Application Note

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Advanced 2D-LC/MS Workflow for the Characterization of Semaglutide and Its Impurities

Using an Agilent 1290 Infinity III bio 2D-LC and an Agilent 6545XT AdvanceBio LC/Q-TOF

Authors

Paramjeet Khandpur, Preeti Bharatiya, and Ashish Pargaonkar

Agilent Technologies, Inc.

Abstract

The characterization and impurity analysis of the GLP-1 receptor agonist peptide semaglutide requires chromatographic separation combined with accurate mass detection. Chromatographic separation methods for peptides and proteins, however, often use MS-incompatible mobile phases. This application note presents the capabilities of the Agilent 1290 Infinity III bio 2D-LC system, coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF and Agilent MassHunter BioConfirm software, version 12.1, to identify and characterize peptide-related impurities in semaglutide. This workflow enables accurate mass-based identification, sequence confirmation, and the detection of impurities resulting from the synthesis process, while achieving impurity separation using a non-MS-compatible buffer system in the first dimension (^{1}D).

Figure 1. Agilent 1290 Infinity III bio 2D-LC coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF.

Introduction

Synthetic peptides are becoming increasingly popular in the pharmaceutical industry for their applications in diagnosis, therapeutics, and drug delivery. They offer advantages such as better potency, high specificity, and low toxicity.[1] Their rising prevalence as therapeutic agents necessitates stringent quality control measures to ensure the resolution and identification of challenging impurities in peptide active pharmaceutical ingredients (APIs), given their structural diversity.

Peptide impurities pose significant concerns during the GMP manufacturing phase, impacting both drug development and clinical applications. These impurities often arise due to amino acid deletions, insertions, or degradation under physiological conditions during storage and transport.[2] Since peptides are typically synthesized through solid-phase methods, impurity management is critical in maintaining product integrity.[3]

GLP-1 receptor agonists—a group of peptide compounds—have gained prominence in the early-stage therapy of type II diabetes and obesity.[4] However, achieving optimal purity for these peptides remains a significant analytical challenge. Among these, semaglutide (MW 4,113.58 Da) is a notable GLP-1 agonist, distinguished by its structural modification with a fatty acid side chain that enhances stability and prolongs its half-life (Figure 2).[4] This modification allows a reduced dosing frequency compared to other GLP-1 receptor agonists, such as liraglutide. However, achieving optimal purity for these peptides remains a significant analytical challenge, as closely related impurities can coelute with the main peak, making their identification difficult using conventional LC/MS approaches.

To address this, a two-dimensional liquid chromatography (2D-LC) strategy was employed in this workflow. The first dimension (^{1}D) achieves high chromatographic resolution using a phosphate buffer-based separation, which is well-suited for peptide analysis, but is incompatible with MS detection. To overcome this limitation, the second dimension (^{2}D) acts as a desalting step while maintaining the separation achieved in the first dimension, allowing efficient impurity identification using mass spectrometry. Multiple heart-cutting (MHC) was used to selectively transfer impurity fractions, ensuring high-resolution separation of structurally similar impurities. This approach enhances sensitivity and provides more detailed impurity characterization compared to a single LC/MS method.

This application note describes a workflow integrating a 1290 Infinity II bio 2D-LC system coupled with a 6545XT AdvanceBio LC/Q-TOF and MassHunter BioConfirm 12.1 software to identify and characterize peptide-related impurities in semaglutide. This system provides enhanced resolution, sensitivity, and robust impurity identification, addressing the analytical challenges associated with peptide therapeutics.

Experimental

Instrumentation

A 2D-LC/Q-TOF MS system was used. It comprised a 1290 Infinity III bio 2D-LC system equipped with MHC and high-resolution sampling options. A 6545XT AdvanceBio LC/Q-TOF system with an Agilent Dual Jet Stream ESI source was used for detection. The system comprised the following modules:

First dimension

- Agilent 1290 Infinity III bio flexible pump (G7131A)
- Agilent 1290 Infinity III bio multisampler (G7137A) with sample thermostat
- Agilent 1290 Infinity III multicolumn thermostat (G7116B) with Agilent InfinityLab QuickConnect heat exchanger (G711660071)
- Agilent 1290 Infinity III variable wavelength detector (G7114B) with an Agilent InfinityLab bio-inert standard flow cell (10 mm)

Second dimension

- Agilent 1290 Infinity III bio high-speed pump (G7132A)
- Agilent 1290 Infinity III multicolumn thermostat (G7116B) equipped with a bio-inert standard flow heat exchanger (G7116-60071)
- Agilent 1290 Infinity III diode array detector (G7117B) with Agilent InfinityLab bio-inert Max-Light cartridge cell LSS, 60 mm (G561560017)

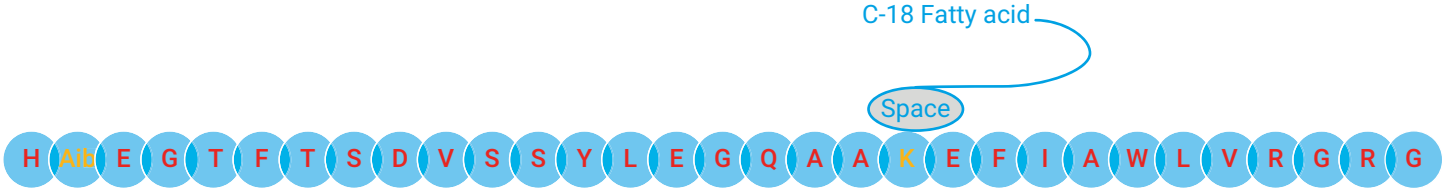


Figure 2. Molecular structure of semaglutide. Aib = α-aminoisobutyric acid (α-methylalanine), modification on Ala2.

2D-LC interface

- Agilent 1290 Infinity valve drive (G1170A) with Agilent InfinityLab bio 2D-LC ASM valve (G5643B)
- Two Agilent 1290 Infinity valve drives (G1170A) with Agilent InfinityLab Multiple Heart-Cutting valve and biocompatible 40 µL loops
- Agilent 2D-LC Pressure release kit (G4236-60010)
- The Agilent 6545XT AdvanceBio LC/Q-TOF equipped with Dual Jet Stream ESI source acquired MS data

Software

- Agilent MassHunter Workstation LC/MS data acquisition software, version 11.0, with Agilent 2D-LC software for MassHunter (G2198AA#410)
- Agilent MassHunterBioConfirm software, version 12.1.

