Application Note

Biopharmaceuticals

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Peptide Drug Stability Analysis Using Agilent InfinityLab LC/MSD and OpenLab CDS Deconvolution

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Abstract

The Federal Drug Administration (FDA) abbreviated new drug application (ANDA) guidelines advise qualitative and quantitative management of peptide-related impurities due to the potential for their immunogenicity. This application note highlights the analysis and identification of impurities arising from stability assays with GLP-1 agonist peptides. The tests were conducted for Liraglutide and Semaglutide under acidic, basic, and oxidative conditions. The analysis was conducted using the Agilent 1260 Infinity II Prime Bio LC, InfinityLab LC/MSD system, and InfinityLab Poroshell 120 EC-C18 1.9 µm column. In addition, the deconvolution capability of Agilent OpenLab CDS software, version 2.8, to generate the masses of the products was demonstrated. Through these tests, we were able to determine the molecular weights of major forced degradation impurities of Liraglutide and Semaglutide.

Introduction

Traditional peptide drug products have been manufactured using recombinant DNA technology. More recently, several producers of therapeutic peptides have applied large-scale synthesis technology, laying the foundation for the production of high-purity synthetic peptides for bulk active pharmaceutical ingredients (APIs). In line with the development of new manufacturing methods, the FDA has published the ANDA guidance for synthetic peptide drug products, aiding API producers in developing their testing and analysis procedures.[1]

However, the shift toward synthetic peptides raises concerns about changes in clinical effects and safety profiles due to changes in the manufacturing methods. Duplicated amino acids or side-chain modifications may lead to impurities that are difficult to separate from the API and may exhibit immunogenicity.[2] Therefore, the FDA guidelines advise that peptide-related impurities in generic peptide drug products do not exceed those of the reference listed drug (RLD), or, considering immunogenicity, new peptide-related impurities do not exceed 0.5% of the main compound.[1] To achieve this goal, analytical methods are necessary for separating and detecting impurities adequately.

Controlling impurities generated during the synthesis process of synthetic peptide drug products is crucial. In addition, it is required to analyze impurities generated by excipients, the drug product manufacturing process, and storage conditions. Although long-term accelerated stability studies are typically conducted, accelerated stability indicating tests are necessary to ensure early identification of the suitability of a formulation, API characteristics, and potential impurity profiles. High-resolution MS is required for identifying sequence information for each impurity in the early stages of drug development. On the other hand, monitoring impurities in drug formulations, stability studies, and quality control can be achieved using single quadrupole MS. Also, the deconvolution feature of OpenLab CDS 2.8 facilitates the interpretation of multiple charges and adducts of peptide impurities.[3]

In this application note, examples for stability-indicating tests were demonstrated using Liraglutide and Semaglutide with an InfinityLab LC/MSD, confirming the intact molecular weight obtained through deconvolution from the raw MS spectrum of major impurities. The test was conducted using two single quadrupole MS instruments—the InfinityLab LC/MSD iQ and XT—based on their availability. Through this, in confirming the molecular weight of peptide-related impurities, both the InfinityLab LC/MSD iQ and InfinityLab LC/MSD XT demonstrate sufficient performance.

Experimental

Instrumentation

- Agilent 1260 Infinity II Bio flexible pump (G7131C)
- Agilent 1290 Infinity II Bio multisampler (G7137A) with sample thermostat
- Agilent 1290 Infinity II multicolumn thermostat (G7116B) with Quick Connect bio heat exchanger standard flow (G7116-60071)
- Agilent 1290 Infinity II diode array detector (DAD) HS (G7117B) with Bio-Inert Max-Light cartridge cell, 60 mm (G5615-60017)
- Agilent InfinityLab LC/MSD iQ (G6160A)
- Agilent InfinityLab LC/MSD XT (G6135C)

Reagents

Difluoroacetic acid (DFA) and hydrochloric acid (HCl) were purchased from Sigma-Aldrich; sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2) were purchased from Merck; and acetonitrile (ACN) was purchased from B&J.

Samples

Liraglutide was purchased through Kairos Tech in Korea. Liraglutide was dissolved in water to a concentration of 1 mg/mL. The solution of Liraglutide 1 mg/mL (1 mL) was heated at 60 °C after adding 100 µL of 1 M HCl, 1 M NaOH, or 2% H_2O_2. Samples were taken immediately, and on the first and second days. After sampling, 100 µL of 1 M NaOH and 1 M HCl were added to neutralize the samples in 0.1 M HCl and 0.1 M NaOH conditions, respectively.

Semaglutide was donated by a local customer. Semaglutide was dissolved in 30% ACN at a concentration of 1 mg/mL. Then, 1 mL of the solution was taken, and 100 µL of 1 M HCl was added. The mixture was heated at 80 °C for one day, followed by neutralization with 100 µL of 1 M NaOH.

Columns

The columns used in this application note include:

- Agilent AdvanceBio Peptide Mapping column, 2.1 × 150 mm, 2.7 µm (part number 653750-902)
- Agilent InfinityLab Poroshell 120 EC-C18 column, 2.1 × 100 mm, 1.9 µm (part number 695675-902)
- Agilent InfinityLab Poroshell 120 EC-C18 column, 2.1 × 150 mm, 1.9 µm (part number 693675-902)

Methods

Table 1. Agilent 1260 Infinity II Prime Bio LC and Agilent InfinityLab LC/MSD iQ method parameters for method confirmation.

