Application Note

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Characterization of Forced Degradation Impurities of Glucagon-Like Peptide-1 Agonists by LC/Q-TOF Mass Spectrometry

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Abstract

Peptide biotherapeutics have gained increased attention due to their many therapeutic uses. This application note presents a liquid chromatography/quadrupole time-of-flight mass spectrometry (LC/Q-TOF MS) method to study the forced chemical degradation of glucagon-like peptide-1 (GLP-1) agonists. The results obtained are helpful for the GLP-1 agonists characterization during development.

Introduction

GLP-1 agonists are an emerging class of biotherapeutics. These synthetic peptides mimic the GLP-1 hormone to trigger the action through GLP receptors. GLP-1 agonists help to lower blood sugar levels and can lead to weight loss.[1] Currently, various GLP-1 agonists are available in the market for medication.

During the biotherapeutics development and manufacturing process, studies are required to assess the active pharmaceutical ingredient (API) impurity profiles. The presence of impurities can play an important role in the efficacy and safety aspects of biotherapeutics. The impurities associated with the product constitute a significant risk associated with adverse immunological reactions.[2] These impurities may arise from various sources throughout the product life cycle, including production processes, degradation, storage conditions, and even during delivery. It is crucial to identify and quantify these impurities to ensure that the therapeutic proteins perform as intended without causing adverse effects.

Forced degradation or stress testing studies are used to understand the chemical and physical changes of biopharmaceuticals. According to International Conference on Harmonisation (ICH) Q5E, manufacturers should evaluate the stability data through stress conditions to provide potential product-related impurities.[3] Therefore, it is essential to have analytical methods that can detect and identify the degradation products. Liquid chromatography/mass spectrometry (LC/MS) is well suited for effectively identifying and characterizing synthetic peptides.

This study used an LC/MS method to identify and characterize GLP-1 agonist impurity products under oxidative and pH stress conditions. The LC/Q-TOF MS identifies mass changes of GLP-1 agonists with oxidative and deaminated conditions. The results demonstrate the suitability of the LC/MS method for determining the GLP-1 agonist impurities and may be applied to routine quality control analysis.

Experimental

Reagents and chemicals

Semaglutide, liraglutide, and tirzepatide peptides were purchased from MedChemExpress (Monmouth Junction, NJ) and stored according to the manufacturer's instructions. Difluoroacetic acid (DFA), Trizma base, tris HCl, and 30% (v:v) hydrogen peroxide (H_2O_2) were procured from Sigma (St. Louis, MO); LC/MS-grade acetonitrile (ACN) was obtained from Fisher (Waltham, MA). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Billerica, MA).

Analytical equipment

Agilent 1290 Infinity II Bio LC system including:

- Agilent 1290 Infinity II Bio high-speed pump (G7120A)
- Agilent 1290 Infinity II Bio multisampler (G7137A)
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549AA)

Software and data processing

- Agilent MassHunter data acquisition software, version 11.0
- Agilent MassHunter BioConfirm software, version 12.1

Sample preparation

Liraglutide, semaglutide, and tirzepatide peptides were dissolved to 1.0 mg/mL in 30% ACN. For oxidative stress, stock solutions were diluted to 0.5 mg/mL and incubated at different concentrations of the oxidizing agent H_2O_2 (0.05%, 0.5%, 1%, and 2% v:v) overnight at room temperature. For a deamidation test, diluted samples were kept at a temperature of 37 °C in a tris buffer pH 8.9 for 3, 6, 12, and 24 days.

LC/MS analysis

The LC separation was performed on an Agilent AdvanceBio Peptide Mapping column (part number 653750-902) using a 30-minute gradient (Table 1). The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF (Table 2) and data analysis was performed in MassHunter BioConfirm version 12.1.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II Bio LC System	
Parameter	**Value**
Column	Agilent AdvanceBio peptide mapping, 2.1 × 150 mm, 2.7 µm, 120 Å
Sample Thermostat	10 °C
Mobile Phase A	0.1% DFA
Mobile Phase B	0.1% DFA in ACN
Gradient	Time (min) %A %B 0.00 80 20 1.00 80 20 20.00 40 60 25.00 10 90 25.10 80 20 30.00 80 20
Stop Time	30 min
Column Temperature	40 °C
Flow Rate	0.4 mL/min

