Application Note

Biopharmaceuticals

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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 liquid chromatograph (LC) coupled with the Agilent 6545XT AdvanceBio liquid chromatography/quadrupole time-of-flight mass spectrometer (LC/Q-TOF MS) 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]

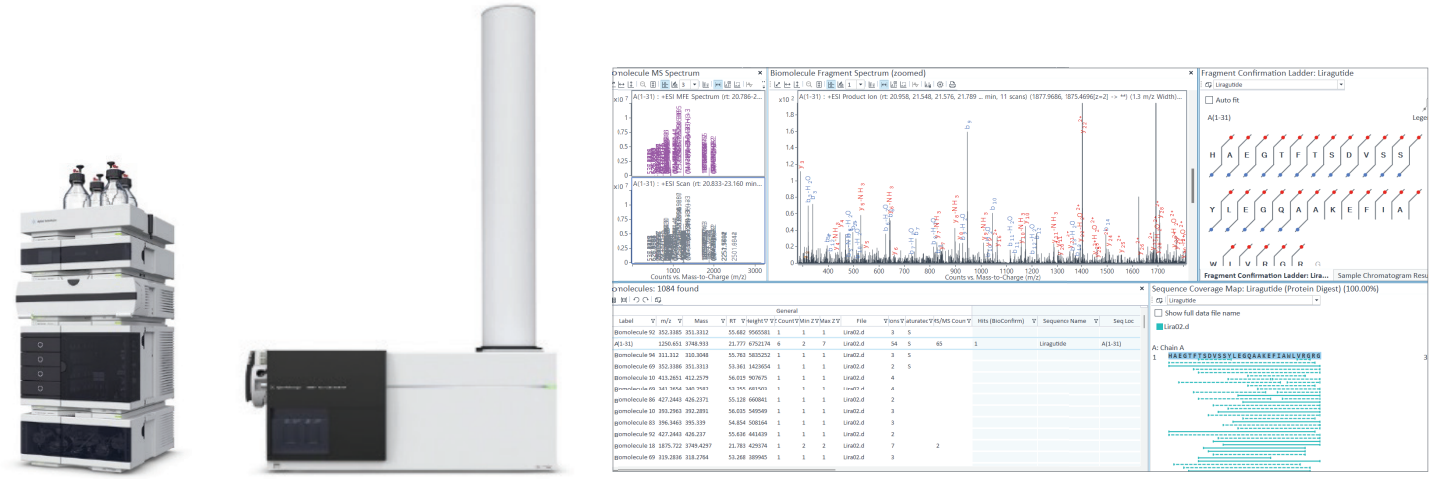


Figure 1. Agilent 1290 Infinity II LC coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF and BioConfirm software.

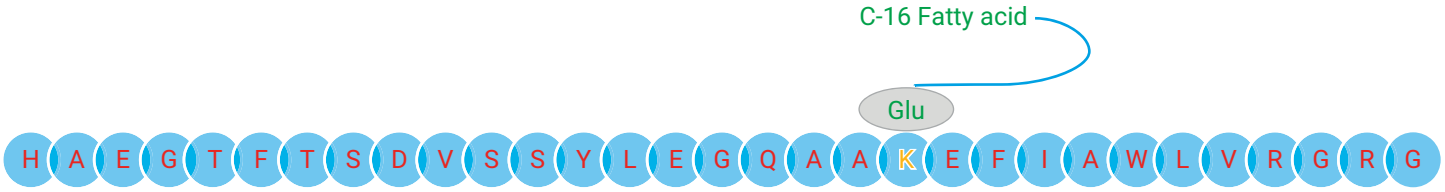


Figure 2. Structure of liraglutide.

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)

Instrument configuration

Table 1. Liquid chromatography parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Plus, 2.1 × 150 mm, 2.7 µm (part number 695775-949)
Thermostat	8 °C
Solvent A	0.01% TFA in 95:5 Milli-Q water:acetonitrile
Solvent B	0.01% TFA in 95:5 acetonitrile:Milli-Q water
Gradient	Time (min) %B 0 10 3 10 6 40 50 50 55 65 55.1 10 60 10
Stop Time	60 min
Column Temperature	45 °C
Flow Rate	0.4 mL/min
Injection Volume	1 µL

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

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).