Parameter	Value
Agilent 1260 Infinity II Prime Bio LC	
Column	Agilent AdvanceBio Peptide Mapping column, 120 Å, 2.1 × 150 mm, 2.7 µm
Flow	0.4 mL/min
Column Temperature	40 °C
Injection Volume	5 µL
Mobile	A: 0.1% DFA in water B: 0.1% DFA in ACN
Gradient—Column Screening	Time %A %B 0 80 20 1 80 20 20 30 60 25 10 90 25.1 80 20 30 80 20
Detector	UV 280 nm (DAD HS with Bio-Inert Max-Light cartridge cell, 60 mm)
Agilent InfinityLab LC/MSD iQ Parameters	
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: 300 to 1,450 *m/z* Fragmentor: 150 V Scan time: 997 ms (1 Hz) Gain: 5 Storage: Centroid

Table 2. Agilent 1260 Infinity II Prime Bio LC and Agilent InfinityLab LC/MSD iQ method parameters for stability indicating test.

Parameter	Value
Agilent 1260 Infinity II Prime Bio LC	
Column	Agilent InfinityLab Poroshell EC-C18 column, 120 Å, 2.1 × 250 mm, 1.9 µm (connected in series with 100 and 150 mm)
Flow	0.4 mL/min
Column Temperature	40 °C
Injection Volume	5 µL
Mobile	A: 0.1% DFA in water B: 0.1% DFA in ACN
Gradien—Column Screening	Time %A %B 0 80 20 1 80 20 120 30 60 125 10 90 130 10 90 130.1 80 20 140 80 20
Detector	UV 280 nm (DAD HS with Bio-Inert Max-Light cartridge cell, 60 mm)
Agilent InfinityLab LC/MSD iQ Parameters	
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: 300 to 1,450 *m/z* Fragmentor: 150 V Scan time: 997 ms (1 Hz) Gain: 5 Storage: Profile

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

Parameter	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: 300 to 1,450 *m/z* Fragmentor: 150 V Scan time: 997 ms (1 Hz) Gain: 1 Storage: Profile

Table 4. MS spectral deconvolution settings.

Parameter	Value
Basic Settings	
Use *m/z* Range	Not selected
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%

Software

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

Results and discussion

Setting up shallow gradient conditions to enhance the resolution of impurities

To analyze Liraglutide impurities, LC/MS analysis was conducted using 0.1% DFA as an acidic modifier and the AdvanceBio Peptide Mapping column. In the analysis of Liraglutide at 1 mg/mL, impurity peaks in both the UV and MS trace were visible. The MS spectrum of intact Liraglutide was derived from the main peak in the total ion chromatogram (TIC) (Figure 1). However, insufficient chromatographic resolution between the impurities and the main compound in this analysis poses challenges for the qualitative and quantitative assessment of the impurities.

To enhance resolution, the column length was increased, and the particle size of the stationary phase was decreased. In addition, the slope of the gradient was decreased. To separate the peaks observed between 16 and 20 minutes in Figure 1, the column length was increased by connecting an InfinityLab Poroshell 120 EC-C18 column, 1.9 µm, 2.1 × 100 mm, and 150 mm in series. The slope of the gradient was changed to approximately 0.34% B/min. As shown in Figure 2, impurity resolution was improved compared to the previous experiment.

