Application Note

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Impurity Profiling of Tirzepatide Under Stress Conditions

Using Agilent Pro iQ Plus

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Abstract

The increased demand and use of glucagon-like peptide-1 (GLP-1) agonists prompts a need for robust analytical methods to screen for impurities that may impact their safety and efficacy. Synthetic GLP-1 peptides are susceptible to degradation through pathways such as oxidation under stress conditions.[1] In this study, a high-performance liquid chromatography (HPLC) system coupled with an Agilent InfinityLab Pro iQ Plus mass detector was used to monitor tirzepatide impurities under various pH and storage conditions. This study demonstrates that a high-sensitivity single quadrupole LC/MS system can be used to streamline the detection and monitoring of low-level peptide impurities, making it suitable for implementation in routine quality control (QC) and quality assurance (QA) environments.

Introduction

Glucagon-like peptide-1 (GLP-1) agonists are a class of compounds utilized to treat type-2 diabetes mellitus (T2DM) and obesity. The function of GLP-1 agonists is to lower serum glucose levels and thereby manage metabolism in affected patients. GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) are both incretin hormones inactivated by dipeptidyl peptidase-4. These hormones stimulate insulin secretion after an oral glucose load through the incretin effect.[2,3] In T2DM, this process can become blunted or absent; however, pharmacological levels of GLP-1 can restore insulin secretion.

The US Food and Drug Administration (FDA) considers the types and amounts of impurities present in a proposed generic drug compared to its reference listed drug (RLD). According to the FDA, a proposed generic synthetic peptide should not contain (i) impurities at levels greater than those found in the RLD nor (ii) any new specified peptide-related impurity that is greater than 0.5% of the drug substance.[4]

Tirzepatide, a dual GIP and GLP-1 receptor agonist, offers promising therapeutic potential but presents analytical challenges due to its susceptibility to chemical degradation, including deamidation, oxidation, and peptide backbone cleavage. Monitoring such degradation pathways is essential for ensuring product integrity, safety, and efficacy.

The Agilent InfinityLab Pro iQ Plus single quadrupole mass spectrometer (MS) offers enhanced sensitivity and dynamic range for low-abundance (sub-ppm) impurity detection. The Agilent 1290 Infinity II LC system coupled with the Pro iQ Plus LC/MS instrument brings a cost-effective mass detection workflow for routine analysis to QC laboratories. This application note details the results from analyzing tirzepatide and its related impurities under varying pH and storage conditions.

Experimental

Chemicals and standards

- Formic acid, 98% (LC-MS grade LiChropur), Millipore Sigma (part number 5.33002)
- InfinityLab acetonitrile (LC/MS grade), 1 × 1 liters, Agilent (part number 5191-5101-001)
- Tirzepatide, AstaTech (part number AT40456)
- Ammonium formate (LC-MS grade LiChropur), Millipore Sigma (part number 70221)
- Ammonium hydroxide, for HPLC, 35% solution in water, Thermo Fisher Scientific (catalog number 460801000)

Sample preparation

Tirzepatide stock solution was prepared at 2 mg/mL in 15% acetonitrile/de-ionized water. Working solutions were diluted to 50 ng/µL in buffer solutions adjusted to pH 5, 7, and 9. Samples were injected directly without additional purification. Aliquots were stored in the autosampler at 5 °C and analyzed on different days after storage for up to seven days.

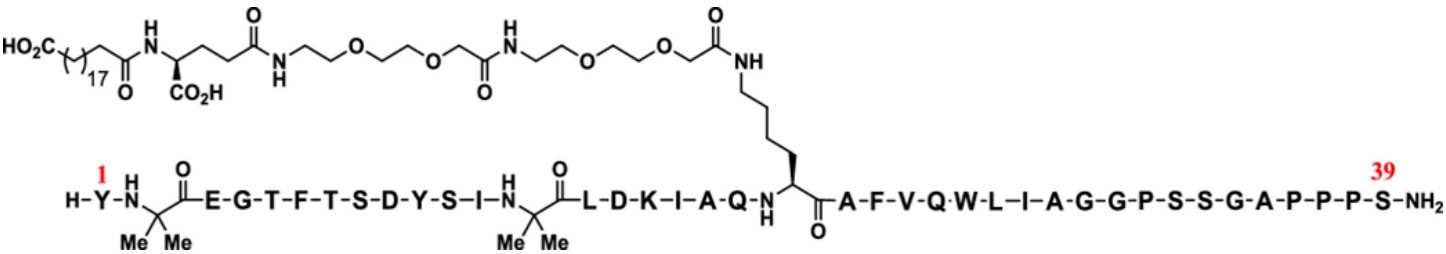

Figure 1. Tirzepatide amino acid composition.[5]