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.

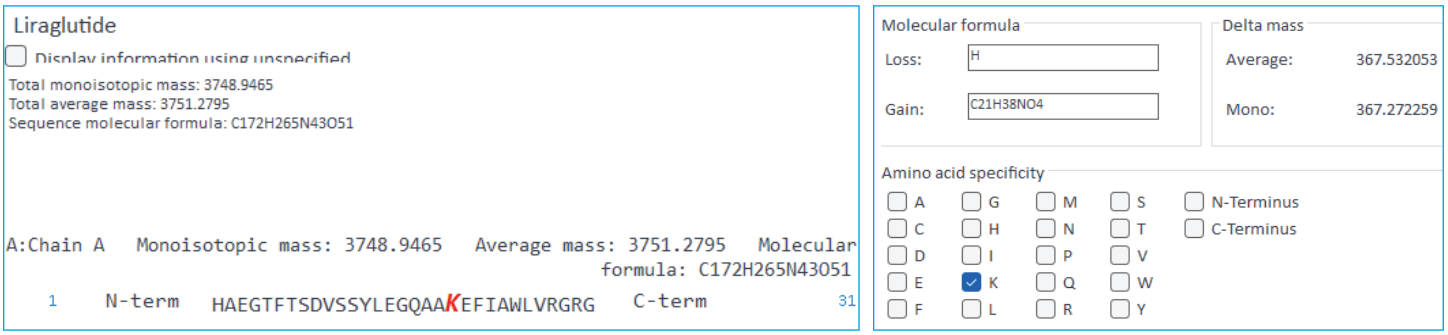


Figure 3. Setting up of chemical modification at lysine 25 (K25) sequence of liraglutide in Agilent MassHunter BioConfirm Sequence Manager. Additional impurities were included in the BioConfirm Sequence Manager as modifications.

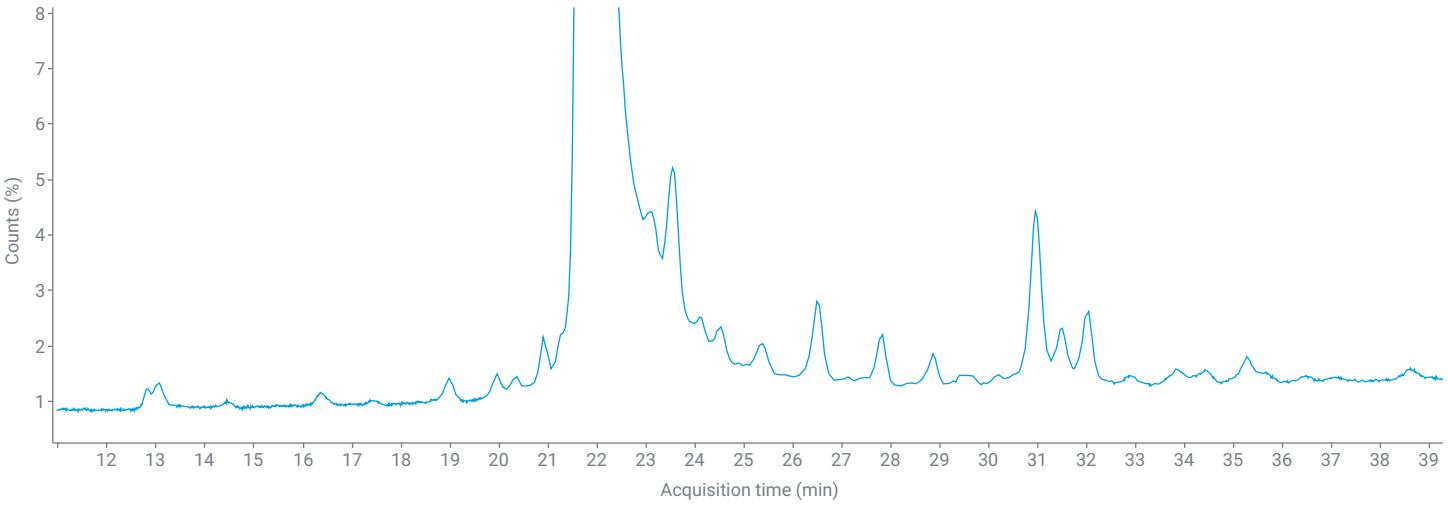
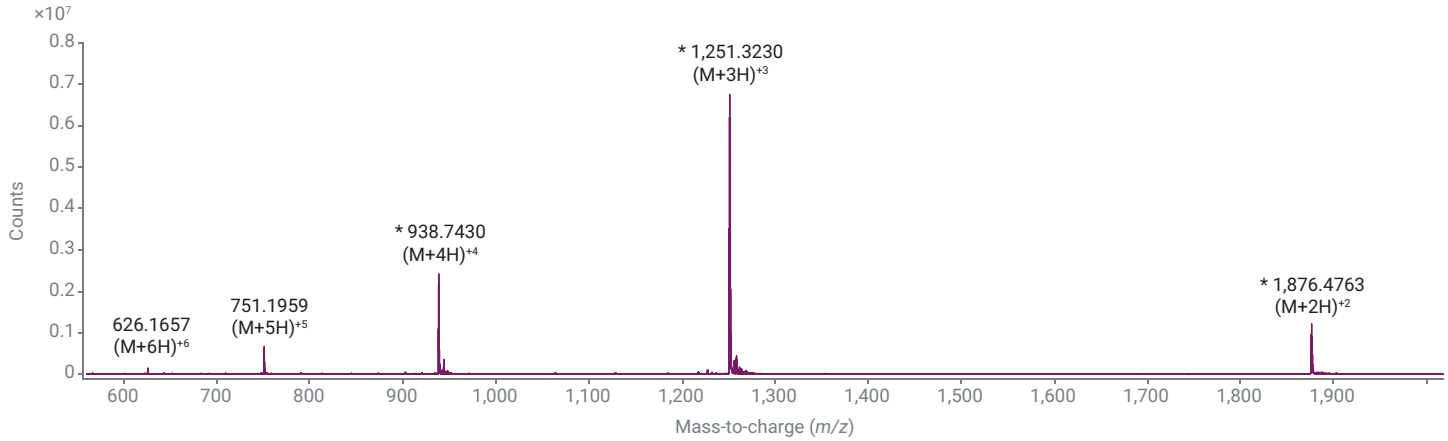


