Application Note

Biopharmaceuticals

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

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Abstract

This application note presents the analysis of peptide-related impurities for Semaglutide using an Agilent 1290 Infinity II bio 2D-LC 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).

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 (^{1}D) and second (^{2}D) 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 ^{2}D before MS detection with the LC/MSD XT. Also, peaks coeluting in ^{1}D were successfully separated in ^{2}D. The mass spectra acquired from the LC/MSD XT were used to determine molecular weights using the deconvolution feature in OpenLab CDS 2.8.

Experiment

Instrumentation

The following instrumentation was used in this application note:

- Agilent 1290 Infinity II flexible pump (part number G7131A)
- Agilent 1290 Infinity II 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 (^{1}D):** Agilent AdvanceBio Peptide Plus, 2.1 × 250 mm, 2.7 µm (part number 693775-949)
- **Second dimension (^{2}D):** Agilent InfinityLab Poroshell 120 CS-C18, 2.1 × 100 mm, 2.7 µm (part number 695775-942)

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 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 2. ^{2}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 3. Agilent InfinityLab LC/MSD XT data acquisition 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

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%

Software

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

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.

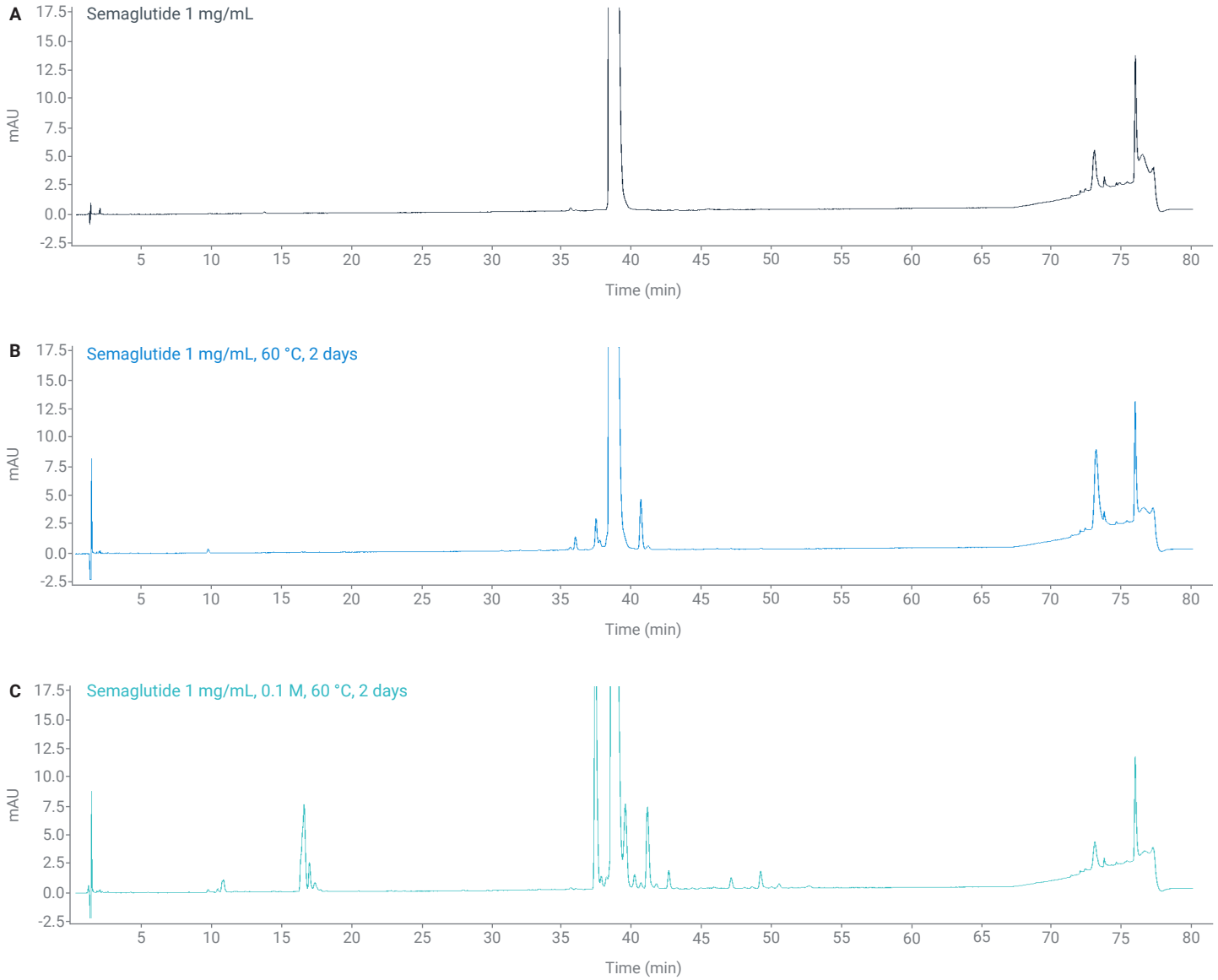

Figure 1. UV chromatogram of a 1 mg/mL Semaglutide solution (A), heated at 60 °C for two days (B), and a Semaglutide solution heated in 0.1 M HCl conditions for two days (C).

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 ^{2}D 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 ^{2}D 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 ^{2}D conditions, while cut numbers 5 and 6 displayed reversed retention order (Figure 3).

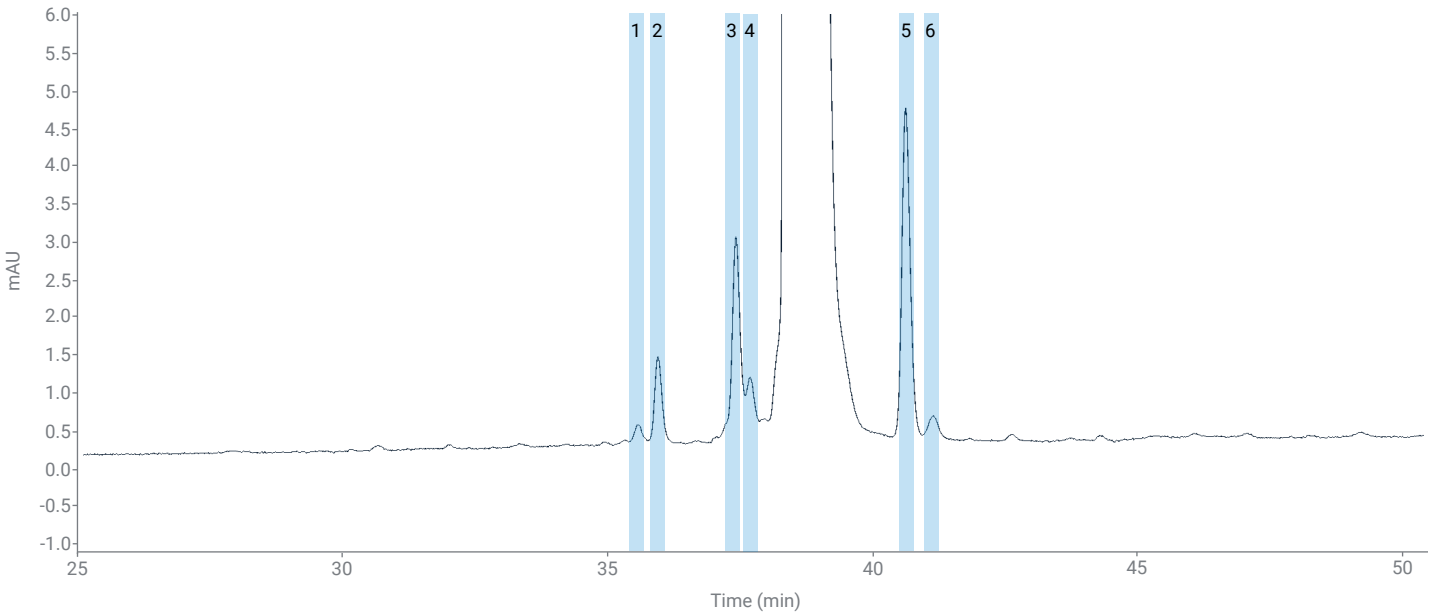

Figure 2. UV chromatogram of a 1 mg/mL Semaglutide solution heated at 60 °C for two days, with indication of the position of the ^{2}D cut.