Instrumentation

LC/MS analysis was performed using the Agilent 1290 Infinity II bio LC system coupled with the Pro iQ Plus mass detector. Chromatographic separation was achieved on an Agilent ZORBAX RRHD 300 Å StableBond C18 column. The detailed LC and MS operating parameters are summarized in Tables 1 and 2.

Table 1. LC method parameters.

1290 Infinity II Bio LC System	
Column	ZORBAX RRHD 300 Å StableBond C18, 2.1 × 150 mm, 1.8 µm (p/n 863750-902)
Mobile Phase A	LC/MS-grade water + 0.1% formic acid
Mobile Phase B	Acetonitrile + 0.1% formic acid
Flow Rate	0.400 mL/min
Injection	Standard
Injection Volume	1 µL
Column Temperature	60 °C
Gradient Program	Time (min) %B 0 20 5 48 10 58 11 60 12 80 14 80 14.1 20 15 20

Table 2. MS parameters.

Pro iQ Plus Single Quadrupole LC/MS	
Source	
Ion Source	Agilent Jet Stream ESI source
Polarity	Positive
Gas Temperature	300 °C
Drying Gas Flow	11 L/min
Nebulizer	30 psi
Sheath Gas Temperature	250 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	0 V
Pro iQ Plus	
Fragmentor	95 V
Scan Type	Scan
Scan Time	500 ms
Data Storage	Profile
MS Spectrum Range	*m/z* 500–2,500

Software and data analysis

The Pro iQ Plus LC/MS system was operated using Agilent OpenLab CDS 2.8 software, which includes built-in spectral deconvolution capabilities for processing GLP-1 peptide data. The deconvolution algorithm in OpenLab CDS is specifically optimized to simplify complex spectra generated from multiply charged ions, particularly those acquired using unit mass resolution instruments. Table 3 outlines the specific data processing parameters used in this study.

Table 3. Data processing parameters used in OpenLab CDS 2.8 for GLP-1 peptide analysis.

Parameter	Value
Spectrum Extraction Type	Peak apex spectrum
Background Mode	Spectrum at peak start and end
Use *m/z* Range	Disabled
Run Automatic Deconvolution	Enabled
Low Molecular Weight	500
High Molecular Weight	10,000
Maximum Charge	6
Minimum Peaks in Set	3
MW Agreement (0.01%)	5
Absolute Noise Threshold	1,000
Relative Abundance Threshold (%)	10
MW Algorithm	Curve Fit
MW Algorithm Threshold	40
Envelope Threshold	50

Results and discussion

Tirzepatide degradation products were highly dependent on pH and storage duration. The Pro iQ Plus single quadrupole LC/MS system successfully detected trace levels of impurity-related products.

Peptide analysis using OpenLab CDS

OpenLab CDS software provides a unified platform for peptide characterization and impurity assessment. Figure 2 shows the data review for tirzepatide after seven days of storage at 5 °C and pH 7. As shown in Figure 2, impurity peaks were detected at trace levels, eluting close to the main tirzepatide peak. It should be noted that accurate deconvolution relies on acquiring high-quality mass spectra. The Pro iQ Plus mass detector provides such high-quality mass spectra, even for trace impurities.

The software layout includes helpful tools such as chromatogram view, MS spectrum window, and spectral deconvolution results, allowing for efficient review of both the main peptide and any low-level impurities.

The total ion chromatogram (TIC) shows peaks corresponding to tirzepatide and its related impurity products. The mass spectrum at 7.89 minutes confirms the elution of tirzepatide, with the presence of +3, +4, and +5 charge states (Figure 2). The deconvoluted mass spectrum shows a molecular weight of 4,813.1 Da, consistent with the theoretical average mass of tirzepatide (4,813.5 Da) and within the expected mass accuracy (±0.3 Da) for the instrument.