Figure 4. The zoomed TIC of a liraglutide sample and the separation of the main peak and impurities.

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.

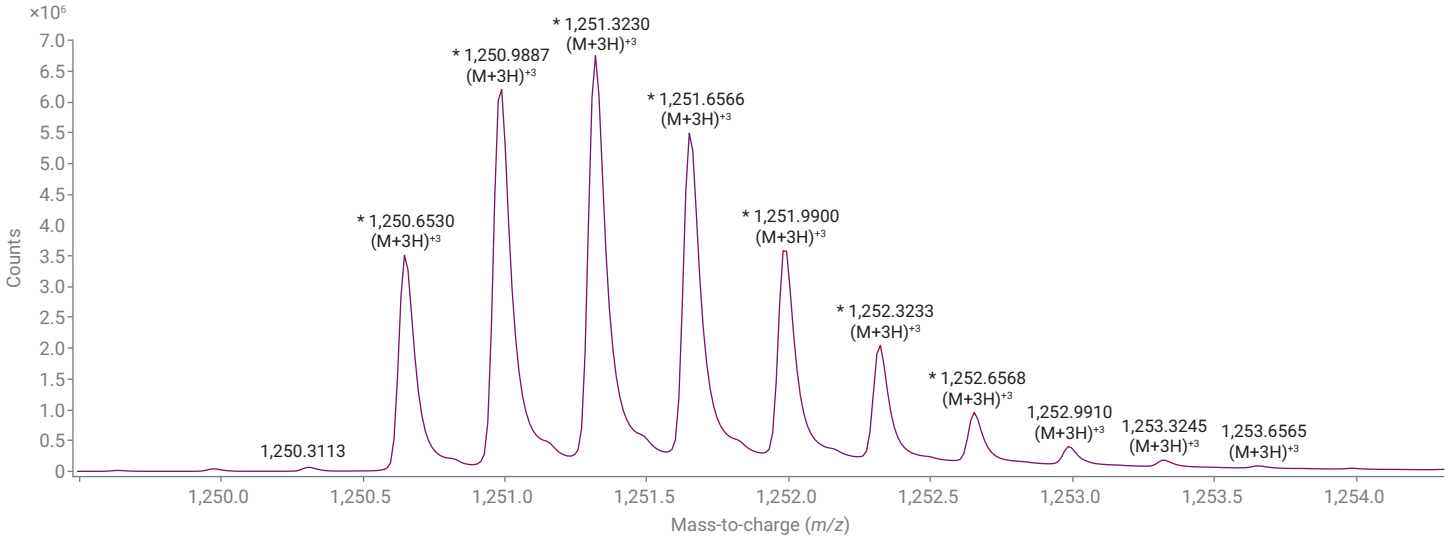


Figure 6. Isotopic fidelity of $(M+3H)^{+3}$ charge state of intact liraglutide.

