected $[M + 5H]^{5+}$ at *m/z* 660.34, $[M + 4H]^{4+}$ at *m/z* 825.42, $[M + 3H]^{3+}$ at *m/z* 1,099.89, and $[M + 2H]^{2+}$ at *m/z* 1,649.34. The results correspond to the full-length amino acids sequence of (GLP-1) 7-36 amide of 3,297.7 Da (Figure 8). For method conditions and instrument parameters, see Table 2.

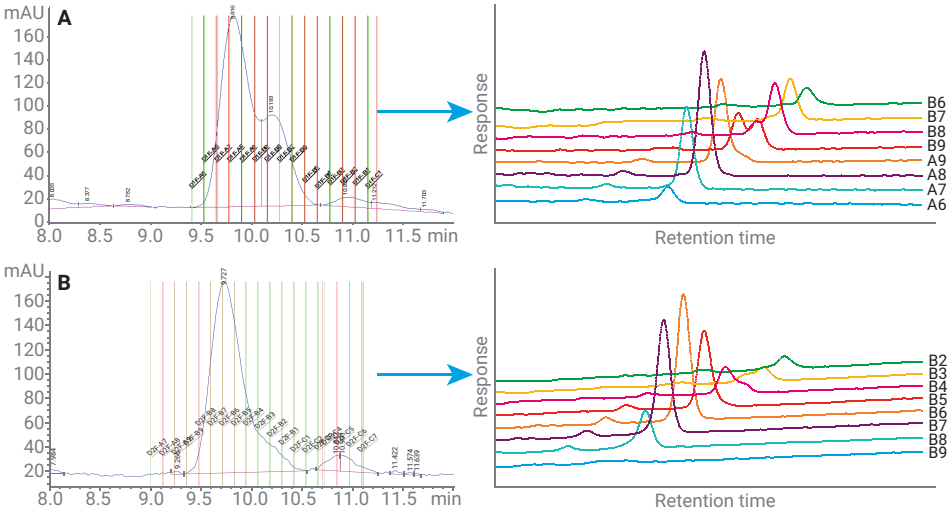

Figure 5. (A) Peptide 1A on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right). (B) Peptide 1B on an Agilent PLRP-S 100 Å column showing fraction reanalysis (right).

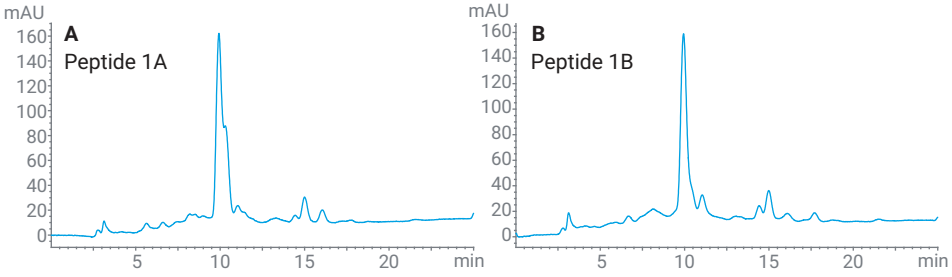

Figure 6. Preparative chromatograms of peptide 1A (A), and peptide 1B (B) on an Agilent PLRP-S 300 Å column.

Return to Contents Section

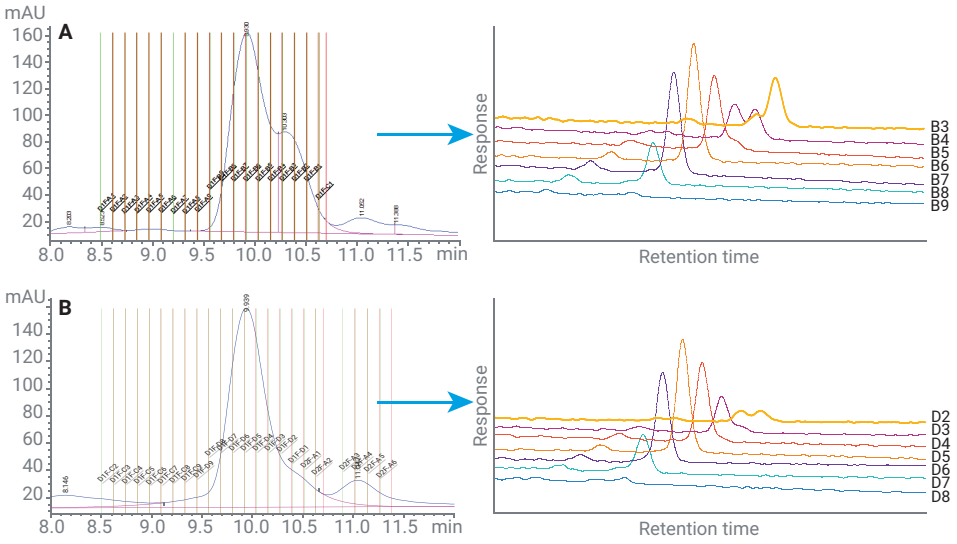

Figure 7. (A) Peptide 1A on an Agilent PLRP-S 300 Å column showing fraction reanalysis (right). (B) Peptide 1B on an Agilent PLRP-S 300 Å column showing fraction reanalysis (right).

