Application Note

Biopharmaceuticals

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Comprehensive Aggregate Profiling of Liraglutide and Semaglutide Using an Agilent 1290 Infinity II Bio 2D-LC and Agilent InfinityLab LC/MSD XT

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Abstract

This application note confirms the aggregates of liraglutide and semaglutide through size exclusion chromatography (SEC) using an Agilent 1290 Infinity II Bio LC system. The Agilent AdvanceBio SEC column was capable of operating not only under high-concentration salt-containing buffer conditions but also under organic solvent conditions, crucially enabling the separation of peptide aggregates. Expanding the same system to operate with the 1290 Infinity II Bio 2D-LC, coupled with High-Resolution Sampling and Multi-inject functions of the 2D-LC along with Agilent InfinityLab LC/MSD XT, allowed molecular weight determination of the aggregates and identification of covalent aggregates containing minor impurities. The complex MS spectra resulting from various impurity combinations were interpreted using the deconvolution feature of Agilent OpenLab CDS 2.8.

Introduction

Aggregation is a critical parameter in the quality control of all biopharmaceuticals, particularly protein therapeutics. According to FDA guidelines, managing aggregates is essential to drug safety and efficacy.[1] Similarly, assessing aggregation in synthetic peptides is crucial due to their immunogenic potential. Both FDA and EMA guidelines suggest that drug product formulation can induce aggregation and recommend comparing generic and reference listed drug (RLD) products to identify these effects.[2,3] Techniques such as the thioflavin T assay are proposed for detecting fibrillary aggregates. Peptides often form fibrils[4] due to pH changes or chemical degradation, with some peptides more prone to aggregation at high concentrations depending on their net charge.[5]

Aggregation can occur through salt bridges formed by hydrogen bonds between oppositely charged amino acids, such as aspartic acid and lysine. This noncovalent aggregation is reversible, but covalent bonds between these residues can create irreversible aggregates.[6] SEC is a common method used to detect these aggregates. The AdvanceBio SEC column is designed to minimize interactions with biomolecules, providing high-resolution separation under SEC conditions. Peptide drugs often require organic solvents and acids in the mobile phase due to their hydrophobicity. This application note demonstrates that the Agilent AdvanceBio SEC 300Å column provides excellent separation for evaluating liraglutide and semaglutide aggregates under conditions of 50% acetonitrile and 15% acetic acid.

However, distinguishing between reversible and irreversible aggregates using SEC alone is challenging. Using the 1290 Infinity II Bio 2D-LC system and InfinityLab LC/MSD XT, the MS spectra of aggregates in semaglutide drug products were analyzed. The complex MS spectra were deconvoluted using OpenLab CDS 2.8, allowing the determination of the molecular weights of the aggregates.

Experimental

Instrumentation

- Agilent 1290 Infinity II Flexible Pump (G7131A)
- Agilent 1290 Infinity II High-Speed Pump (G7132A)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Sample Thermostat
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Quick-Connect Heat-Exchanger 1290 Bio Standard Flow (part number G7116-60071)
- 2x Agilent 1290 Infinity II Diode Array Detector (DAD) (G7117B) with Max-Light cartridge cell LSS 10 mm (part number G7117-60020) and Bio-inert Max-Light cartridge cell, 60mm (part number G5615-60017)
- Agilent 1290 Infinity Valve Drive (G1170A) with Agilent InfinityLab Bio 2D-LC ASM Valve (G5643B)
- 2x Agilent 1290 Infinity Valve Drive (G1170A) with Multiple Heart-Cutting Valves and biocompatible 40 µL loops
- Agilent InfinityLab LC/MSD XT (G6135C)

Standards and reagents

Agilent AdvanceBio SEC 130Å Protein Standard (part number 5190-9416) was used for tests using phosphate buffer. Arginine, sodium chloride, sodium phosphate dibasic, sodium phosphate monobasic, and formic acid were purchased from Sigma-Aldrich. Acetonitrile and acetic acid were purchased from B&J.

Samples

Liraglutide was purchased through Kairos Tech in Korea. Semaglutide and Ozempic were donated by a local customer.

Liraglutide was dissolved in water, and semaglutide was dissolved in 30% acetonitrile to prepare a concentration of 1 mg/mL. Both solutions were heated at 80 °C for a specified period, cooled, and then analyzed under the following conditions.

