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Characterization and Quantification of Glucagon Like Peptide-1 Agonists and their Impurities Using Liquid Chromatography/Mass Spectrometry (LC/MS)

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Introduction

Glucagon-like peptide-1 (GLP-1) agonists are an emerging class of therapeutic agents. 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.

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. It is crucial to identify and quantify these impurities to ensure that the therapeutic proteins perform as intended without causing adverse effects. Liquid chromatography/mass spectrometry (LC/MS) is well suited for effectively identifying and characterizing synthetic peptides.

Further monitoring drug stability is crucial to ensure the right dosage for achieving optimal therapeutic levels. Therefore, sensitive analytical methods must be used to characterize and quantify these peptide impurities during the biotherapeutic development life cycle.

In this work, we employed the LC/Q-TOF MS and TQ to study the GLP-1 agonists. LC/Q-TOF MS was used 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.

For quantitation study, a rapid multiple reaction monitoring (MRM)-based method for the highly sensitive quantitative analysis of both unmodified and oxidized Tirzepatide was developed using a triple quadrupole LC/MS. The calibration curve demonstrated a wide dynamic range, spanning four orders of magnitude for unmodified Tirzepatide and three orders of magnitude for mono-oxidized Tirzepatide.

GLP-1 agonist sequence	
Liraglutide	HAEGTFTSDVSSYLEGQAA-{Lys-N6-[N-(1-oxohexadecyl)-L-g-glutamyl]}-EFIAWLVRGRG
Semaglutide	H-{Aib}-EGTFTSDVSSYLEGQAA-{C18 diacid-γ-Glu-(AEEA)2-Lys}-EFIAWLVRGRG
Tirzepatide	Y-{Aib}-EGTFTSDYSIXLDKIAQ-{C20 diacid-gamma-Glu-(AEEA)2-Lys}-AFVQWLIAGGPSSGAPPPS

Experimental

Impurity characterization LC/Q-TOF MS

Agilent 1290 Infinity II Bio LC system

Agilent 6545XT AdvanceBio LC/Q-TOF MS

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 H2O2 (0.05%, 0.5%, 1%, 2% v:v) for overnight at room temperature.

Quantitation LC/TQ

Agilent 1290 Infinity II Bio LC system

Agilent 6495D Triple quadrupole mass spectrometer

Tirzepatide peptide was dissolved in methanol to a concentration of 1.0 mg/mL. Calibration curve samples were prepared by serial dilution using 30% ACN and 2% DFA. Concentrations of the prepared calibration curve samples ranged from 0.025 to 250 ng/mL. Quality control (QC) samples were prepared at 0.75, 7.5, and 75 ng/mL. For oxidative stress, stock solutions were diluted to 0.5 mg/mL in 30% ACN and incubated with the oxidizing agent H_2O_2 (2% v:v) overnight at room temperature.

Refer to Application notes (5994-7794EN and 5994-7992EN) for LC and MS conditions