Table 2. MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF	
Parameter	**Value**
Ion Mode	Positive ion mode, dual AJS ESI
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Sheath Gas Temperature	350 °C
Sheath Gas Flow	12 L/min
Nebulizer	35 psi
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Fragmentor Voltage	175 V
Skimmer Voltage	65 V
Oct RF Vpp	750 V
Reference Mass	922.009798
Acquisition Mode	Data were acquired in extended dynamic range (2 GHz)
MS Mass Range	100 to 1,700 *m/z*
Acquisition Rate	2 spectra/s

Results and discussion

To characterize the synthetic peptides, the workflow comprises the 1290 Infinity II Bio LC, AdvanceBio peptide mapping column, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm version 12.1 (Figure 1). The biocompatibility of the instrument configuration is a critical factor in the analysis of synthetic peptides. Instruments that are compatible with biological materials ensure that the analysis of peptides is accurate and free from interference caused by the instrument itself. This biocompatibility is particularly important when identifying impurities that may affect the quality and efficacy of peptide-based drugs.

To assess the forced chemical degradation products, the GLP-1 agonist was subjected to H_2O_2 and high pH treatment. The treatment conditions were tested at different time points and concentrations of chemical agents. Methionine and tryptophan are the most common oxidation residues in protein and peptide pharmaceuticals. The three GLP-1 agonists employed in this study contain one tryptophan residue in each peptide sequence. Typically, under laboratory conditions, H_2O_2 is used to assess oxidative degradation species. Figure 2 depicts the LC/MS monitoring of GLP-1 agonist profiles following overnight H_2O_2 treatment. The total ion chromatograms (TICs) of GLP-1 agonists can reveal significant insights into their oxidative profiles under stress conditions. Initial experiments were conducted with different time points (data not shown), and overnight incubation resulted in several oxidation forms of the peptides. The observation of multiple peaks with increasing H_2O_2 concentration indicates the formation of various oxidized forms of peptides, which can be effectively separated using the AdvanceBio peptide mapping column. Table 3 shows the expected native and oxidized GLP-1 agonist molecular weight details.

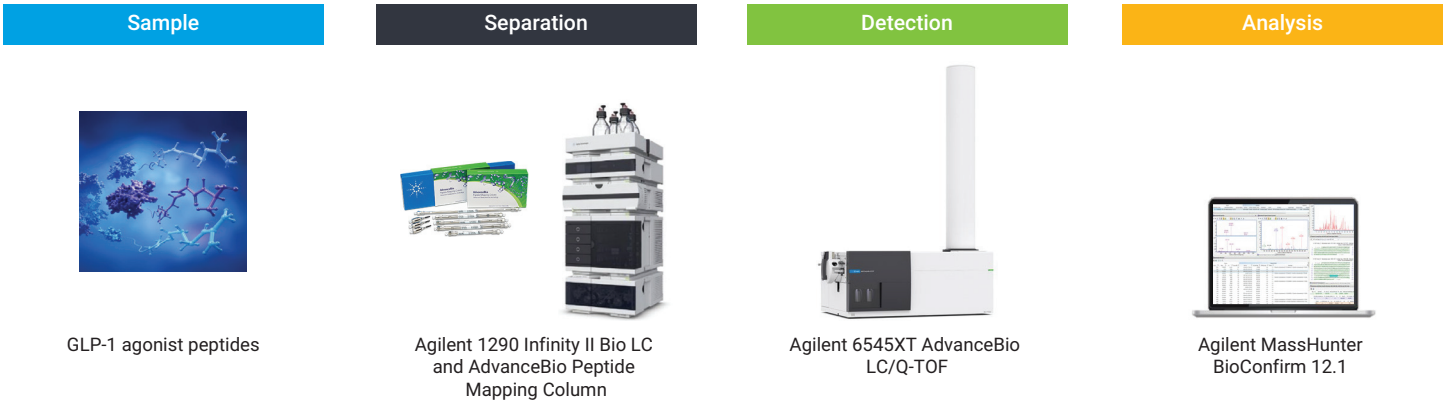

Figure 1. Synthetic peptide characterization workflow configuration.