Mobile phases

The mobile phase for the size exclusion condition used in Figure 1 was prepared by dissolving 18.34 g of sodium phosphate dibasic, 2.48 g of sodium phosphate monobasic, and 17.53 g of sodium chloride in water to make 1 L. The mobile phase used for the ^{1}D condition of other analyses was prepared by mixing arginine solution at a concentration of 1 mg/mL with acetonitrile and acetic acid in a volume ratio of 35:50:15. For the ^{2}D condition, 0.1% formic acid in water and 0.1% formic acid in acetonitrile were used as the mobile phases.

Columns

- **First dimension (^{1}D)**:
  - Agilent AdvanceBio SEC 130Å, 7.8 × 300 mm, 2.7 µm (part number PL1180-5350)
  - Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm (part number PL1180-5301)
- **Second dimension (^{2}D)**: Agilent InfinityLab Poroshell 120 CS-C18, 2.1 × 100 mm, 2.7 µm (part number 695775-942)

Methods

Table 1. ^{1}D parameters.

Parameter	Value
Column	Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm
Flow	0.6 mL/min
Column Temperature	40 °C
Injection Volume	^{1}D only: 5 µL ^{2}D: 20 µL
Mobile Phase	1 mg of arginine in water:acetonitrile:acetic acid = 35:50:15
Detector	UV 280 nm (DAD HS with Max-Light cartridge cell LSS, 10 mm + aperture)

Table 2. ^{2}D parameters.

Parameter	Value
Column	Agilent InfinityLab Poroshell CS-C18 100Å, 2.1 × 100 mm, 2.7 µm
Flow	0.6 mL/min (Idle flow: 0.05 mL/min)
Column Temperature	40 °C
Mobile Phase	A) 0.1% FA in water B) 0.1% FA in acetonitrile
2D-LC Operation Mode	Time-based heart cut hi-res sampling
Gradient	Time (min) %A %B 0 80 20 6 65 35 8 45 55 10 10 90 ^{2}D Run time: 10 min ^{2}D Equilibration: 2 min Cycle time: 12 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.

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	500 to 2,500 *m/z*
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 9,500
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.

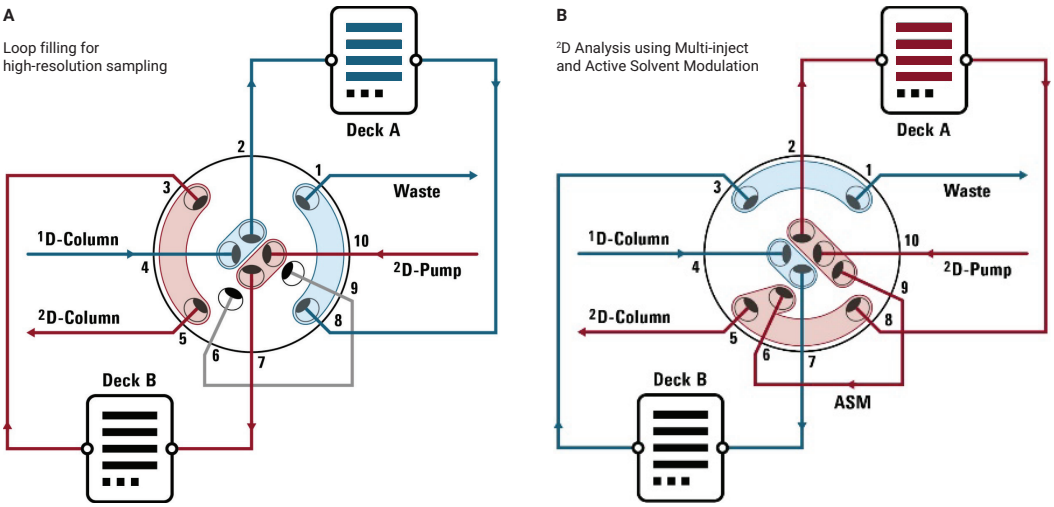
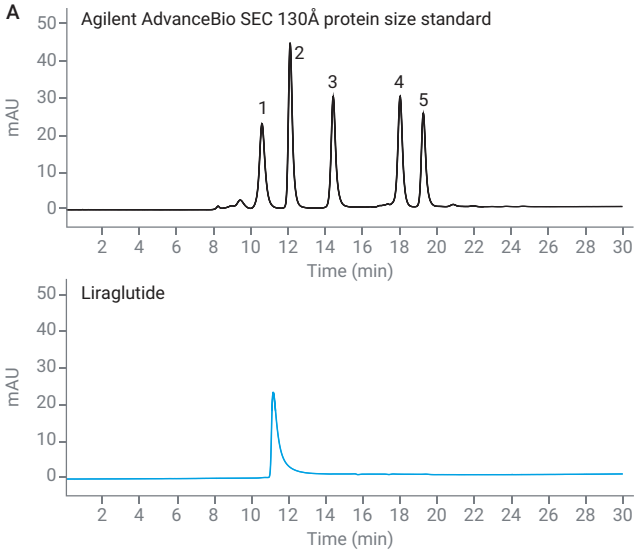


