Application Note

Biopharmaceuticals

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Amino Acid Composition Test of Semaglutide and Liraglutide Using an Agilent 1260 Infinity II Prime Bio LC

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Abstract

In this application note, an amino acid composition analysis of semaglutide and liraglutide was conducted using an Agilent AdvanceBio Amino Acid Analysis (AAA) LC column and an Agilent 1260 Infinity II Prime bio LC system. The precolumn derivatization with ortho-phthalaldehyde (OPA) allowed straightforward and reliable quantification. Notably, 2-aminoisobutyric acid present in semaglutide was accurately quantified.

Introduction

In synthetic peptide production, it is common to synthesize peptides containing fewer than 40 amino acids through solid phase peptide synthesis (SPPS).[1] The sources of amino acids used in synthesis are maintained at high purity levels, and the synthesized peptides are thoroughly purified. Impurities resulting from unintended insertions, deletions, or substitutions of amino acids can readily be identified using high-performance liquid chromatography (HPLC) and liquid chromatography/mass spectrometry (LC/MS).[2] While the advancements in LC/MS/MS enable the use of peptide mapping to confirm amino acid composition, the inability to distinguish between isoleucine and leucine remains a limitation.[3] The United States Pharmacopeia (USP) monograph for exenatide includes amino acid analysis as an identification test[4], and the European Medicines Agency guidelines also mention amino acid analysis.[5] Thus, amino acid analysis remains a valuable tool for confirming the composition of peptides.

To analyze the amino acids in peptides, pretreatment through hydrolysis is required to convert the peptide into free amino acids. Typically, hydrolysis is performed using 6 M hydrochloric acid; however, additives may be included to prevent oxidation and account for disulfide bonds. Tryptophan is particularly sensitive to acidic conditions and requires preparation under alkaline conditions. Various preparation methods, including gas-phase and microwave-assisted hydrolysis, are also used.[6]

Once peptides are hydrolyzed into amino acids, the content of each amino acid can be determined using LC/MS and HPLC. Due to the high polarity and lack of UV chromophore in most amino acids, derivatization is necessary for separation and detection. Several methods, including the use of OPA, fluorenylmethoxycarbonyl (FMOC), and ninhydrin, are used for derivatization and subsequent HPLC analysis.[6] The OPA and FMOC methods in particular offer quantitative and rapid reactivity, as well as higher resolution compared to ion-exchange chromatography due to the use of reversed-phase chromatography. Furthermore, using the sample preparation method available with Agilent autosamplers reduces human error and labor associated with the derivatization process. Performing derivatization immediately before analysis ensures the stability of the derivatized amino acid solution and allows for flexibility in handling unexpected samples.

This application note focuses on the quantification of amino acids present in semaglutide and liraglutide. Using the AdvanceBio AAA LC column and autosampler sample preparation method, the precolumn derivatized amino acids were analyzed. The testing procedure was conducted based on the guidelines outlined in the Agilent "Amino Acid Analysis 'How-to' Guide".[7] The study confirmed the presence of 14 amino acids in semaglutide, including the deliberately substituted 2-aminoisobutyric acid. Excellent linearity was achieved with the Agilent 1260 Infinity II fluorescence detector (FLD), and interference from by-products of the derivatization reagent was minimized.

Experimental

Instrumentation

The following instrumentation was used in this study:

- Agilent 1260 Infinity II bio flexible pump (product number G7131C)
- Agilent 1290 Infinity II bio multisampler (product number G7137A) with sample thermostat
- Agilent 1290 Infinity II multicolumn thermostat (product number G7116B) with Quick-Connect heat exchanger 1290 bio standard flow (product number G7116-60071)
- Agilent 1290 Infinity II diode array detector (product number G7117B) with bio-inert Max-Light cartridge cell, 60 mm (product number G5615-60017)
- Agilent 1260 Infinity II FLD spectra (product number G7121B) with flow cell, 8 µL, 20 bar (product number G1321-60005)