Chemicals and reagents

Formic acid and disodium hydrogen phosphate were purchased from Sigma-Aldrich, USA (Sigma-Aldrich, St. Louis, MO, USA). Ultra-grade LC/MS acetonitrile was purchased from Biosolve (MS grade, Biosolve, Dieuze, France). Water was purified using a Milli-Q IQ 7003 purification system (Merck KGaA, Darmstadt, Germany).

Samples

Semaglutide (1 mg/mL) was dissolved in 1 mL of water:methanol (70:30).

Columns

The following columns were used in this workflow:

- **First dimension:** Agilent AdvanceBio Peptide Plus, 2.1 × 250 mm, 2.7 µm (part number 693775-949)
- **Second dimension:** Agilent AdvanceBio RP-mAb C4 (part number 799775-904)

Methods

The ^{1}D and ^{2}D parameters are displayed in Tables 1 and 2, respectively. The 6545XT AdvanceBio LC/Q-TOF MS data acquisition parameters are shown in Table 3.

Table 1. ^{1}D parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 250 mm, 2.7 µm
Flow	0.6 mL/min
Column Temperature	45 °C
Injection Volume	20 µL
Mobile Phase	Buffer solution: 25 mM disodium hydrogen phosphate, pH 4.5 A) buffer:acetonitrile 70:30 (v:v) B) buffer:acetonitrile 30:70 (v:v)
Gradient	Time (min) %A %B 0 85 15 5 80 20 30 76 24 45 72 28 50 15 85 55 5 95 60 5 95 60.50 85 15 68 85 15
Detector	UV 215 nm

Table 2. ^{2}D parameters.

Parameter	Value
Column	Agilent AdvanceBio RP-mAb C4 column
Flow	0.4 mL/min
Column Temperature	50 °C
Injection Volume	20 µL
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
2D-LC Operation Mode	Time-based heart-cut MHC
Gradient	Time %A %B 0 70 30 5 70 30 6 58 42 15 55 45 15.1 45 55 17 45 55 18 70 30
^{2}D Run Time	18 min
^{2}D Equilibration	5 min
Cycle Time	23 min
ASM Setting	Factor: 3 Flush factor: 5
Sample Loop	40 µL
Detector	UV 280 nm

Table 3. Agilent 6545XT AdvanceBio LC/Q-TOF MS parameters.

Parameter	Value
Instrument	Agilent 6545XT AdvanceBio LC/Q-TOF
Gas Temperature	325 °C
Drying Gas Flow	11 L/min
Nebulizer	35 psig
Sheath Gas Temperature	275 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	4,000 V
Nozzle Voltage Fragmentor Skimmer	500 V 175 V 65 V
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 300 to 3,200
Acquisition Rate	2 spectra/sec
Auto MS/MS Range	*m/z* 50 to 3,200
Minimum MS/MS Acquisition Rate	4 spectra/sec
Isolation Width	Narrow (approximately *m/z* 1.3)
Precursors/Cycle	Three
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 0.01%
Dynamic Exclusion On	Two repeats, then exclude for 0.2 minutes
Precursor Abundance-Based Scan Speed	No
Purity	100% stringency, 30% cutoff
Isotope Model	Peptides
Active Exclusion Enabled	No
Sort Precursors	By abundance only
Charge State Preference	Selected charges 3 2 > 3

High resolution (HiRes) sampling and multi-inject functionality in 2D-LC experiments

In this study, a HiRes sampling mode was employed to capture the entire width of the main peak and impurity peaks, enabling consecutive cutting throughout the peak. This approach ensures that broad peaks can be fractionated and analyzed efficiently, maximizing resolution during the second dimension. The HiRes mode facilitated the collection of all peak cuts, ensuring comprehensive sampling of the analytes for thorough analysis in the ^{2}D column. Additionally, the Multi-Inject function was used in conjunction with HiRes mode to further optimize the experiment. In the Multi-Inject function, the contents of each MHC deck—comprising five loops from Deck A and five loops from Deck B—were transferred to the ^{2}D column before a single ^{2}D cycle was initiated. This feature allowed for more flexible volume adjustments through software settings, eliminating the need for hardware modifications.[5] The combination of HiRes sampling and the Multi-Inject function significantly enhanced the throughput and efficiency of the 2D-LC setup, enabling high-resolution separations with minimal time investment.

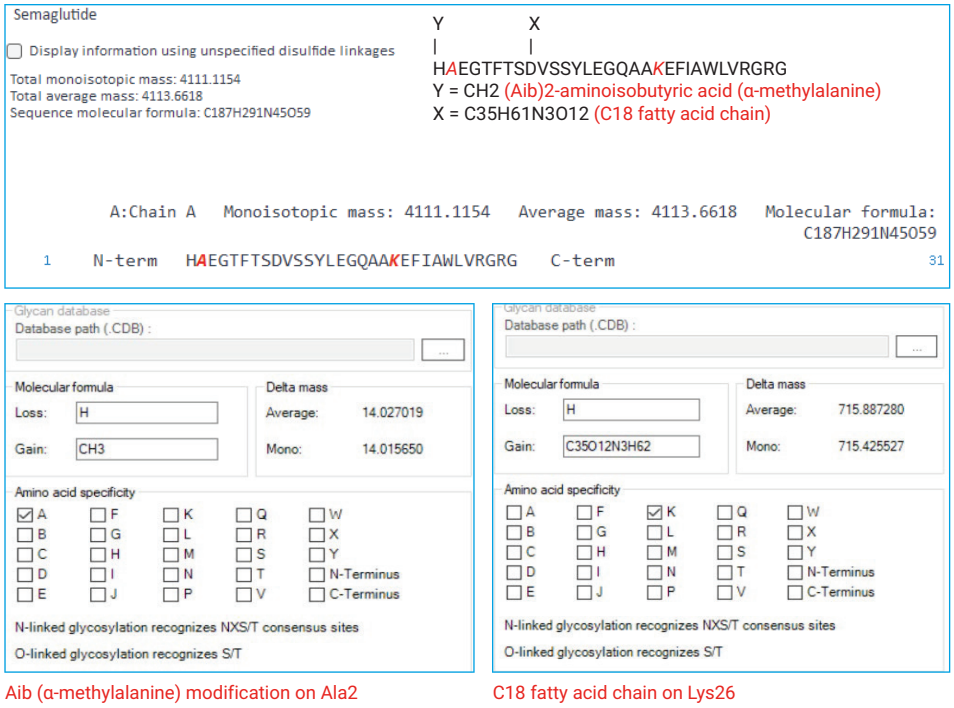
Semaglutide data acquisition and processing using the BioConfirm workflow

