Application Note

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Impurity Analysis of GLP-1 Peptide Retatrutide

Using the Agilent 1290 Infinity III bio 2D-LC and 6230C TOF LC/MS

Authors

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Abstract

LC/UV methods for drug product impurity analysis often use mobile-phase conditions optimized for chromatographic performance that may be incompatible with MS detection. This application note demonstrates impurity analysis of the GLP-1 peptide retatrutide using the multiple heart-cutting Agilent 1290 Infinity III bio 2D-LC coupled to an Agilent 6230C time-of-flight (TOF) LC/MS system. A trifluoroacetic acid (TFA)-based first-dimension separation provided effective resolution of the main product and product-related impurities. Selected peaks were then transferred online through multiple heart-cutting to an MS-compatible, formic acid (FA)-based second-dimension separation for sensitive, accurate-mass detection. Agilent MassHunter BioConfirm software, which now features intact peptide workflows, was used for sequence assignment of retatrutide and its product-related impurities. These results demonstrate a 2D-LC/MS strategy that preserves TFA-based chromatographic selectivity while providing MS-compatible accurate-mass detection and sequence assignment of intact retatrutide and its product-related impurities.

Introduction

GLP-1 receptor agonists are an important class of peptide therapeutics. Characterizing product-related impurities during development and manufacturing is critical because synthetic and degradation impurities can affect product quality. For structurally complex peptides such as retatrutide, closely related sequence variants and synthetic byproducts can be difficult to resolve chromatographically and often require MS characterization for confident identification.[1]

Peptide LC/UV methods are commonly optimized for chromatographic performance using acidic or ion-pairing mobile-phase additives. Trifluoroacetic acid (TFA) provides strong ion pairing that can influence peptide peak shape and chromatographic selectivity but may suppress electrospray ionization and reduce MS sensitivity. In contrast, formic acid (FA) provides better MS compatibility with weaker ion-pairing effects, resulting in different chromatographic selectivity.

To address this challenge, an Agilent 1290 Infinity III bio 2D-LC with an Agilent 6230C time-of-flight (TOF) LC/MS system was used to combine chromatographically optimized separation with MS-compatible detection.[2] A TFA-based first dimension provided separation of retatrutide and its product-related impurities, while selected peaks were transferred online through multiple heart-cutting to an FA-based second dimension for accurate-mass detection using the 6230C TOF LC/MS. This approach enables effective impurity separation and accurate MS detection without constraining the MS analysis to the same mobile-phase conditions. Agilent MassHunter BioConfirm software with intact peptide workflows was used to analyze intact retatrutide and related impurity signals, including peptides containing nonnatural amino acids and long-side-chain modifications.[3] This study demonstrated the ability of the integrated 2D-LC/MS workflow to separate, detect, and assign retatrutide-related impurities while preserving the selectivity of a TFA-based first-dimension LC separation.



1290 Infinity III Bio 2D-LC



6230C TOF LC/MS

Experimental

Instrumentation

- The Agilent 1290 Infinity III bio 2D-LC system comprised the following modules:
  - 1290 Infinity III bio multisampler with thermostat (G7137A)
  - 1290 Infinity III multicolumn thermostat (G7116B)
  - 1290 Infinity III diode array detector (G7117B) with Max-Light cartridge cell (G7117-60020)
  - 1260 Infinity III bio flexible pump (G7131C)
  - 1290 Infinity III bio high-speed pump (G7132A)
  - 1290 Infinity III valve drive (G1170A) with InfinityLab 2D-LC ASM Valve (G5643B)
  - Two 1290 Infinity III valve drives (G1170A) with InfinityLab bio multiple heart cutting valves (G5642-64000) equipped with 40 µL bio loops
- Agilent 6230C time-of-flight (TOF) LC/MS system

Materials

Retatrutide was purchased from Selleckchem, Houston, TX. The standard sample was reconstituted in water to a final concentration of 2 mg/mL.

LC/MS analysis

The sample was analyzed using a 1290 Infinity III bio 2D-LC system coupled to a 6230C TOF LC/MS system. Data were acquired with Agilent MassHunter acquisition software for LC/MS systems (TOF/Q-TOF) using the LC/MS conditions summarized in Tables 1–3. Agilent MassHunter BioConfirm software (version 14.1) and Agilent MassHunter Qualitative Analysis software (version 14.0) were used for data analysis.