Results and Discussion

LC/MS of GLP-1 agonists

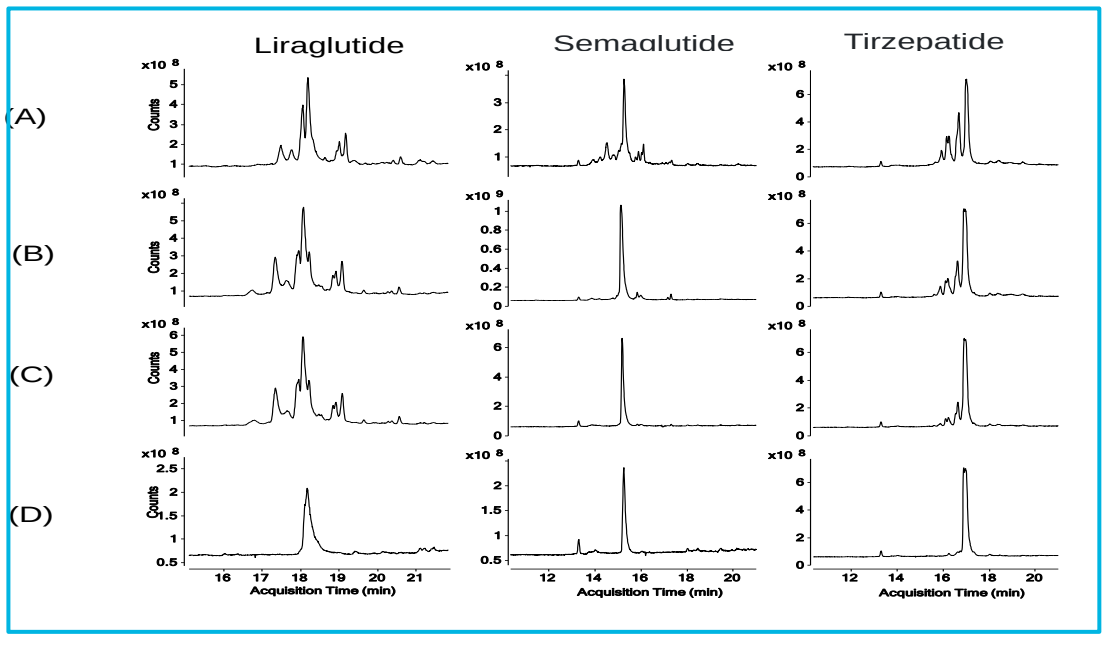

Figure 1. LC/MS monitoring GLP-1 agonists TIC profiles following H_2O_2 stress. (A) 2% H_2O_2, (B) 1% H_2O_2, (C) 0.5% H_2O_2 (D) 0.05% H_2O_2.

GLP-1 agonists peptide oxidation products

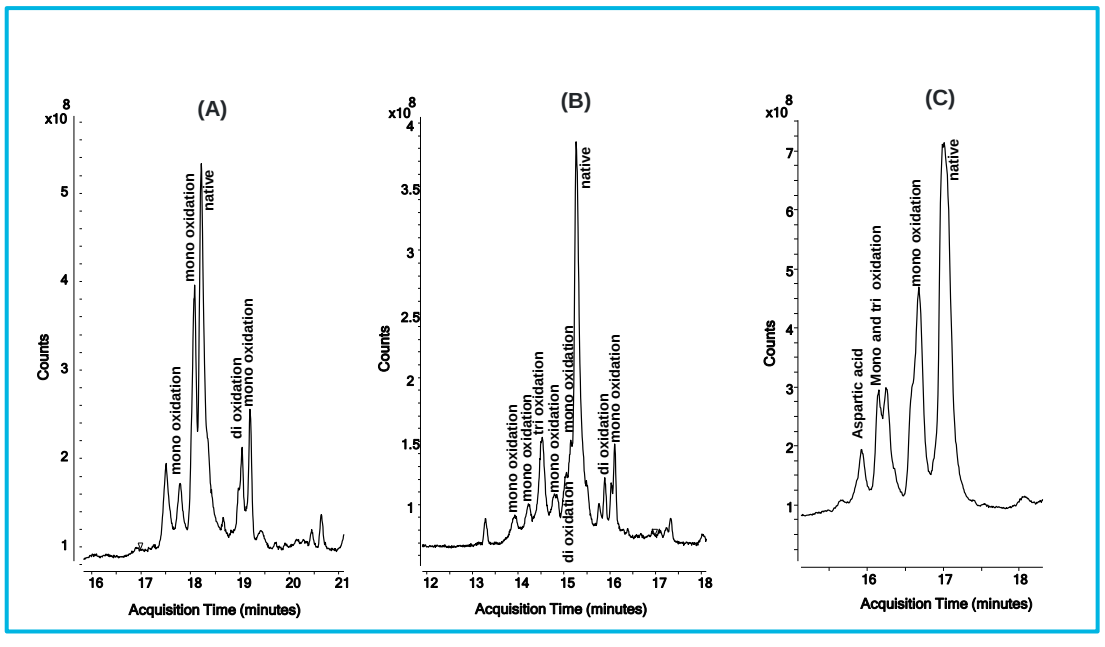

Figure 2. TIC profiles of oxidation of GLP-1 agonists (2% H_2O_2 stress). (A) Liraglutide; (B) Semaglutide; (C) Tirzepatide.