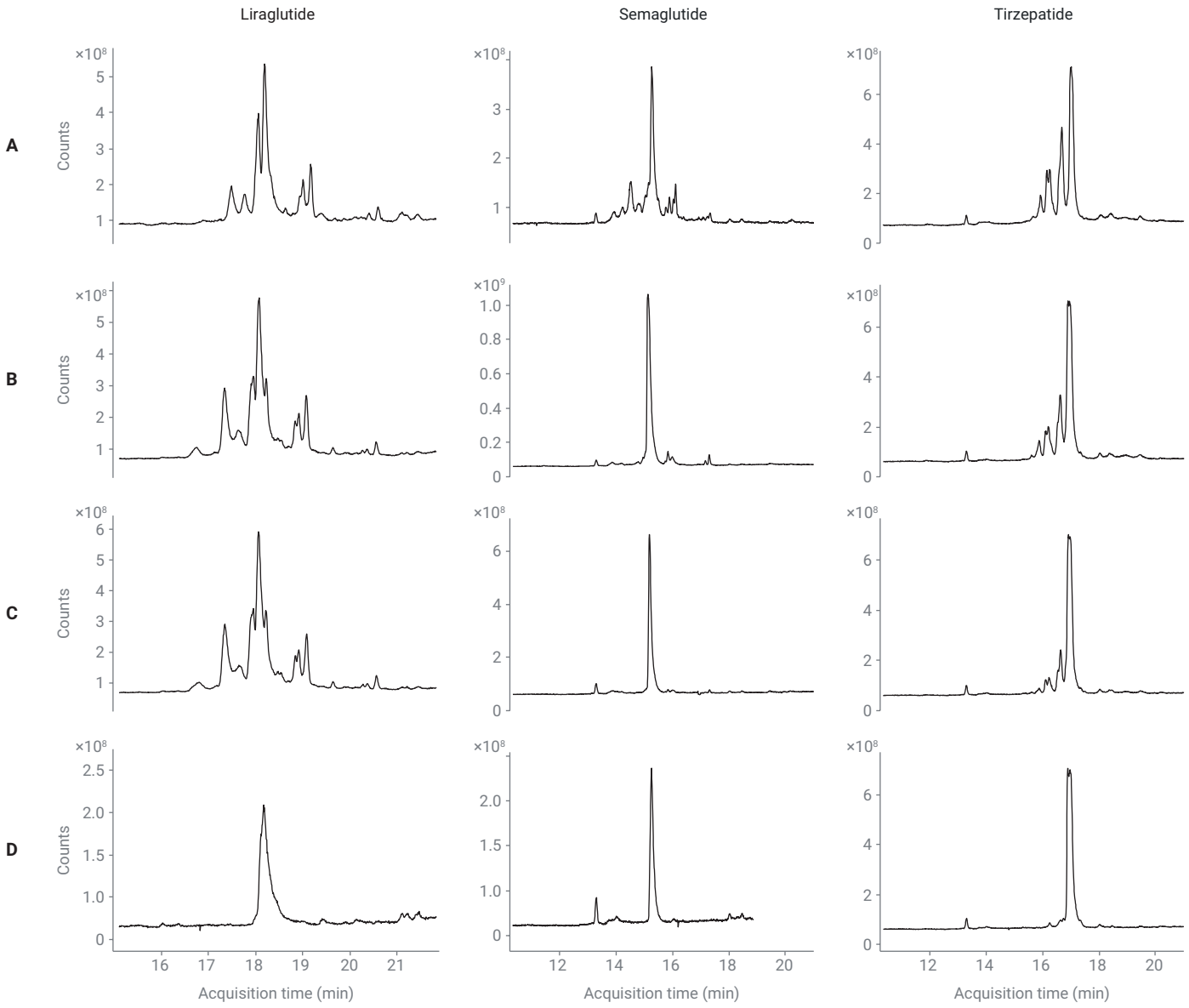

Figure 2. LC/MS monitoring of GLP-1 agonist profiles following H_2O_2 stress. (A) 2% H_2O_2, (B) 1% H_2O_2, (C) 0.5% H_2O_2, (D) 0.05% H_2O_2.

Table 3. GLP-1 agonist sequence and molecular weights.

