

Poster Reprint

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

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Introduction

Synthetic peptides are widely used in therapeutics for their high potency and specificity. However, their structural diversity makes impurity profiling critical, especially during GMP manufacturing, where impurities from deletions, insertions, or degradation can affect safety and efficacy. GLP-1 receptor agonists like semaglutide (MW 4,113.58 Da) are prominent in treating type II diabetes and obesity. Related variants often coelute with the main peak, making their detection challenging in conventional LC/MS workflows. A 2D-LC/MS workflow was implemented using the Agilent 1290 Infinity III Bio 2D-LC system with multiple heart-cutting, coupled to the 6545XT AdvanceBio LC/Q-TOF and BioConfirm software. The phosphate-buffer-based 1D separation offers high resolution, while the 2D step enables MS-compatible desalting and improved impurity detection.

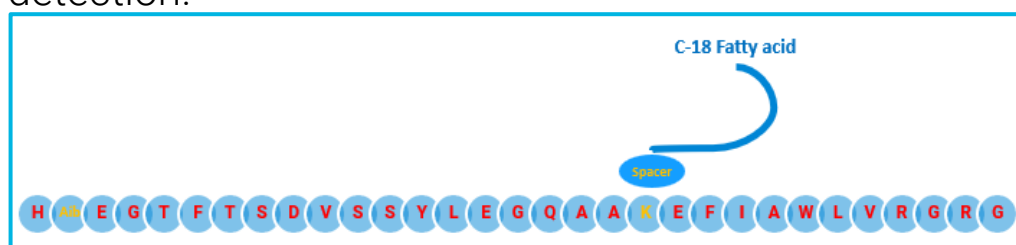


Figure 1. Semaglutide structure with Aib at position 2 (α -methylalanine).

Instrumentation

1290 Infinity III Bio 2D-LC & 6545XT LC/Q-TOF includes:

- 1290 Infinity III bio flexible pump (G7131A)
- 1290 Infinity III bio high-speed pump (G7132A)
- 1290 Infinity III bio multisampler (G7137A)
- 1290 Infinity III multicolumn thermostat (G7116B)
- 1290 Infinity III variable wavelength detector (G7114B)
- 1290 Infinity III diode array detector (G7117B)
- 2D-LC System with multiple heart cutting set up