Table 1. First-dimension (^{1}D) parameters.

Parameter	Value
Column	Agilent Altura Peptide Plus column, 2.1 × 150 mm, 2.7 µm (part number 227215-903)
Sampler Temp.	4 °C
Injection Volume	2 µL
Column Temp.	80 °C
Mobile Phase	A) 0.1% TFA in water B) 0.1% TFA in acetonitrile
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0.0 30 2.0 30 22.0 47 22.1 80 24.0 80 25.0 90 27.0 90 27.1 30
UV Detection	220 nm

Table 2. Second-dimension (^{2}D) parameters.

Parameter	Value
Column	Agilent Altura Peptide Plus column, 2.1 × 50 mm, 2.7 µm (part number 227205-903)
Column Temp.	80 °C
Mobile Phase	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile
Flow Rate	0.4 mL/min
2D-LC Mode	Time-based multiple heart-cutting Time (min) Parameter 19.16 High-resolution 1 × 5.7 s 19.51 High-resolution 1 × 5.7 s 19.79 High-resolution 3 × 5.3 s, Multi-Inject No 20.12 High-resolution 1 × 5.7 s
Gradient	Time (min) %B 0.0 30 0.5 30 1.5 60 1.6 80 2.0 80 2.1 30 ^{2}D gradient run time: 2.5 min ^{2}D gradient equilibration: 0.5 min
ASM Setting	Off

Table 3. Agilent 6230C TOF LC/MS system data acquisition parameters.

Parameter	Value
Ion Source	Agilent Jet Stream Technology source (AJS)
Gas Temp.	325 °C
Drying Gas	8 L/min
Nebulizer Gas	30 psi
Sheath Gas Temp.	350 °C
Sheath Gas Flow	11 L/min
Capillary	3,500 V
Nozzle	400 V
Octopole RF	750 V
Fragmentor	175 V
Skimmer	45 V
Acquisition Mode	MS only, *m/z* 100–3,200
Acquisition Rate	5 spectra/sec

Results and discussion

Comparison of first-dimension separations

To evaluate the effect of the mobile-phase modifier on chromatographic selectivity for retatrutide and its product-related impurities, reversed-phase LC/UV separations using TFA and FA were compared on an Agilent Altura Peptide Plus column under identical gradient conditions. The same LC method shown in Table 1 was used, with TFA replaced by FA for the FA-based separation. The Altura Peptide Plus column features a hybrid C18 stationary phase modified to provide a charged surface, which gives the stationary phase an additional selectivity mechanism for peptides beyond hydrophobicity alone. In this experiment, the TFA-based method provides improved resolution between retatrutide and product-related impurities (Figure 1). Three impurity peaks (P1–P3) are baseline separated from the main retatrutide product peak (M) with TFA, whereas P2 coelutes with the main product peak (M) when FA is used. These results demonstrate the chromatographic advantage of using TFA in the separation of retatrutide and its product-related impurities on the Altura Peptide Plus column.

