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アプリケーションノート

Comprehensive Characterization and Impurity Profiling of the GLP-1 Analogue Tirzepatide Using the Xevo™ G3 QTof High Resolution Mass Spectrometer

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Abstract

Forced degradation studies on glucagon-like peptide-1 (GLP-1) agonists (*e.g.*, semaglutide, liraglutide, and tirzepatide) are essential to identify degradation pathways, validate stability-indicating analytical methods, and ensure product safety and efficacy.

In this application note, tirzepatide is chemically stressed under oxidative conditions at elevated temperatures while performing comprehensive characterization and profiling using the Xevo G3 QTof quadrupole time-of-flight Mass Spectrometer coupled to the ACQUITY™ UPLC™ I-Class System with liquid chromatograph. Data processing was carried out using the high-resolution screening platform UNIFI™ Application as part of the waters_connect™ Software platform.

Benefits

- Routine sub 2 ppm mass accuracy profiling of GLP-1 analogues for confident decision making
- Flexible, ready-made workflows within the UNIFI Application for easy data screening and visualization,

providing automated mass confirmation and comprehensive impurity profiling and sequencing

- ACQUITY Premier CSH C18 Peptide Column for optimal separation of peptide and related impurities

Introduction

GLP-1 receptor agonists have emerged as a game-changing treatment for type-2 diabetes mellitus and obesity.[1-3] However, for analytical chemists supporting pharmaceutical and biotechnology manufacturing, these large peptide therapeutics come with a considerable level of complexity, presenting significant challenges for analytical methodologies designed to comprehensively characterize and profile these drugs. Their complex structures, susceptibility to modification,[4-6] and intricate behavior under stress create ongoing challenges in liquid chromatography – mass spectrometry (LC-MS) workflows.

The high resolution and specificity of high resolution mass spectrometry (HRMS) plays a vital role in the characterization of complex therapeutics to enable, for example, peptide mapping and identification of modifications such as fatty acid side-chains.[7] Product stability is also impacted by aggregation, with size-exclusion chromatography (SEC) combined with HRMS used to monitor, separate and identify this critical quality attribute (CQA).[8] Additionally, with GLP-1 drugs coming off patent, the expiration of the intellectual property protection permits other drug manufacturers to legally produce and sell generic versions of the drug. In this scenario, HRMS is critical for assessing the uniformity of a synthetic generic peptide compared to the original recombinant DNA (rDNA) peptides.[9,10]

Forced degradation studies are a critical component of pharmaceutical development for GLP-1 peptide drugs. Unlike real-time stability testing, forced degradation deliberately subjects the drug substance and drug product to extreme conditions to accelerate degradation and reveal potential impurity pathways. Regulatory agencies rely on these studies to evaluate molecular stability, validate analytical methods, and ensure that all clinically relevant impurities have been identified and controlled, and the necessary analytical methodologies are in place.[11-13]

Tirzepatide, a dual GIP (glucose-dependent insulinotropic polypeptide) and GLP-1 receptor agonist, offers promising therapeutic potential[14] 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.

Experimental

Tirzepatide reference material was prepared at 0.1 mg/mL in water containing 0.1% formic acid. Forced degradation was carried out by adding 1% (v/v) H_2O_2 to the sample and incubating at 40 °C. Injections were made at time points 0, 0.5, 1, 1.5, 2, 2,5, 3, 4, 5, 6, 9, and 12 hours to investigate and characterize degradation products over time.

LC Conditions

LC system:	Waters ACQUITY Premier UPLC I-Class System
Vials:	Waters QuanRecovery™ MaxPeak™ 12 x 32 mm Polypropylene 300 µL Screw Cap Vials (p/n: 186009186)
Column:	ACQUITY Premier CSH C_{18} Peptide 130 Å 1.7 µm 2.1 x 100 mm Column (p/n: 186009652)
Column temperature:	60 °C
Sample temperature:	40 °C
Injection volume:	1 µL
Flow rate:	0.4 mL/min
Mobile phase A1:	H_2O 0.1% formic acid
Mobile phase B1:	ACN 0.1% formic acid

Gradient Table

Time (min)	Flow (mL/min)	%A	%B	Curve
0.00	0.4	75	25	Initial
1.00	0.4	75	25	6
2.50	0.4	65	35	6
25.00	0.4	50	50	6
25.50	0.4	25	75	6
26.50	0.4	25	75	6
28.00	0.4	65	35	6
28.25	0.4	75	25	6
30.00	0.4	75	25	6

MS Conditions

MS system:	Waters Xevo G3 QTof Mass Spectrometer
Mode:	Sensitivity
Mass range:	50-2000 *m/z*
Polarity:	Positive
Scan rate:	10 *Hz*
Cone voltage:	30 V
Source temperature:	120 °C
Desolvation temperature:	350 °C

Capillary voltage:	2.8 kV
MS^E low energy (fixed):	6 V
MS^E elevated energy ramp:	30-55 V