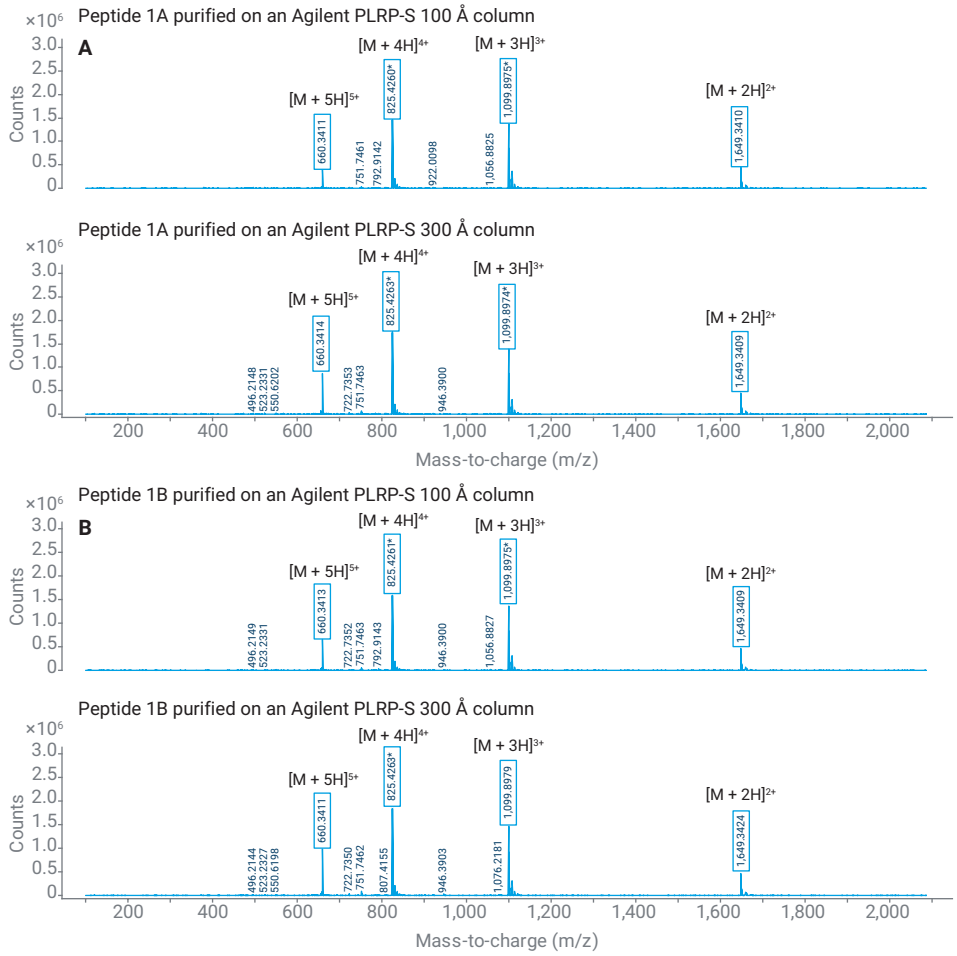

Figure 8. Mass spectral results of purified peptides analyzed by LC/MS on an Agilent AdvanceBio Peptide Mapping column (for method conditions, see Table 2).

Return to Contents Section

Conclusion

This application note demonstrates that crude peptide purity may depend on synthesis conditions, including choice of solid phase resin. However, Agilent PLRP-S columns are an ideal choice for ion-pair reversed-phase purification of synthetic peptides. The smaller pore size 100 Å particles with higher available surface area can potentially offer higher purification capacity. Wider pore sizes can lead to better mass transfer and sharper peaks for larger species.

By coupling the Agilent PLRP-S preparative HPLC columns to the Agilent 1290 Infinity II preparative LC system, you can achieve efficient separations. Finally, for the LC/MS method, an Agilent AdvanceBio Peptide Mapping column was successfully used with formic acid as a mobile phase modifier to confirm the identity of the molecule.

Application Note

Purification Solutions

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Efficient Purification of Synthetic Peptides at High and Low pH

Author

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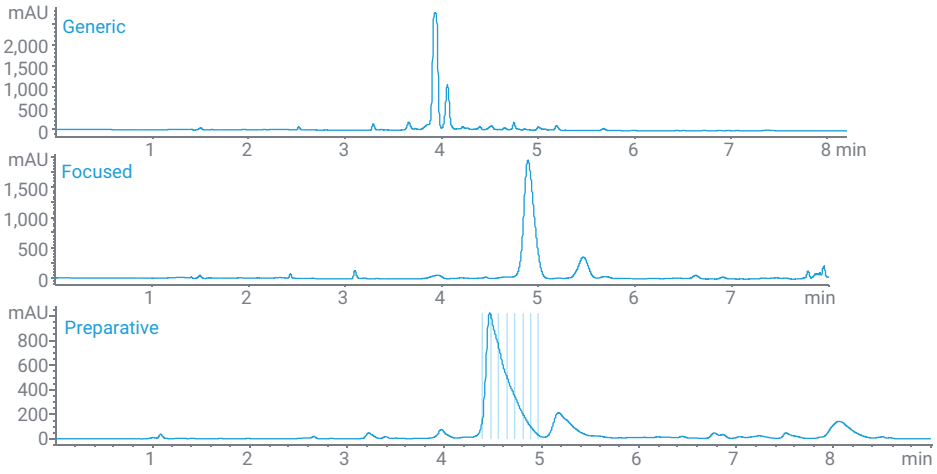
Abstract

Synthetic peptides play an increasingly important role as therapeutics. To analyze and isolate pure products from a synthesis mixture, high-performance liquid chromatography (HPLC) at low pH is an established tool. Some peptides, however, do not dissolve at low pH and thus must be separated under basic conditions. This application note presents a workflow for analytical method development and preparative purification using a single HPLC system. Valve automation enables fast and easy switching between pH modes and scales by a simple method parameter. A crude synthetic peptide is purified using both high and low pH, with automated fraction reanalysis for purity assessment. Fraction slices with purity of typically more than 95% were collected, enabling fraction pooling depending on purity requirements.



Return to Contents Section

Introduction

Since the introduction of insulin as the first therapeutic peptide in the 1920s, the number of peptide-based drugs approved by the FDA has constantly increased to about 80 in 2021.1 Already in the early development of new therapeutic peptides, potential drug candidates undergo multiple tests for activity, side effects, solubility, and more. For these tests, the compounds need to be clean from any residues from the synthesis, as well as product-related impurities. This cleanup can be done using preparative HPLC.

Owing to the amphoteric nature of amino acids, the building blocks of peptides, each synthetic peptide has a pH at which the net charge is zero. At this so-called isoelectric point, the peptide is least soluble. In solution, depending on the pH, amino acid side chains are dissociated and charged. In HPLC analyses, this behavior may cause poor peak shape due to ionic interactions with free silanols, in addition to the hydrophobic interactions with the nonpolar stationary phase. To achieve sharp signals, a low pH is typically chosen to protonate all carboxylate groups of the peptide. Many HPLC methods use trifluoroacetic acid for this purpose. Depending on the amino acid sequence of a peptide, however, it can be beneficial to adjust the pH to higher levels, for example by adding ammonia or an ammonium bicarbonate buffer.

This application note demonstrates the workflow of a peptide purification under both acidic and basic conditions. A crude desalted synthetic peptide is analyzed using an HPLC system with UV and mass selective detection (MSD). Valve automation enables automated switching between solvents and columns to run analyses in two pH ranges without hardware changes. The Agilent 1290 Infinity II Autoscale Preparative LC/MSD System further allows seamless transfer from analytical to preparative conditions. The analytical method can thus be developed on analytical scale and then transferred to preparative scale without moving to another instrument.