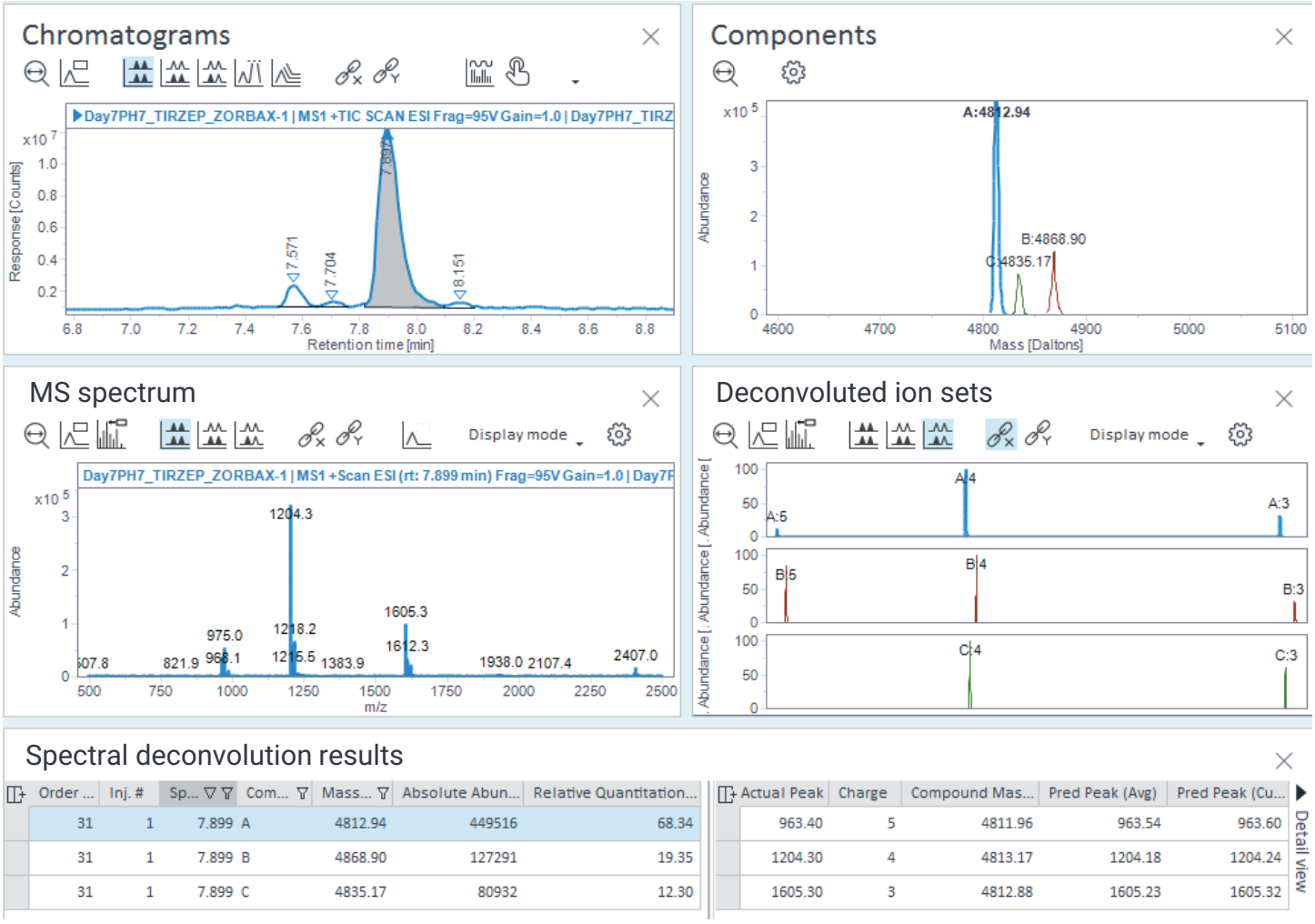

Figure 2. Data review for tirzepatide after seven days of storage at 5 °C and pH 7.

The Pro iQ Plus LC/MS system enables sensitive detection and characterization of low-level impurities in tirzepatide samples. Figure 3 shows the mass spectra of two impurities eluting at 7.57 and 7.71 minutes (as seen in Figure 2), with deconvoluted masses of 4,845.2 and 4,817.9 Da, respectively. These species correspond to mass shifts of +32 and +4.4 Da relative to the theoretical average mass of native tirzepatide (4,813.5 Da). The mass shifts are consistent with oxidative modifications, which are common degradation pathways for peptides under stress conditions. Amino acids such as methionine, tryptophan, and histidine are particularly susceptible to oxidation. Notably, tryptophan oxidation can lead to a +4 Da shift due to specific structural modifications ($+O_2-CO$).[6]

Table 4 summarizes the deconvoluted molecular weights of tirzepatide and its related impurities, including oxidized forms and an unknown impurity.