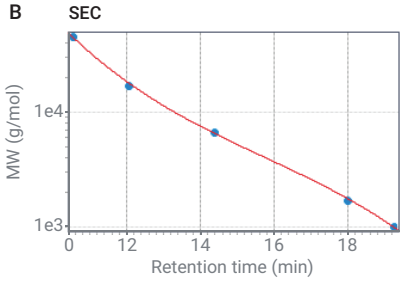
Figure 1. Diagram of the flow path through the Bio 2D-LC ASM Valve during loop filling for high-resolution sampling (A) and ^{2}D analysis using Multi-inject and Active Solvent Modulation (B).

Results and discussion

Size exclusion analysis of liraglutide using phosphate buffer

Using a phosphate buffer commonly used in protein aggregate analysis, AdvanceBio SEC 130Å column conditions were employed to analyze AdvanceBio SEC 130Å protein size standard and liraglutide. Calibration using the OpenLab CDS GPC add-on confirmed a coefficient of determination of 0.9996 over the range of 1,000 Da to 45,000 Da (Figure 2). However, peaks corresponding to liraglutide monomers showed tailing, attributed to its relatively high hydrophobicity.





Peak	Compound	MW (Da)
1	Ovalbumin (chicken)	45,000
2	Myoglobin (horse heart)	17,000
3	Aprotinin (bovine lung)	6,700
4	Neurotensin	1,700
5	Angiotensin II (human)	1,000

Curve fit

Order of curve fit 3

Curve fit equation $y = -0.00192354x^3 + 0.090646x^2 - 1.57347x + 13.4126$

Curve fit statistics

Residual sum of squares	0.000772
Coefficient of determination	0.999589
Linear correlation coefficient	−0.996454

Figure 2. UV chromatograms (A) and calibration curve (B) of Agilent AdvanceBio SEC 130Å protein standard and 1 mg/mL liraglutide analyzed using an Agilent AdvanceBio SEC 130Å column with 150 mM sodium phosphate, pH 7.4 + 300 mM sodium chloride as the mobile phase.

Optimization of size exclusion conditions for liraglutide

The performance of size exclusion chromatography hinges crucially on the column used. The AdvanceBio SEC 130Å column separates proteins up to 120 kDa, while the AdvanceBio SEC 300Å extends this range to 1,250 kDa, making both columns suitable for evaluating peptide aggregates. Peak tailing was observed when using phosphate as the mobile phase, as shown in Figure 2. To address this issue, the mobile phase composition was adjusted, adding arginine to mitigate ionic interactions caused by the silica stationary phase and using acetonitrile to reduce hydrophobic interactions with the analyte. By analyzing liraglutide under the conditions specified in Table 1 with both the AdvanceBio SEC 130Å and SEC 300Å columns, significant improvements in peak tailing were achieved (Figures 3 and 4) facilitating the identification of aggregate peaks. While aggregate peaks were detected under both column conditions, the resolution between aggregates was notably better with the AdvanceBio SEC 300Å column.