GLP-1 Agonist	Sequence	Native Peptide Molecular Weight (Da)	Oxidized Peptide Molecular Weight (Da)		
			Mono	Di	Tri
Liraglutide	HAEGTFTSDVSSYLEGQAA-{Lys-N6-[N-(1-oxohexadecyl)-L-g-glutamyl]}-EFIA**W**LVRGRG	3,751.27	3,767.27	3,783.27	3,799.27
Semaglutide	H-{Aib}-EGTFTSDVSSYLEGQAA-{C18 diacid-γ-Glu-(AEEA)2-Lys}-EFIA**W**LVRGRG	4,113.66	4,129.66	4,145.66	4,161.66
Tirzepatide	Y-{Aib}-EGTFTSDYSIXLDKIAQ-{C20 diacid-gamma-Glu-(AEEA)2-Lys}-AFVQ**W**LIAGGPSSGAPPPS	4,813.55	4,829.55	4,845.55	4,861.55

Tryptophan (W) oxidation is highlighted in bold.

Multiply charged spectra corresponding to different oxidation forms were observed under 2% H_2O_2 treatment (Figure 3). Quadruple charge ($[M + 4H]^{4+}$) is the most abundant charge state in all three GLP-1 agonist samples. The charge states were corresponding to the expected $[M + 3H]^{3+}$, $[M + 4H]^{4+}$, $[M + 5H]^{5+}$, and $[M + 6H]^{6+}$ charge states of oxidized and nonoxidized peptide forms.

The mass spectrum was deconvoluted using a Maximum Entropy deconvolution algorithm in MassHunter BioConfirm software. It converts the multiply-charged spectrum to the zero-charge mass spectrum. Figure 3 shows the results obtained from the deconvolution. The deconvolved mass confirms the multiple oxidation forms of the GLP-1 agonist. A mass shift of +16 Da, +32 Da, and +48 Da corresponds to mono, di, and tri oxidation products, respectively.