Standards and reagents

Sodium phosphate dibasic, disodium tetraborate decahydrate, sodium azide, and hydrochloric acid (HCl) were purchased from Sigma-Aldrich, while sodium hydroxide (NaOH) was purchased from Merck. Phosphoric acid was obtained from Fujifilm. 2-Aminoisobutyric acid was donated by a local customer. Acetonitrile (ACN) and methanol (MeOH) were purchased from B&J. AAA standards and a derivatization reagent were used as per the following product information:

- Agilent AdvanceBio AAA standard, 1 nmol/µL (part number 5061-3330)
- Agilent AdvanceBio AAA supplement kit (part number 5062-2478)
- Agilent borate buffer, 0.4 N in water, pH 10.2 (part number 5061-3339)
- Agilent OPA reagent, 10 mg/mL in 0.4 M borate buffer and 3-mercaptoproprionic acid (part number 5061-3335)

Samples

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

Preparation of standard solutions

AAA standards were prepared by dilution in 0.1 N HCl to achieve various standard concentrations. Glutamine, asparagine, and 2-aminoisobutyric acid were dissolved in 0.1 N HCl to a concentration of 100 mM and subsequently diluted for the experiment.

Preparation of sample solutions

Liraglutide was dissolved in water, and semaglutide was dissolved in 30% ACN to achieve a concentration of 10 mg/mL. Each solution (100 µL, corresponding to 1 mg of peptide) was transferred into a vacuum hydrolysis tube, followed by the addition of 100 µL water to collect any residual peptides from the tube walls. Subsequently, 200 µL of 12 M HCl was added to achieve a final concentration of 6 M HCl. The tube was subjected to three cycles of vacuum evacuation and nitrogen purging through the side arm to remove residual oxygen. The vacuum-sealed tube was then heated at 110 °C in a heating block for 24 hours, with occasional gentle agitation.

After hydrolysis, the tube was allowed to cool to room temperature before carefully releasing the vacuum. To dilute and neutralize the 6 M HCl, 4.2 mL of 0.1 M HCl and 400 µL of 6 M NaOH were added, resulting in a final peptide concentration of 1 mg per 5 mL. The sample was analyzed under conditions below without further filtration. To prevent contamination of the column by impurities in the sample solution, an Agilent InfinityLab Quick Change inline filter (2.1 mm id, 0.2 µm pore size, part number 5067-1603) was installed at the outlet port of the multisampler.

Mobile phases and injection diluent

The following mobile phases and injection diluents were used in this study:

- **Mobile phase A:** 2.8 g sodium phosphate dibasic, 7.6 g of disodium tetraborate decahydrate, and 0.4 g of sodium azide were dissolved in 2 L of water. Then, 1.5 mL of HCl was added, and the pH was adjusted to 8.2 by adding HCl in small increments. The solution was filtered through a 0.22 µm polyethersulfone filter.
- **Mobile phase B:** ACN:MeOH:water (45:45:10, v/v/v).
- **Injection diluent:** mobile phase A, 100 mL + phosphoric acid (85%), 0.4 mL.

Column

Agilent AdvanceBio AAA LC column, 4.6 × 100 mm, 2.7 µm (part number 655950-802)

Methods

Table 1. Agilent 1260 Infinity II Prime bio LC method parameters.

Parameter	Value
Column	Agilent AdvanceBio AAA, 4.6 × 100 mm, 2.7 µm
Flow	1.5 mL/min
Column Temperature	30 °C
Gradient	Time (min) %A %B 0 98 2 0.5 98 2 1.5 88 12 6 78 22 7.5 65 35 11 59 41 11.1 0 100 13.1 0 100 13.2 98 2 14.5 98 2
UV Detector	Signal: 338 nm (bandwidth 10 nm) Reference: 390 nm (bandwidth 20 nm) Data rate: 40 Hz
FLD	Excitation: 340 nm Emission: 450 nm Photomultiplier gain: 10 Data rate: 37.04 Hz

Table 2. Sample preparation method for precolumn derivatization of amino acids.