Data acquisition was performed using the 6545XT AdvanceBio LC/Q-TOF system and MassHunter BioConfirm 12.1 software. Optimized MS source and acquisition parameters ensured reliable data collection. The protein digest workflow within the software enabled comprehensive acquisition and analysis of semaglutide samples. Peptide and impurity identification were achieved through sequence-matching algorithms, facilitating intact mass detection and identification of peptide sequences and impurities. These impurities included missing or additional amino acids, deamidation, and other posttranslational modifications, ensuring accurate assessment of the therapeutic peptide's integrity. Specific processing parameters were as follows.

1. Peptide sequence and modifications: Semaglutide, a GLP-1 analog peptide, has modifications that are critical for its biological efficacy. The BioConfirm workflow begins by entering the peptide sequence of semaglutide, which is given in Figure 3. The key modifications of interest include Aib (2-aminoisobutyric acid) at position Ala2 for stability and a C18 fatty acid chain at Lys26 to extend half-life, facilitating its therapeutic effect.

2. Data acquisition setup and peak identification: The LC/Q-TOF system acquires MS and MS/MS data (Table 3), which are used to confirm the presence of the peptide and its modifications. Collision energies were optimized for semaglutide due to their relatively higher values for GLP-1 peptides.[6] The settings capture a wide mass range, ensuring the detection of semaglutide and potential impurities or isoforms. These settings enable the comprehensive analysis of semaglutide, including identifying potential modifications.

3. MassHunter BioConfirm protein digest workflow setup: Semaglutide was analyzed under nonreduced conditions with nonspecific, undigested enzyme settings to preserve its native structure and ensure accurate detection of intact peptide sequences and modifications, such as deamidation and terminal truncations (Figure 4). These parameters provided a comprehensive assessment, capturing all relevant peptide forms, isoforms, and truncated impurities associated with synthesis and degradation, ensuring the robust characterization of semaglutide.



Aib (α-methylalanine) modification on Ala2

C18 fatty acid chain on Lys26

Figure 3. Setup of chemical modifications at alanine 2 (A2) and lysine 26 (K26) in the amino acid sequence of semaglutide using Agilent BioConfirm sequence manager.

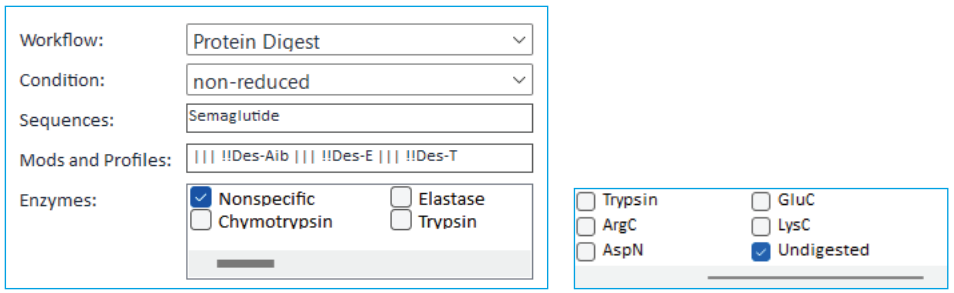


Figure 4. Protein digestion workflow setup for semaglutide analysis using Agilent MassHunter BioConfirm software.

The modifications can be customized and specificity to amino acids can be defined in the BioConfirm software, as shown in Figure 5.

4. Mass matching and data identification: Data processing involved setting mass tolerance windows of ± 10 ppm for MS and ± 20 ppm for MS/MS spectra, ensuring reliable peptide matching. The peptide length range (2 to 70 amino acids) ensured the detection of both intact and truncated peptides from degradation or modifications. Charge state settings were optimized for accurate mass determination, which is crucial for the precise identification of semaglutide and its variants.