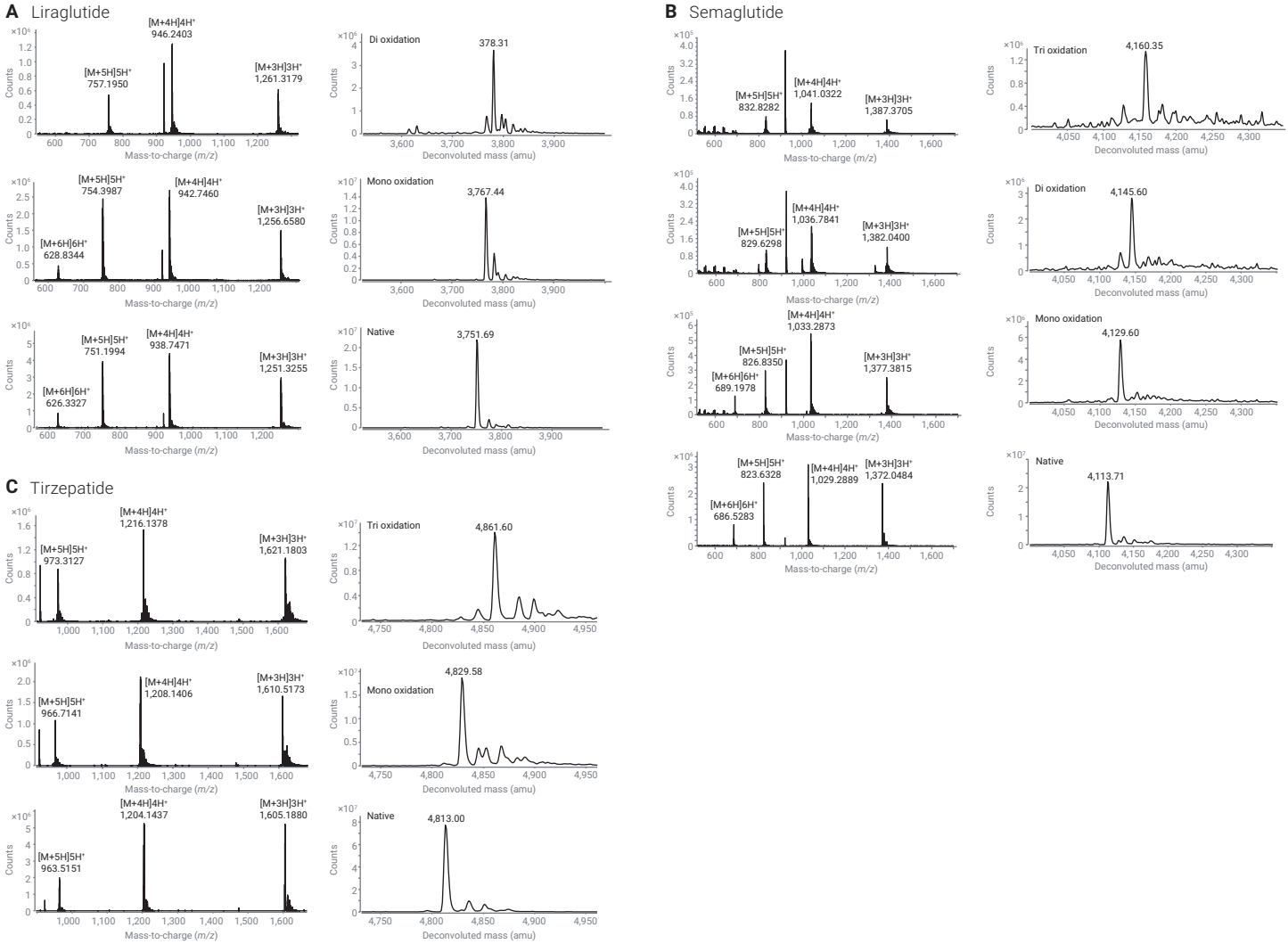

Figure 3. The charge state envelopes and deconvoluted spectra of oxidized and native GLP-1 agonists. (A) Liraglutide, (B) semaglutide, and (C) tirzepatide.

Tryptophan is most susceptible to oxidation and can produce isomeric peptide oxidation products.[4] Based on deconvolved mass, different peptide oxidation products are assigned and shown in Figure 4. Partial separation of singly, doubly, and triply oxidized isomeric peptides was achieved by reversed-phase chromatography.