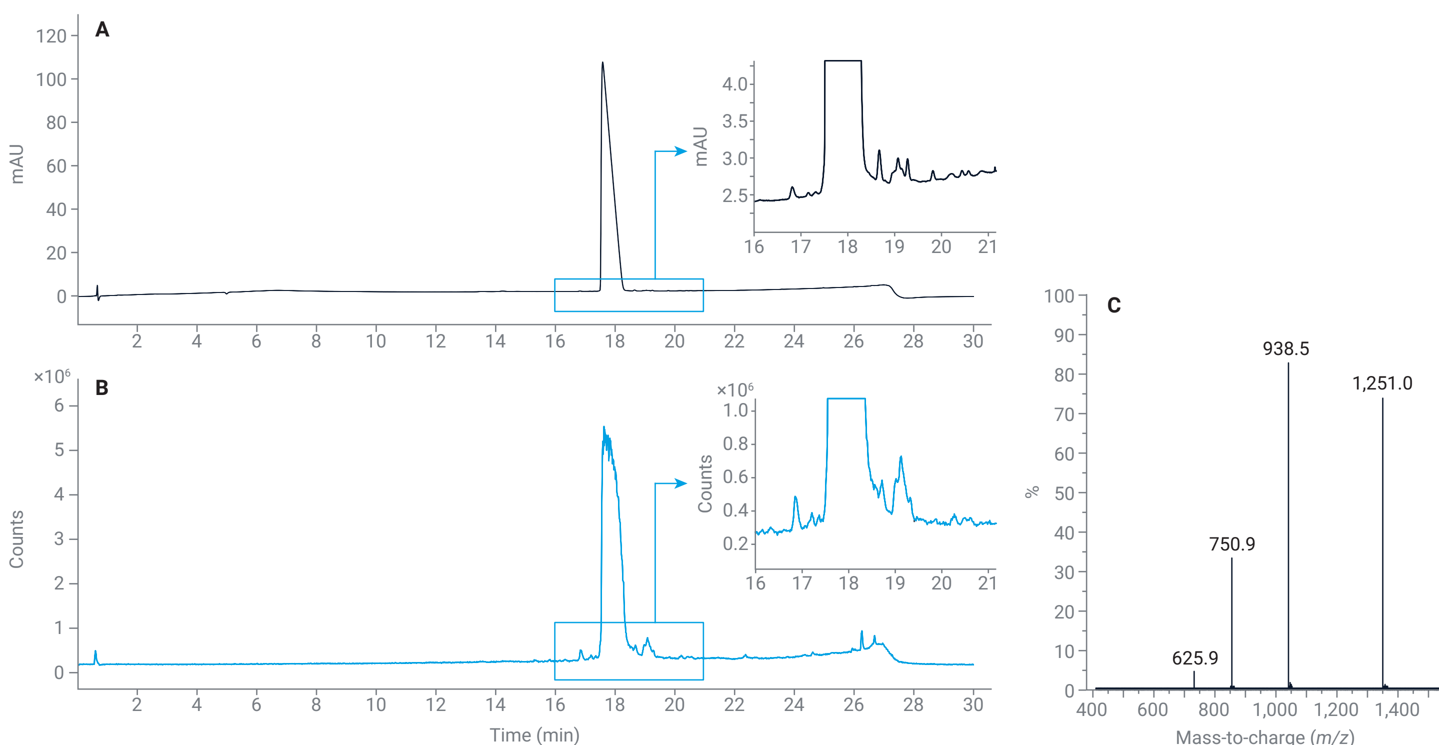


Figure 1. UV chromatogram (A). MS TIC of Liraglutide 1 mg/mL (B). MS spectrum of Liraglutide (C).

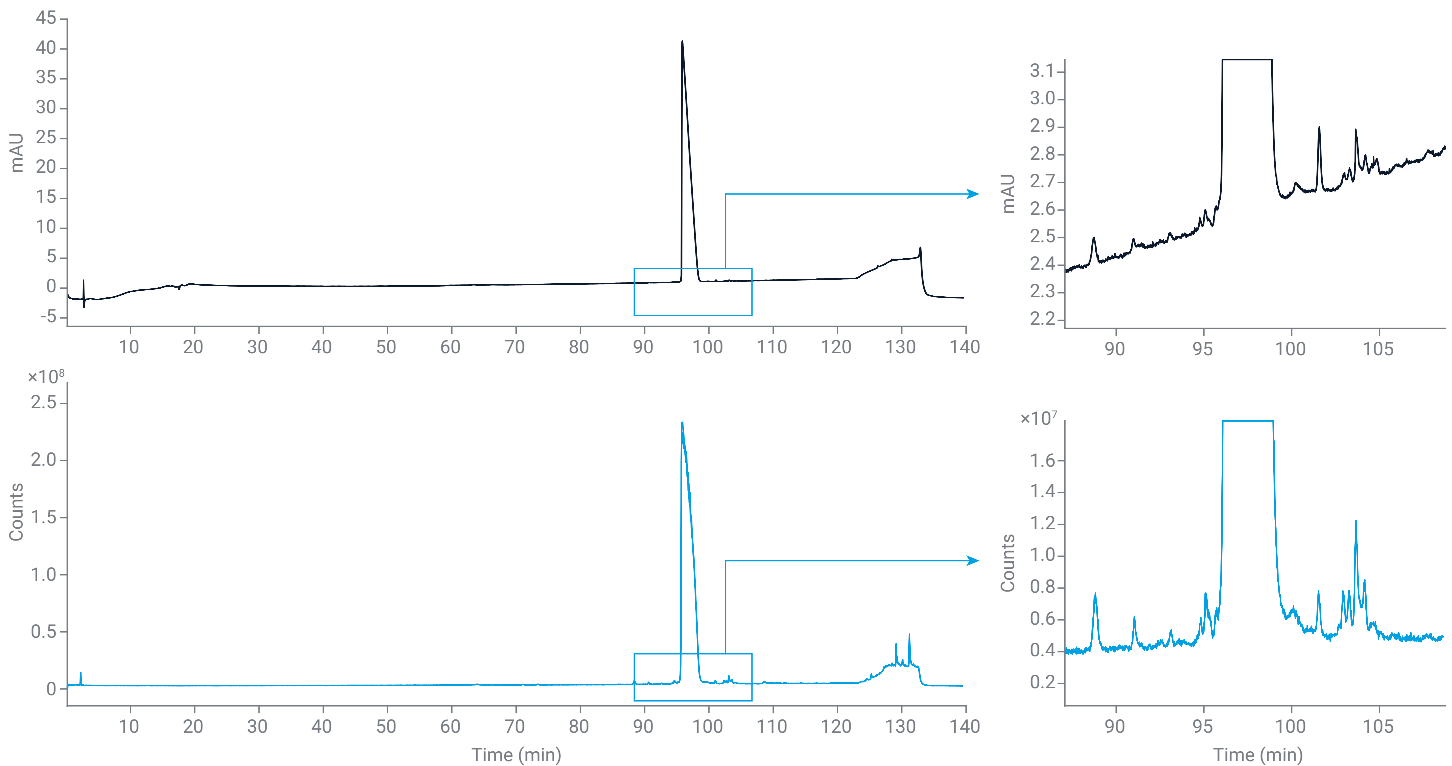


Figure 2. Chromatogram of Liraglutide using an Agilent InfinityLab Poroshell 120 EC-C18 column, 1.9 µm, 2.1 × 250 mm (100 + 150 mm). (Black: UV; blue: TIC.)

Stability test of Liraglutide

To analyze potential impurities from the degradation of Liraglutide, the sample was exposed to acidic conditions using 0.1 M HCl, basic conditions using 0.1 M NaOH, and oxidative conditions using 0.2% H_2O_2. Samples were collected immediately, and after one and two days of exposure. Immediately after each sampling point, the samples were neutralized with equivalent amounts of NaOH and HCl, and then analyzed according to the method outlined in Table 2 for forced degradation profiling (Figure 3). Under basic conditions, degradation of Liraglutide was most prominent. Under acidic and oxidative conditions, various impurity peaks were observed next to the main peak. Peaks with different retention times under acidic and oxidative conditions indicated different impurities. This was confirmed by differences observed in the TIC, indicating different types of impurity profiles for the different conditions.