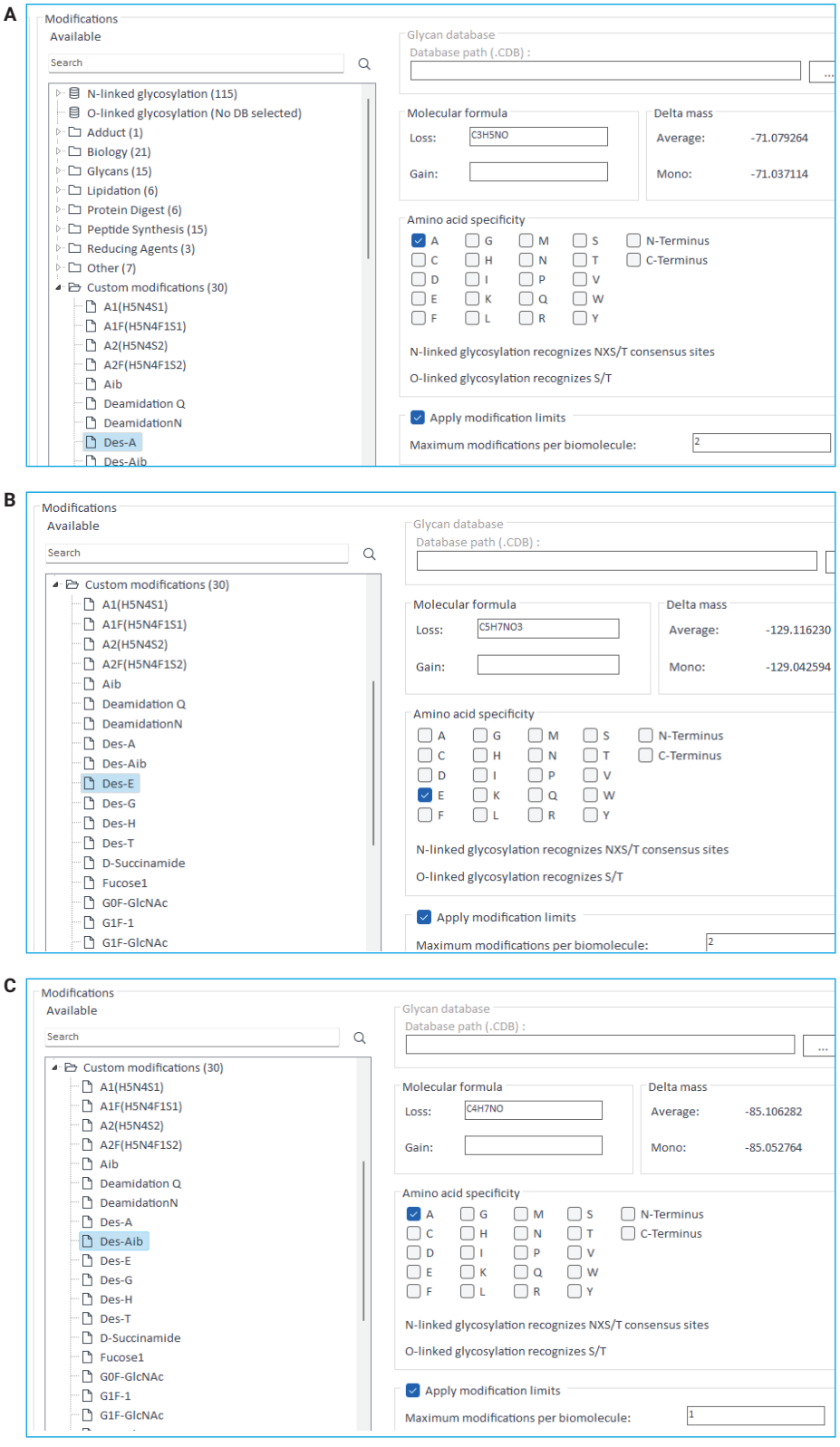


Figure 5. Agilent MassHunter BioConfirm software processing parameters for synthetic peptide modifications.

Results and discussion

^{2}D chromatography for peptide separation

The 2D-LC workflow employed orthogonal columns to maximize the separation and resolution of semaglutide and its impurities. In the first dimension, the LC method used the AdvanceBio Peptide Plus column with a phosphate buffer to achieve better separation of coeluting peaks. However, this buffer system is not compatible with MS ionization. To address this, 2D-LC was used for online coupling to MS, with the second dimension serving as an effective desalting tool. The second dimension used the AdvanceBio RP-mAb C4 column with a volatile solvent system containing formic acid, enabling compatibility with mass spectrometry for impurity separation and desalting. The hybrid charged silica surface of the AdvanceBio Peptide Plus column provided superior resolution and selectivity, while the low hydrophobicity and wide-pore design of the RP-mAb C4 column enhanced separation efficiency under shallow gradients.

The combination of these two columns maximized separation efficiency, allowing detailed characterization of semaglutide impurities. This orthogonal column setup, along with the 2D-LC/Q-TOF MS system, enabled the identification of the therapeutic peptide through accurate mass and sequence matching, and the detection of related impurities based on mass differences due to missing or additional amino acids, deamidation, or other modifications.

Detailed characterization of pre-main (impurities), main, and post-main peaks across two experiments

A HiRes 2D-LC sampling approach, combined with multi-injection, was employed for the analysis of the semaglutide sample, as illustrated in Figure 6. Two separate experiments were conducted: Experiment 1 focused on the peaks before the main peak, and Experiment 2 covered the main peak and peaks eluting after it.

Although the same semaglutide sample was used, it was injected twice, as only 10 HiRes cuts can be marked in a single run. To ensure comprehensive coverage of different regions of interest in the chromatogram, HiRes cuts were selected from the pre-main, main, and post-main peak areas. This high-resolution sampling approach, coupled with multi-injection technology, enabled detailed and efficient data collection in the second dimension of analysis.

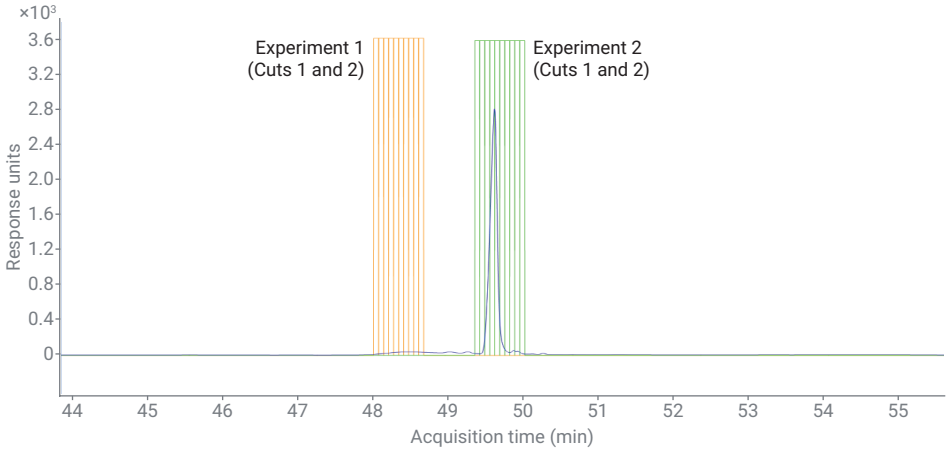


Figure 6. Overlay of ^{1}D chromatograms with HiRes cut markers: Experiment 1 (pre-main impurities) and Experiment 2 (main and post-main peaks).