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

Experimental

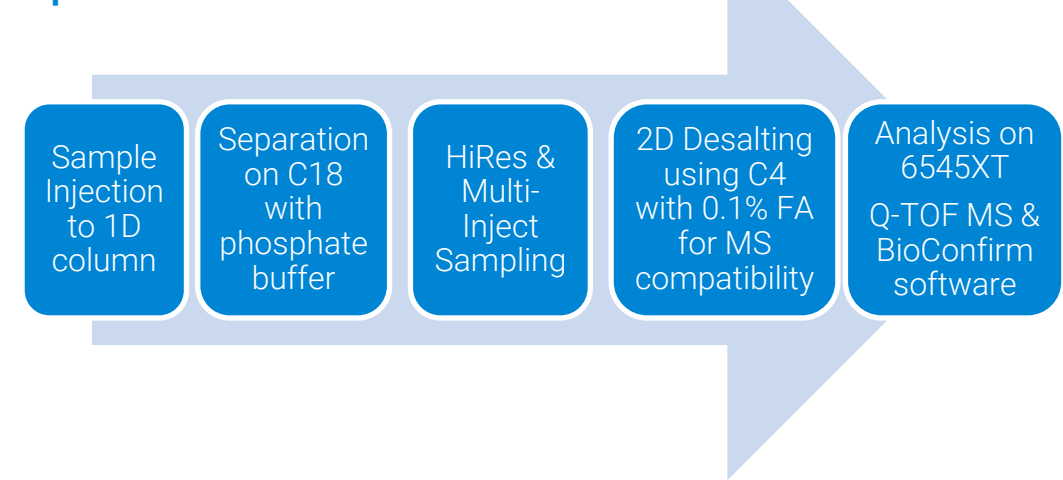
Table 1: 1D and 2D Method Parameters

Parameters	Details									
First-dimension										
Mobile Phase	Buffer : 25 mM disodium hydrogen phosphate, pH 4.5 Mobile phase A: buffer: acetonitrile 70:30 Mobile phase B: buffer: acetonitrile 30:70									
Flowrate	0.6 ml/min									
Gradient	Time (min)	0	5	30	45	50	55	60	60.5	68
	B (%)	15	20	24	28	85	95	95	15	15
Injection Vol	20 μ L									
Column	AdvanceBio Peptide Plus, 2.1 \times 250 mm, 2.7 μ m									
Column Temp	45 $^{\circ}$ C									
Detector	UV 215 nm									
Second- dimension										
Mobile Phase	Mobile phase A: 0.1% formic acid in water Mobile phase B: 0.1% formic acid in acetonitrile									
Flowrate	0.4 ml/min									
Gradient	Time (min)	0	5	6	15	15.1	17	18		
	B (%)	30	30	42	45	55	55	30		
Column	AdvanceBio RP-mAb C4 column									
Column Temp	50 $^{\circ}$ C									
Flowrate	0.6 ml/min									
2D Cycle Time	23 mins									
ASM Setting	Factor: 3 ; Flush factor: 5									

Table 2: MS Parameters

System	6545XT AdvanceBio LC/Q-TOF
Gas Temp	325 $^{\circ}$ C
Drying Gas	11 L/min
Nebulizer	35 psi
Sheath Gas Temp	275 $^{\circ}$ C
Sheath Gas Flow	11 L/min
VCap	4000 V
Nozzle Voltage	500 V
Fragmentor	175 V
Mass Range	m/z 300 to 3,200
Auto MS/MS Range	m/z 50 to 3,200
Isotope Model	Peptides
Sort Precursors	By abundance only

Optimized 2D-LC/MS Workflow



Results and Discussion

Semaglutide Characterization Using BioConfirm

Step 1: Acquisition Setup 6545XT LC/Q-TOF

- MS & MS/MS data collected
- Wide mass range & optimized Collision energy used

Step 2: Define Sequence and Modifications

- Input semaglutide sequence into BioConfirm with Aib at position 2 & C18 chain at Lys20
- Define modifications to enable accurate identification of related impurities

Step 3: BioConfirm Protein Digest Workflow

- Non-reduced, undigested setup
- Detect intact peptides, isoforms, truncated variants and post-translational modifications (PTMs)

Step 4: Mass Matching and Identification

- Tolerance set to MS: ± 10 ppm & MS/MS: ± 20 ppm
- Detects peptides (2–70 amino acids long) and confirms variants, isoforms, degradants etc.

Figure 3. Chemical modifications at alanine 2 & lysine 20 in semaglutide sequence set up in BioConfirm.

Figure 4. Protein digest workflow for semaglutide analysis in BioConfirm.

2D-LC Separation and HiRes Sampling Strategy

- Orthogonal 2D-LC using Peptide Plus (1D) and RP-mAb C4 (2D) enabled high-resolution separation and MS-compatible desalting.
- HiRes sampling captured full peak profiles across pre-main, main, and post-main regions.
- Multi-inject allowed combined transfer of heart-cuts, improving throughput and efficiency.

A high-resolution sampling approach was applied across two targeted experiments:

- Experiment 1:
 - Focused on pre-main peak impurities
 - Targeted HiRes cuts captured early-eluting variants
- Experiment 2:
 - Focused on main and post-main peak regions
 - Enabled detailed profiling of product and late-eluting impurities
 - Multi-inject transferred 10 heart-cuts per run, ensuring high-resolution separation and full MS data acquisition across all key regions.