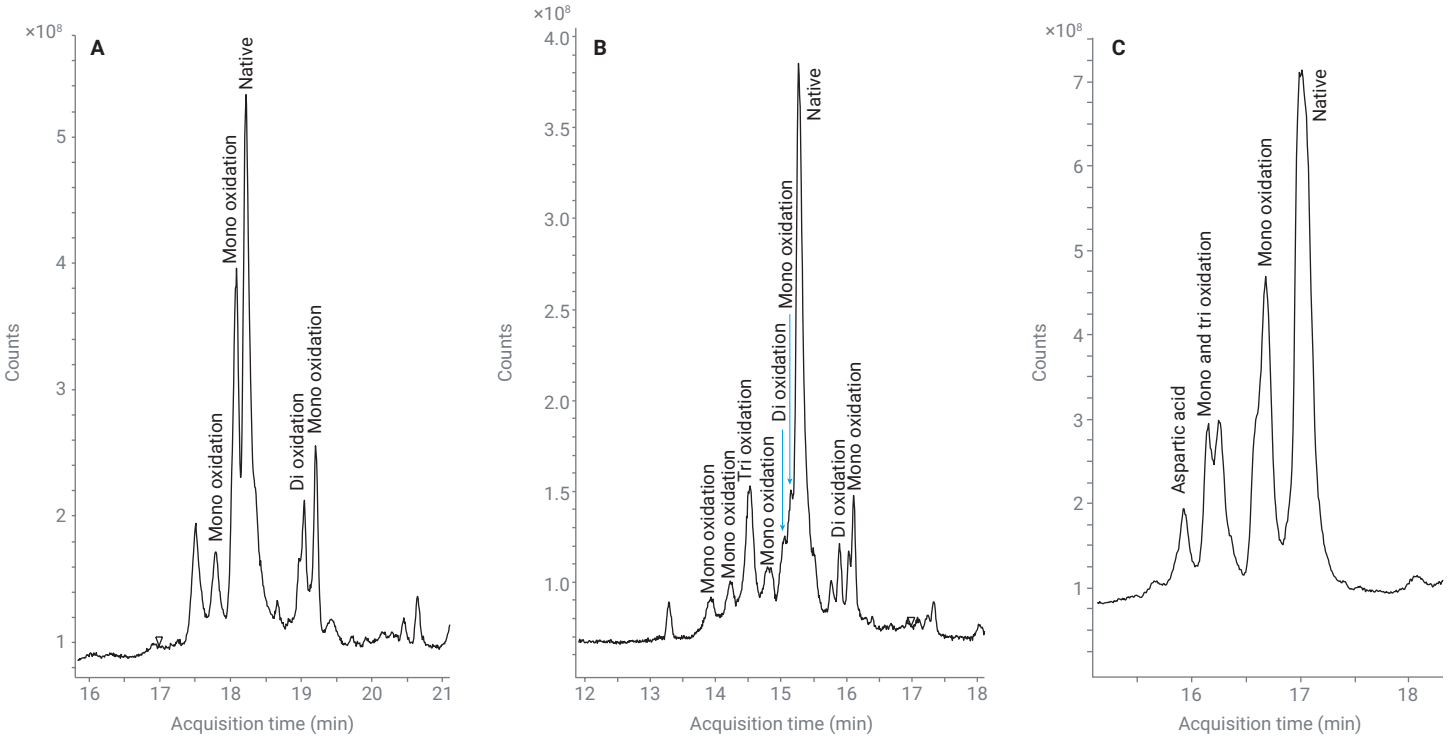

Figure 4. Oxidation of GLP-1 agonists. (A) liraglutide, (B) semaglutide, and (C) tirzepatide.

Figure 5 shows the representative MS/MS spectrum for both unmodified and oxidized liraglutide. Inspection of the y ion shows an increase of *m/z* ~8 in oxidized MS/MS spectra, suggesting oxidation of the tryptophan residue.

Progression of the oxidation reaction was monitored using extracted ion chromatograms (EICs) for different identified oxidized species (Figure 6). The analysis of the forced oxidation samples showed that the mono-oxidation state is highly abundant in all three molecules.