LC/MS of GLP-1 agonists

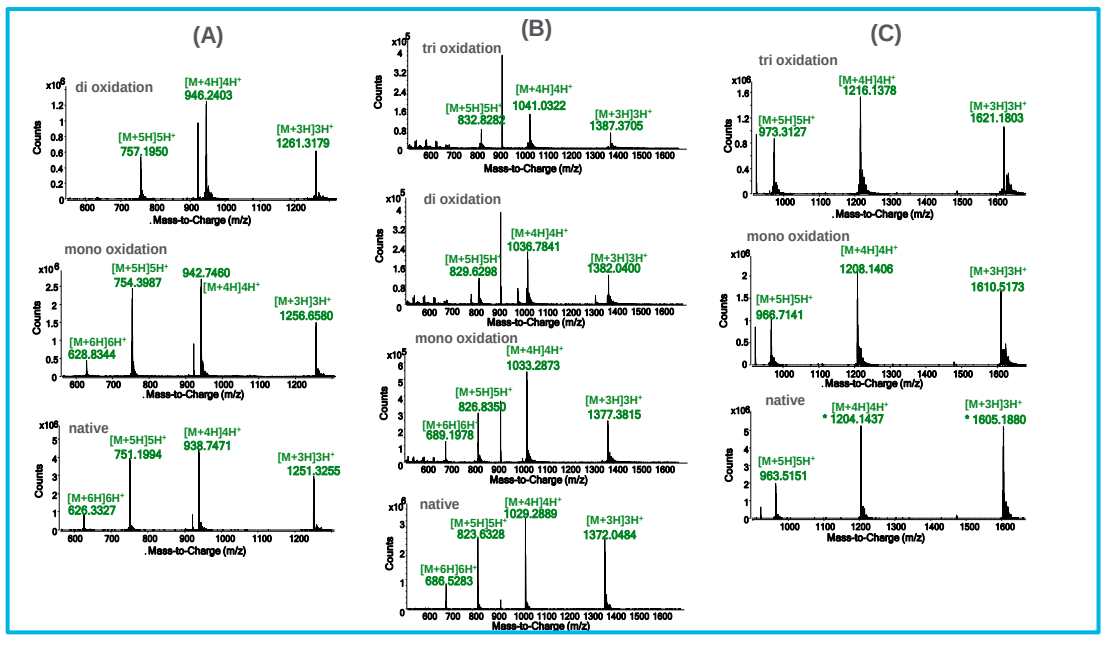

Figure 3. Charge state distribution of unmodified and oxidized GLP-1 agonists. (A) Liraglutide; (B) Semaglutide; (C) Tirzepatide.