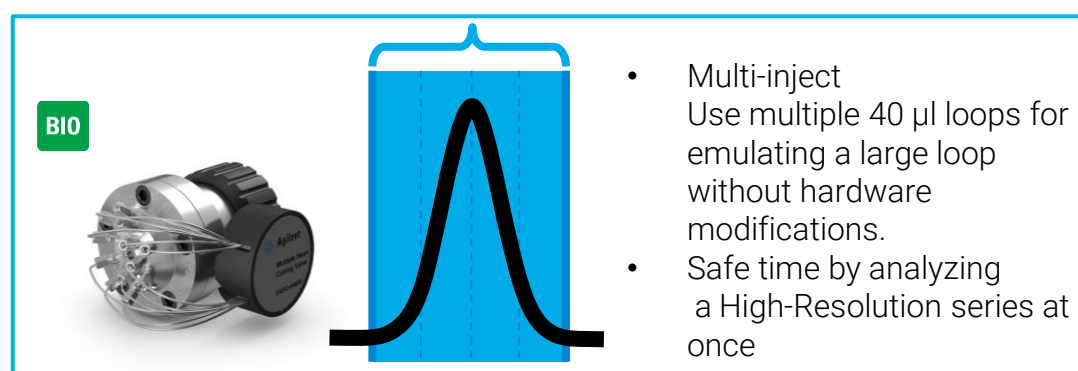


Figure 5. Multi-Inject with Bio MHC Valve & Biocompatible Loops

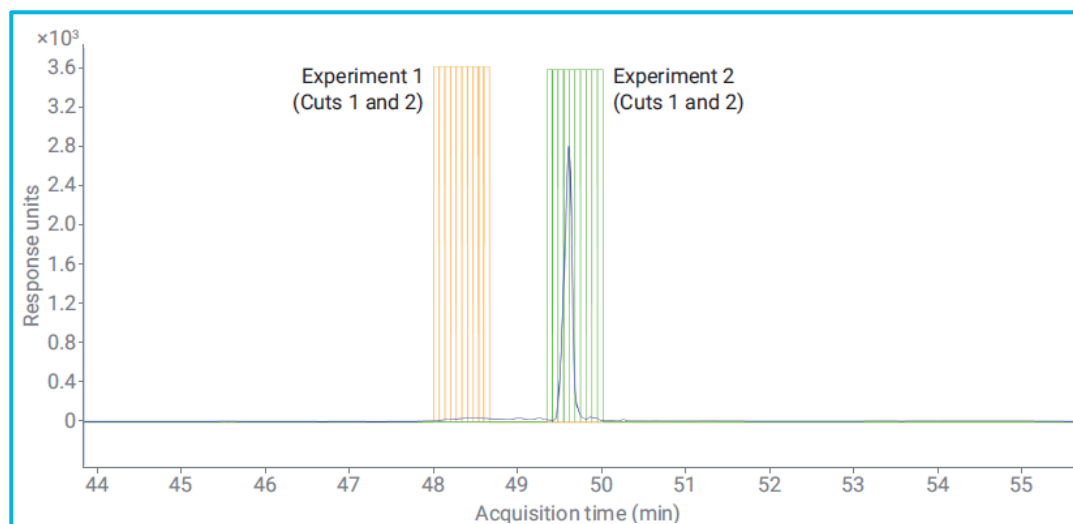


Figure 6. Overlay of 1D chromatograms with HiRes cut markers: Experiment 1 (pre-main impurities) & Experiment 2 (main and post-main peaks).