Function	Parameter
Draw	Draw 2.50 µL from location "Borate buffer" with default speed using default offset
Draw	Draw 1.00 µL from sample with default speed using default offset
Mix	Mix 3.50 µL from air with default speed five times
Wait	Wait 0.2 minutes
Draw	Draw 0.50 µL from location "OPA reagent" with default speed using default offset
Mix	Mix 4.00 µL from air with default speed ten times
Draw	Draw 32.00 µL from location "Injection diluent" with default speed using default offset
Mix	Mix 20.00 µL from air with default speed eight times
Inject	Inject
Wait	Wait 0.35 minutes
Valve	Switch valve to "Bypass"
Wait	Wait 11 minutes
Valve	Switch valve to "Mainpass"

Software

The software used in this application note was Agilent OpenLab CDS software, version 2.7.

Results and discussion

When analyzing the 16-component amino acid standard mixture according to the method outlined in Table 1, the resulting chromatograms were obtained for both UV and FLD detection, as shown in Figure 1. The amino acid standard mixture included cystine and proline. Cystine exhibited high sensitivity at a UV wavelength of 338 nm, while proline, being a secondary amine, does not derivatize with OPA and is instead recommended to be derivatized with FMOC. Since the amino acids constituting semaglutide and liraglutide do not include cystine or proline, the experiments were conducted using FLD, which offers superior sensitivity and specificity.

During the hydrolysis of peptides using 6 M HCl, glutamine undergoes deamidation and is completely converted to glutamic acid, resulting in the absence of detectable glutamine in the sample solution. Additionally, tryptophan is unstable under acidic conditions and requires separate preparation for accurate analysis. In Figure 2, the retention times for glutamine, tryptophan, asparagine, and 2-aminoisobutyric acid were examined to reference the positions of their respective peaks.