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.

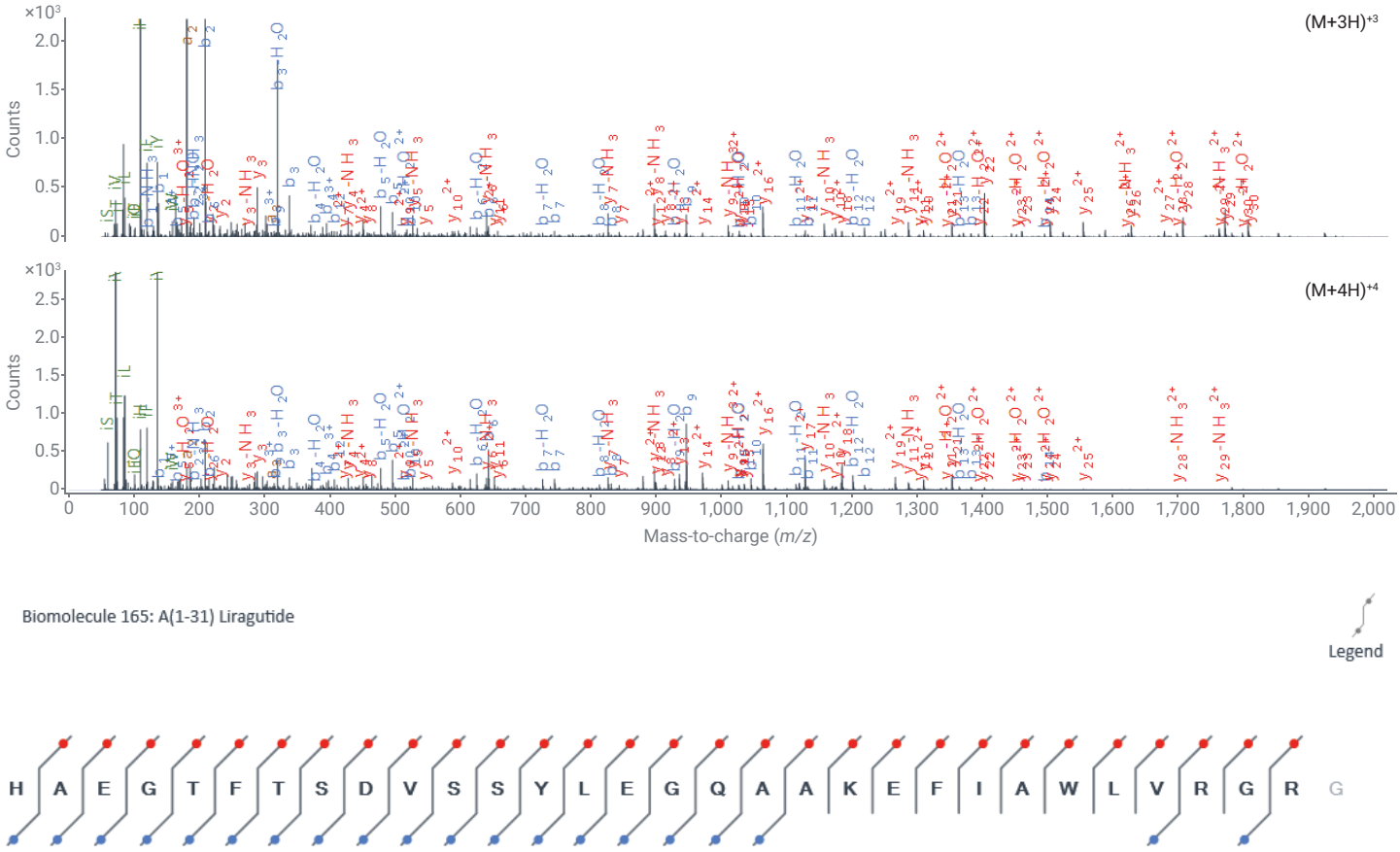


Figure 7. The MS/MS fragmentation pattern for the liraglutide peptide (z = 3, 4) with coverage of both b and y ion series. The fragmentation ladder annotates the identified b/y series (z = 3) for the sequence.