Experiment 1: Characterization of impurity peaks before the main peak

Figure 7 shows the semaglutide ^{1}D chromatogram reference with overlaid HiRes series. HiRes cuts were made at an impurity peak before the main peak using a multi-injection approach. The cuts were transferred to the ^{2}D column through MHC decks, which facilitated efficient fraction collection and transfer. MHC decks consist of two sets of loop-based storage systems, decks A and B, each containing six loops. These decks allow sequential collection of specific impurity fractions from a ^{1}D separation, ensuring precise and targeted transfer to the ^{2}D column. The use of MHC decks enables the simultaneous analysis of multiple fractions in a single ^{2}D cycle, improving resolution and throughput. To streamline the analysis, the software grouped the collected fractions into two sets. The first five collected fractions were injected and analyzed together into the second-dimension column and labeled as Cut 1, while the next five fractions were processed as Cut 2. This structured grouping allowed for a more detailed characterization of impurity peaks eluting prior to the main peak.

The fractions, labeled Cut 1 and Cut 2, were separated on the ^{2}D column for MS analysis, as shown in Figure 8.

The MS analysis of Cuts 1 and 2 was matched with the sequence of semaglutide and its truncated impurities. The presence of a mass corresponding to the mass of semaglutide in Cut 1, which is well separated from the main semaglutide peak, suggests an isomeric impurity of semaglutide. Table 4 summarizes the exact match of semaglutide and truncated impurities in both Cuts 1 and 2.

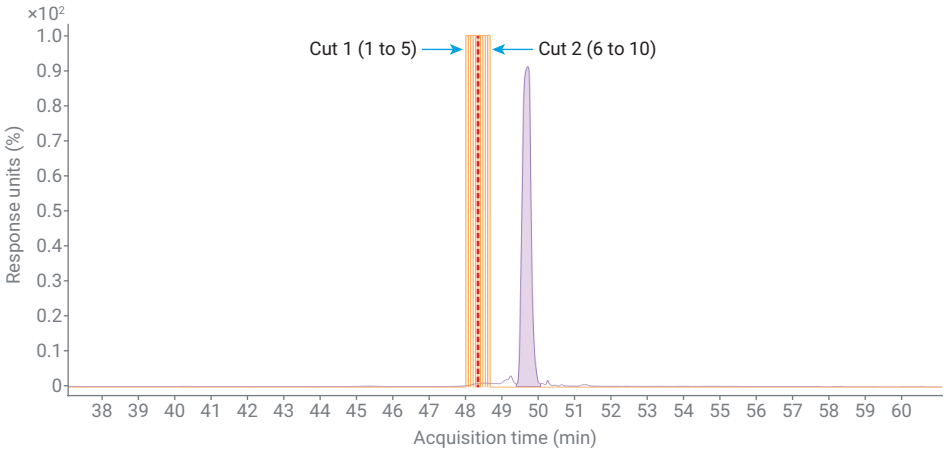


Figure 7. HiRes fraction collection from 1D-LC to 2D-LC at an impurity peak before the main peak.

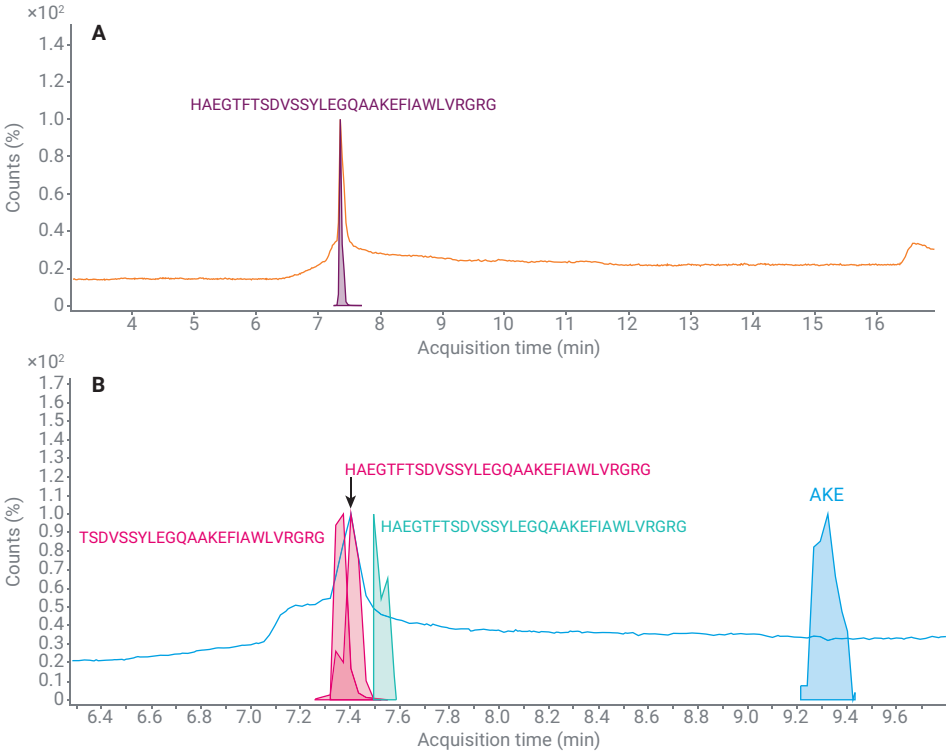


Figure 8. Pre-main impurity peaks from the 2D-LC extracted ion chromatogram of (A) Cut1 and (B) Cut 2. They were overlaid on the ^{2}D total ion chromatogram, highlighting the chromatogram where the intact peptide of the semaglutide was identified using Agilent MassHunter BioConfirm software.

In Cut 2, in addition to the identification of the mass of intact semaglutide at the correct retention time (RT), masses corresponding to truncated impurities with amino acid sequences 7 to 31 and 19 to 21 were also detected. The analysis also revealed slight separation of multiple masses corresponding to semaglutide at different retention times than the main peak, suggesting the presence of isomeric impurities coeluting with the main peak in the ^{1}D chromatogram. The sequence for semaglutide and the impurities were also confirmed by MS/MS analysis. Figures 9 to 11 represent the identification based on well-resolved charge states and MS/MS fragmentation patterns specific to semaglutide and its impurity sequence.