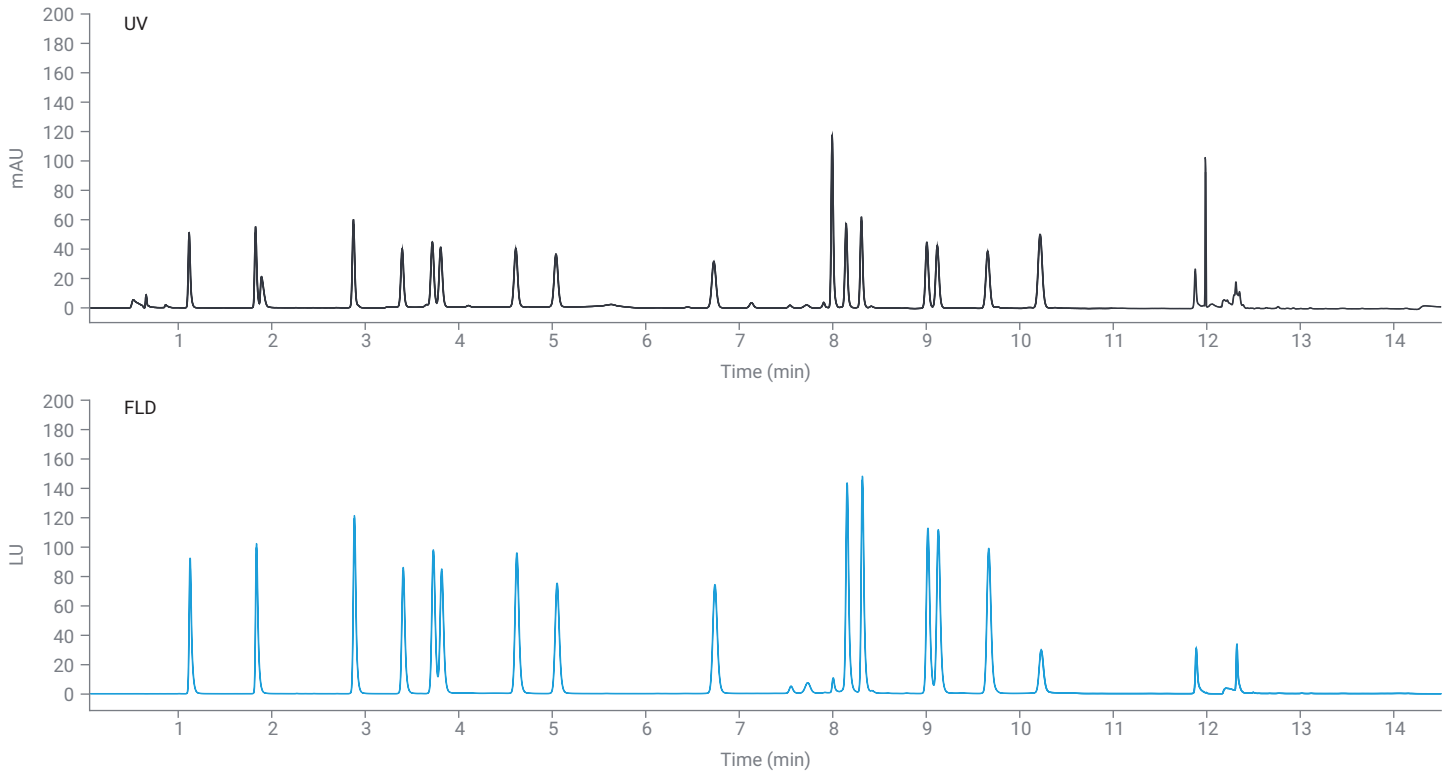

Figure 1. UV and FLD chromatograms of 100 µM amino acid standard mixture derivatized with OPA, analyzed using the Agilent AdvanceBio AAA LC column.