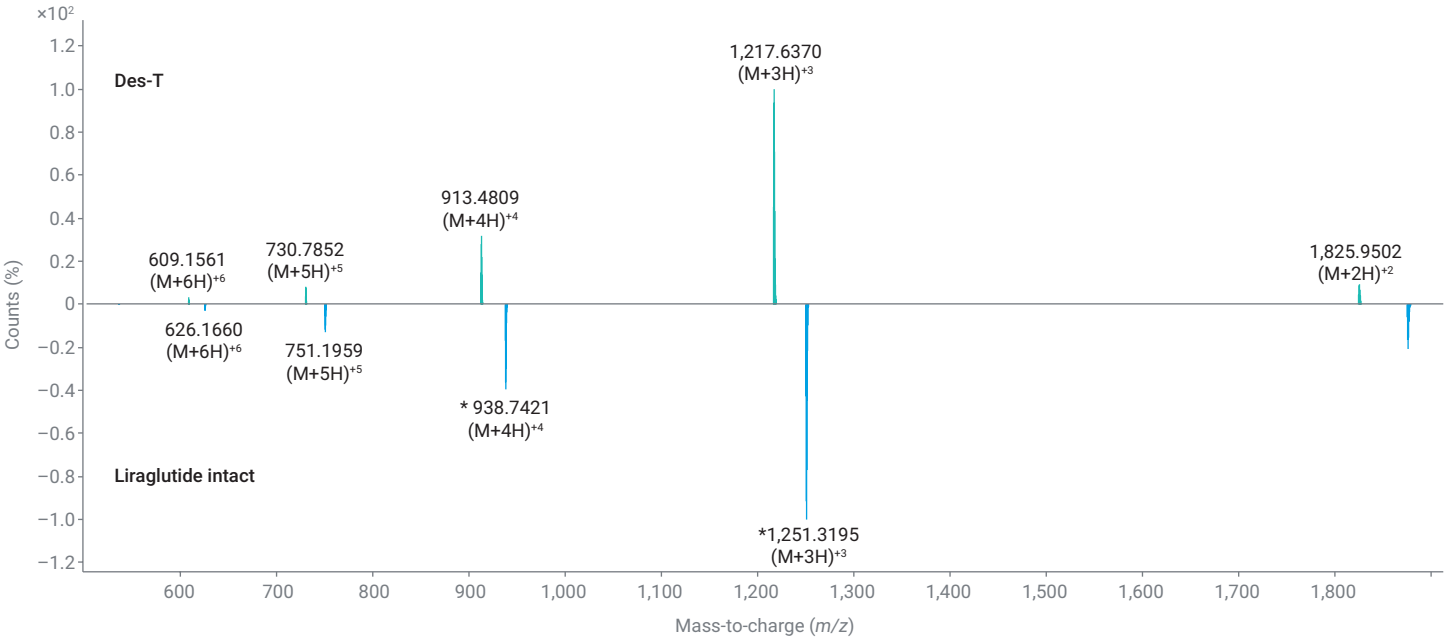


Figure 8. The different charge states of impurities compared by mirror plots of Agilent MassHunter BioConfirm software.

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.