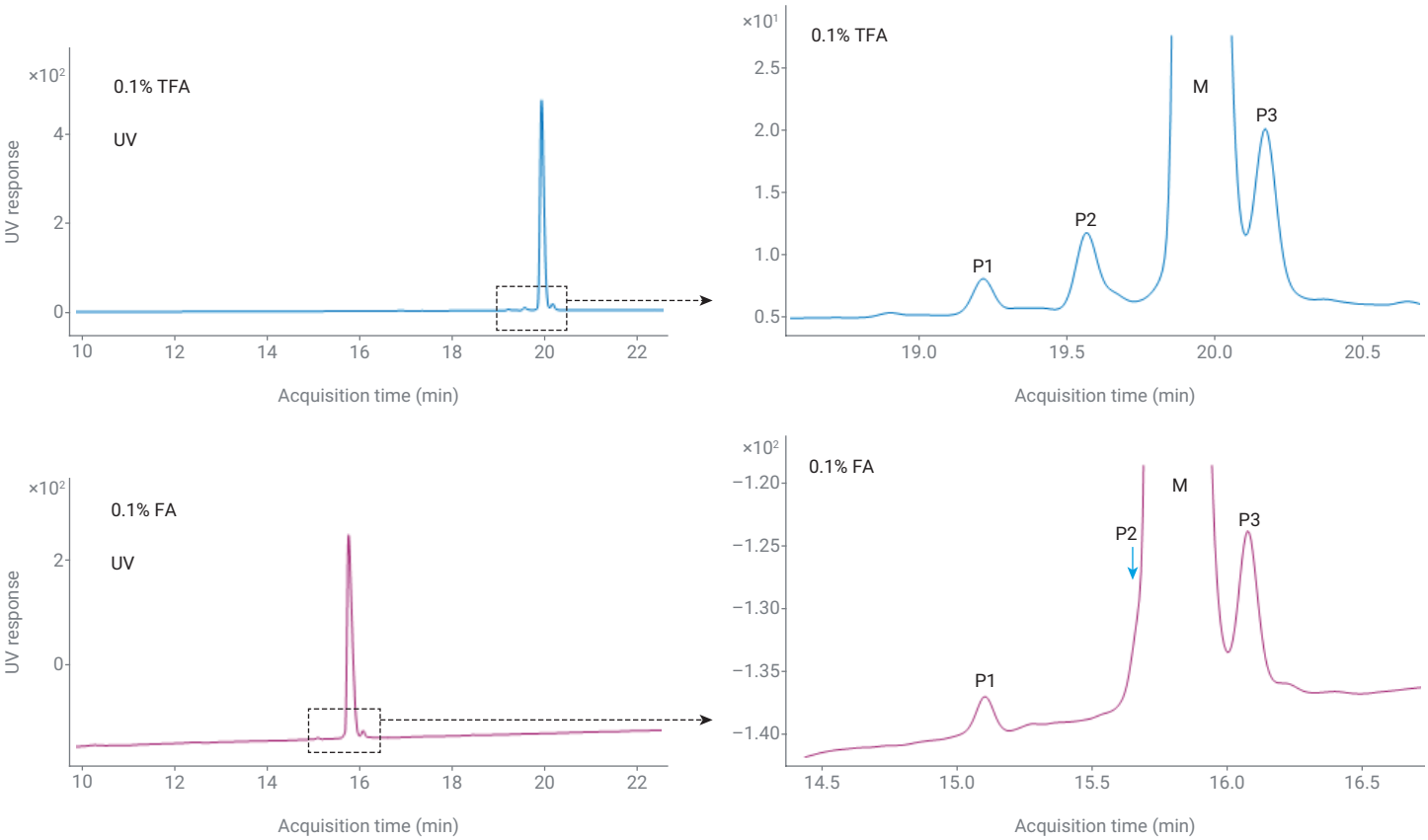


Figure 1. Comparison of one-dimensional reversed-phase LC/UV chromatograms obtained for retatrutide impurity analysis using TFA and FA as mobile-phase modifiers on an Agilent Altura Peptide Plus column under identical LC gradient conditions. TFA baseline resolved impurity peaks P1–P3 from the main product (M), whereas P2 coeluted with the main product under FA-based conditions.

Online multiple heart-cutting for 2D-LC/MS analysis

Because TFA can suppress electrospray ionization, Agilent 2D-LC with multiple heart-cutting was used to transfer selected peaks online from a TFA-based first-dimension (^{1}D) separation to an MS-compatible second-dimension (^{2}D) LC method for accurate-mass detection. Figure 2 illustrates the multiple heart-cutting of retatrutide and its impurities during 2D-LC/MS analysis. Figure 2A shows the relative UV peak area percentages (%) of the three impurity peaks and the main product peak normalized to the four labeled peaks, with P1 (0.89%), P2 (1.64%), P3 (2.90%), and the main product M (94.58%). Figure 2B shows the six heart-cutting window markers overlaid with the ^{1}D UV signal using Agilent MassHunter Qualitative Analysis software. The online-transferred fractions were analyzed using a three-minute MS-compatible LC/MS method, with representative overlaid total ion chromatogram (TIC) and extracted ion chromatogram (EIC) traces for cut 2 shown in Figure 2C. The amount of sample transferred to the ^{2}D method provided sufficient MS signal abundance for the low-abundance impurity peaks. In contrast, three consecutive cuts across the high-abundance main product peak (M) reduced the amount transferred in each cut, minimizing MS signal saturation, as shown in Figure 3.