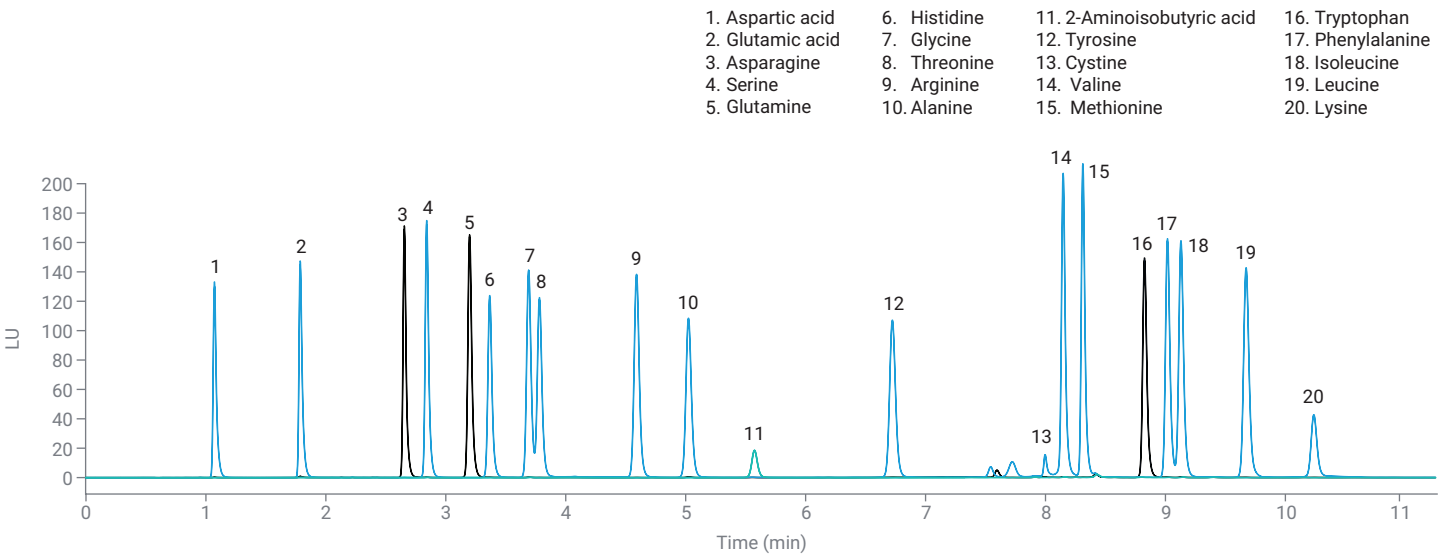

Figure 2. FLD profile of 100 µM standard solution of 20 amino acids.

Calibration for 15 amino acids in the composition of semaglutide was performed using the analysis of amino acid standard solutions at various concentrations. As shown in Figure 3 and Table 3, the calibration curves for both semaglutide and liraglutide achieved R^2 values exceeding 0.999 within the quantifiable range. Unlike in amino acid analysis for food samples, the molar ratios of amino acids in semaglutide and liraglutide are precisely predictable, negating the need for a broad calibration range. However, it is crucial to avoid excessively high peptide concentrations during hydrolysis, as this can lead to incomplete hydrolysis. In this study, a peptide concentration of approximately 50 µM was used, enabling quantification of amino acids up to 250 µM.

Semaglutide and liraglutide, each with a final concentration of approximately 50 µM, were hydrolyzed and analyzed using the same method. All 15 amino acids were detected. As expected, glutamine was fully deamidated and thus undetectable in the sample solutions of both peptides. Additionally, 2-aminoisobutyric acid was identified in semaglutide, along with an early-eluting peak (retention time 6.5 minutes) that appeared before tyrosine. Further testing revealed that this peak at 6.5 minutes was sensitive to column temperature and gradient slope changes, demonstrating its separation from tyrosine. According to the analysis conditions outlined in the "How-To" Guide[7], this peak typically overlaps with tyrosine. It is hypothesized that this peak is related to a compound derived from the side chain of semaglutide. The analytical conditions were optimized to separate this peak, resulting in the conditions specified in Table 1.