Table 4. Deconvoluted molecular weights of tirzepatide and its related impurities.

GLP-1 Peptide	Native Peptide Molecular Weight (Da)	Impurity Product		
		Oxidation ($+O_2$)	Oxidation (O_2-CO)	Unknown Impurity
Tirzepatide	4,813.1	4,845.2	4,817.9	4,689.6

In addition to the oxidation-related impurities, an unknown species was detected at a retention time of 8.15 minutes. The deconvoluted mass spectrum of this species indicates a molecular weight of ~ 4,689.6 Da, corresponding to a mass difference of +124 Da relative to native tirzepatide. Further MS characterization (that is, MS/MS) is required to identify this impurity.

The high sensitivity and dynamic range of the Pro iQ Plus LC/MS instrument allows for confident detection of both major and trace-level species in a single run. Importantly, the MS successfully detects impurities present at a relative peak area < 2%, highlighting the suitability of the Pro iQ Plus LC/MS for monitoring minor degradants in peptide samples. Furthermore, the integrated spectral deconvolution within OpenLab CDS streamlines data analysis by simplifying interpretation of multiply charged spectra, eliminating the need for external software.

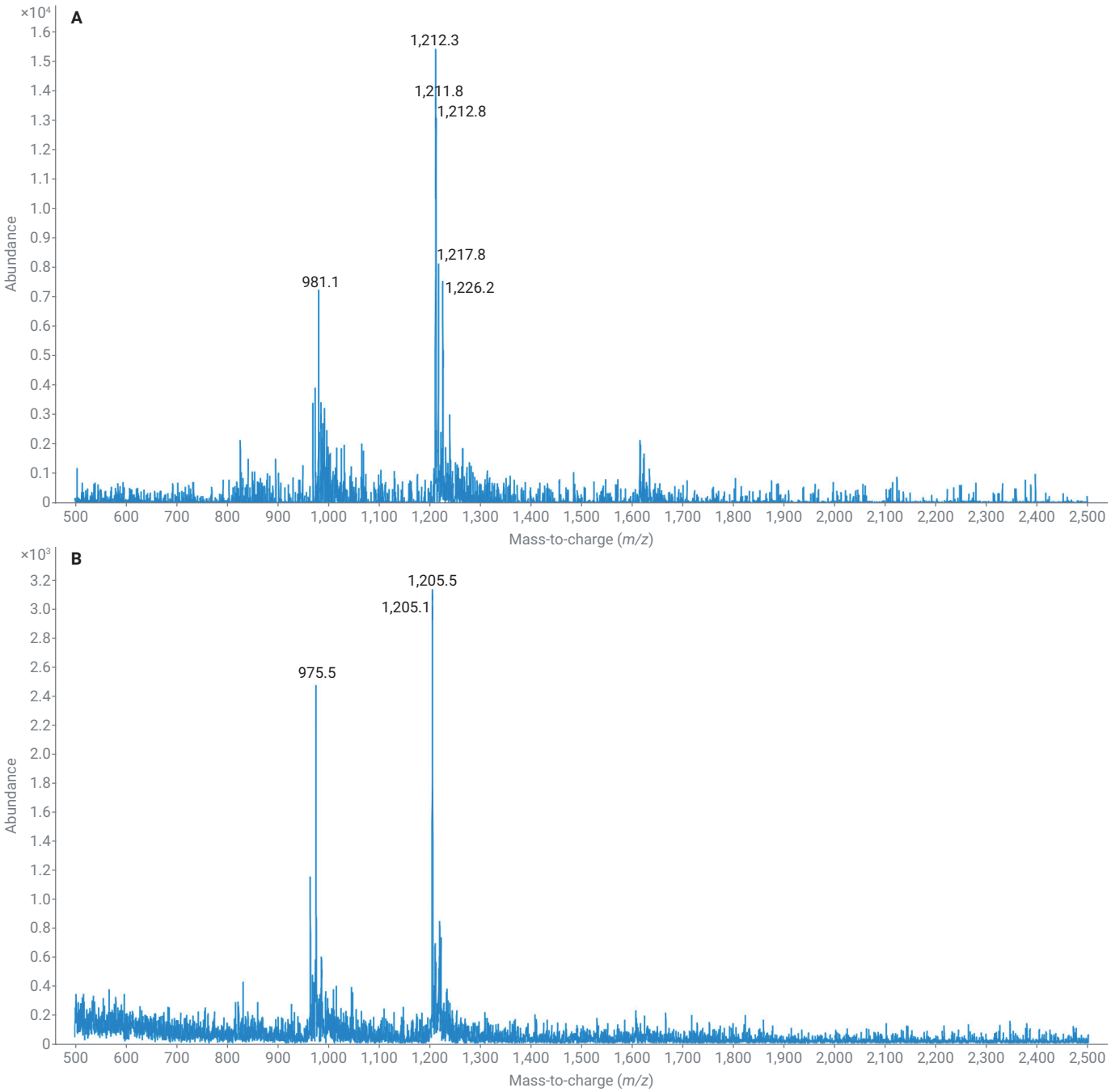

Figure 3. Mass spectra of tirzepatide impurities eluting at retention times 7.57 minutes (Panel A) and 7.71 minutes (Panel B). Mass spectral deconvolution reveals molecular weights of 4,845.2 Da for the impurity in Panel A and 4,817.9 Da for the impurity in Panel B.

Temporal monitoring of impurity generation