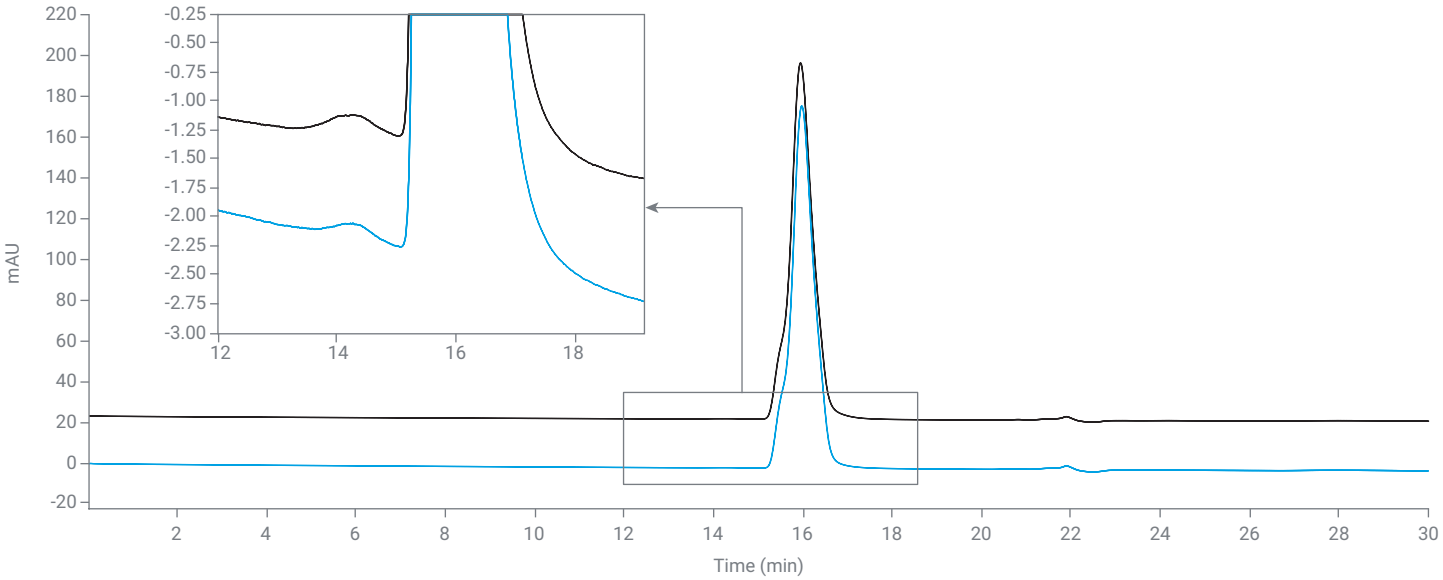


Figure 3. UV chromatogram of 1 mg/mL liraglutide (blue) and 1 mg/mL liraglutide heated at 80 °C for 2 hours (black) under Agilent AdvanceBio SEC 130Å conditions.

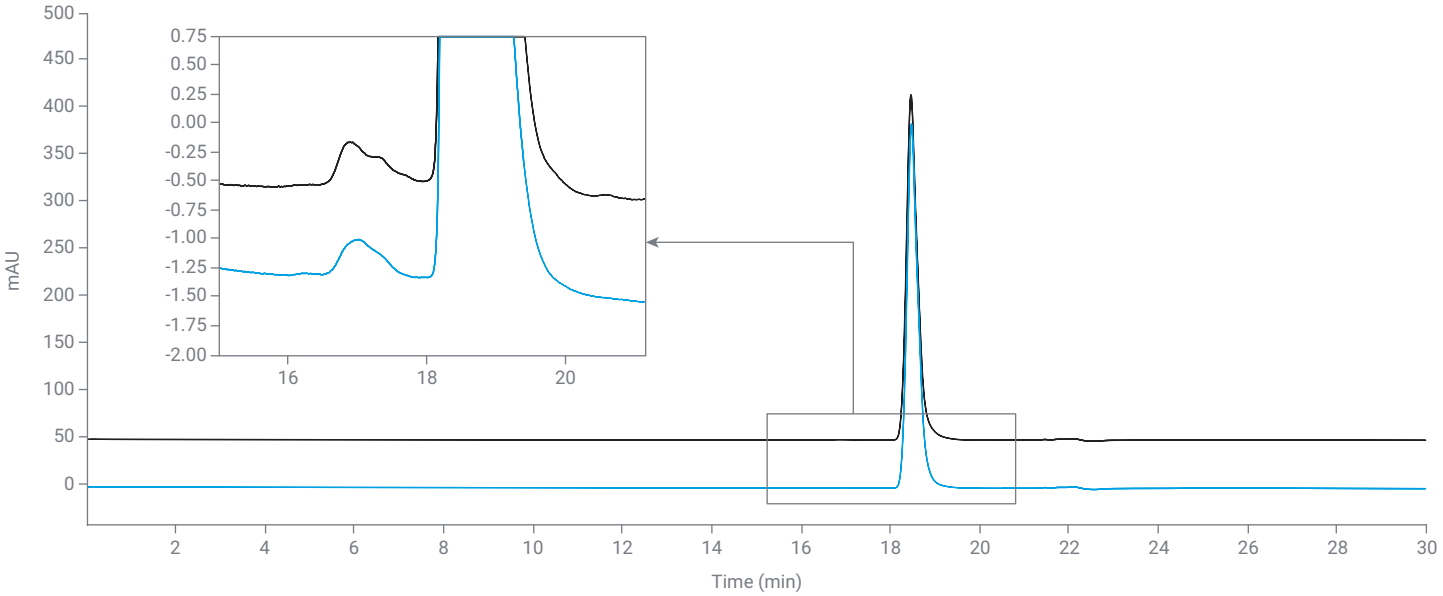


Figure 4. UV chromatogram of 1 mg/mL liraglutide (blue) and 1 mg/mL liraglutide heated at 80 °C for 2 hours (black) under Agilent AdvanceBio SEC 300Å conditions.