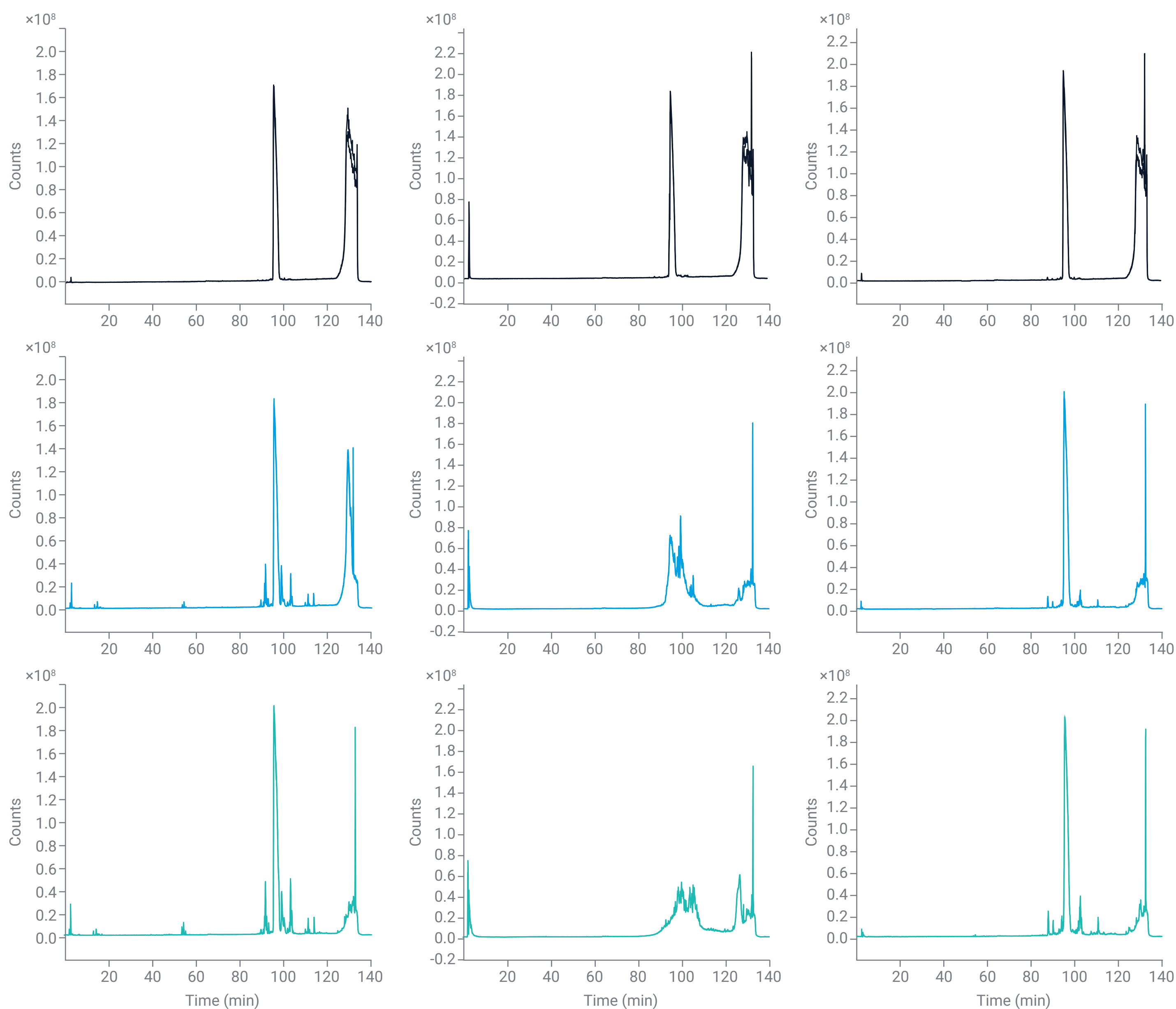
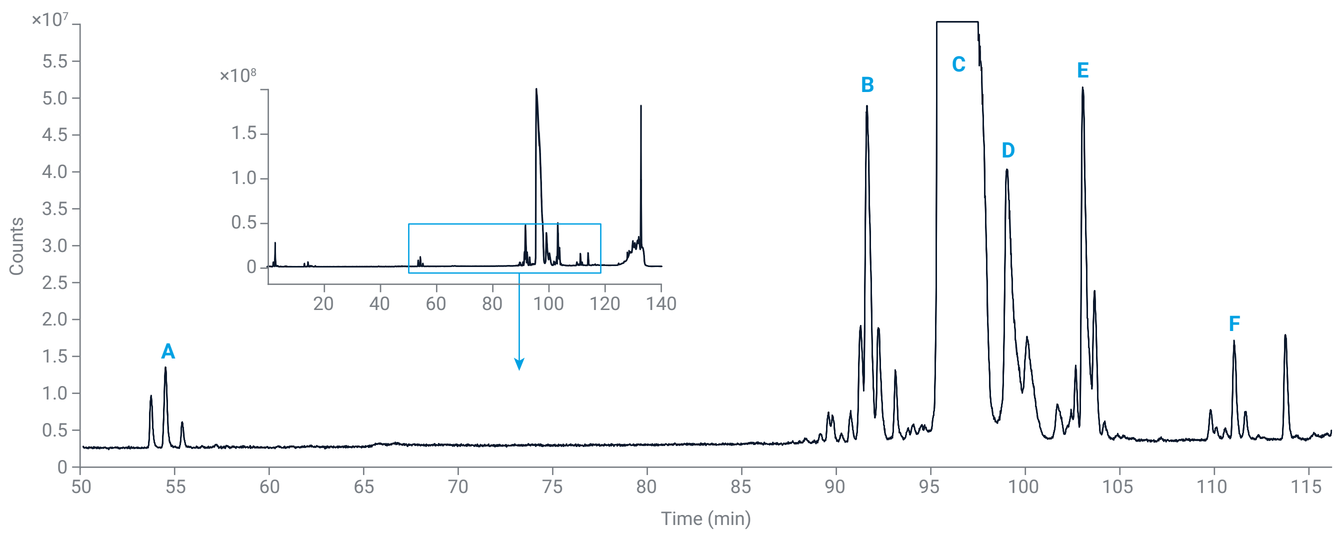
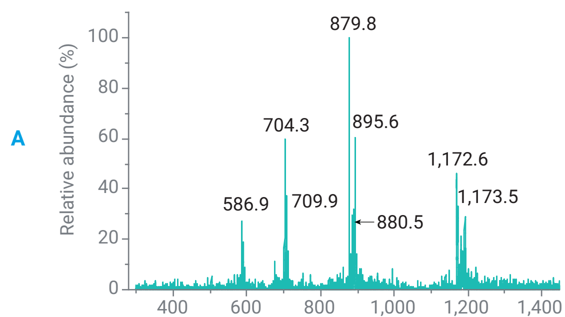