Proper storage and handling of GLP-1 medications are essential to preserve their stability, potency, and overall effectiveness. Inappropriate storage conditions can lead to degradation, reduced therapeutic efficacy, and potential safety risks. Therefore, evaluating how different storage durations and conditions influence the formation of degradation products is critical for ensuring product quality and patient safety.

Figure 4 shows temporal plots for the generation of the oxidation products of tirzepatide at various solution pH values. The data in Figure 4 suggest that tirzepatide is least stable at pH 5 and can undergo significant oxidation, even at 5 °C.

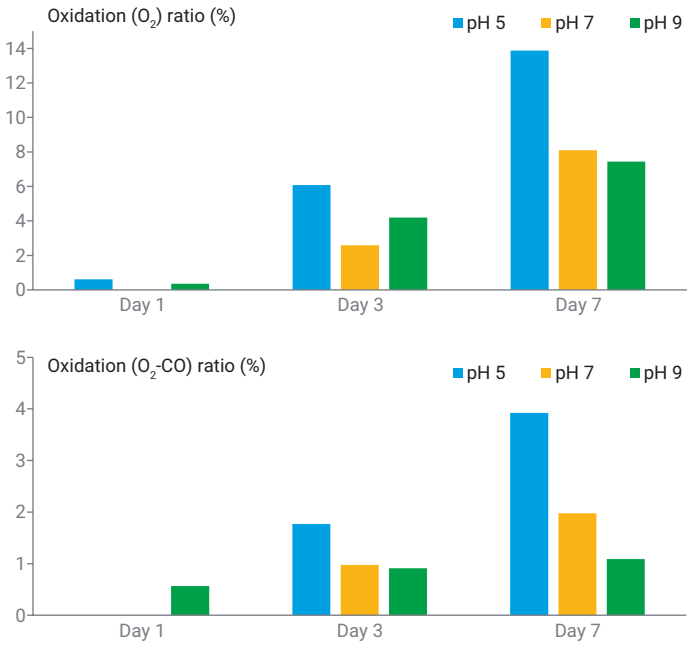

Figure 4. Oxidation ratio of tirzepatide related impurities over time under different pH conditions.

Conclusion

In conclusion, impurity profiling of tirzepatide under stress conditions using the Agilent Pro iQ Plus mass detector demonstrates the effectiveness of this analytical method for detecting and monitoring low-level peptide impurities. Notably, the method was able to detect impurities with relative peak areas less than 2%, showcasing its sensitivity for low-level impurity identification. The study highlights the susceptibility of tirzepatide to degradation through oxidation, particularly under varying pH and storage conditions. These findings underscore the importance of proper storage and handling to maintain the stability and efficacy of GLP-1 medications. The high sensitivity, cost-effectiveness, and simplicity of this single quadrupole LC/MS make it a practical and accessible tool for routine pharmaceutical QC and QA workflows.

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