Using the method from Table 1, liraglutide was analyzed at 1 mg/mL and liraglutide solution heated at 80 °C for 2 hours and 1 day, respectively. Aggregates of liraglutide were observed at 17 minutes (Figure 5).

[1]D Size exclusion test results of semaglutide and Ozempic

To confirm the molecular weight of aggregates through 2D-LC analysis, the injection volume was increased to 20 µL using the method described in Table 1: 1 mg/mL semaglutide solution heated at 80 °C for 1 day, and Ozempic diluted to 1 mg/mL in 30% acetonitrile, were analyzed. Semaglutide eluted 0.14 minutes earlier than liraglutide, showing similar patterns in aggregate and monomer peaks. Ozempic exhibited two types of aggregate peaks at 13.7 minutes and 14.3 minutes (Figure 6).

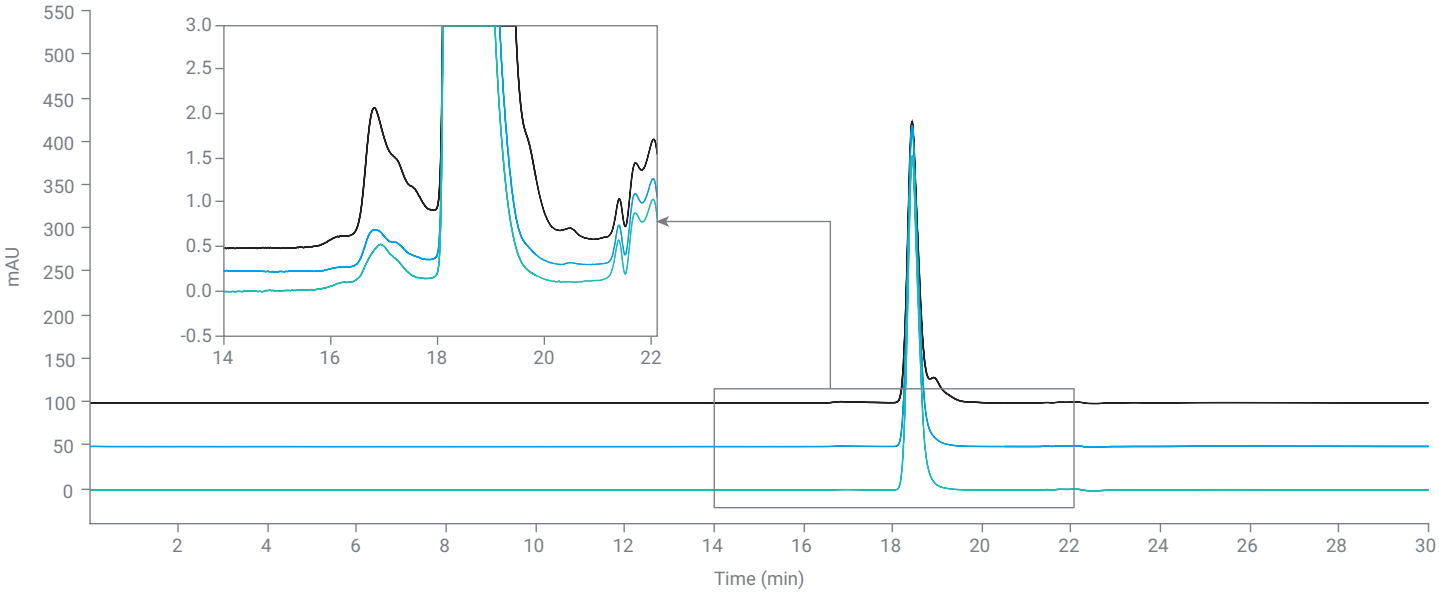


Figure 5. UV chromatogram of 1 mg/mL liraglutide (green) and 1 mg/mL liraglutide heated at 80 °C for 2 hours (blue) and 1 day (black) under Agilent AdvanceBio SEC 300Å conditions.