Experimental

Instrumentation

All experiments were conducted on an Agilent 1290 Infinity II Autoscale Preparative LC/MSD System:

- Agilent 1260 Infinity II Quaternary Pump (G7111B) with active seal wash (option #030) and active inlet valve (option #032)
- Agilent 1290 Infinity II Preparative Binary Pump (G7161B) with 200 mL pump heads (option #206)
- Agilent 1290 Infinity II Preparative Open-Bed Sampler/ Collector (G7158B)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A) with 0.3 mm preparative flow cell (option #024)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with 10 mm standard flow cell (option #018)
- Agilent 1290 Infinity II MS Flow Modulator (G7170B)
- Three Agilent 1290 Infinity II Valve Drives (G1170A), equipped with:
  - 2-position/14-port preparative-scale valve (G4738A)
  - 4-position/10-port valve (G4237A)
  - 6-position/14-port preparative-scale valve (G4734B)
- Agilent 1290 Infinity II Preparative Column Compartment (G7163B)
- Agilent 1260 Infinity II Delay Coil Organizer (G9324A) with delay coils for 15–40 mL/min flow (option #210)
- Agilent InfinityLab LC/MSD XT (G6135B)

Columns

Analytical columns:

- Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 4 µm (part number 683970-902)
- Agilent InfinityLab Poroshell HPH-C18, 4.6 × 150 mm, 4 µm (part number 693970-702)

Preparative columns:

- Agilent InfinityLab Poroshell 120 SB-C18, 21.2 × 150 mm, 4 µm (part number 670150-902)
- Agilent InfinityLab Poroshell 120 HPH-C18, 21.2 × 150 mm, 4 µm (part number 670150-702)

Return to Contents Section

Software

Agilent OpenLab CDS ChemStation edition for LC and LC/MS Systems, Rev. C.01.10 [272] or later versions

Solvents

HPLC gradient grade acetonitrile (ACN) was purchased from VWR (Darmstadt, Germany). Ammonia solution 25%, ammonium bicarbonate >99.5%, LC/MS grade formic acid, and reagent grade trifluoroacetic acid were obtained from Merck (Darmstadt, Germany). Agilent InfinityLab Ultrapure LC/MS methanol (part number 5191-4497) was used to prepare make-up solvent for the MSD. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak).

Sample

Angiotensin I (sequence DRVYIHPFHL) was purchased from Proteogenix (Schiltigheim, France) with quality grade "desalted, crude" (purity approximately 51%, according to delivery note). Sample solutions were created with acetonitrile:water 15:85 (v:v).

Method settings

Table 1. Chromatographic conditions of analytical and preparative runs at low pH..

Parameter	Analytical Runs	Preparative Runs
Mobile Phase	A1) 0.1% trifluoroacetic acid in water B1) 0.1% trifluoroacetic acid in ACN	
Flow Rate	1.5 mL/min	32 mL/min
Gradient	Time (min) %B 0 9 1 25 7 35 8 99 10 min stop time 2.5 min post time	Time (min) %B 0 9 1 9 2 25 7 35 7.1 99 8.1 99 8.2 9 9 min stop time
Injection Volume	5 µL	1,000 µL
Sampler Method Preset	Preset 1: Polar sample matrix	Preset 1: Polar sample matrix
Temperature	Ambient	Ambient
UV Detection	Signal A: 220 nm 10 Hz data rate	Signal A: 220 nm 5 Hz data rate
MS Detection	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500
Split Ratio to MSD	Full flow	10,000:1 (mode M7) Turn on after 1.00 min
Fraction Collection	Not applicable	Peak-based, UV and MSD UV threshold: 20 mAU UV upslope: 70 mAU/s UV downslope: 2 mAU/s MSD threshold: 5,000 cps

Return to Contents Section

Table 2. Chromatographic conditions of analytical and preparative runs at high pH.

Parameter	Analytical Runs	Preparative Runs
Mobile Phase	A2) 10 mM ammonium bicarbonate, pH 9.8 in water B2) 10 mM ammonium bicarbonate, pH 9.8 in ACN:water 90:10 (v:v)	
Flow Rate	1.5 mL/min	32 mL/min
Gradient	Time (min) %B 0 9 1 22 6 32 7 99 8 min stop time 2.5 min post time	Time (min) %B 0 9 1 9 2 22 7 32 7.1 99 8.1 99 8.2 9 9 min stop time
Injection Volume	5 µL	1,000 µL
Sampler Method Preset	Preset 1: Polar sample matrix	Preset 1: Polar sample matrix
Temperature	Ambient	Ambient
UV Detection	Signal A: 220 nm 10 Hz data rate	Signal A: 220 nm 5 Hz data rate
MS Detection	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500	Signal 1: positive scan *m/z* 300 to 1,500 Signal 2: negative scan *m/z* 300 to 1,500
Split Ratio to MSD	Full flow	10,000:1 (mode M7) Turn on after 1.00 min
Fraction Collection	Not applicable	Peak-based, UV and MSD UV threshold: 20 mAU UV upslope: 70 mAU/s UV downslope: 10 mAU/s MSD threshold: 10,000 cps

Table 3. MSD spray chamber and fraction collection settings.

Parameter	Value
Make Up Solvent	0.1% formic acid in methanol/water 70/30
Make Up Flow	1.5 mL/min
Ionization Source	Agilent Jet Stream Electrospray
Nebulizer Pressure	35 psig
Drying Gas Temperature	300 °C
Drying Gas Flow	13.0 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12.0 L/min
Capillary Voltage	±2,500 V
Nozzle Voltage	±600 V
Target Mass	1,295.7 *m/z*
Ion Species	$[M+H]^+$, multiple charge states: 2, 3

Return to Contents Section

Results and discussion

Diluted samples were analyzed with separation methods using generic gradients from 9 to 99% B, both with low and high pH eluents (Tables 1 and 2). Method parameters for the MSD were the same for both pH ranges (Table 3). The retention time of the target peak was used to calculate shallow, focused gradients to enhance the resolution around the target peak.[2] Figure 1 shows chromatograms of the separation by a generic and a focused gradient at high pH. The resolution between the target peak (1) and the major impurity (2) increased noticeably when the focused gradient was applied. High resolution of the target peak is a prerequisite to increase the sample load on the column on preparative scale. A similar increase in resolution was achieved by optimizing the low pH method (Figure 2).

To identify the target peak and confirm the trigger masses and ion species, a mass spectrum was extracted from the main peak. Using the integrated deconvolution tool in Agilent ChemStation, three prominent signals of the mass spectrum were successfully assigned to the target molecule. The software identified the signals as single, double, and triple charged species of the angiotensin I ion (Figure 3).