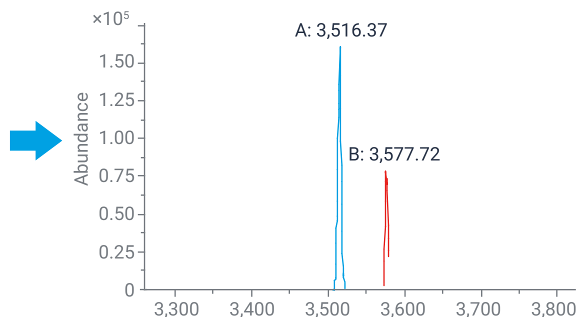
Figure 3. Forced degradation TIC profile of Liraglutide using an Agilent InfinityLab LC/MSD iQ. The top, middle, and bottom rows indicate sample collection immediately, after one day, and after two days, respectively. Left to right columns indicate exposure to acidic (0.1 M HCl), basic (0.1 M NaOH), and oxidative (0.2% H_2O_2) conditions, respectively.

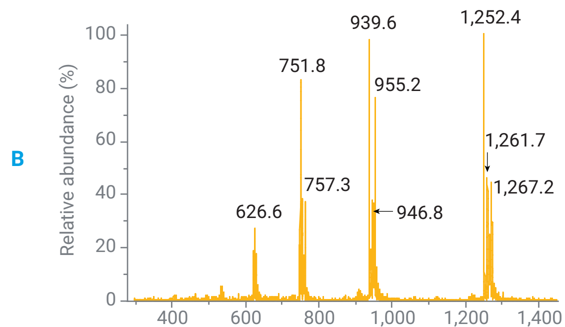
Confirmation of Liraglutide impurity MS spectrum using the deconvolution feature of OpenLab CDS 2.8

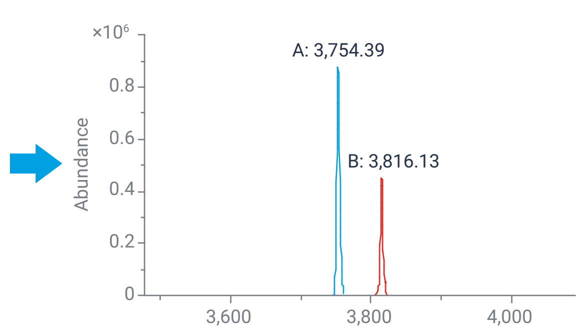
For illustrative purposes, the MS spectrum and the molecular weight of the major peaks were extracted from the MS TIC of the sample heated at 60 °C for two days under acidic conditions, using the deconvolution feature of OpenLab CDS 2.8. The deconvolution results for the six representative peaks are shown in Figure 4 and Table 5.

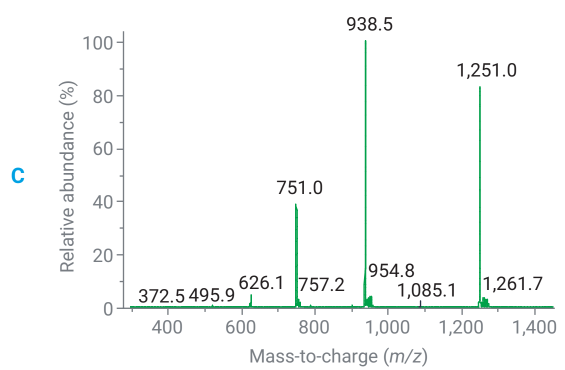

×10^7
Counts
Time (min)
A
B
C
D
E
F
×10^8

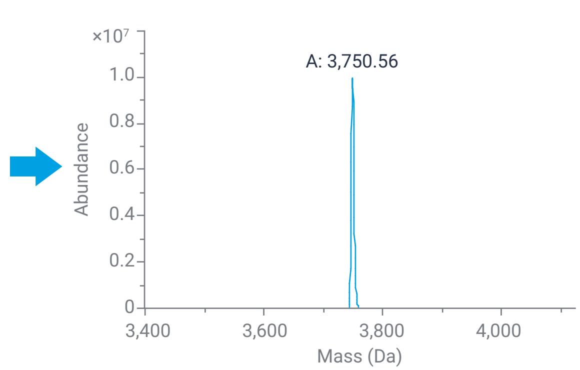

A
Relative abundance (%)
879.8
704.3
895.6
1,172.6
586.9
709.9
1,173.5
880.5


×10^5
Abundance
A: 3,516.37
B: 3,577.72


B
Relative abundance (%)
939.6
1,252.4
751.8
955.2
1,261.7
757.3
1,267.2
626.6
946.8