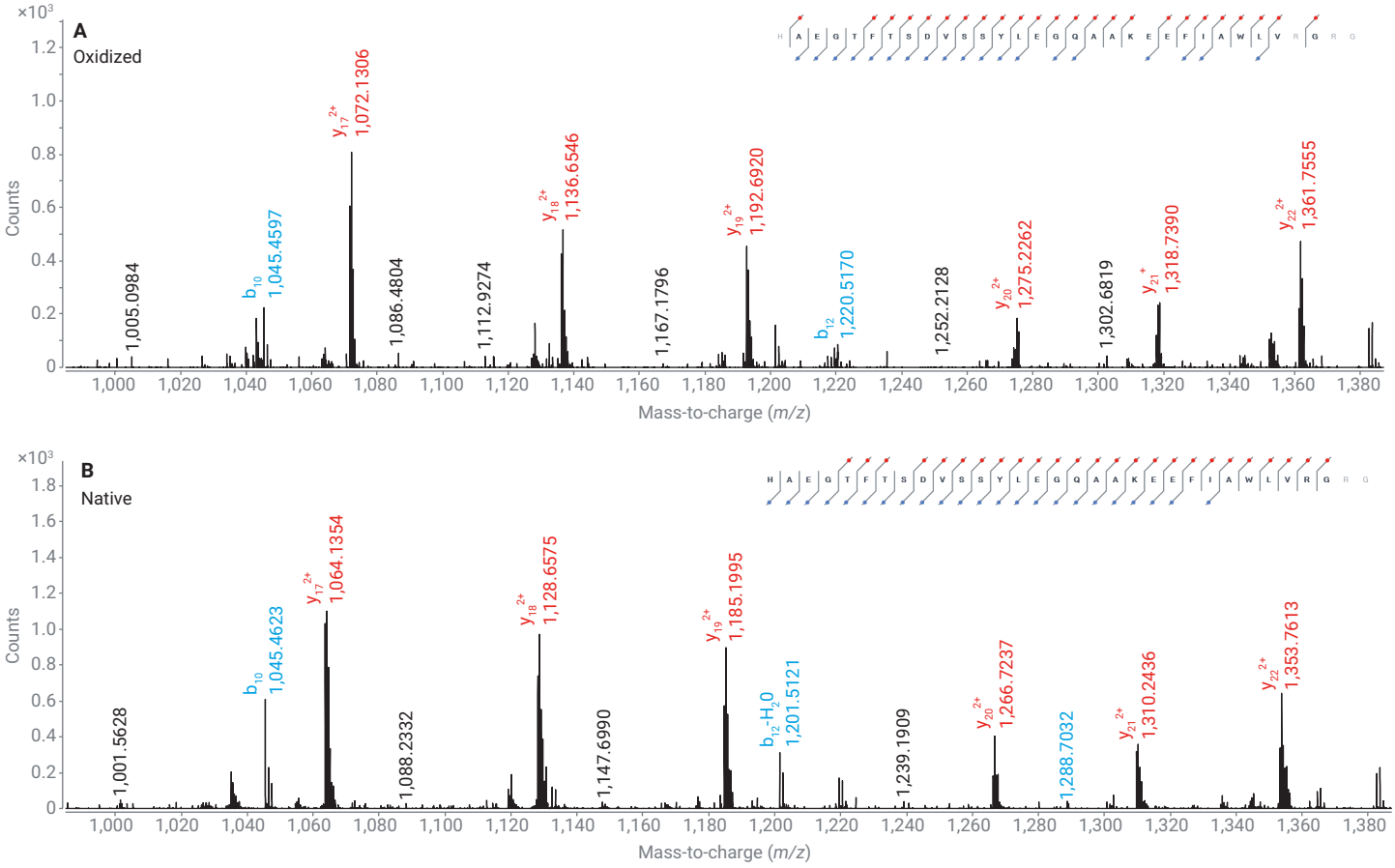

Figure 5. MS/MS spectra of unmodified and oxidized liraglutide.

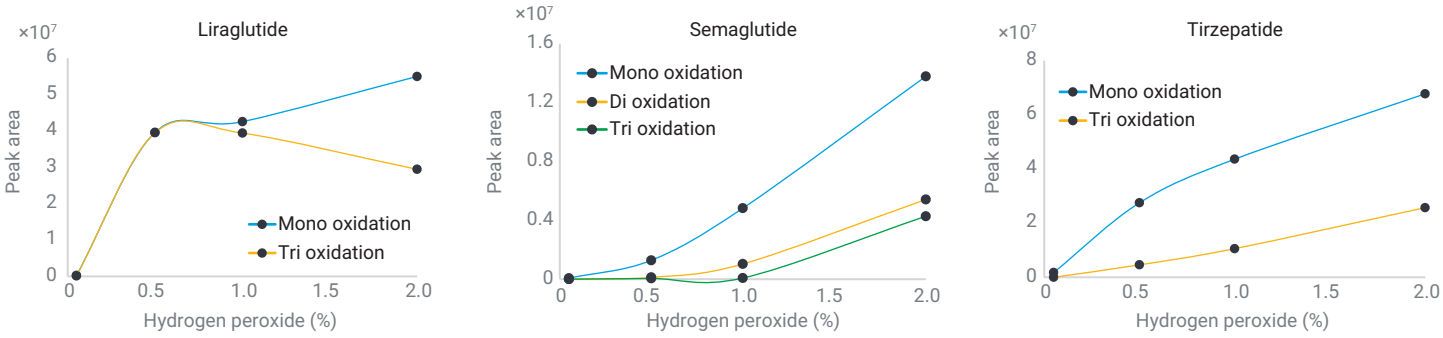

Figure 6. Oxidation responses of GLP-1 agonists. EIC peak area of quadruple charged ions.

The intact level analysis of forced degradation conditions for 24 days (at high pH) indicates no formation of deamidation species (Figure 7). It is noteworthy that all three peptides contain only glutamine as a possible deamidation site, which happens to have a much slower rate of deamidation than asparagine.