The optimized analytical methods and focused gradients were then transferred to preparative scale using the Agilent HPLC Advisor App. The gradient method translation tool recalculated the flow rate and gradient to account for the wider column diameter and larger dwell volume of the preparative system path. Sample load on column was scaled up 200-fold to between 20 and 30 mg per run. Fraction collection was enabled and set to a peak-based trigger with collection of time slices to facilitate selection of the purest part of the peak.

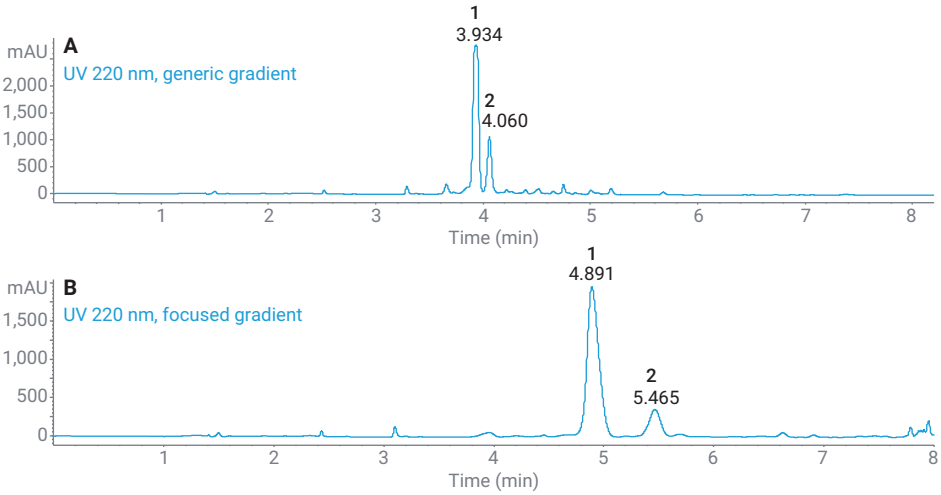


Figure 1. Separation of crude angiotensin I (1) with generic (A) and focused gradient (B) at high pH. Note the increase in resolution between angiotensin and the main impurity (2).

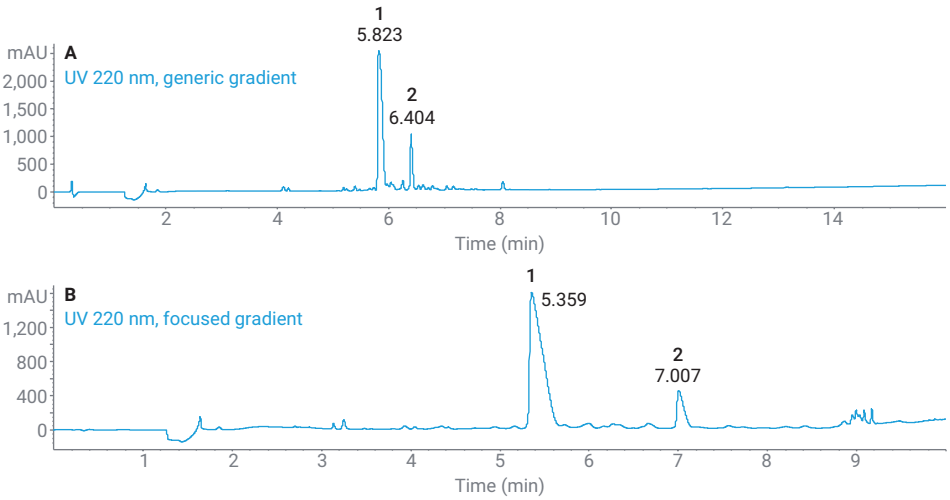


Figure 2. Separation of crude angiotensin I (1) with generic (A) and focused gradient (B) at low pH. Note the increase in resolution between angiotensin and the main impurity (2).

Return to Contents Section

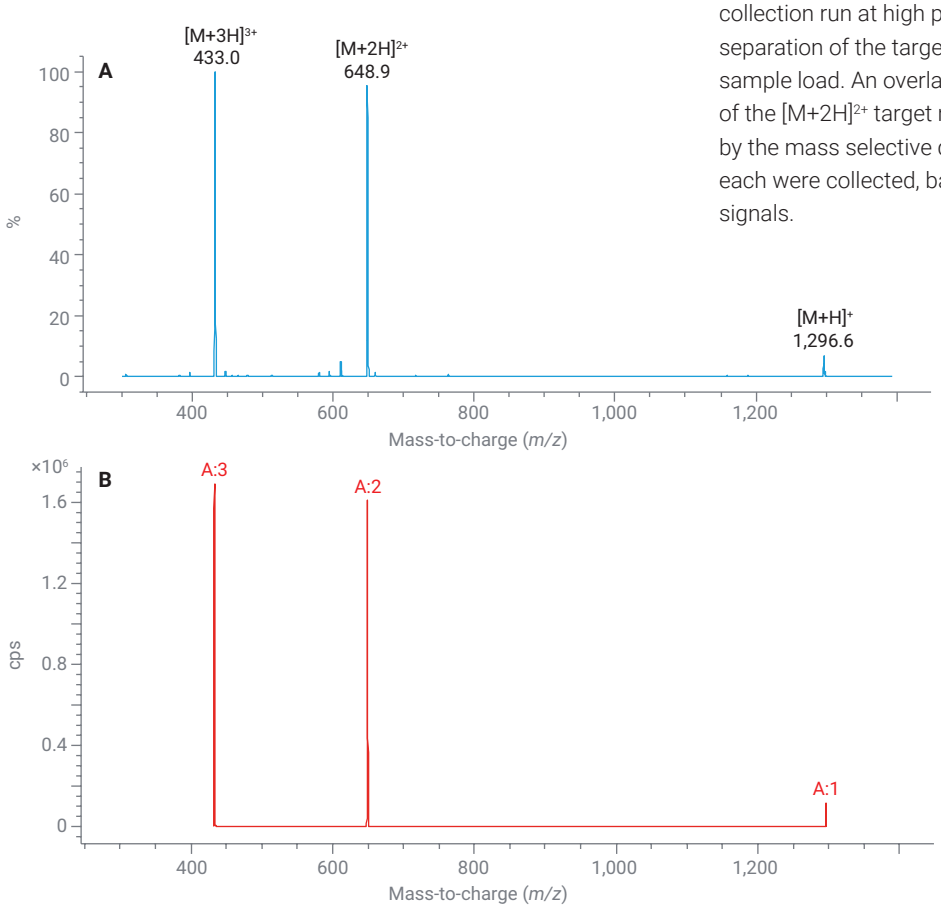


Figure 4 shows the preparative chromatogram of the fraction collection run at high pH. The focused gradient enabled separation of the target peak from the impurities despite the high sample load. An overlay of the extracted ion chromatogram (EIC) of the $[M+2H]^{2+}$ target mass underlines the specificity gained by the mass selective detector. Seven fractions of six seconds each were collected, based on a combination of the UV and MSD signals.