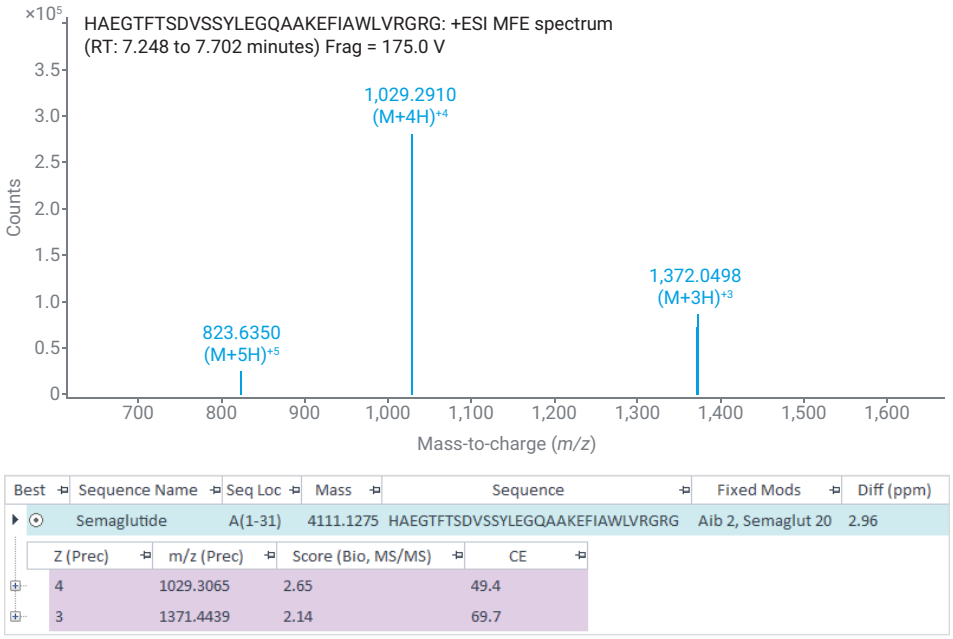


Figure 9. The intact mass charge state distribution and sequence identification by Agilent MassHunter BioConfirm software for semaglutide.

Table 4. The exact match of the semaglutide sequence and truncated impurities from Cuts 1 and 2 in Experiment 1 with excellent mass accuracy.

^{1}D Cuts	Mass	^{2}D RT	Sequence Name	Sequence Location	Sequence	Fixed Modifications	Variable Modifications	Difference (Bio, ppm)
Cut 1	4,111.128	7.355	Semaglutide	(1- 31)	H**A**EGTFTSDVSSYLEGQAA**K**EFIAWLVRGRG	Aib 2, Semaglutide 20		2.96
Cut 2	3,454.836	7.36	Semaglutide	(7 - 31)	TSDVSSYLEGQAA**K**EFIAWLVRGRG	Semaglutide 14		3.72
Cut 2	4,111.129	7.411	Semaglutide	(1 - 31)	H**A**EGTFTSDVSSYLEGQAA**K**EFIAWLVRGRG	Aib 2, Semaglutide 20	1*Des-Aib	3.32
Cut 2	4,111.126	7.514	Semaglutide	(1 - 31)	H**A**EGTFTSDVSSYLEGQAA**K**EFIAWLVRGRG	Aib 2, Semaglutide 20		2.59
Cut 2	932.571	9.314	Semaglutide	(19 - 21)	A**K**E	Semaglutide 2	Des-E 3	3.02

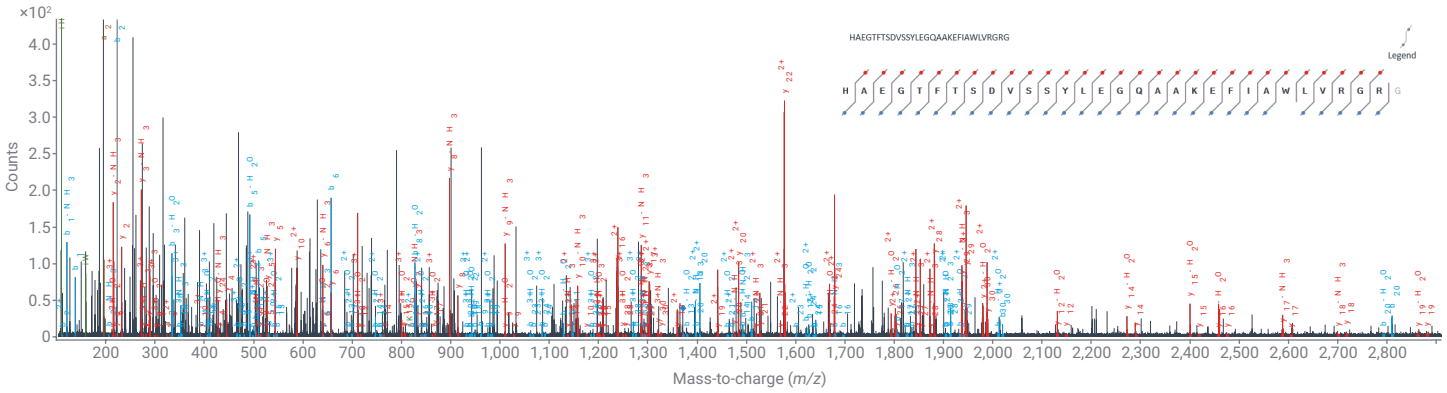


Figure 10. The MS/MS fragmentation pattern for the semaglutide peptide (z = 3) with coverage of both b and y ion series. The fragmentation ladder annotates the identified b/y series (z = 3) for the sequence.