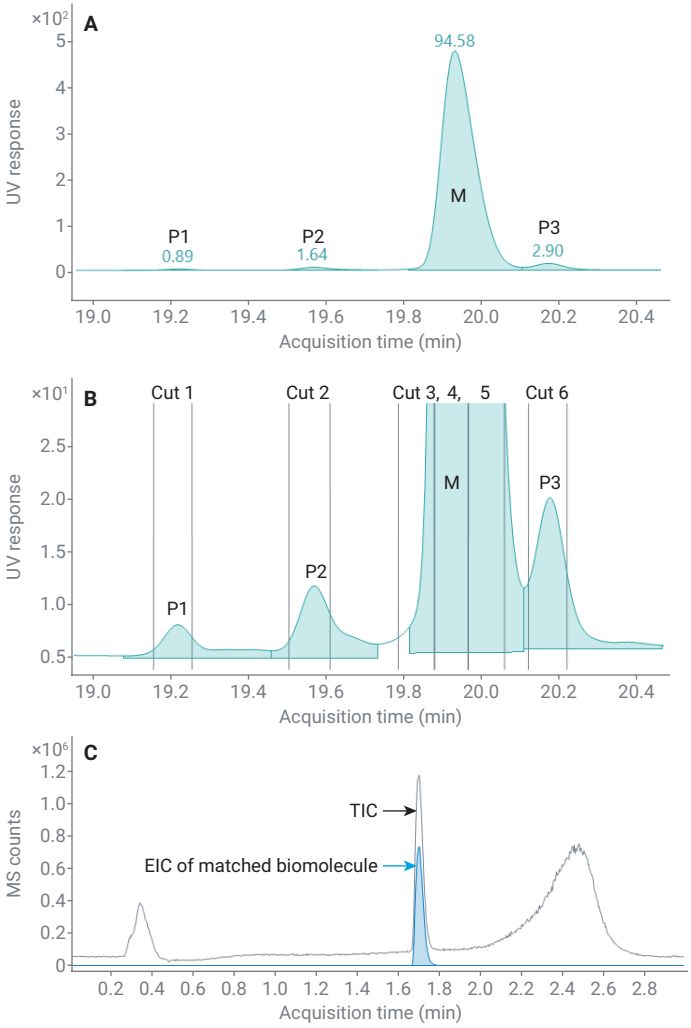


Figure 2. Multiple heart-cutting of retatrutide and its impurities during 2D-LC/MS analysis. (A) ^{1}D UV chromatogram showing baseline separation of the retatrutide main product (M) and impurity peaks P1–P3, with each peak labeled with its UV-based relative peak area (%). (B) Heart-cutting windows defined in the ^{1}D UV chromatogram for transfer of selected fractions to the ^{2}D method for LC/MS analysis. (C) Total ion chromatogram (TIC) from the ^{2}D LC/MS analysis of cut 2, overlaid with an extracted ion chromatogram (EIC) of the biomolecule with a matched sequence.