Results and Discussion

Experiment 1: Pre-Main Impurities

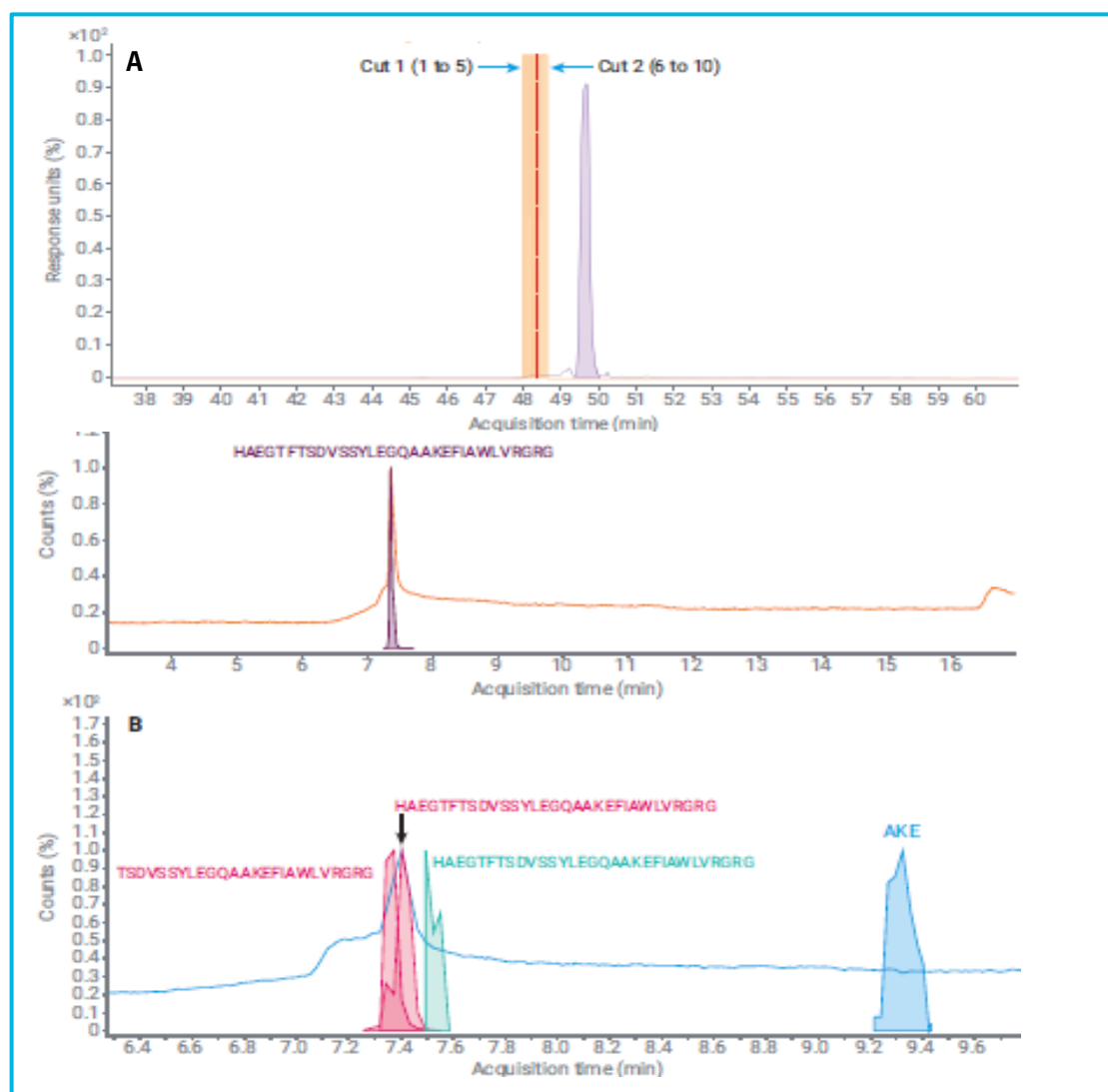


Figure 7. (A) HiRes fractions of pre-main impurities (Cuts 1 & 2); (B) 2D EICs (m/z 4,111.13) showing clear separation of semaglutide isomer & truncated fragments.