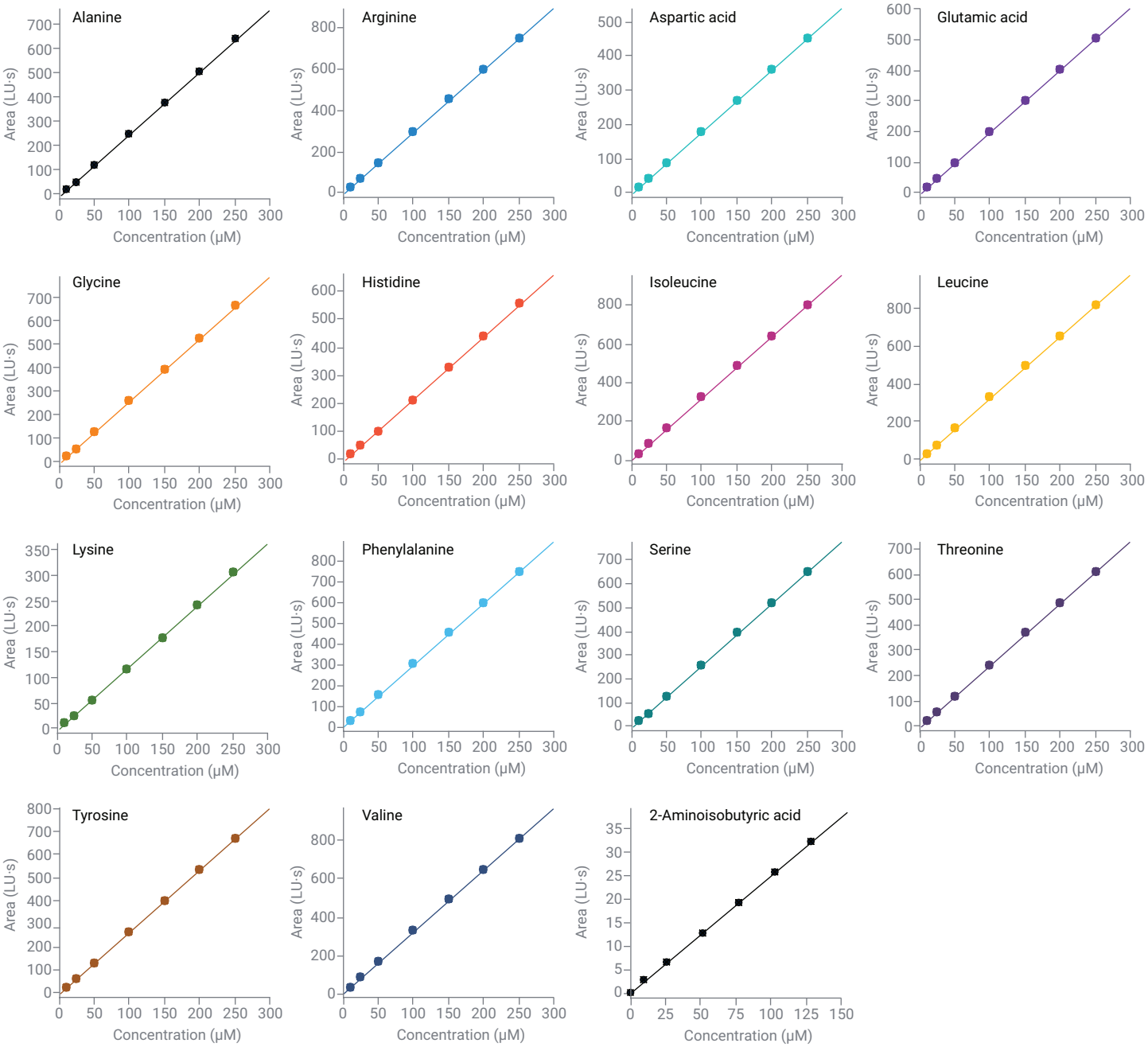

Figure 3. Calibration curves for 15 amino acids obtained using the Agilent AdvanceBio AAA column and the Agilent 1260 Infinity II FLD.

Table 3. Calibration range and R^2 values for 15 amino acids.

Peak Number	Compound	Retention Time (min)	Calibration Range (µM)	R^2
1	Aspartic acid	1.069	10 to 250	0.99990
2	Glutamic acid	1.784	10 to 250	0.99990
3	Serine	2.838	10 to 250	0.99988
4	Histidine	3.358	10 to 250	0.99970
5	Glycine	3.680	10 to 250	0.99976
6	Threonine	3.770	10 to 250	0.99991
7	Arginine	4.579	10 to 250	0.99990
8	Alanine	5.007	10 to 250	0.99986
9	2-Aminoisobutyric acid	5.560	1 to 125	0.99987
10	Tyrosine	6.700	10 to 250	0.99996
11	Valine	8.122	10 to 250	0.99986
12	Phenylalanine	8.987	10 to 250	0.99991
13	Isoleucine	9.099	10 to 250	0.99992
14	Leucine	9.639	10 to 250	0.99995
15	Lysine	10.202	10 to 250	0.99937

The theoretical concentrations of each amino acid in semaglutide and liraglutide were calculated from their molar concentrations and compared with the observed values, yielding the results presented in Table 4. While this hydrolysis method may not be optimized for absolute accuracy, it is sufficient for understanding the relative quantities of each amino acid. Unlike LC/MS/MS sequence mapping, which is hard to distinguish between isoleucine and leucine, this method allows for clear differentiation between these amino acids. Additionally, it can identify intentionally modified amino acids, such as 2-aminoisobutyric acid, which is included to prolong the peptide's half-life in the body. Notably, the amount of glutamic acid in semaglutide reflects not only the combined quantity of glutamine incorporated into the peptide but also any additional glutamic acid present in the side chain. Not all amino acid contents were precisely determined. The losses are presumed to be due to degradation of amino acids during the preparation process and incomplete hydrolysis. A more optimized hydrolysis process may be able to improve recovery.