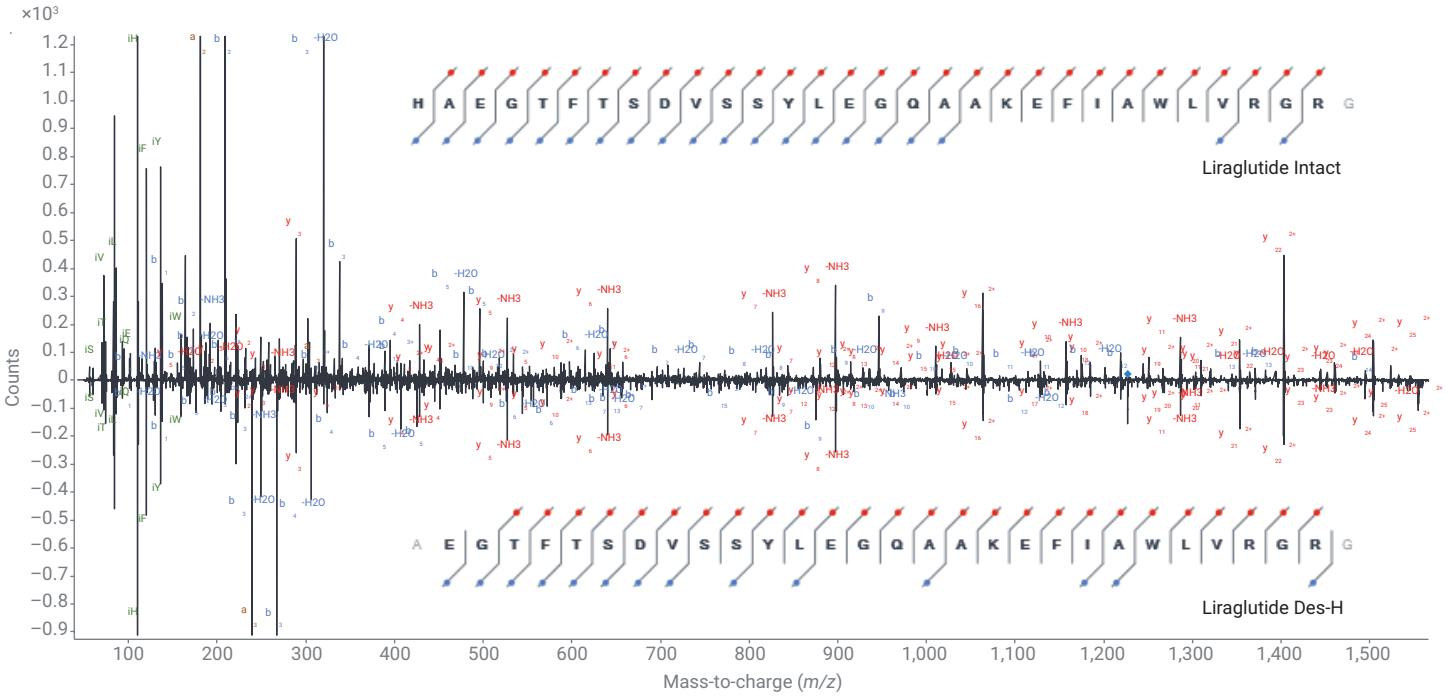


Figure 9. Missing H (1) impurity in liraglutide: Comparison of the y and b ions from the MS/MS spectrum for modified b1 ions shows an effective change over those ions in unmodified peptide, suggesting that histidine (H) is missing at position 1.

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.