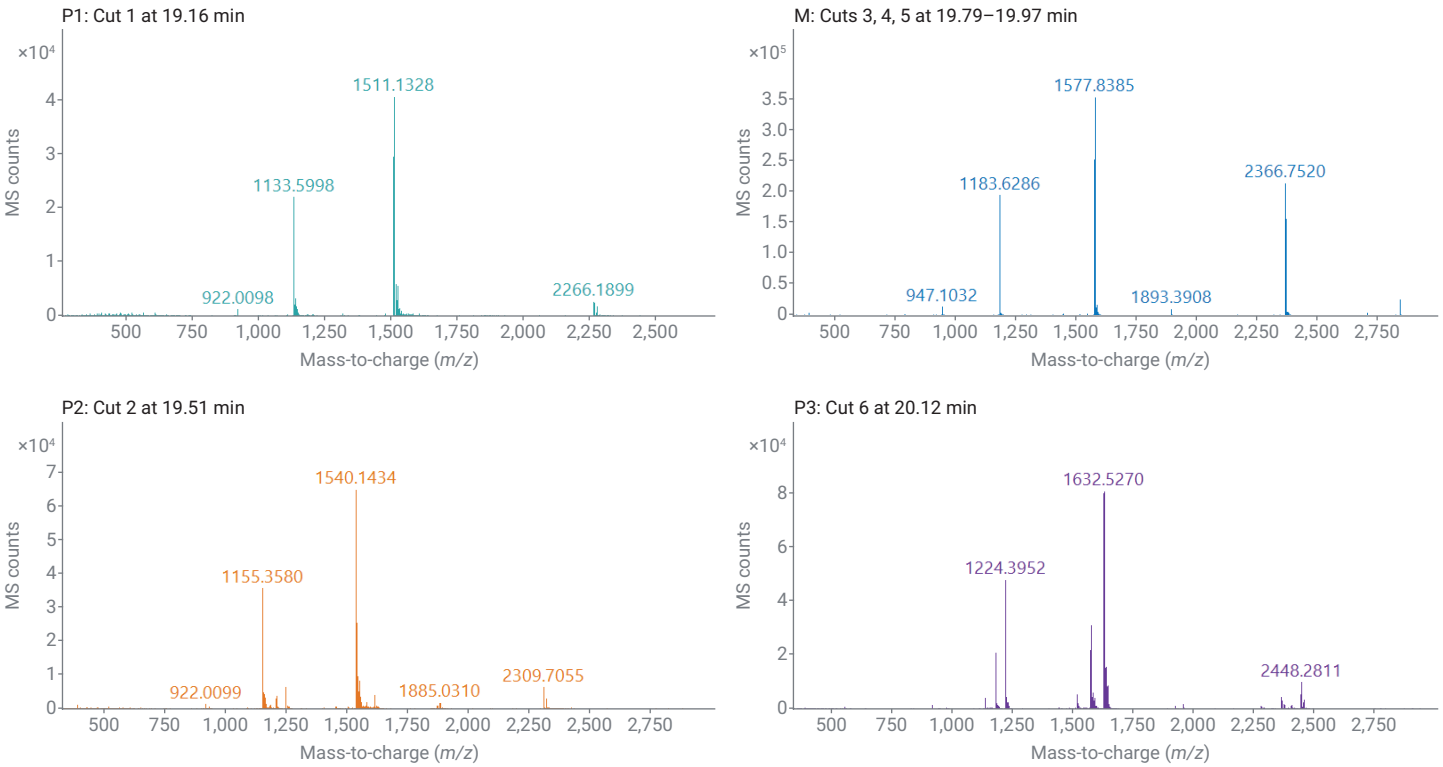


Figure 3. Representative MS spectra and corresponding heart-cutting times for the retatrutide main product (M) and impurity peaks P1–P3.

Sequence assignment of intact peptide and candidate impurities

To assign potential sequences for the targeted retatrutide intact peptide and the related impurity peaks, Agilent MassHunter BioConfirm software 14.1 was used to process 2D-LC/MS data. The file splitter utility in Agilent MassHunter acquisition software for LC/MS systems automatically splits 2D-LC/MS data according to the order of cuts from the 1D UV chromatogram. Therefore, data analysis can be conducted separately for each 2D-LC cut. This enables straightforward analysis of 2D-LC/MS data using MassHunter BioConfirm software.

BioConfirm software can define synthetic peptides containing nonnatural amino acids and long-side-chain modifications, providing the following two workflows for intact peptide sequence assignment:

- **MS-based Target Plus Impurities (TPI) workflow:** isotopically resolved deconvolution and impurity screening
- **MS/MS-based Peptide Identification (PI) workflow:** sequence-level characterization of the target and impurities

In this study, the TPI workflow was used for data analysis. The TPI workflow supported candidate assignments for retatrutide and its product-related impurities based on accurate mass and isotopic pattern matching. Figure 4 shows the biomolecules with sequence matches across the six 2D-LC/MS cuts and their potential assignments to the target retatrutide sequence and related impurity sequences. In addition to the main retatrutide product peptide, five candidate impurity assignments were obtained across the six cuts:

- Deletion of serine and isoleucine (Del:11–12 SI) in cut 1
- Deletion of isoleucine (Del:12 I) in cut 2
- Deletion of proline (Del:31 P) in cut 5
- Deletion of alanine and proline (Del:35–36 AP) in cut 6
- Insertion of tyrosine (Ins:10 Y) in cut 6

Because these sequence assignments are based solely on MS1 mass accuracy and isotope distribution matching, the specific amino acid positions assigned to the impurities may require further confirmation by MS/MS analysis. Such confirmation can be performed using the intact peptide PI workflow.