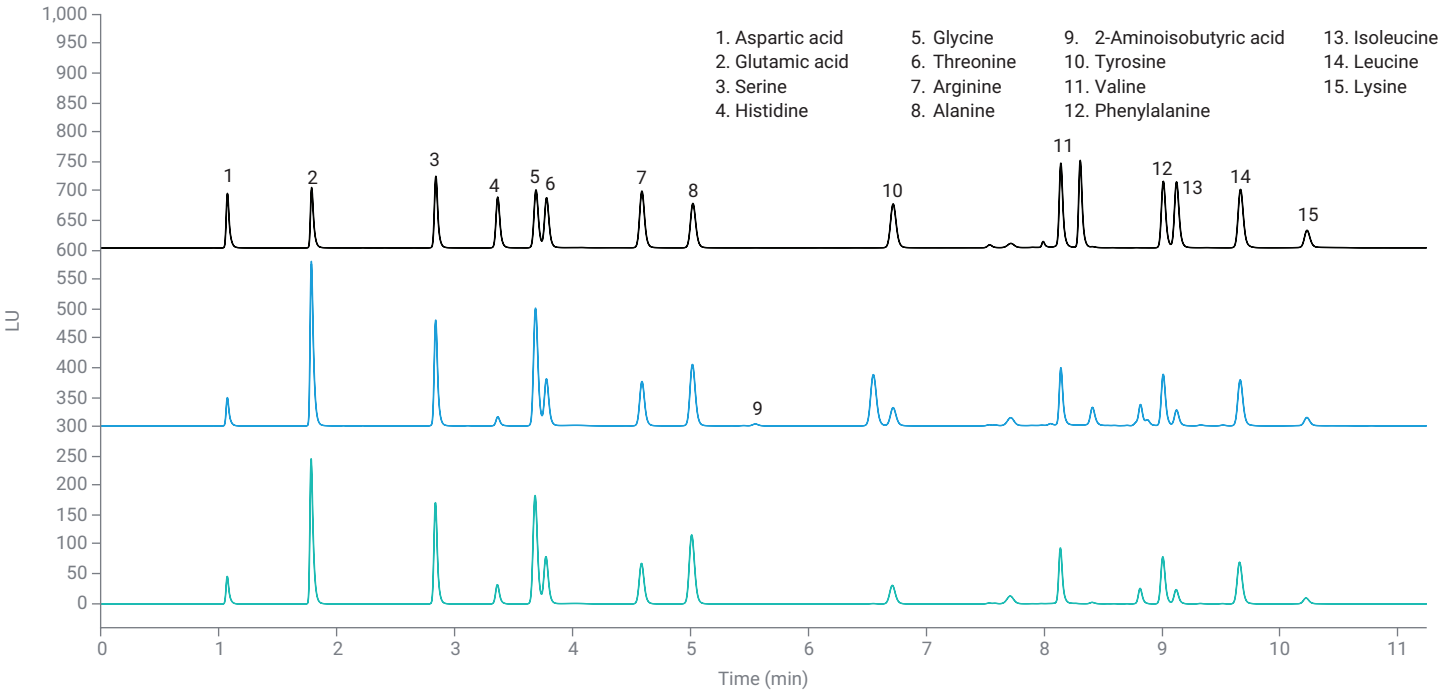

Figure 4. FLD chromatograms of hydrolyzed semaglutide and liraglutide solutions derivatized with OPA using the Agilent AdvanceBio AAA column (black = amino acid standard 100 µM; blue = semaglutide; green = liraglutide).