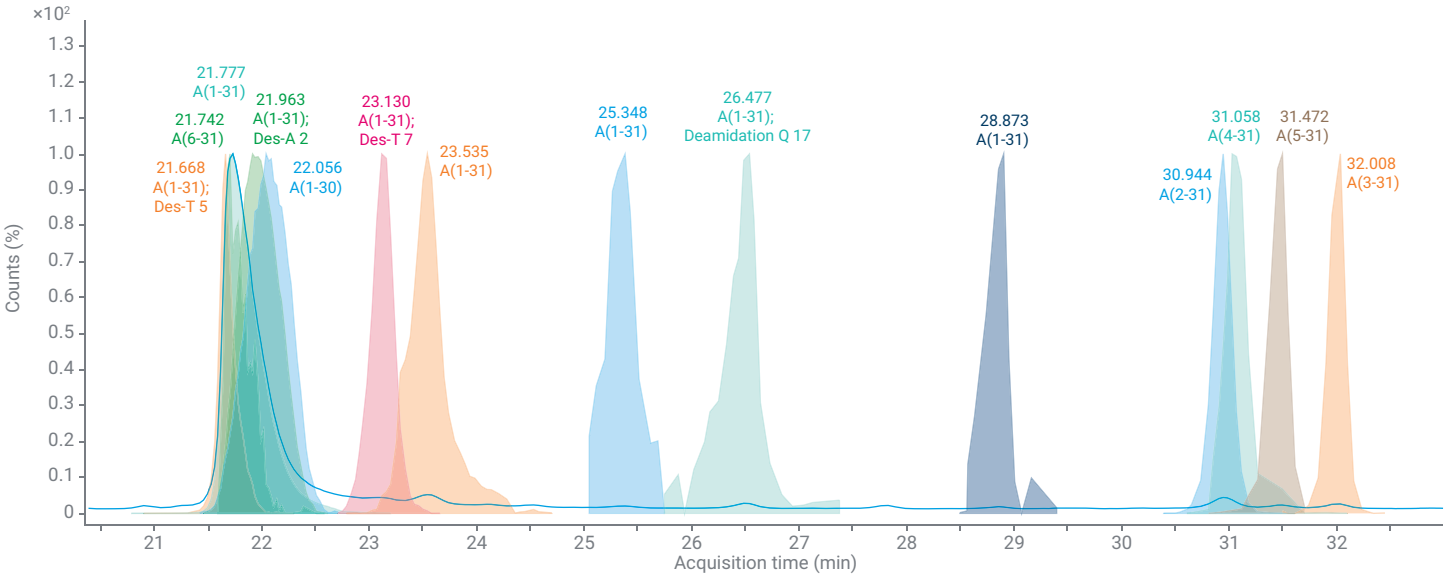


Figure 10. Overlaid chromatograms for EIC of different impurities separated at different retention times (RTs).

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

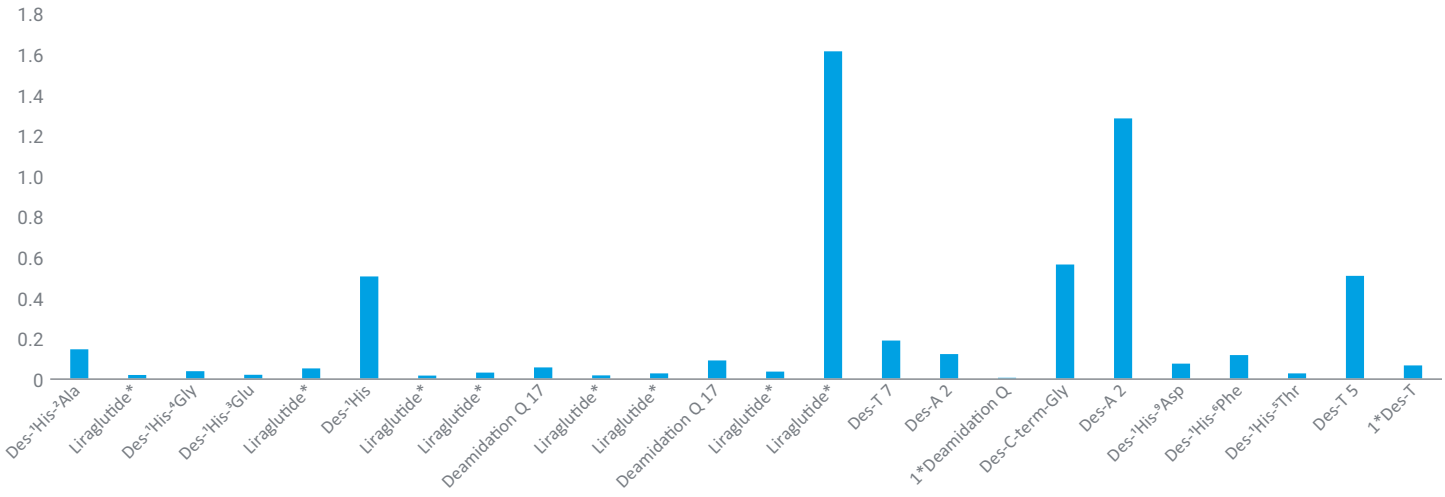


Figure 11. Relative percentage of identified impurities with respect to intact liraglutide. The isomeric impurities separated chromatographically are marked with an asterisk (*).

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-MS. 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.

References

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DE-000344

Printed in the USA, September 25, 2024
5994-7727EN

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