General							Sequence Match						Ion Scoring
Formula	Height	Area	File	Z Count	Mass	Tgt Seq Mass	Sequence Name	Sequence	Score (Bio)	Impurity Rules	Diff (Bio, ppm)	Quality Score
C212 H326 N44 O65	463402	1118915	2D_RPxRP_6cut_4ug- Cut01 at 19.16 min.d	3	4528.3388	4528.3557	Retatrutide	Y/Aib/QGTFTSDY/aMel	87.07	{Del:11-12 SI}	-3.72	40.4
C215 H331 N45 O67	734370	1887576	2D_RPxRP_6cut_4ug- Cut02 at 19.51 min.d	3	4615.3662	4615.3877	Retatrutide	Y/Aib/QGTFTSDYS/aM	80.51	{Del:12 I}	-4.66	49.5
C221 H342 N46 O68	3630379	1443284	2D_RPxRP_6cut_4ug- Cut03 at 19.79 min.d	4	4728.448	4728.4718	Retatrutide	Y/Aib/QGTFTSDYSI/aM	77.71		-5.02	39
C221 H342 N46 O68	3777522	2115051	2D_RPxRP_6cut_4ug- Cut04 at 19.88 min.d	3	4728.452	4728.4718	Retatrutide	Y/Aib/QGTFTSDYSI/aM	83.97		-4.18	42.3
C216 H335 N45 O67	26078	91953	2D_RPxRP_6cut_4ug- Cut05 at 19.97 min.d	1	4631.4074	4631.419	Retatrutide	Y/Aib/QGTFTSDYSI/aM	93.93	{Del:31 P}	-2.5	33.2
C221 H342 N46 O68	3496414	1317874	2D_RPxRP_6cut_4ug- Cut05 at 19.97 min.d	4	4728.4543	4728.4718	Retatrutide	Y/Aib/QGTFTSDYSI/aM	87.25		-3.69	39.4
C213 H330 N44 O66	50907	131761	2D_RPxRP_6cut_4ug- Cut06 at 20.12 min.d	2	4560.3675	4560.3819	Retatrutide	Y/Aib/QGTFTSDYSI/aM	90.58	{Del:35-36 AP}	-3.15	49.4
C221 H342 N46 O68	325561	1055911	2D_RPxRP_6cut_4ug- Cut06 at 20.12 min.d	3	4728.4503	4728.4718	Retatrutide	Y/Aib/QGTFTSDYSI/aM	81.42		-4.53	51.2
C230 H351 N47 O70	902874	2578496	2D_RPxRP_6cut_4ug- Cut06 at 20.12 min.d	3	4891.515	4891.5351	Retatrutide	Y/Aib/QGTFTSDYYSI/aI	84.43	{Ins:10 Y}	-4.11	55.7

Figure 4. MS1-based sequence assignment of retatrutide and product-related impurities across six 2D-LC/MS cuts using the BioConfirm intact peptide Target Plus Impurities (TPI) workflow.

Figure 5 shows the raw and deconvoluted mass spectra of the retatrutide main product (M) processed using BioConfirm software. The raw mass spectrum exhibits multiple charge states and dimeric species, with the most abundant isotope ions observed at *m/z* 1183.62265, 1577.83020, and 2366.24133, corresponding to the 4+, 3+, and 2+ charge states, respectively (Figure 5A). Following deconvolution, a monoisotopic mass of 4728.4635 Da is determined, matching the theoretical monoisotopic mass of 4728.4718 Da for the target retatrutide sequence, with a mass error of −1.8 ppm. As shown in Figure 5B, the green rectangular outlines overlaid on each isotope cluster indicate the predicted relative isotopic abundances, enabling direct visual comparison between the experimental and theoretical isotope patterns.