Figure 3. Mass spectrum and deconvolution of the main peak. The three most abundant signals are different charge states of the angiotensin I ion.

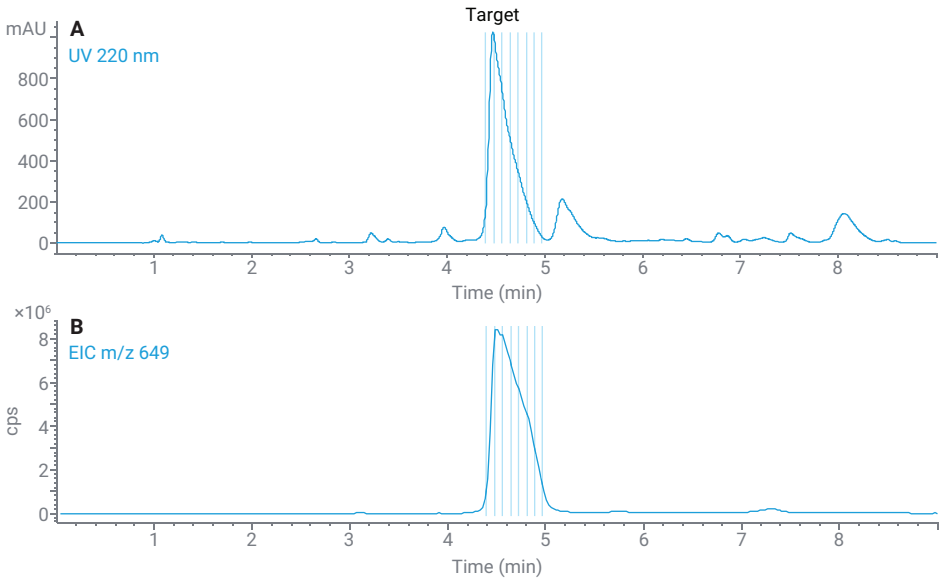


Figure 4. Chromatogram of the preparative purification at high pH. The target peak was collected into seven fractions, triggered by the UV signal and a target mass of m/z 649.

Return to Contents Section

The separation at low pH was also successfully scaled up. Figure 5 displays a chromatogram overlay of the UV and MSD signals. Like on analytical scale, the target peak is wider and less Gaussian-shaped compared to the separation at high pH. Judging by the broad shoulder, the UV peak appears to be coeluting with another compound. The EIC, however, shows that the target mass was present throughout the entire peak. Therefore, 12 fractions of 6 seconds each were collected.

Finally, all collected fractions were reanalyzed by switching back to the analytical path of the system and using the optimized analytical methods. Depending on workflow requirements, a minimum purity of the final product must be observed. By collecting slices of the target peak, only the fractions that meet purity requirements can be picked and pooled. Fractions containing impurities can be repurified without having to discard the entire collection.

Figures 6 and 7 show chromatogram overlays of the reanalyses of the fraction collection at high and low pH, respectively. Of the seven fractions collected at high pH, the first one clearly contains an impurity coeluting with the front of the target peak. All other fractions are more than 95% pure (by UV signal), with a small impurity occurring at the later fractions. A peculiar impurity was present at fraction 4, eluting before the target peak, but not present in any other fraction. It seems like this compound was associated with angiotensin I during the preparative injection and could only be separated during fraction reanalysis. Mass spectral information on this peak suggests that the impurity is a deletion of valine or proline from the angiotensin I sequence.

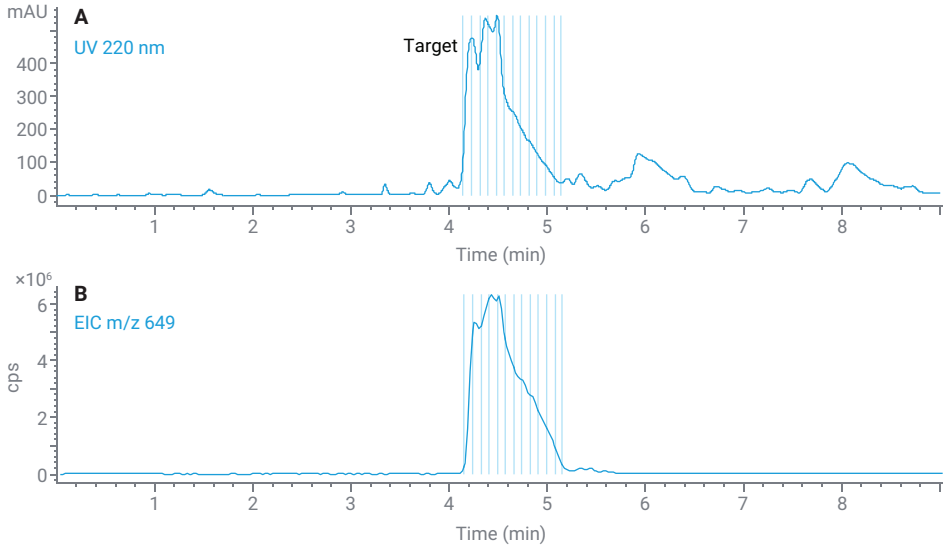


Figure 5. Chromatogram overlay of the preparative purification at low pH. The target peak was collected into 12 fractions, triggered by the UV signal and a target mass of m/z 649.

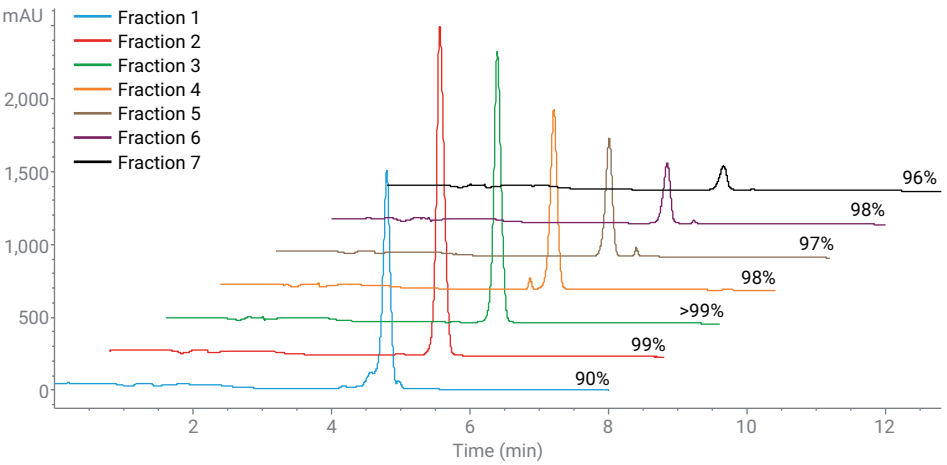


Figure 6. Reanalysis results of the fractions collected at high pH.