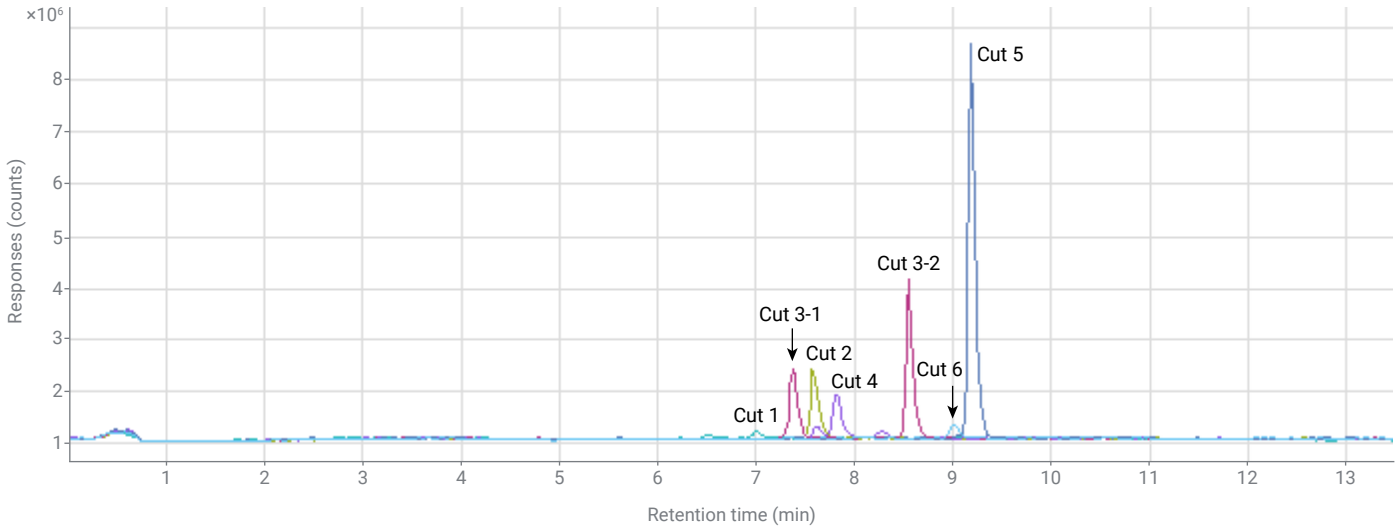

Figure 3. ^{2}D TIC illustrating the analysis of impurities caused by thermal degradation using an Agilent InfinityLab Poroshell CS-C18 column with 0.1% FA as the mobile phase additive.

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.