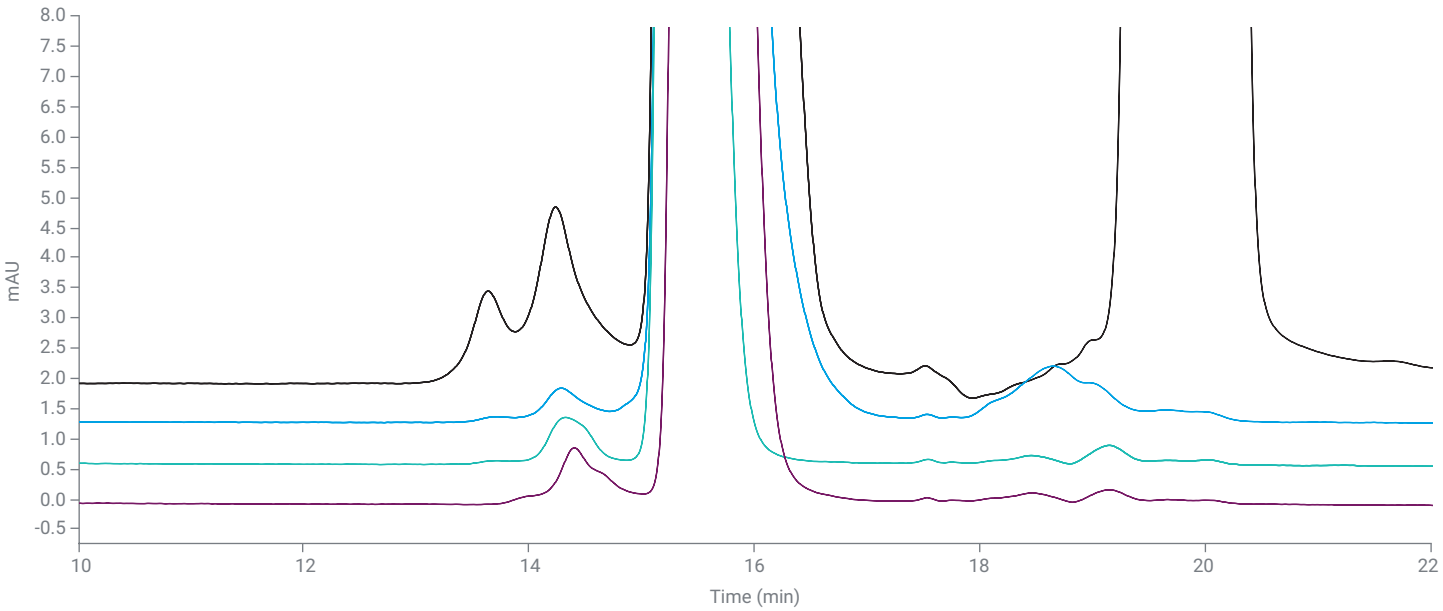


Figure 6. UV chromatogram of 1 mg/mL liraglutide (purple), 1 mg/mL semaglutide (green), 1 mg/mL semaglutide heated at 80 °C for 1 day (blue), and Ozempic (black) with injection volume of 20 µL under Agilent AdvanceBio SEC 300Å conditions.

Aggregate analysis by 2D-LC/MS

To determine the molecular weight of aggregate peaks observed under size exclusion conditions, the analysis was performed according to the conditions outlined in Table 2. Multiple heart cutting was used with a 40 µL sample loop to fill the loop with multiple cuts and analyze them under mass-friendly reversed-phase conditions, thereby facilitating the acquisition of molecular weight information for the desired peaks. However, the standard 40 µL cut volume configuration allows only partial parking of the target peak. To increase sensitivity by accommodating a wider peak range, the Multi-inject function of high-resolution sampling was used (Figure 7). By injecting four 3.2-second cuts for both Cut 1 and Cut 2 in a single ^{2}D analysis cycle, a total fraction volume of 128 µL (80% of the 160 µL loop's physical volume) was achieved. The solution filling the loop under ^{1}D conditions contained approximately 50% acetonitrile and 15% acetic acid, which complicated the retention of the target compound on the ^{2}D column. The Active Solvent Modulation (ASM) function was used in the ^{2}D setup to minimize the solvent effects of the loop solvent under these conditions.

Figure 8 illustrates the ^{2}D analysis results of Ozempic. Various impurity peaks were identified through the TICs of Cut 1 and Cut 2. When both cuts were analyzed under ^{2}D conditions, a common peak for semaglutide at 7.2 minutes was observed, with peaks C and D specifically detected in Cut 2. The MS raw spectrum for each peak was deconvoluted using OpenLab CDS 2.8. Peaks A and B, which have weak ionic bonds, dissociated easily into semaglutide under ^{2}D conditions. In contrast, peaks C and D were identified as impurities with molecular weights approximately twice that of semaglutide (Figure 9). These impurities are considered to originate from the aggregation of semaglutide and form covalent bonds. Unlike reversible aggregates formed by salt bridges and dipole interactions, covalent aggregates are irreversible.