Additional Tune Page Settings

StepWave			
StepWave RF (V)	250		
Body Gradient (V)	20		
Manual MS Profile			
Mass (*m/z*)	400	500	600
Dwell Time (% scan time)	25	25	
Ramp time (%scan time)	25	25	

Results and Discussion

Main Species Characterization

Using the BayesSpray™ algorithm enables the deconvolution of a multiply charged species to a singly charged form. BayesSpray differs from maximum entropy (MaxEnt) deconvolution by using prior information provided in the form of sequence and modifications within the processing method to create the expected data and then comparing to the observed data. This approach is better suited to targeted workflows and produces cleaner spectra. A resulting monoisotopic mass measurement can then be calculated as part of compound identification and characterization.

In this work, tirzepatide was confidently identified with a mass error of 0.3 ppm with 57 primary fragments confirmed (Figure 1-2). The peptide mapping workflow in UNIFI Application was set up to identify the Linker

and C20 fatty di-acid moiety present on K20 (highlighted in red), presenting as a modifier to the imported tirzepatide amino acid sequence.

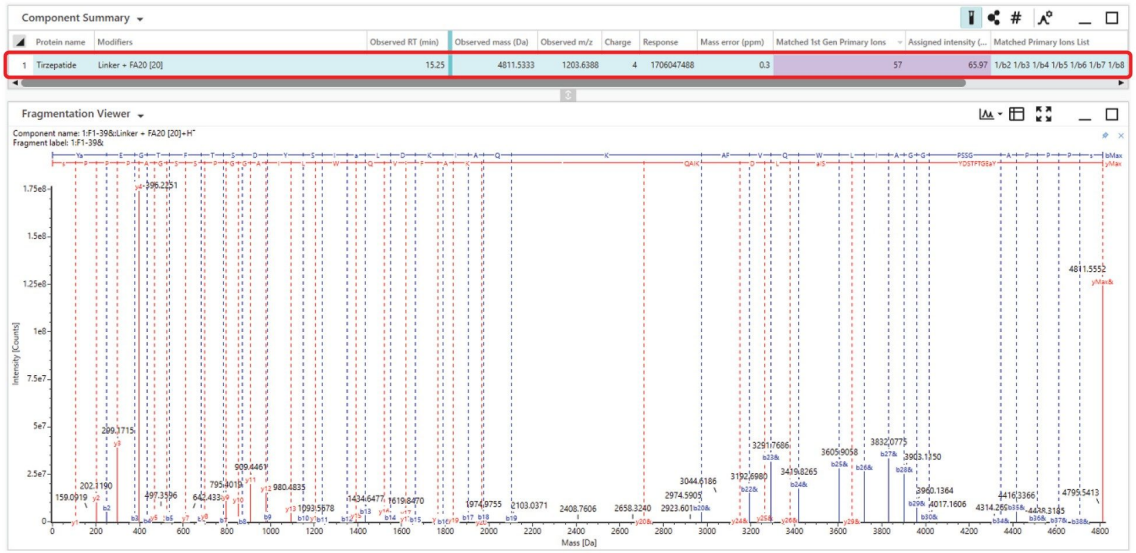


Figure 1. Intact mass calculation of tirzepatide with fragmentation viewer visualizing fragment coverage.