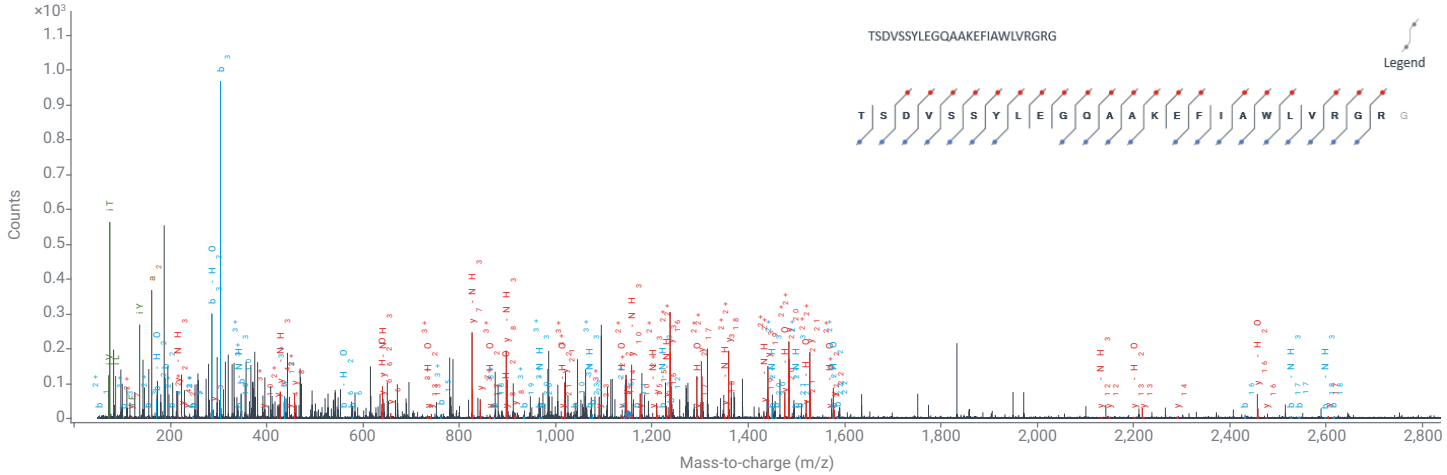


Figure 11. MS/MS fragmentation confirmation of the truncated peptide (7 to 31) impurity.

Experiment 2: Analysis of the main peak and post-main impurities

In this experiment, 10 HiRes cuts were made across the main peak, along with an impurity peak that eluted after the main peak, as shown in Figure 12. To capture different regions of interest in the chromatogram, multiple runs were conducted, selecting HiRes cuts from various parts of the chromatographic profile. By using this high-resolution sampling approach and multi-injection technology, detailed information was gathered about the sample while achieving substantial time savings in the second dimension.

The cuts, labeled Cut 1 and Cut 2, were analyzed on a 2D-LC column and by mass spectrometry to improve resolution and characterization. The MS analysis confirmed the peptide sequence in the semaglutide sequence and variable modifications in the detected impurities, as detailed in Table 5.

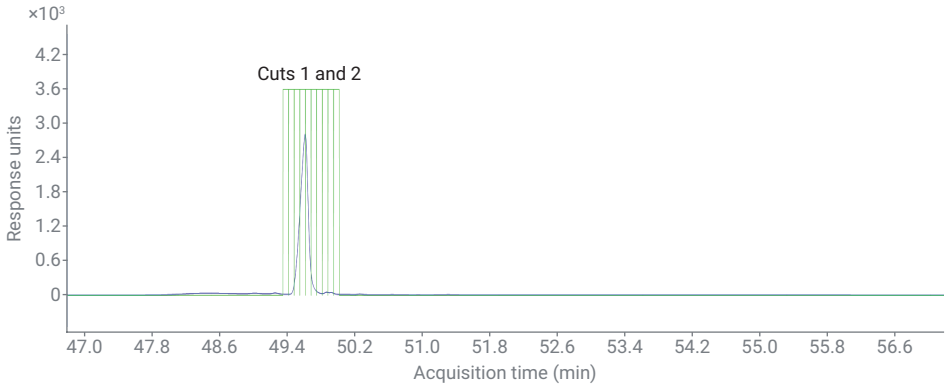


Figure 12. Semaglutide 1D chromatogram, 10 HiRes cuts (five each for Cut 1 and Cut 2) were made across the main peak, along with an impurity peak that eluted after the main peak.

Table 5. The list of impurities identified from both cuts in Experiment 2.

Cut	Sequence	Precursor Mass	Mass (Bio)	RT	Height	Difference (Bio, ppm)	Sequence Location	Fixed Modifications	Variable Modifications
1	VSSYLEGQAAKEFIAWLVRGRG	3,151.7291	3,151.7169	7.146	2,203	3.86	(10 - 31)	Semaglut 11	
1	EGQAAKEFIAWLVRGRG	2,602.4438	2,602.437	7.152	2,062	2.61	(15 - 31)	Semaglut 6	
1	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	4,111.1296	4,111.1154	7.208	4,047,569	3.45	(1 - 31)	Aib 2, semaglut 20	
1	AKEFIAWLVRGRG	2,217.2797	2,217.2773	7.247	3,385	1.1	(19 - 31)	Semaglut 2	
1	AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	3,889.0114	3,889.0037	7.44	20,908	1.98	(2 - 31)	Aib 1, semaglut 19	1*Des-Aib
1	AEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	3,974.0597	3,974.0565	7.487	8,945	0.82	(2 - 31)	Aib 1, semaglut 19	
1	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	4,111.1308	4,111.1154	7.493	13,301	3.75	(1 - 31)	Aib 2, semaglut 20	
2	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	4,111.1314	4,113.747	7.331	3,431,598	3.89	(1 - 31)	Aib 2, semaglut 20	
2	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	4,111.1138	4,113.6081	7.469	2,556	-0.39	(1 - 31)	Aib 2, semaglut 20	
2	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	4,111.1229	4,113.2259	7.504	28,352	1.82	(1 - 31)	Aib 2, semaglut 20	
2	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR	4,054.1116	4,056.6052	7.361	279,151	4.36	(1 - 30)	Aib 2, semaglut 20	
2	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGR	4,054.1021	4,056.5528	7.47	6,389	2.02	(1 - 30)	Aib 2, semaglut 20	
2	AKEFIAWLVRGRG	2,217.2744	2,218.4606	7.341	2,966	−1.3	(19 - 31)	Semaglut 2	

A significant finding of Des-HA and Des-H impurities were separated and identified in the ^{2}D analysis of Cut 1. The separation of these impurities is illustrated in Figure 13, where the extracted ion chromatograms (EICs) of the identified impurities were overlaid on the total ion chromatogram (TIC). The impurities eluted at retention times of 7.44 and 7.48 minutes, respectively.