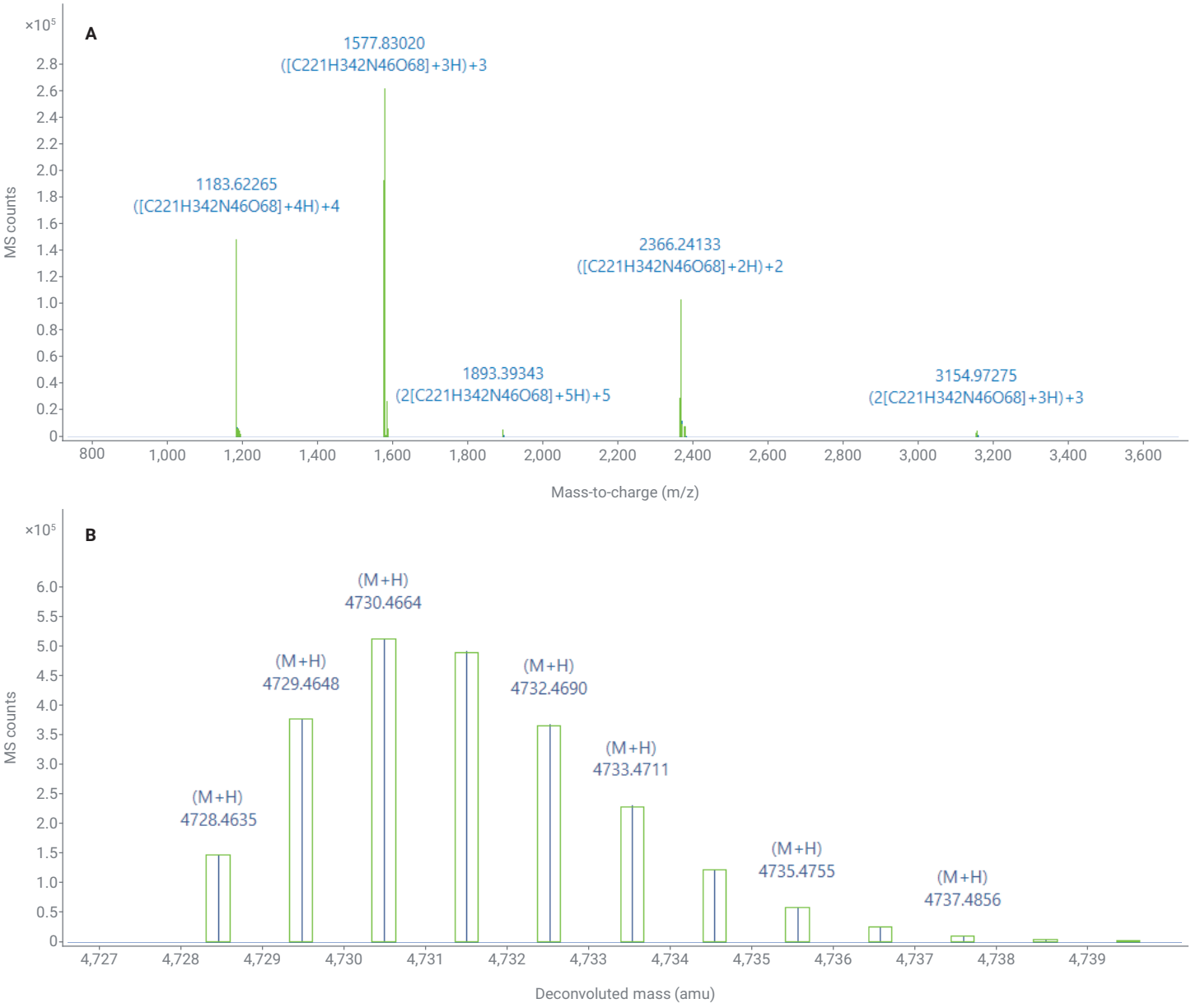


Figure 5. MS analysis of the retatrutide main product using Agilent MassHunter BioConfirm software. (A) Raw mass spectrum showing the major multiply charged ions of retatrutide and its dimeric species. (B) Deconvoluted mass spectrum showing the isotopic mass distribution of retatrutide. Green rectangular outlines indicate the predicted isotope distribution.

Conclusion

TFA-based reversed-phase LC provided improved separation of retatrutide and product-related impurities compared with FA-based separation on an Agilent Altura Peptide Plus column. By combining multiple heart-cutting 2D-LC with MS detection, the workflow retained the selectivity of the TFA-optimized first-dimension LC/UV method while enabling selected peaks to be transferred to an MS-compatible second dimension for accurate-mass analysis. This approach enables targeted online MS investigation of retatrutide and its product-related impurities without offline fraction collection or extensive redevelopment of the original LC/UV separation. Agilent MassHunter BioConfirm software further supported sequence assignments for the intact retatrutide peptide and its product-related impurities. Overall, this integrated 2D-LC/MS workflow extends a TFA-optimized LC/UV impurity method with targeted MS detection and sequence assignment for the intact peptide and its product-related impurities.

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Printed in the USA, November 6, 2026
5995-0093EN

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