Table 4: Identified sequences from HiRes fractions

ID Cuts	Mass	ID RT	Sequence Name	Sequence Location	Sequence	Fixed Modifications	Variable Modifications	Difference (Bio, ppm)
Cut 1	4,111.128	7.355	Semaglutide	(1-31)	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	Alb 2, Semaglutide 20		2.96
Cut 2	3,454.836	7.36	Semaglutide	(7-31)	TSDVSSYLEGQAAKEFIAWLVRGRG	Semaglutide 14		3.72
Cut 2	4,111.129	7.411	Semaglutide	(1-31)	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	Alb 2, Semaglutide 20	1*Des-Alb	3.32
Cut 2	4,111.126	7.514	Semaglutide	(1-31)	HAEGTFTSDVSSYLEGQAAKEFIAWLVRGRG	Alb 2, Semaglutide 20		2.59
Cut 2	932.571	9.314	Semaglutide	(19-21)	AKE	Semaglutide 2	Des-E 3	3.02

- Table 4 summarizes the exact match of semaglutide and truncated impurities in both Cuts 1 and 2.
- Cut 1 identified intact semaglutide (1–31), while Cut 2 revealed truncated peptides (7–31) and (19–21).
- Isomeric impurities coeluting in 1D were resolved by slight retention time shifts.

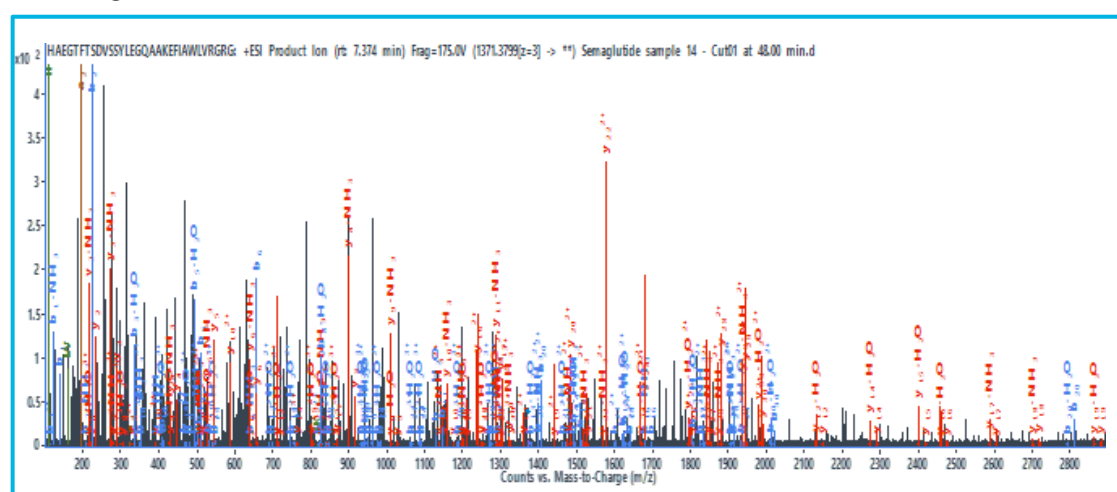


Figure 8. MS/MS fragmentation ladder of semaglutide (z = 3) showing b/y ion series coverage.

<https://www.agilent.com/en/promotions/asms>

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Experiment 2: Main & Post-Main Impurities