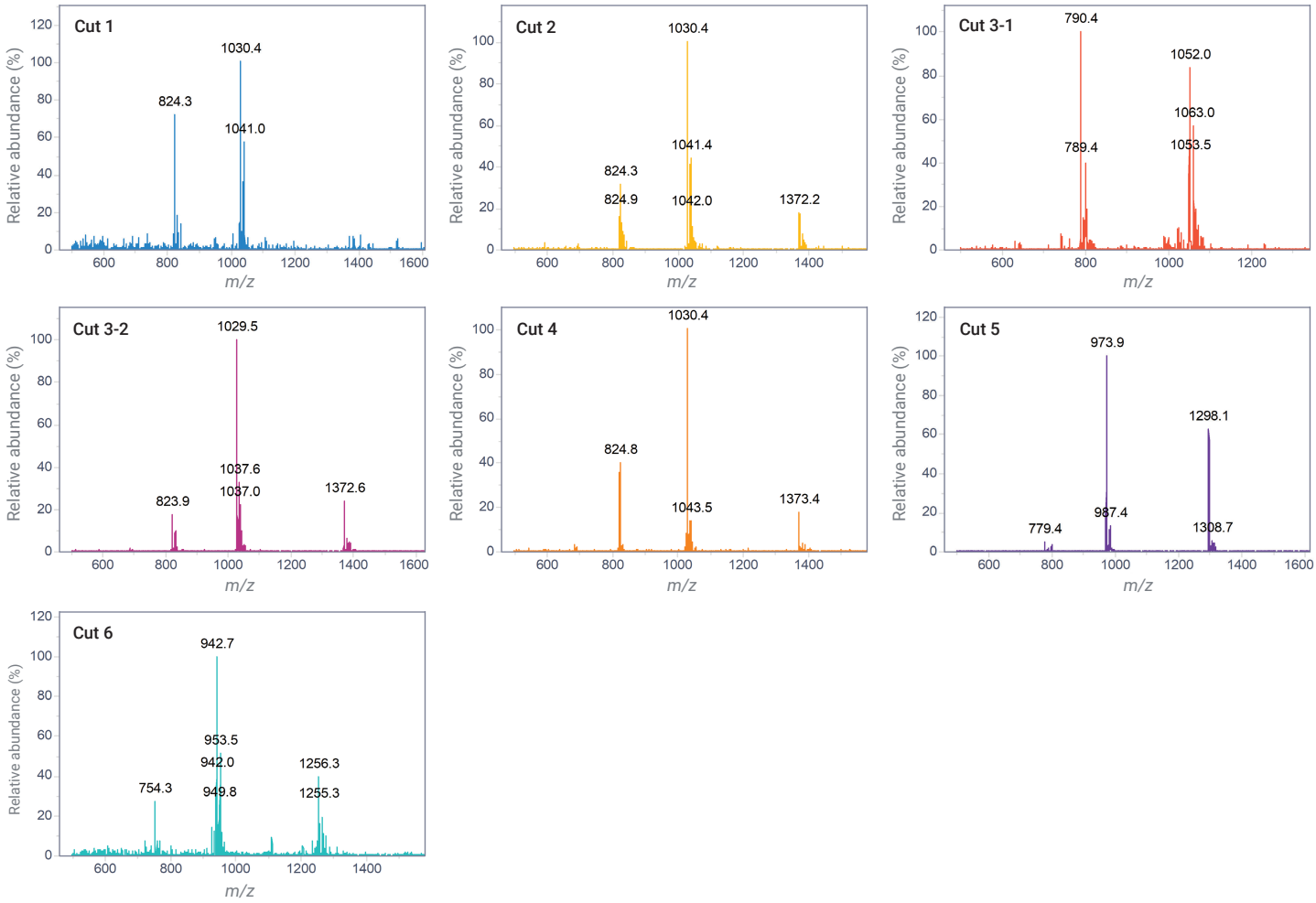

Figure 4. MS spectra of thermal degradation impurities of Semaglutide.