Figure 14 illustrates the comparison of the triply charged states of two truncated impurities: one impurity missing both a histidine and Aib-modified alanine residue (missing HA), and the other impurity missing only the histidine residue (missing H).

This analysis revealed two significant biomolecule compounds with impurities present in Cut 1 at 49.35 minutes of the main peak. Both impurities were identified as fragments of the semaglutide peptide: impurity 1 (3 to 31): EGTFTSDVSSYLEGQAAKEFIAWLVRGRG, with a loss of Aib and histidine, and impurity 2 (2 to 31): AEGTFTSDVSSYL EGQAAKEFIAWLVRGRG, with a loss of histidine. Figure 14 shows the comparison of the triply charged states of both truncated impurities with distinct mass differences due to the missing respective amino acids.

The precise mass differences and the retention times confirm the identity and structural differences between the impurities. This analysis is critical for ensuring quality control in biopharmaceutical therapeutic peptide development.

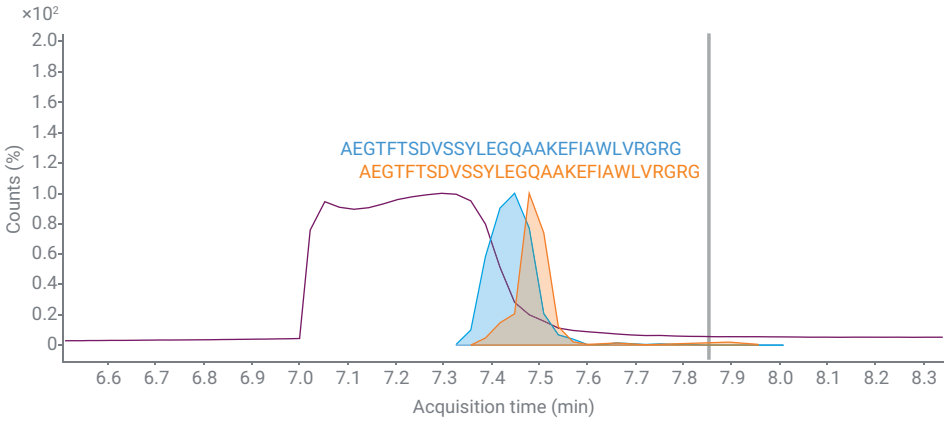


Figure 13. EIC of Des-H and Des-HA impurities in the ^{2}D analysis of Cut 1 overlaid onto the TIC.

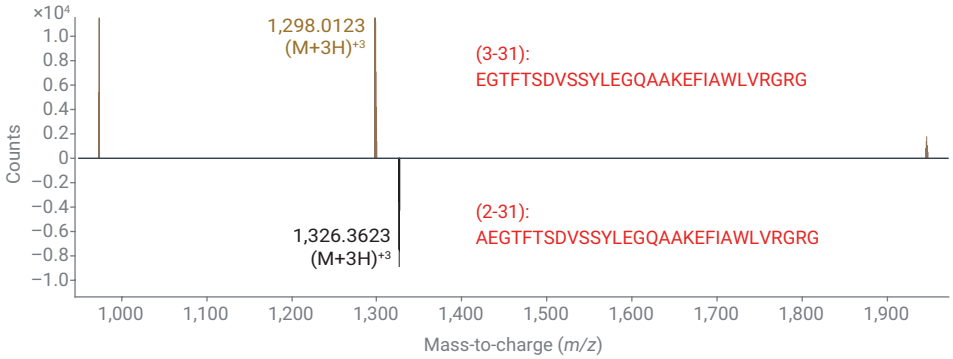


Figure 14. Comparison of the triply charged states of two truncated impurities in Cut 1 from Experiment 2.

Table 6. Agilent MassHunter BioConfirm software sequence confirmation for truncated impurities from the Cut 2 analysis.

Cut	Mass	RT	Sequence Name	Sequence	Fixed Modifications	Variable Modifications	Difference (Bio, ppm)
Cut 2	3,889.0114	7.44	EGTFTSDVSSYLEGQAA**K**EFIAWLVRGRG	(3-31)	Semaglutide 18		1.98
Cut 2	3,974.0597	7.487	**A**EGTFTSDVSSYLEGQAA**K**EFIAWLVRGRG	(2-31)	Aib 1, semaglutide 19	1*Des-Aib	0.82

Conclusion

This study demonstrates a comprehensive workflow for the identification and characterization of therapeutic peptides using an Agilent 1290 Infinity III bio 2D-LC system coupled with an Agilent 6545XT AdvanceBio LC/Q-TOF using Agilent MassHunter BioConfirm software, version 12.1. The workflow effectively confirmed peptide sequences and identified low-abundance impurities.

The 2D-LC system, integrated with a Q-TOF mass spectrometer, enabled the separation of product-related impurities closely eluting with the API peak. In the first dimension, an Agilent AdvanceBio Peptide Plus column ensured effective separation, while in the second dimension, an Agilent AdvanceBio RP-mAb C4 column provided desalting while preserving the enhanced resolution from the first dimension. High-resolution sampling with multi-injection offered unique capabilities for superior separation efficiency and faster automated analysis. The 6545XT AdvanceBio LC/Q-TOF system delivered accurate mass and MS/MS data, facilitating the identification of low-abundance impurities.

MassHunter BioConfirm 12.1 software streamlined data analysis by automating the identification of peptide products and related impurities using advanced sequence matching algorithms and intact mass detection. This robust workflow provided reliable solutions for precise impurity characterization and sequence confirmation of synthetic peptide therapeutics.

The integration of 2D-LC with high-resolution sampling, multi-injection technology, the advanced detection capabilities of the AdvanceBio Q-TOF system, and the peptide workflow of BioConfirm software provides a comprehensive, all-in-one solution for peptide characterization, driving the development and quality control of therapeutic peptides.

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