×10^6
Abundance
A: 3,754.39
B: 3,816.13


C
Relative abundance (%)
938.5
1,251.0
751.0
954.8
372.5
495.9
626.1
757.2
1,085.1
1,261.7
Mass-to-charge (m/z)


×10^7
Abundance
A: 3,750.56
Mass (Da)

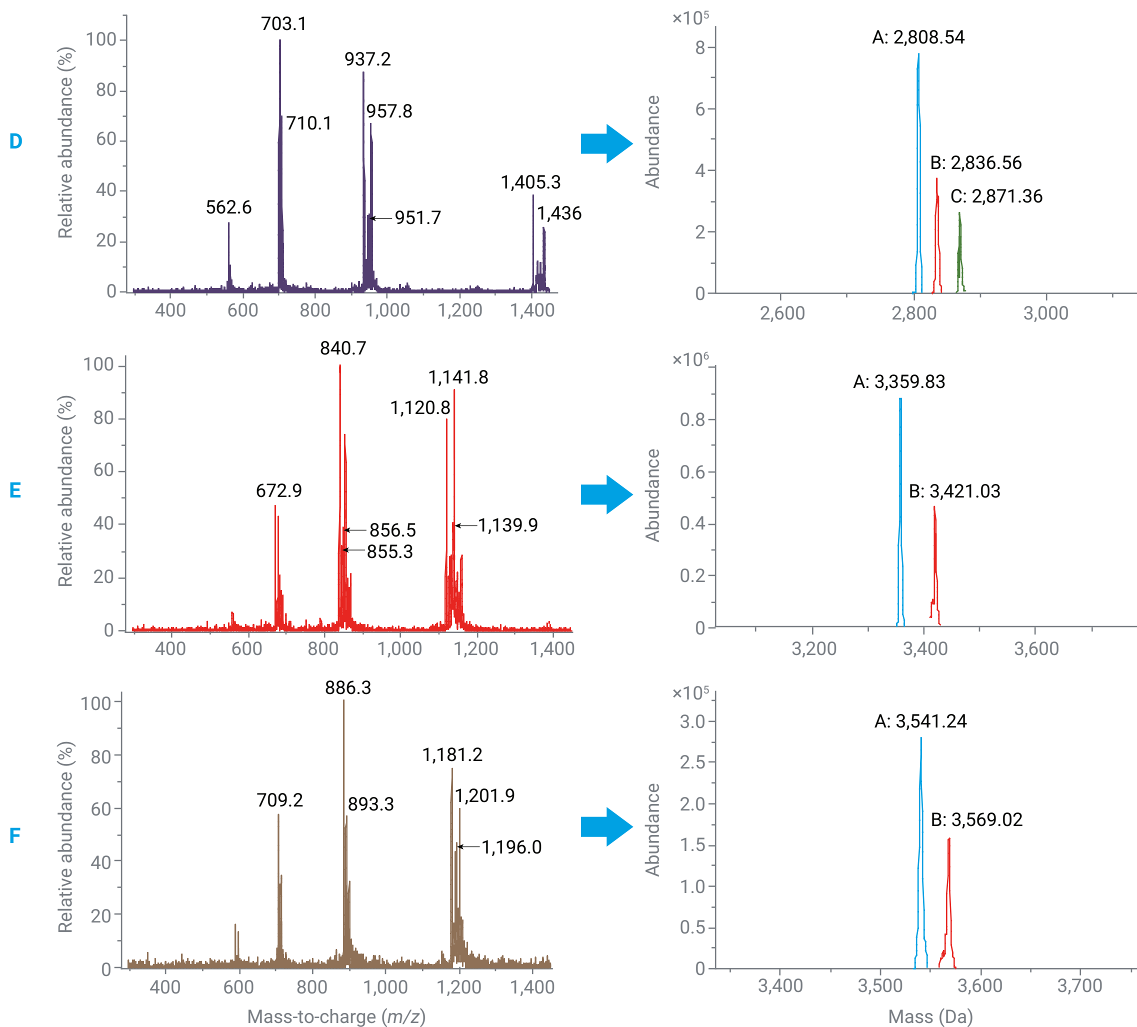


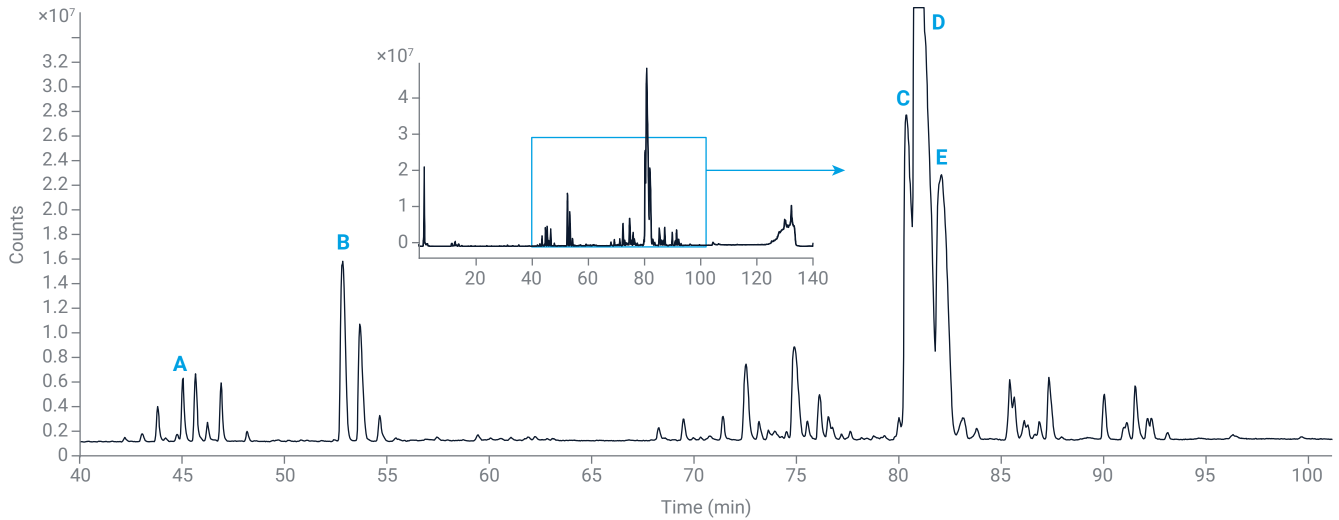
Figure 4. TIC of Liraglutide sample heated for two days under acidic conditions (top) using an Agilent InfinityLab LC/MSD iQ, and the MS raw spectrum (A to F, left) and the deconvoluted spectrum (A to F, right) for each of the six peaks.

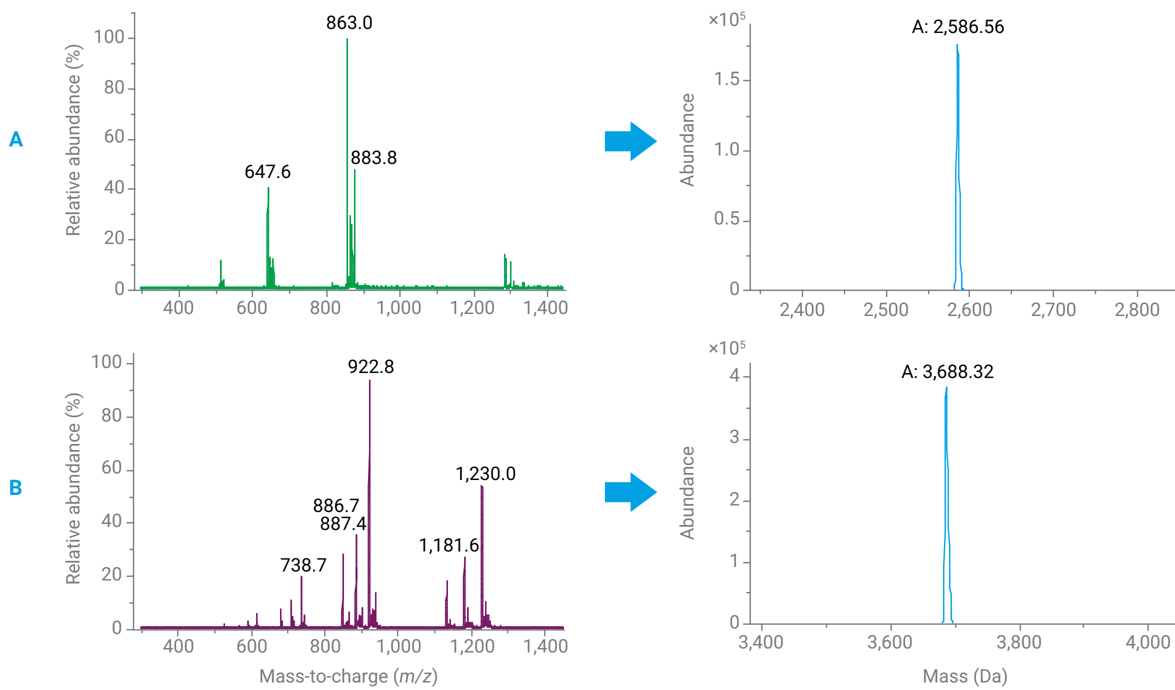
Table 5. The deconvolution results from the mass spectrum of the Liraglutide sample heated for two days under acidic conditions and the mass difference compared to Liraglutide using an Agilent InfinityLab LC/MSD iQ and OpenLab CDS 2.8 deconvolution.

Peak	Spectrum RT (min)	Component	Mass (Da)	Absolute Abundance	Mass Difference (Da)
A	54.372	A	3,516.37	159,683	−234
	54.372	B	3,577.72	78,012	−173
B	91.635	A	3,754.39	875,778	4
	91.635	B	3,816.13	453,879	66
C	95.557	A	3,750.56	9,934,175	(Liraglutide)
D	99.057	A	2,808.54	777,637	−942
	99.057	B	2,836.56	370,460	−914
	99.057	C	2,871.36	259,511	−879
E	103.088	A	3,359.83	876,644	−391
	103.088	B	3,421.03	462,119	−330
F	111.134	A	3,541.24	279,455	−209
	111.134	B	3,569.02	156,874	−182

Confirmation of Semaglutide impurity MS spectrum

Similarly, Semaglutide was analyzed under the conditions outlined in Table 3 after being heated at 80 °C for one day in 0.1 M HCl. Applying deconvolution, the molecular mass at 81.0 minutes indicated Semaglutide for peak D, and between 40 to 95 minutes, various impurity peaks confirmed by TIC showed their MS spectra and molecular weights (Figure 5 and Table 6).