Return to Contents Section

Why this molecule would be coeluting with the intact peptide, however, remains to be clarified. High-resolution MS analyses would be the next logical step to shed some light on this conundrum.

A similar pattern was observed for the fractions collected at low pH. All but the first and last fractions were 95% pure or higher. Fraction 2 already was more than 99% pure; in fractions 3 to 5, however, an impurity coeluting with angiotensin I was present like in the high pH fraction 4. Again, this impurity elutes with the heart of the target peak and can only be separated during reanalysis of the collected fraction. By collecting slices of the target peak, this suspected degradation product could now be separated from the pure fractions and investigated with high-resolution MS or another technique. Fractions that meet purity requirements may already be pooled and dried for later use in the downstream process.

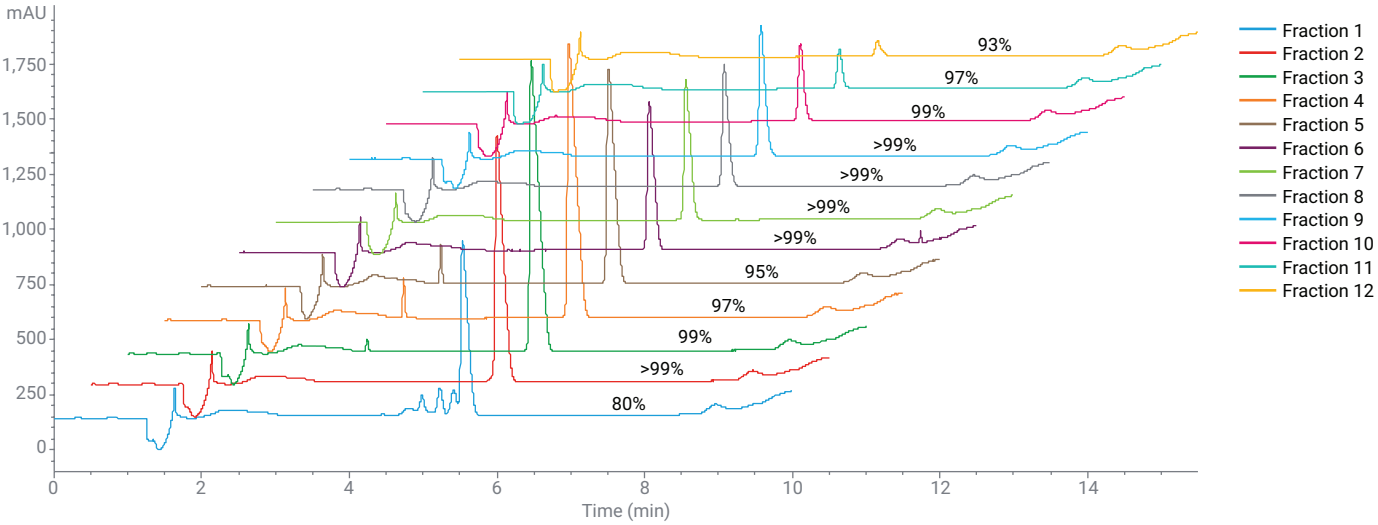


Figure 7. Reanalysis results of the fractions collected at low pH.

Return to Contents Section

Conclusion

A crude synthetic peptide was successfully purified using preparative HPLC under high and low pH conditions. Both methods were developed on an analytical scale and then transferred to preparative conditions using a method calculator. Switches between analytical and preparative scale, as well as low and high pH, were automated using built-in solvent and column selection valves. The target compound was successfully isolated using UV and MSD signals. Instead of a single fraction, time slices were collected and reanalyzed for purity. Purity was 95% or higher for all but the first and last slices, giving the option of pooling only the purest fractions. This workflow of method scale-up; column and solvent selection; and fraction reanalysis on a single system clearly facilitates the workflow for successful, confident purification of synthetic peptides.

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Bioanalysis Studies

Introduction

Bioanalysis is essential in pharmaceutical development for quantifying therapeutic peptides and metabolites in biological matrices (plasma, serum, whole blood, urine). These measurements underpin pharmacokinetics (PK), pharmacodynamics (PD), bioavailability, and exposure-response assessments. Peptide analysis in such complex matrices is challenged by matrix effects, low analyte levels, non-specific interactions, and limited stability, all of which can affect accuracy and reproducibility.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) in multiple reaction monitoring (MRM) mode is the gold standard, offering high specificity, sensitivity, broad dynamic range, multiplexing, and rapid method development. Robust implementation requires optimizing sample preparation (e.g., SPE, 2D-LC), mitigating matrix suppression/enhancement, and applying reliable calibration strategies (e.g., isotopically labeled internal standards). As therapeutic modalities expand to more complex peptides and biologics, robust LC-MS/MS methods are increasingly critical to support both preclinical and clinical studies.

Application Note

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Quantification of Therapeutic Peptide Exenatide in Rat Plasma

Using Agilent 1290 Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems

Author

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Agilent Technologies, Inc.

Abstract

In recent years, pharmaceutical research and development has shifted focus from small molecule drugs to biologic therapeutics such as large peptide drugs, antibody-drug conjugates, recombinant fusion proteins, monoclonal antibodies (mAb), and oligonucleotide drugs, among others. The traditional analytical method for these large molecules is the ligand binding assay (LBA) due to its high sensitivity, high throughput, low cost, and ease-of-automation. Over the past two decades, liquid chromatography/mass spectrometry (LC/MS) has become an alternative method to analyze these large molecules due to its high specificity, sensitivity, wide dynamic range, and fast method development.[1] At the same time, LC/MS can avoid cross-reactivity, and has proven to overcome reagent availability compared to traditional LBA.

Exenatide is a large therapeutic peptide approved to treat diabetes mellitus type 2.[2] It is a synthetic version of Exendin-4, a 39 amino acid hormone found in the saliva of the Gila monster reptile with a molecular weight of 4,186.6 Da.[3] Traditionally, the plasma concentration of exenatide was determined by LBA, which needs time to develop the antibody, and lacks selectivity and specificity. This application note demonstrates an LC/MS method to quantify exenatide in rat plasma using the Agilent Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems (Figure 1). The results show that this LC/MS assay is easy, simple, and can be used for exenatide quantitative analysis with excellent sensitivity and reproducibility.