Resolved isotope deconvoluted spectra

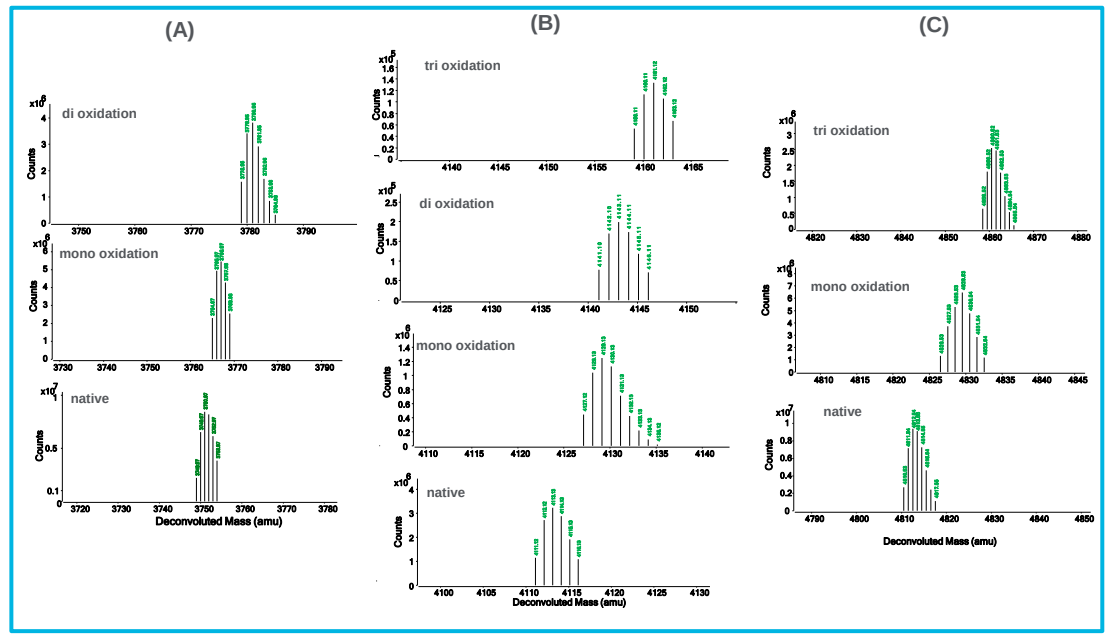

Figure 4. Resolved isotope deconvoluted spectra of oxidized and unmodified GLP-1 agonists. (A) Liraglutide; (B) Semaglutide; (C) Tirzepatide.

LC/MS/MS of Liraglutide

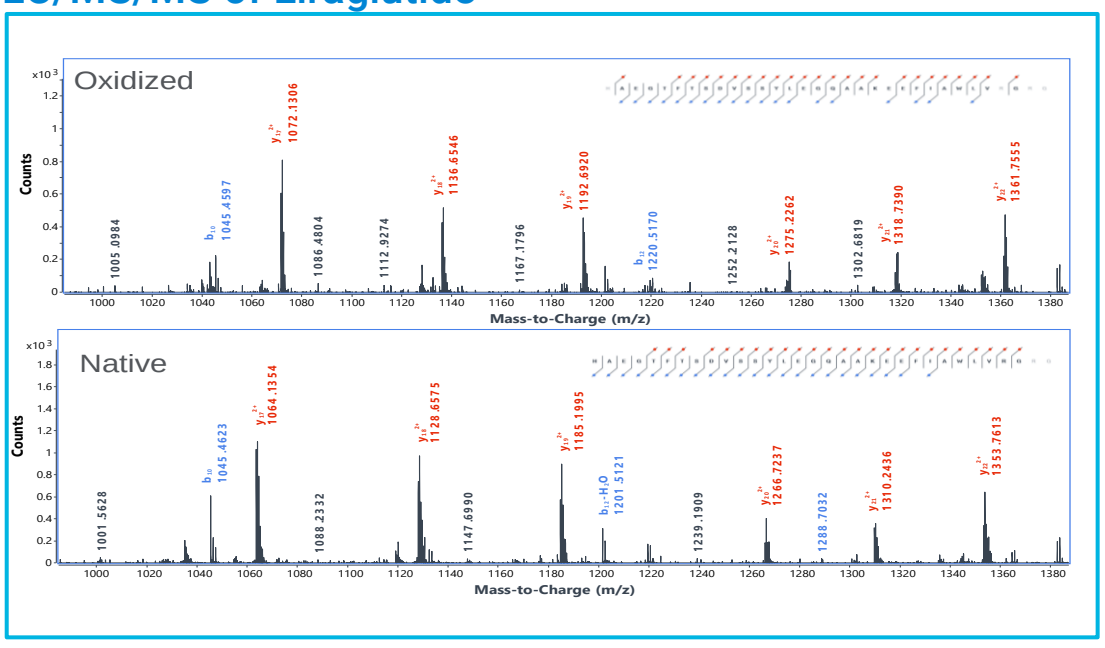

Figure 5. MS/MS spectrum of unmodified and oxidized Liraglutide (2% H_2O_2 stress).

- The total ion chromatograms (TICs) of GLP-1 agonist reveals multiple peaks with increasing H_2O_2 concentration, indicating the formation of various oxidized forms of peptides (figure 1 and figure 2).
- 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 (figure 4).
- MS/MS analysis (precursor m/z 942.2468) shows an increase of m/z ~8 (y ion) in oxidized MS/MS spectra, suggesting oxidation of the tryptophan residue (figure 5).