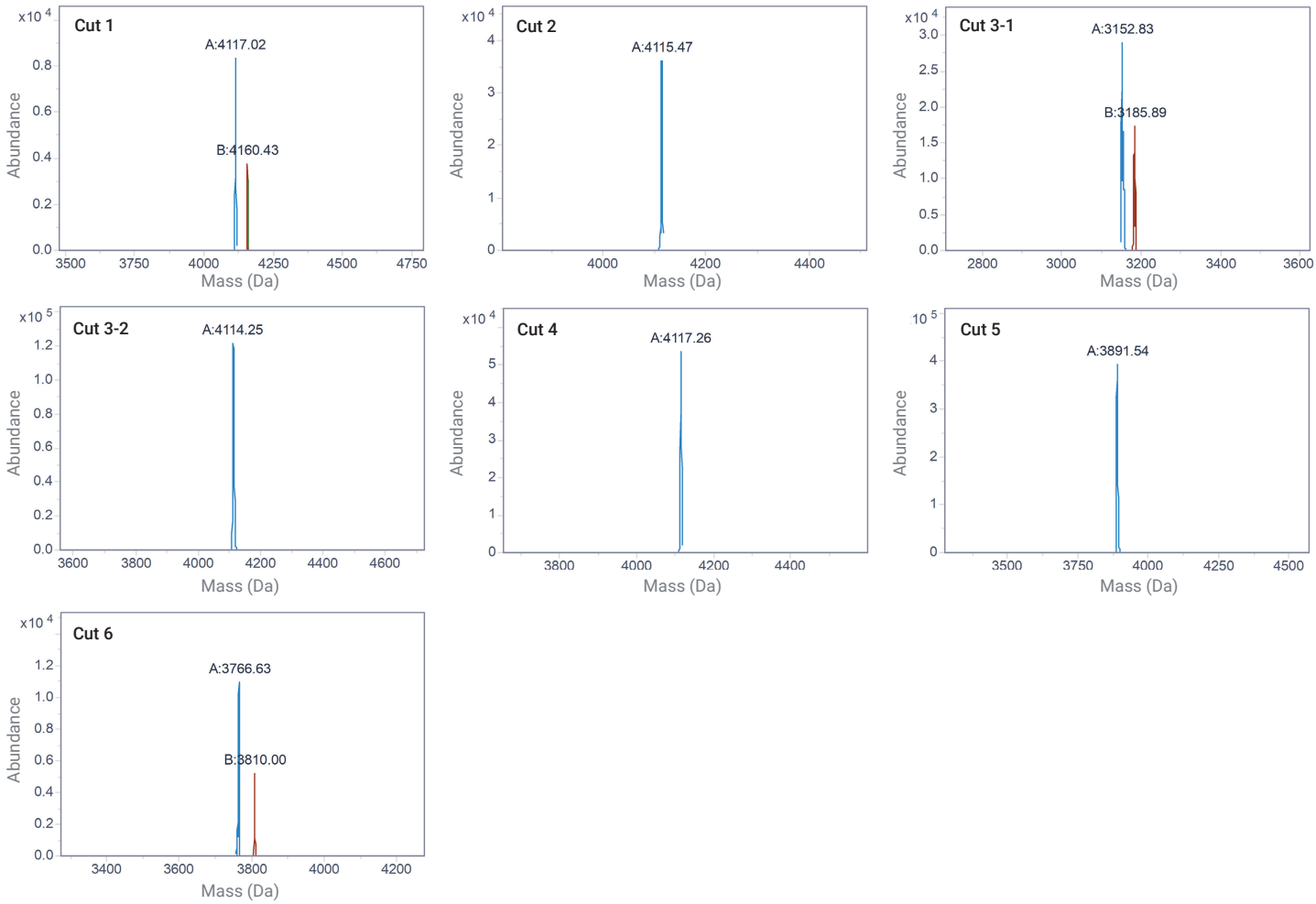

Figure 5. Deconvoluted mass spectra of thermal degradation impurities of Semaglutide.

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).

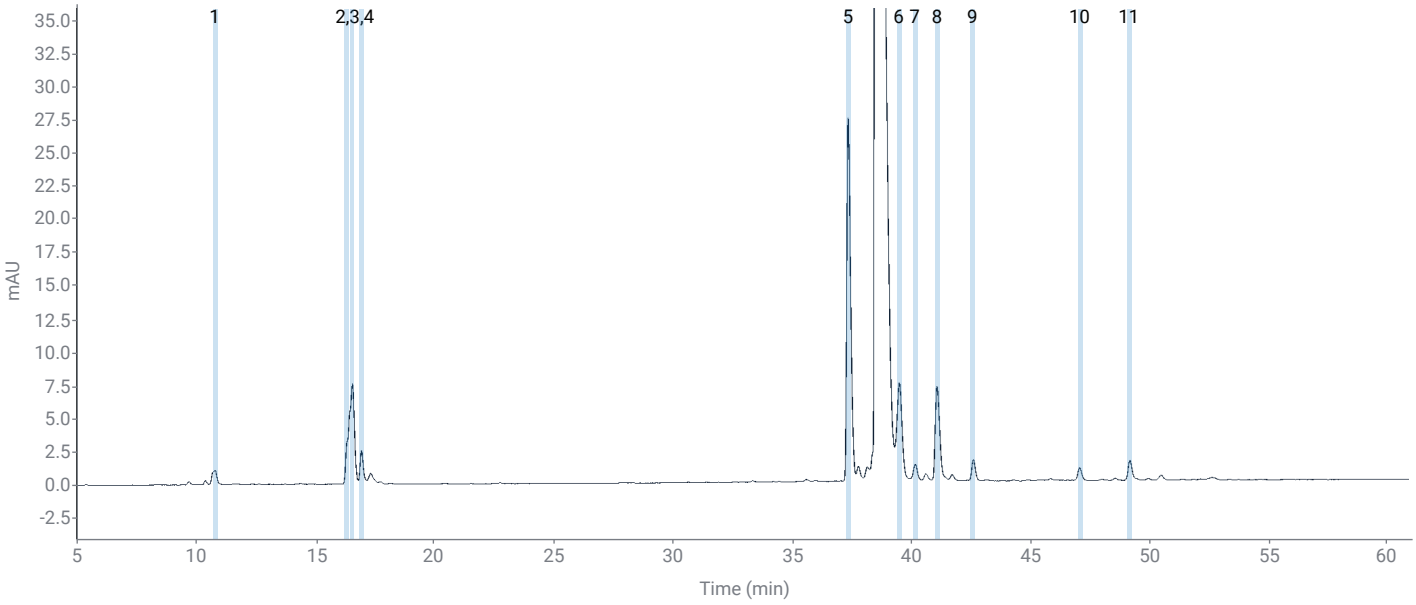

Figure 6. UV chromatogram of a 1 mg/mL Semaglutide solution heated at 60 °C for two days under 0.1 M HCl conditions, along with the location of the ^{2}D cut.