Return to Contents Section

Experimental

Materials and methods

Exenatide was purchased from MedChemExpress (Monmouth Junction, NJ, USA). Formic acid (FA), was purchased from Sigma-Aldrich (St. Louis, MO, USA). LC/MS-grade acetonitrile was purchased from Agilent Technologies, and 96-well protein LoBind plates were purchased from Eppendorf USA (Hauppauge, NY, USA).

Instrumentation

- Agilent 1290 Infinity II Bio UHPLC system including:
- Agilent 1290 Infinity II Bio high speed pump (G7132A)
- Agilent 1290 Infinity II Bio multisampler (G7137A)
- Agilent 1290 Infinity II thermostat column compartment (G7116A) equipped with a Standard Flow Quick Connect bio heat exchanger (G7116-60071)
- Agilent 6495 triple quadrupole LC/MS system

Sample preparation

Acetonitrile (600 µL with 1% formic acid) was added to 200 µL of rat plasma aliquots fortified with different concentrations of exenatide. This mixture was vortexed for 5 minutes, then spun down at 16,000 g for 10 minutes. The supernatant was transferred to a 96-well protein LoBind plate, then dried down under nitrogen gas with heating. After drying down, 100 µL of 20% acetonitrile (0.1% FA) was added to reconstitute, and 20 µL was injected into LC/MS for analysis.

Table 1 Liquid chromatography parameters.

LC Conditions	
Column	AdvanceBio Peptide Mapping, 120 Å, 2.1 × 150 mm, 2.7 µm (p/n 653750-902)
Column Temperature	60 °C
Injection Volume	20 µL
Autosampler Temperature	4 °C
Needle Wash	5 seconds in wash port (50/50 water/methanol)
Mobile Phase	A: Water + 0.1% formic acid B: Acetonitrile + 0.1% formic acid
Flow Rate	0.5 mL/min
Gradient Program	Time %B 0 10 8 65 8.2 95 9.2 95 9.3 10 12 10
Stop Time	12 min

Figure 1. Agilent 1290 Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems.

LC/MS analysis

Data acquisition was performed using an Agilent 1290 Infinity II Bio UHPLC coupled to an Agilent 6495 triple quadrupole LC/MS system with Agilent Jet Stream source. Separation was obtained with an Agilent AdvanceBio Peptide Mapping column (2.1 × 150 mm, 120 Å, 2.7 µm). Tables 1 and 2 list the LC and MS parameters used for this workflow.

Positive electrospray ionization of exenatide yielded $[M+5H]^{5+}$ signal at m/z 838.3 as the most intense ion. MRM transitions were optimized and 838.3 and 948.8 was chosen as the quantifier, while 838.3 and 396.3 was chosen as the qualifier with optimal collision energy.

Data processing

All MS data were processed using Agilent MassHunter Quantitative Analysis software.

Table 2. MS acquisition parameters.

MS Conditions	
Gas Temperature	290 °C
Drying Gas Flow	18 L/min
Nebulizer Gas	35 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,500 V
Nozzle Voltage	1,000 V
High Pressure RF	150 V
Low Pressure RF	80 V

Return to Contents Section

Results and discussion

Method optimization for exenatide quantitative analysis

To improve the sensitivity and reproducibility for exenatide quantitative analysis in rat plasma, the sample preparation, LC conditions, and MS conditions were all optimized to achieve best sensitivity and reproducibility.

Acetonitrile with 0, 0.1, 0.5, and 1% formic acid were evaluated to precipitate proteins from rat plasma. The data are shown in Figure 2. As the extracted ion chromatogram (EIC) of MRM transition showed that 1% formic acid in acetonitrile produced the best analyte response, 1% formic acid in acetonitrile was later used for all sample preparations.

The UHPLC method was optimized using the peptide map column function, with the temperature set at 60 °C and a flow rate of 0.5 mL/min to achieve the best reproducibility. The column temperature reduced pump pressure, and improved the MS sensitivity significantly under these conditions.

Quantitative analysis of exenatide from rat plasma

Agilent MassHunter Quantitative Analysis software was used to perform quantitative analysis of the calibration curve and quality control samples. With blank rat plasma and an exenatide lower limit of quantification of 0.02 ng/mL (as shown in Figures 3A and 3B), the calibration curve was from 0.02 to 20 ng/mL with quadratic fit and 1/x2 weight (as shown in Figure 4 and Table 3).

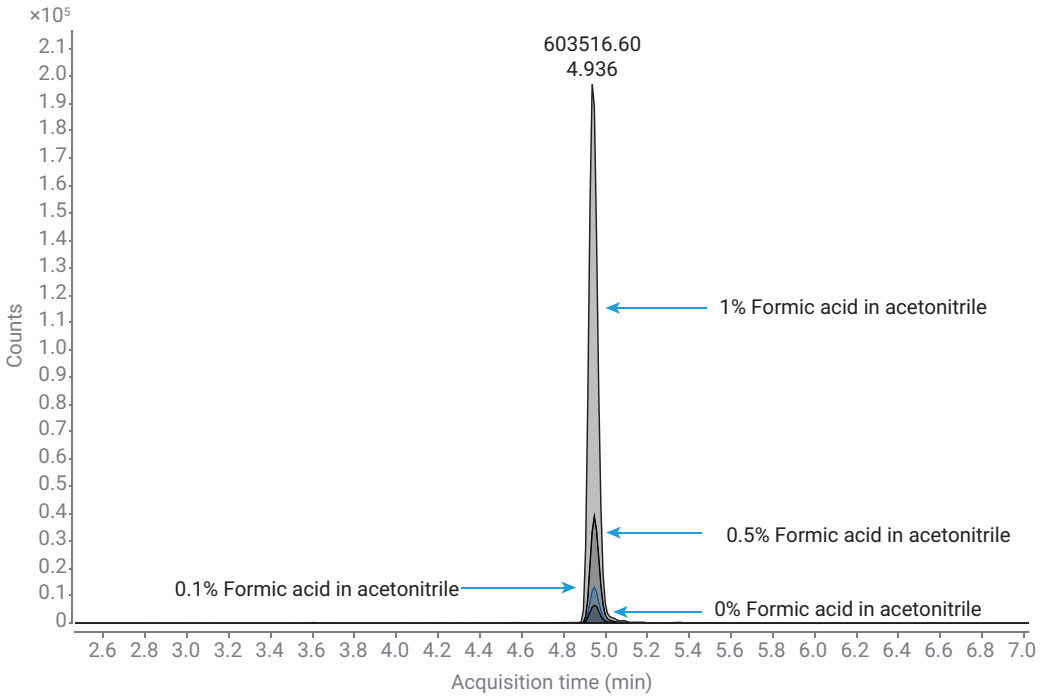

Figure 2. Effects of different concentrations of formic acid in acetonitrile for sample preparation.