Results and Discussion

MRM chromatograms

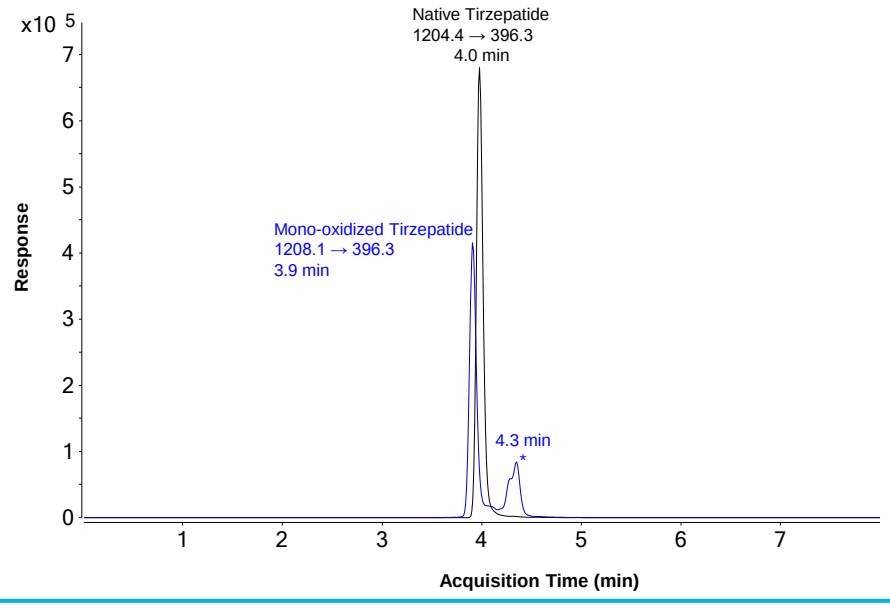

Figure 6. MRM chromatograms of unmodified (black) and mono-oxidized Tirzepatide (2% H_2O_2 treated, blue). * unknown peak

MRM chromatograms and standard curves

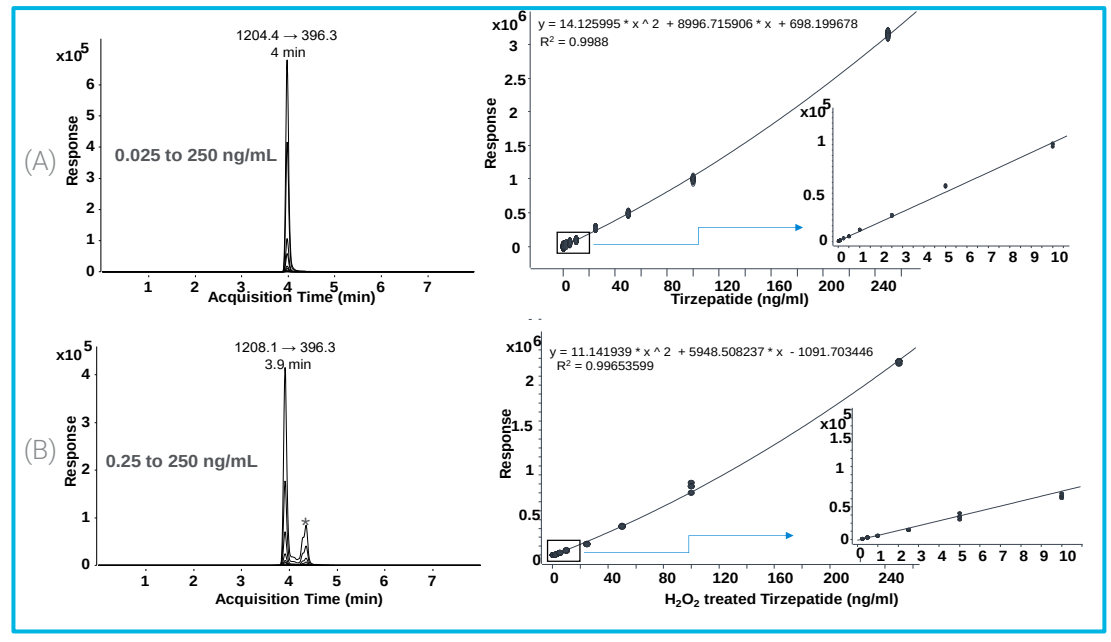

Figure 7. Quantitative performance of (A) unmodified and (B) tryptophan mono-oxidized Tirzepatide. Left: overlays of MRM chromatograms Right: the standard curves