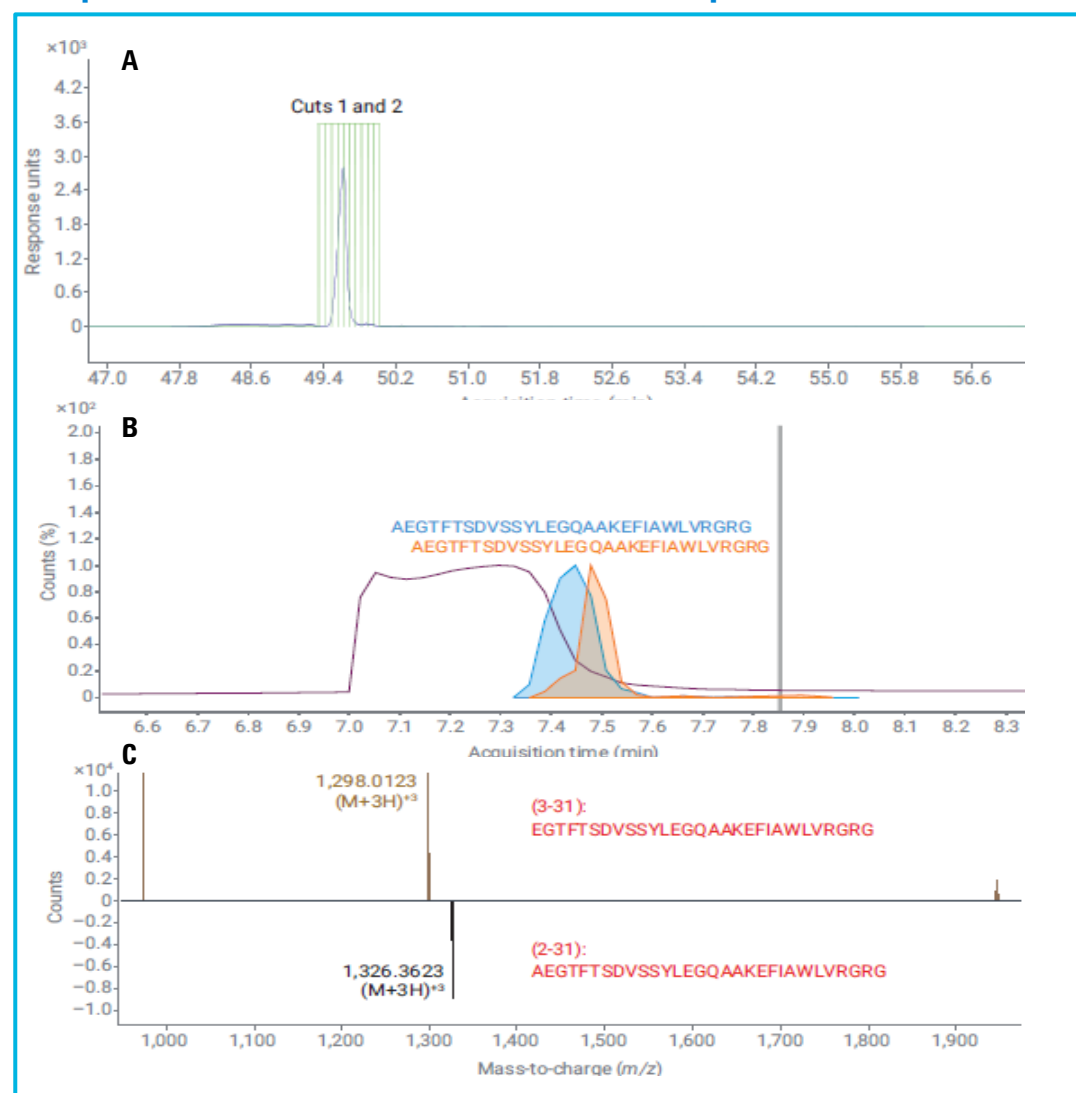


Figure 9: (A) Ten HiRes fractions across the main peak and one post-peak impurity; (b) 2D-LC/MS identification of variants, including Des-HA (2–31) and Des-H (3–31) fragments; (c) comparison of the triply charged states of two truncated impurities.

Conclusions

- A 2D-LC/MS workflow enabled effective separation and identification of coeluting impurities.
- HiRes sampling with multi-injection improved resolution and throughput while preserving 1D separation for MS-compatible desalting.
- AdvanceBio LC/Q-TOF delivered accurate mass and MS/MS data for low-abundance impurity detection.
- BioConfirm software enabled automated sequence confirmation and impurity identification.
- This integrated platform offers a robust solution for peptide characterization in therapeutic development.

References

Khandpur, P.; Bharatiya, P.; Pargaonkar, A. Advanced 2D-LC/Q-TOF Workflow for Impurity Characterization of GLP-1 Therapeutic Peptide Semaglutide, Agilent Technologies application note, publication number 5994-8288EN, 2025.