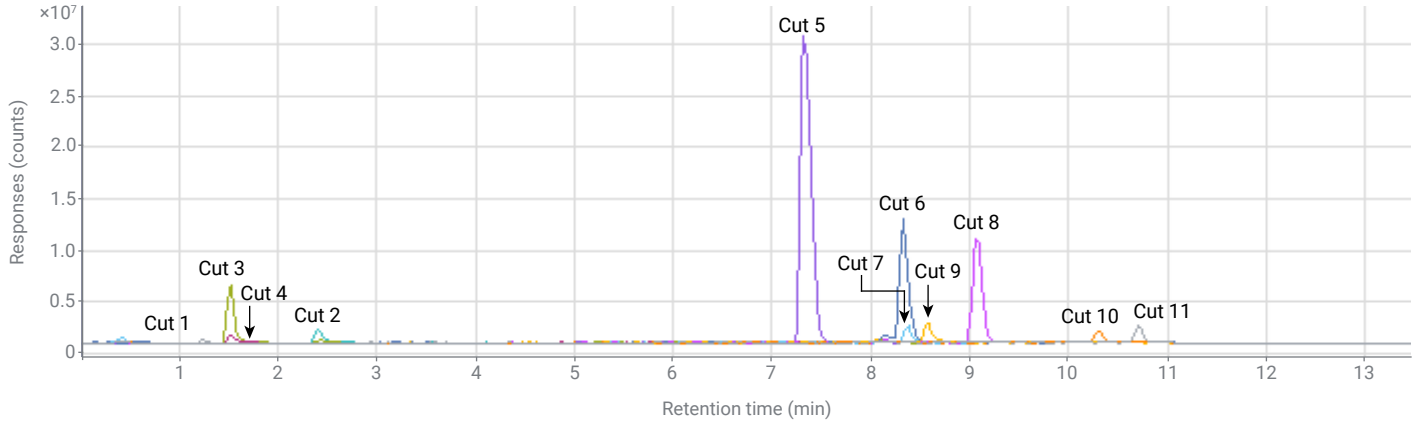

Figure 7. ^{2}D TIC illustrating the analysis result of acid degradation impurities using an Agilent InfinityLab Poroshell 120 CS-C18 column with 0.1% FA as the mobile phase.