MRM chromatograms

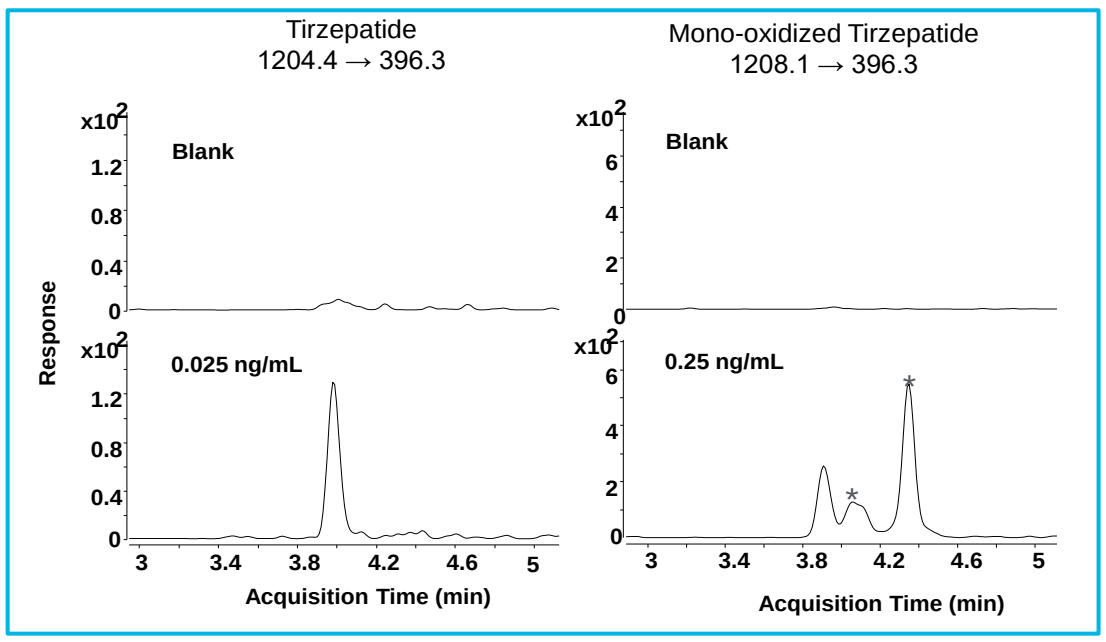

Figure 8. Extracted ion chromatograms of blank samples and samples at the lowest concentration levels.

- MRMs verify the peptide identity and demonstrate the use of MRM to monitor PTMs such as oxidation.
- Calibration curves: The correlation coefficients (R2) were 0.997 and 0.996 for the unmodified and mono-oxidized forms, respectively.
- Precision and accuracy were excellent at all levels, with percent relative standard deviation (%RSD) < 6% and accuracy ranging from 81 to 115%.
- The method achieves an LOQ of 0.025 ng/mL for unmodified Tirzepatide and 0.25 ng/mL for mono-oxidized Tirzepatide.

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Conclusions

Impurity characterization LC/Q-TOF MS

- This study demonstrates the analysis of GLP-1 agonists forced chemical degradation products using the 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 MassHunter BioConfirm software is easy to use and provides an integrated environment for the analysis of synthetic peptide.

Quantitation LC/TQ

- Development of rapid multiple reaction monitoring (MRM)-based method for the highly sensitive quantitative analysis of both unmodified and oxidized Tirzepatide.
- The calibration curve demonstrated a wide dynamic range, spanning four orders of magnitude for unmodified Tirzepatide and three orders of magnitude for mono-oxidized Tirzepatide.

References

[1]Characterization of Forced Degradation Impurities of Glucagon-Like Peptide-1 Agonists by LC/Q-TOF Mass Spectrometry. Agilent application note 5994-7992EN.

[2]Quantification of Glucagon-Like Peptide-1 Agonist Tirzepatide Using an Agilent 6495D Triple Quadrupole LC/MS System. Agilent application note 5994-7992EN.

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