Table 4. Comparison of theoretical and observed amino acid composition for semaglutide and liraglutide.

Number	Compound	Semaglutide (48.6 µM)			Liraglutide (53.3 µM)		
		Theoretical Concentration (µM)	Observed Concentration (µM)	Recovery (%)	Theoretical Concentration (µM)	Observed Concentration (µM)	Recovery (%)
1	Aspartic acid	48.6	50.8	104.4	53.3	50.7	95.2
2	Glutamic acid	243.1	271.9	111.8	266.6	243.8	91.4
3	Serine	145.9	144.7	99.2	159.9	141.8	88.7
4	Histidine	48.6	20.4	41.9	53.3	43.3	81.3
5	Glycine	194.5	199.6	102.6	213.3	188.6	88.4
6	Threonine	97.2	92.3	94.9	106.6	95.5	89.5
7	Arginine	97.2	76.0	79.1	106.6	71.6	67.2
9	2-Aminoisobutyric acid	48.6	42.0	86.4	–	–	–
10	Tyrosine	48.6	41.3	85.0	53.3	43.0	80.7
11	Valine	97.2	67.8	69.7	106.6	68.5	64.2
12	Phenylalanine	97.2	76.1	78.3	106.6	72.3	67.8
13	Isoleucine	48.6	23.2	47.7	53.3	21.5	40.3
14	Leucine	97.2	76.4	78.5	106.6	71.3	66.8
15	Lysine	48.6	44.4	91.3	53.3	36.9	69.2

* Glutamine was calculated as glutamic acid, and tryptophan was not included in the calculation.

Conclusion

While the amino acid composition of synthetic peptides can be indirectly determined using LC/MS/MS, it is not possible to distinguish between isoleucine and leucine. This remains an important quality attribute in characterization analyses. Using the Agilent AdvanceBio AAA solution with OPA for precolumn derivatization, the amino acid composition of synthetic peptides was successfully identified, including isoleucine and leucine, and quantified modified amino acids. Chromatographic interference caused by amines from intentionally added structures was overcome by optimizing the HPLC method.

The use of the Agilent AdvanceBio AAA column enabled rapid and high-resolution analysis of the amino acid composition of GLP-1 analogues. Furthermore, the automation of precolumn derivatization using the autosampler sample preparation method of the Agilent 1260 Infinity II Prime bio LC system minimized human error, streamlined the derivatization process, and ensured reliable results.

References

1. Stawikowski M, Fields GB. Introduction to Peptide Synthesis. Curr Protoc Protein Sci. **2002**, 18:18.1.1–18.1.13.
2. Chae-Young, R. Peptide Drug Stability Analysis Using Agilent InfinityLab LC/MSD and OpenLab CDS Deconvolution. *Agilent Technologies application note*, publication number 5994-7500EN, **2024**.
3. Edwards, H. M.; Wu, H.; Julian, R. R.; Jackson, G. P. Differentiation of Leucine and Isoleucine Residues in Peptides Using Charge Transfer Dissociation Mass Spectrometry (CTD-MS). *Rapid Comm. Mass Spectrom.* **2022**, *36(5)*.
4. Exenatide, USP monograph, *US Pharmacopeia*.
5. European Medicines Agency, Guideline on the Development and Manufacture of Synthetic Peptides, **2023**.
6. Dai, Z.; Wu, Z.; Jia, S.; Wu, G. Analysis of Amino Acid Composition in Proteins of Animal Tissues and Foods as Pre-Column o-Phthaldialdehyde Derivatives by HPLC with Fluorescence Detection. *J. Chromatog. B* **2014**, *964*, 116–127,
7. Amino Acid Analysis "How-to" Guide, Accurate Results with AdvanceBio End-to-End Solution. *Agilent Technologies user guide*, publication number 5991-7694EN, **2017**.

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