Return to Contents Section

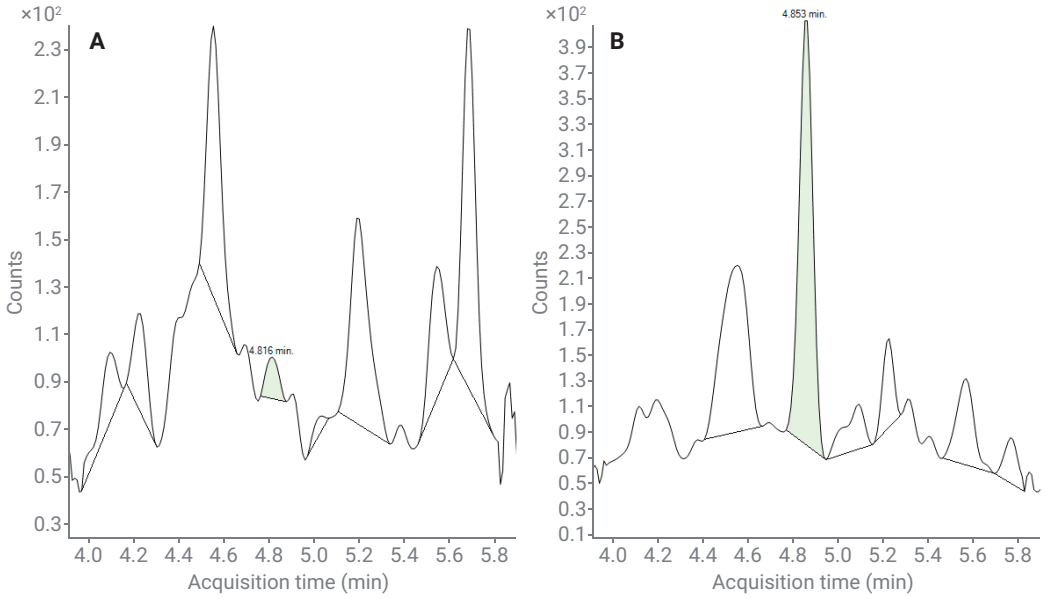

Figure 3. (A) EIC of blank rat plasma. (B) EIC of exenatide lowest calibration point.

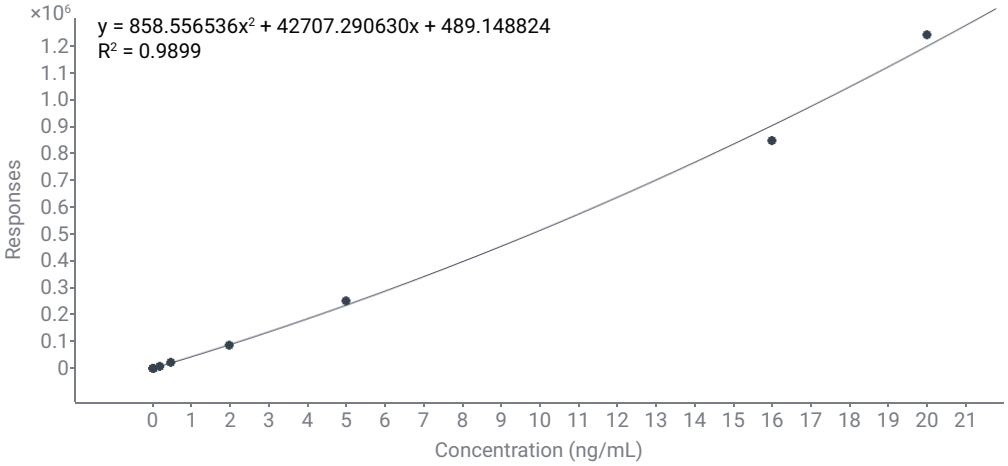

Figure 4. Calibration curve of exenatide from 0.02 to 20 ng/mL in rat plasma.

The intraday and interday analytical precision and accuracy were determined from three independent preparations, performed over 3 days. The precision and accuracy result of exenatide in rat plasma is shown in Table 4. All levels of quality control samples (n = 6) met acceptance criteria of 20% (25% for LLOQ), as recommended by the US Food and Drug Administration. The results demonstrated excellent assay performance using the 1290 Infinity II Bio LC and 6495 triple quadrupole LC/MS system for large peptide quantification.

Return to Contents Section

Table 3. Exenatide calibration curve performance over three runs.

Calibration (ng/mL)	0.020	0.040	0.200	0.500	2.000	5.000	16.000	20.000
Mean	0.021	0.044	0.201	0.538	1.978	5.315	15.922	19.855
% Bias	5.25	9.00	0.30	7.62	-1.11	6.31	–0.49	–0.72
% CV	3.70	15.57	12.48	5.55	1.37	0.12	6.63	4.90

Table 4. Precision and accuracy of quality control samples in mouse plasma (n = 6).

	QC Concentration (ng/mL)	0.02 (LLOQ)	0.06 (Low)	1.00 (Mid)	15.0 (High)
Run 1	Mean	0.0181	0.0649	1.19	15.3
	% Bias	–9.5	8.2	19.0	2.1
	% CV	12.8	12.0	6.7	3.5
Run 2	Mean	0.0225	0.0593	1.04	15.2
	% Bias	12.3	–1.1	3.7	1.6
	% CV	17.0	15.2	4.5	9.8
Run 3	Mean	0.0218	0.0623	1.10	14.8
	% Bias	9.0	3.8	10.0	–1.3
	% CV	14.6	13.7	8.3	8.6
Interday	Mean	0.021	0.0622	1.11	15.1
	% Bias	3.9	3.6	10.9	0.8
	% CV	11.8	4.7	7.7	1.8

Conclusion

The Agilent Infinity II Bio LC and Agilent 6495 triple quadrupole LC/MS systems are ideal for large peptide quantitative analysis with excellent assay reproducibility. This application note demonstrates a sensitive LC/MS method for the quantitative analysis of exenatide from rat plasma. This method is simple and fast. In this method, the lower limit of quantification is 0.02 ng/mL from 200 µL of rat plasma, which is equivalent to other solid phase extraction sample preparations. In three qualification runs, intraday and interday QC sample precision and accuracy all met regulatory acceptance criteria, demonstrating excellent assay performance and reproducibility.

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Return to Contents Section

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