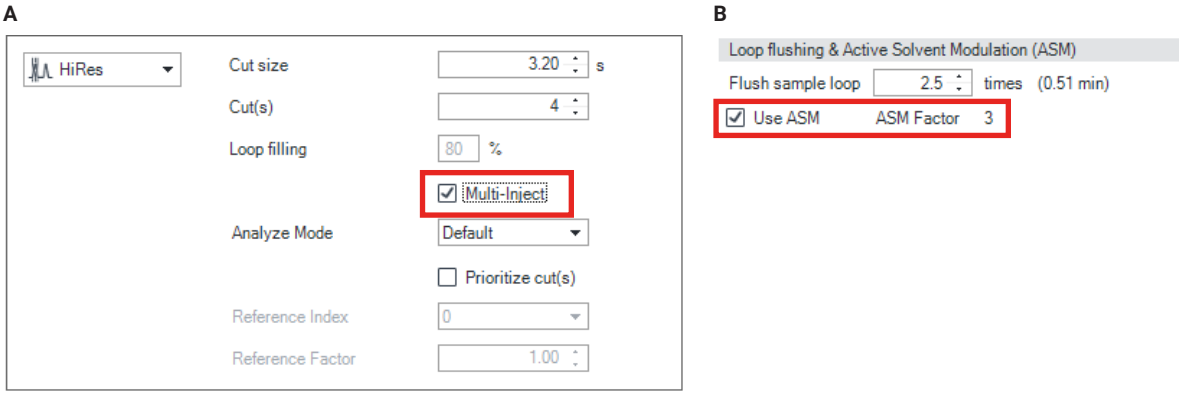


Figure 7. Agilent OpenLab CDS Acquisition snapshot of the high-resolution sampling settings configured for multi-injection (A) and ASM settings (B).

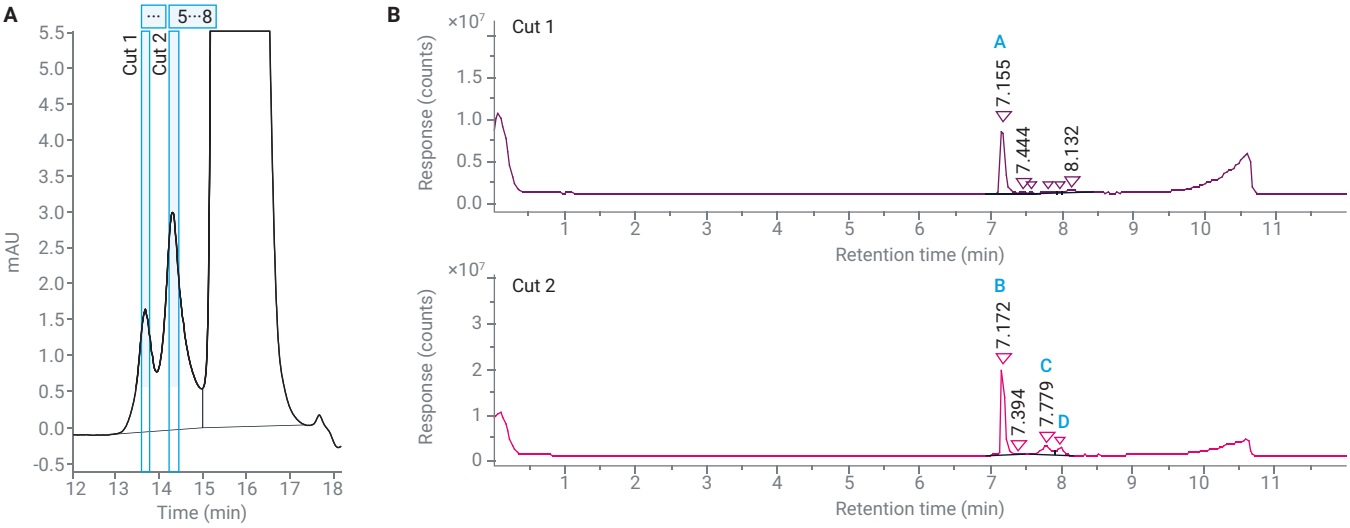


Figure 8. ^{1}D cut annotation (A) and the total ion chromatogram (TIC) of LC/MSD XT analyzing the cut in ^{2}D (B).