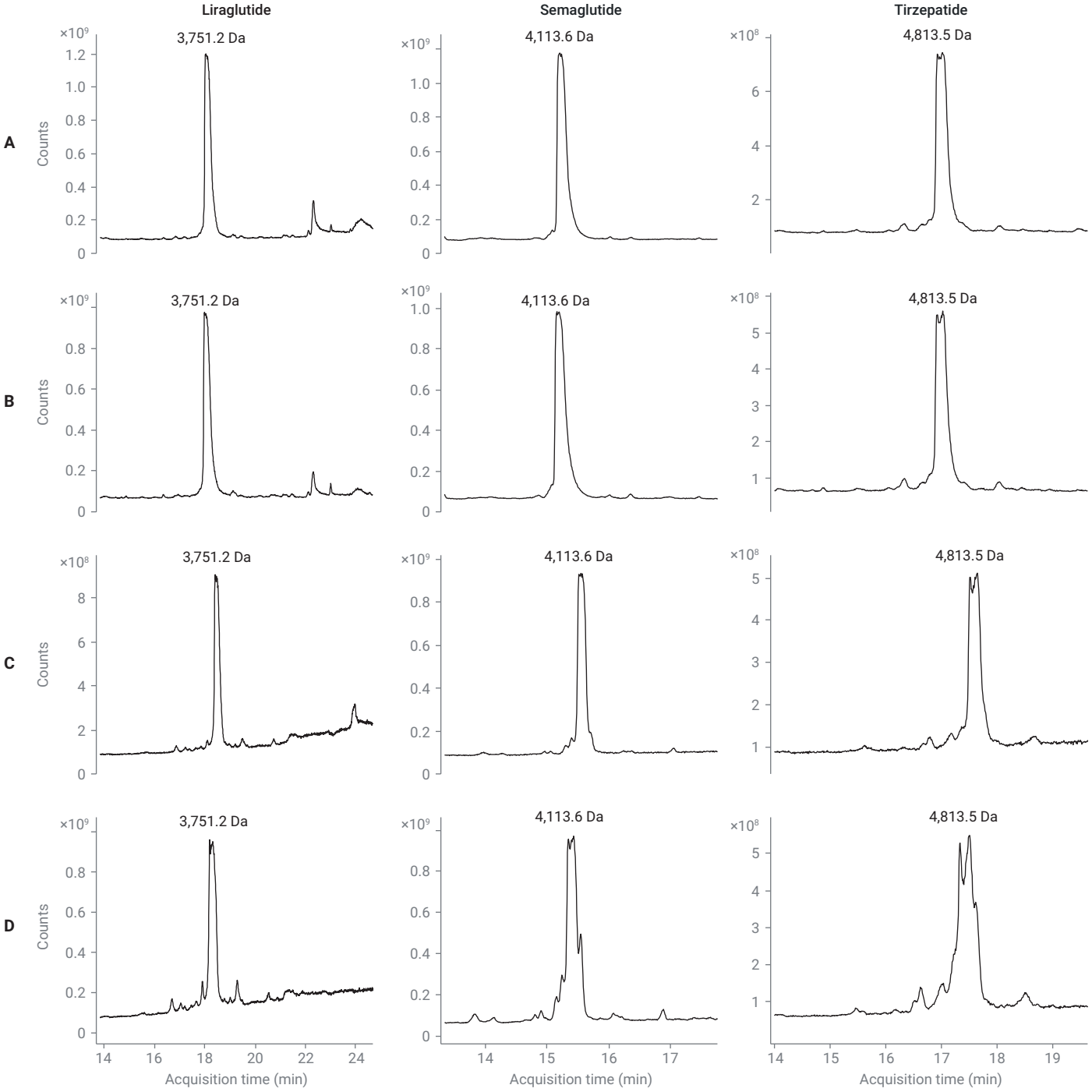

Figure 7. LC/MS monitoring of GLP-1 agonists at pH 8.9. (A) 3 days, (B) 6 days, (C) 12 days, and (D) 24 days. Deconvoluted masses are presented.

Conclusion

This application note demonstrates the analysis of GLP-1 agonist forced chemical degradation products using an Agilent LC/Q-TOF system. Precise characterization of forced chemical degradation of GLP-1 was achieved with high-efficiency chromatographic separation and high-quality MS spectra. The Agilent MassHunter BioConfirm software is easy to use and provides an integrated environment for the analysis of synthetic peptides. The developed LC/MS method demonstrates the applicability of the method in peptide drug research and manufacturing.

References

1. Müller, T. D.; *et al.* Glucagon-Like Peptide 1 (GLP-1). *Mol. Metab.* **2019** *30*, 72–130.
2. Kiese, S.; Pappenberger, A.; Friess, W.; Mahler, H-S. Shaken, Not Stirred: Mechanical Stress Testing of an IgG1 Antibody. *J. Pharm. Sci.* **2008**, *97(10)*, 4347–4366.
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