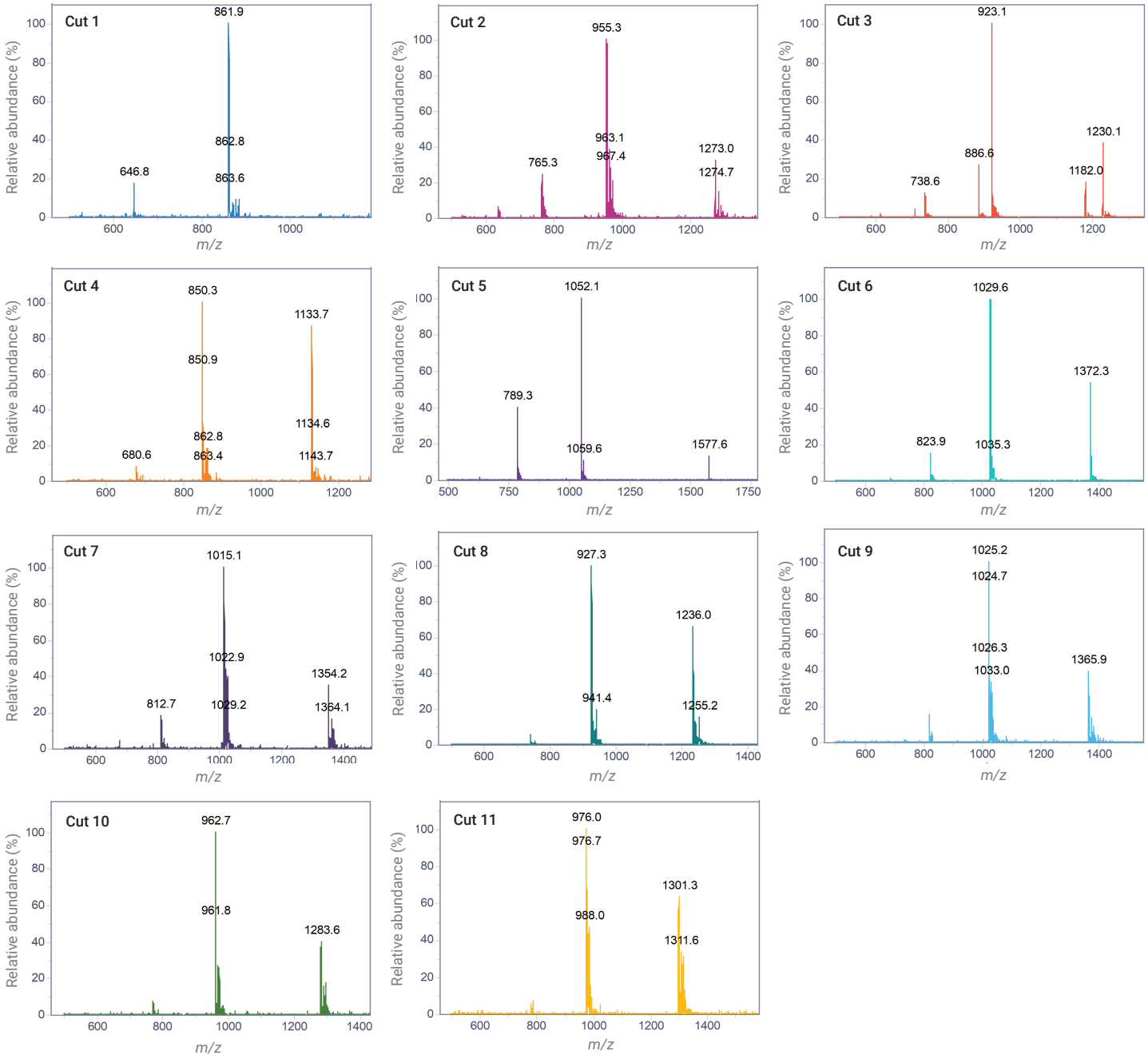

Figure 8. MS spectra of acid degradation impurities of Semaglutide.