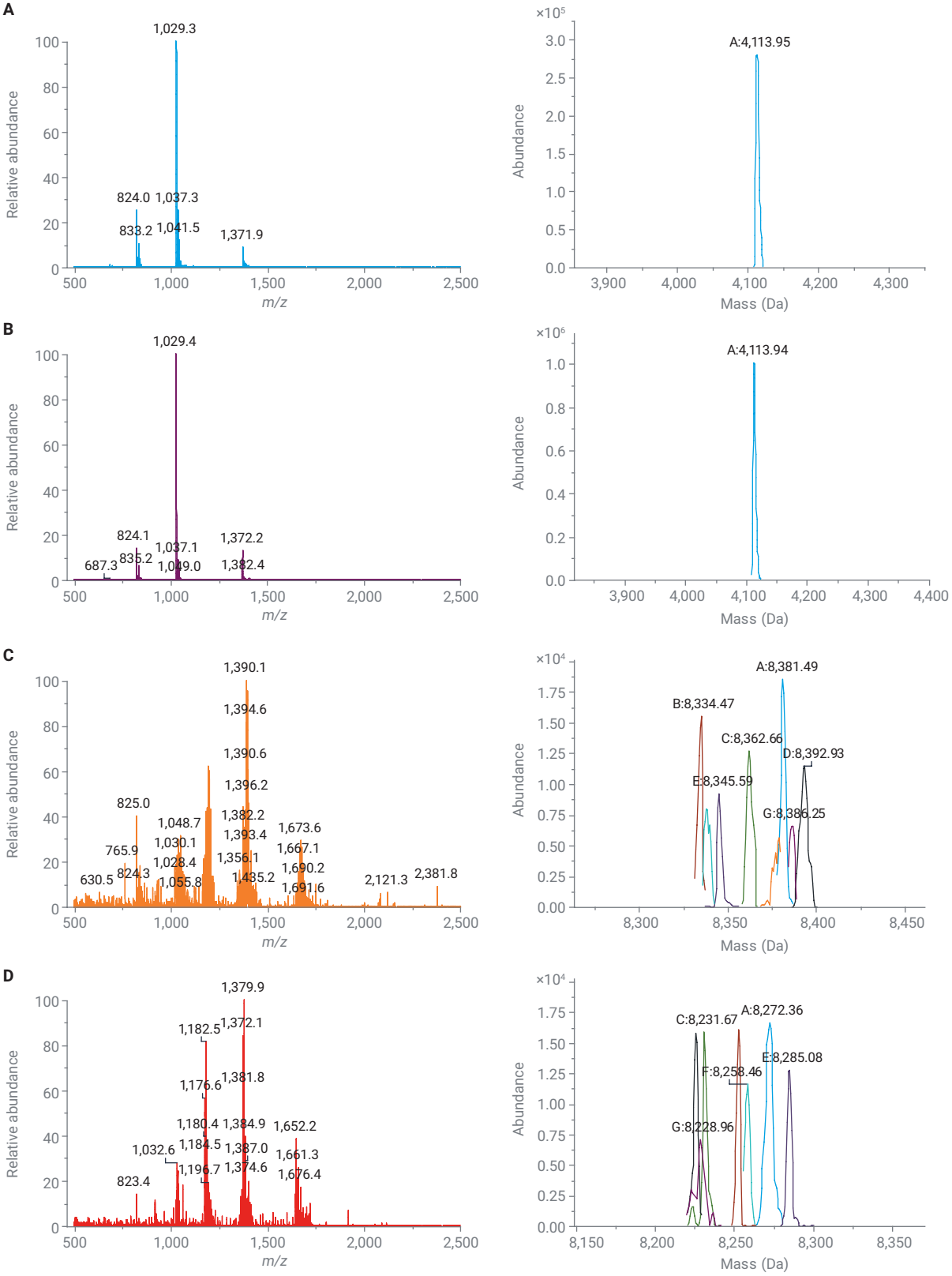


Figure 9. Raw spectrum (left) and deconvoluted spectrum (right) of the peak obtained from the ^{2}D TIC in Figure 8.

Conclusion

Unlike size exclusion chromatography approaches for proteins, evaluating peptide aggregates involves deep interactions between the column and analyte. Organic solvents were used as the mobile phase to analyze aggregates of semaglutide and liraglutide, employing an Agilent AdvanceBio SEC 300Å column for separation. The mobile phase conditions used for SEC cannot directly be applied for MS analysis. To determine the molecular weight of the aggregates, an Agilent 1290 Infinity II Bio 2D-LC system and Agilent InfinityLab LC/MSD XT were used, enabling aggregate analysis under MS-friendly conditions. Covalently bonded aggregates were characterized by SEC method retention times, and through the use of Multi-inject in high-resolution sampling and active solvent modulation technologies in 2D-LC, molecular weight information was obtained through deconvolution in OpenLab CDS 2.8.

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