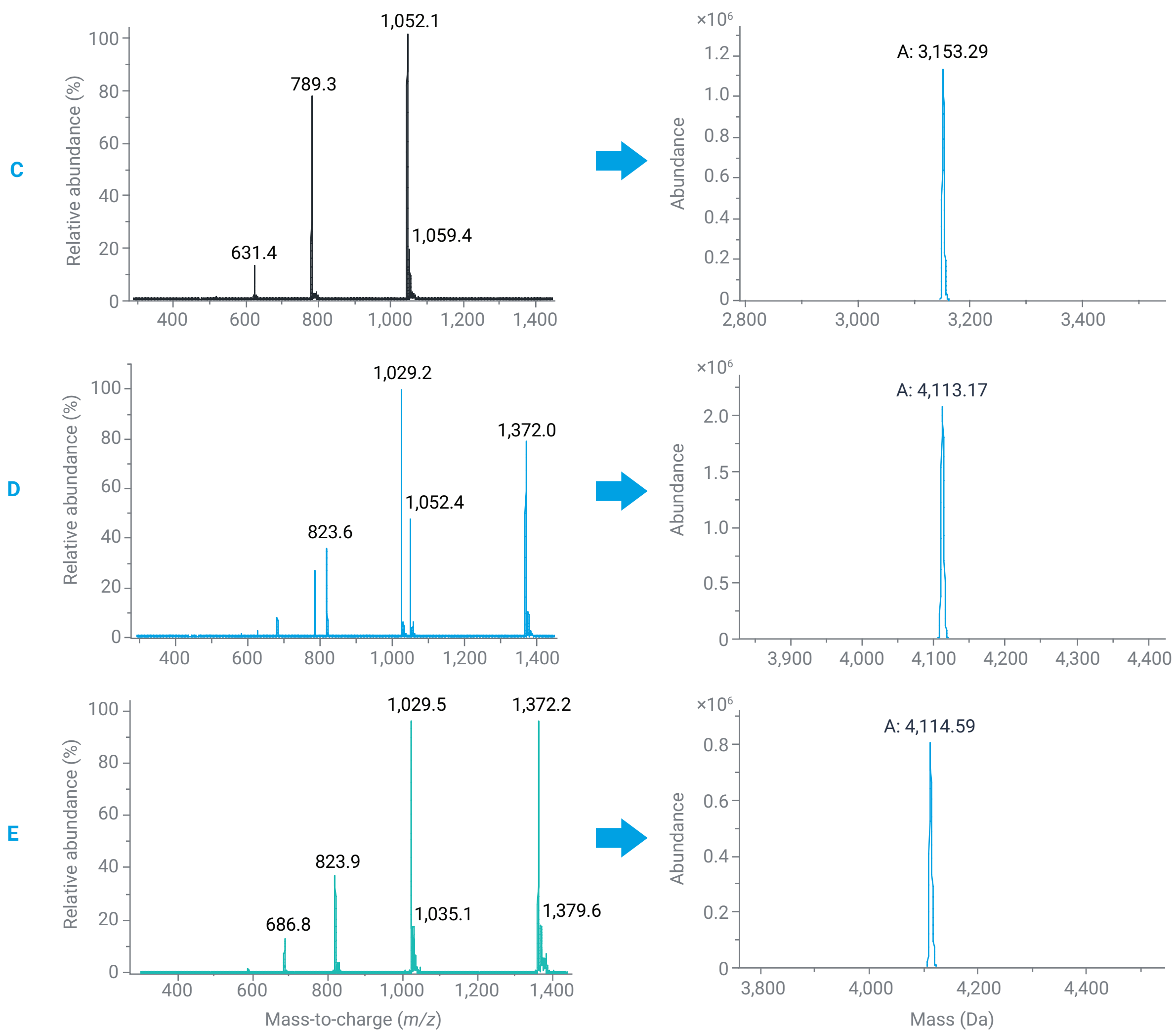


Figure 5. TIC (top) and MS spectra of Semaglutide 1 mg/mL heated for a day under acidic conditions with an Agilent AdvanceBio Peptide Mapping column and the Agilent InfinityLab LC/MSD XT (A to E, left: raw spectrum; A to E, right: deconvoluted spectrum).

Table 6. The deconvolution results from the MS spectrum of the Semaglutide sample heated for a day under acidic conditions and the mass difference compared to Semaglutide using Agilent InfinityLab LC/MSD XT and OpenLab CDS 2.8 deconvolution.

Peak Name	Spectrum RT (minutes)	Component	Mass (Da)	Absolute Abundance	Mass Difference (Da)
A	45.044	A	2,586.56	177,605	−1,527
B	52.855	A	3,688.32	383,321	−425
C	80.392	A	3,153.29	1,125,716	−960
D	81.000	A	4,113.17	2,080,250	(Semaglutide)
E	82.094	A	4,114.59	799,997	1

Conclusion

The MS spectra obtained from forced degradation studies of Liraglutide and Semaglutide, analyzed with the Agilent 1260 Infinity II Prime Bio LC, InfinityLab LC/MSD iQ, and InfinityLab LC/MSD XT, were easily converted to neutral masses by applying the deconvolution feature of Agilent OpenLab CDS software, version 2.8. Evaluation of impurities of peptide formulations generated in degradation studies should prioritize ease of use for late-stage development and quality control. The InfinityLab LC/MSD iQ or XT used with OpenLab CDS 2.8 and deconvolution represents an ideal tool for ease of use with informative high-quality analysis. This setup enables flexible monitoring of impurities arising from degradation of therapeutic peptides such as GLP-1 agonists.

Furthermore, the Agilent AdvanceBio Peptide Mapping column and InfinityLab Poroshell 120 EC-C18 1.9 µm column facilitate straightforward separation of multiple impurities generated under various forced degradation conditions for Liraglutide and Semaglutide. This allows for simple nominal mass determination of main compound and impurities and simplifies the comparison with RLDs.

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