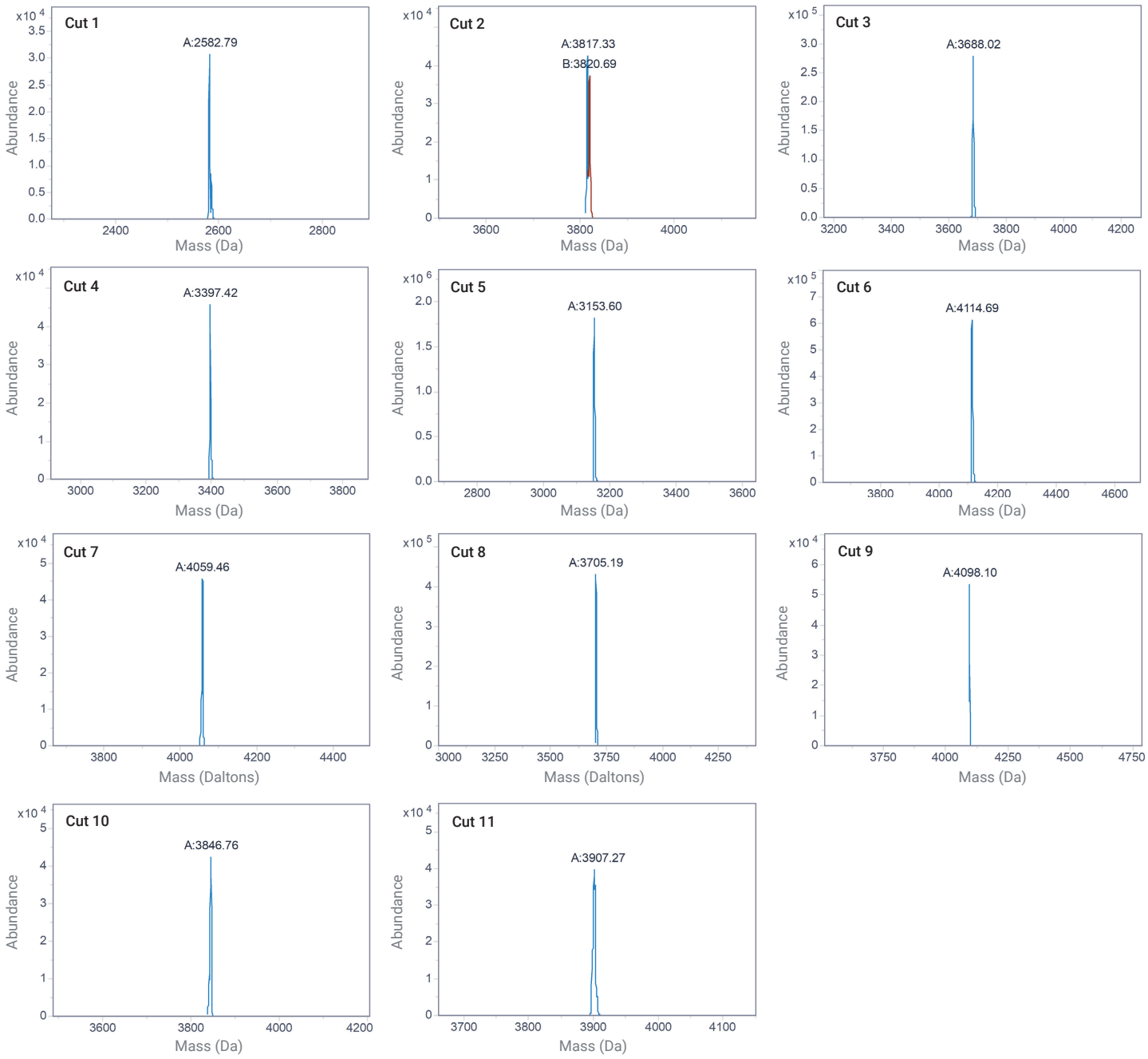

Figure 9. Deconvoluted mass spectra of acid degradation impurities of Semaglutide.

The molecular weights of the impurities from Semaglutide generated under heat and acidic conditions, as determined, are listed in Table 5. For further identification of the impurities, the exact formulas of these results need to be confirmed against known impurity information. An analysis using the Agilent AdvanceBio 6545XT LC/Q-TOF under the same 2D-LC conditions would aid to provide comprehensive sequence information.

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).

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.

References

1. ANDAs for Certain Highly Purified Synthetic Peptide Drug Products That Refer to Listed Drugs for rDNA Origin, Guidance for Industry, *U.S. Department of Health and Human Services, FDA, CDER*, **2021**.
2. Guideline on the Development and Manufacture of Synthetic Peptides, *European Medicines Agency*, **2023**.
3. Pradhan, R.; *et al.* Glucagon-Like Peptide 1 Receptor Agonists and Risk of Anaphylactic Reaction Among Patients With Type 2 Diabetes: A Multisite Population-Based Cohort Study. *Am. J. Epidemiol.* **2022**, *191(8)*, 1352–1367.
4. Liu, X.; Zhai, A.; Zhang, B. A Case Report of Severe Adverse Reaction of Exenatide: Anaphylactic Shock. *Medicine* **2022**, *101(39)*, e30805.
5. Zhang, X.; Belousoff, M. J.; Liang, Y. L.; Danev, R.; Sexton, P. M.; Wootten, D. Structure and Dynamics of Semaglutide- and Taspoglutide-Bound GLP-1R-Gs Complexes. *Cell Reports* **2021**, *36(2)*, 109374.
6. Sanz-Nebot, V.; Benavente, F.; Toro, I; Barbosa, J. Separation and Characterization of Complex Crude Mixtures Produced in the Synthesis of Therapeutic Peptide Hormones by Liquid Chromatography Coupled to Electrospray Mass Spectrometry (LC–ES-MS). *Analytica Chimica Acta* **2004**, *521(1)*, 25–36.
7. De Spiegeleer, B.; Vergote, V.; Pezeshki, A.; Peremans, K; Burvenich, C. Impurity Profiling Quality Control Testing of Synthetic Peptides Using Liquid Chromatography-Photodiode Array-Fluorescence and Liquid Chromatography-Electrospray Ionization-Mass Spectrometry: the Obestatin Case. *Analytical Biochemistry* **2008**, *376(2)*, 229–234.
8. Chae-Young, R. Efficient Method Optimization of Semaglutide Analysis Using an Agilent 1260 Infinity II Bio Prime LC System and Blend Assist. *Agilent Technologies application note*, publication number 5994-7414EN, **2024**.
9. Chae-Young, R. Rapid Confirmation of GLP-1 Analog (Liraglutide) Using Agilent InfinityLab LC/MSD iQ. *Agilent Technologies application note*, publication number 5994-7415EN, **2024**.

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