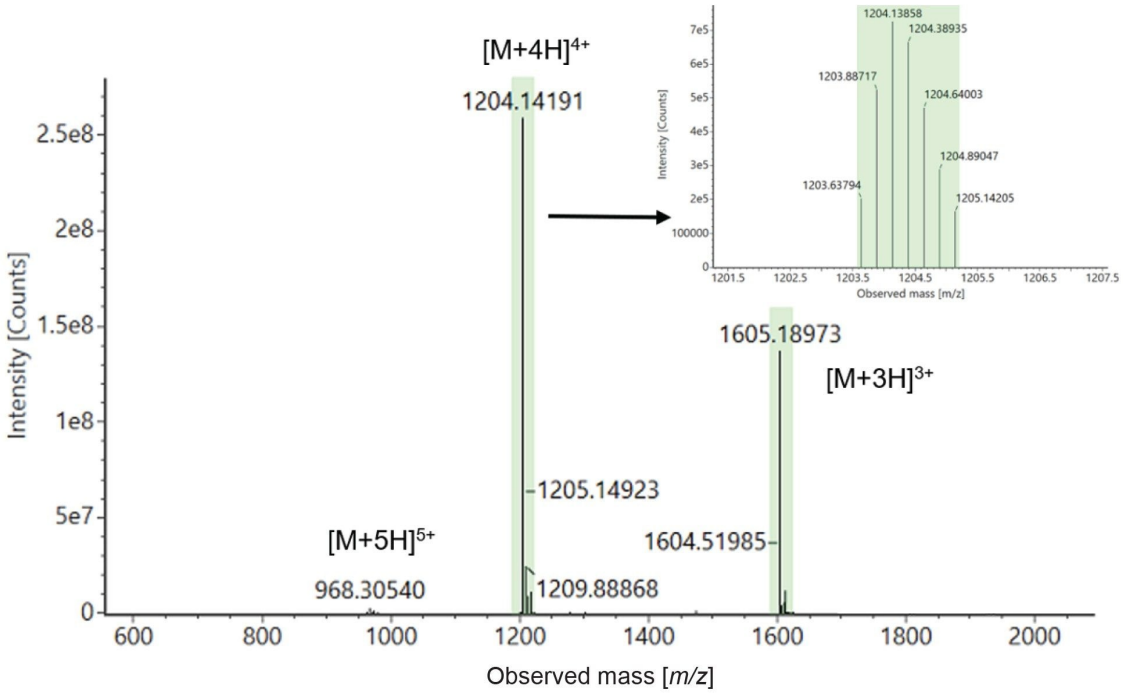


Figure 2. Example of deconvolved tirzepatide mass spectra with zoomed inset showing the isotopic distribution envelope of spectral peaks for the [M+4H]$^{4+}$ charge state.

Impurity Profiling

Stability testing is a critical aspect of peptide therapeutic development, essential for quality assurance, therapeutic efficacy and safety testing. Adequate storage of GLP-1 medications is imperative for ensuring safety, purity and reducing toxicity possible through formation of impurities and degradants.[15] Evaluating the effects of degradation through a stress study highlights the importance of preserving these medications correctly, with initial appraisal of the chemically and thermally stressed tirzepatide sample revealing multiple degradation products (Figure 3). Reduction in the parent compound at 15.29 minutes is evident with multiple degradation species being generated.

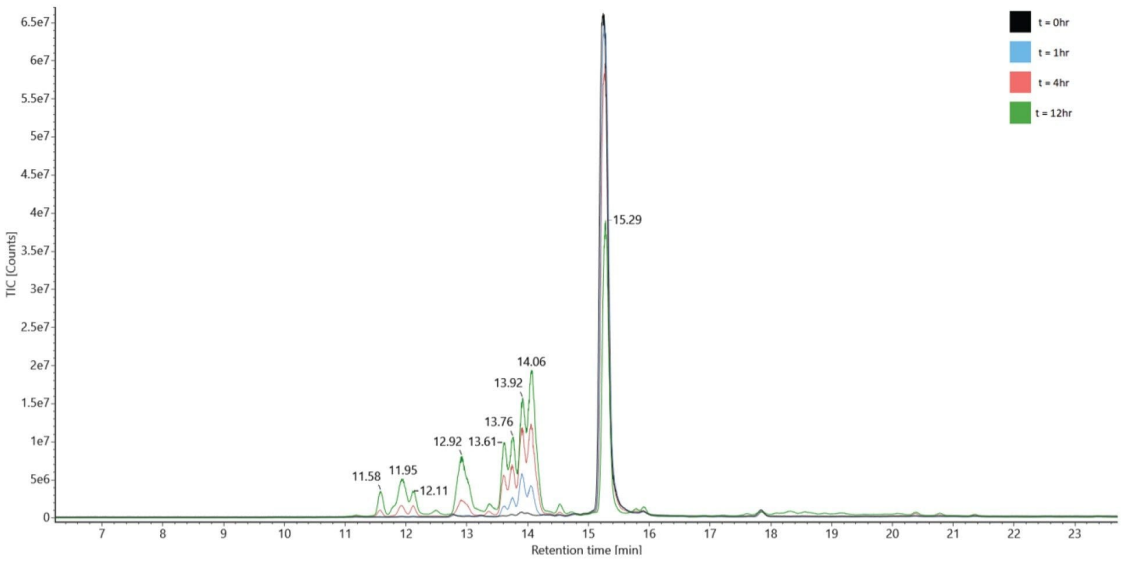


Figure 3. Overlay of tirzepatide degradation timepoints t = 0, 1, 4 and 12 hours demonstrating a reduction in drug substance with a concomitant increase in degradation products over time.

To further scrutinize the detected degradants, analytical tools within the UNIFI Application were employed to characterize the unknown peaks. Sequence information, fatty acid chains, and potential modifications were imported into the processing method and applied to the raw data for identification and characterization.

Monitoring of Tirzepatide Isomers

The presence of isomeric forms of the active compound has a potentially significant impact on product safety, efficacy, and stability. While unintentional isomers are generally considered impurities, intentional combinations, or the presence of related substances - common in compounded products - can lead to altered pharmacokinetic profiles, increased risk of adverse events, and higher immunogenicity.[16]

For this reason, the ability to confidently separate and identify GLP-1 isomers is crucial for safe and efficacious GLP-1 production. the UNIFI Application identified four components with the modifier ‘Linker + FA20’, each with distinct retention times and low mass error *i.e.* -1.4 – 1.5 (Figure 4).

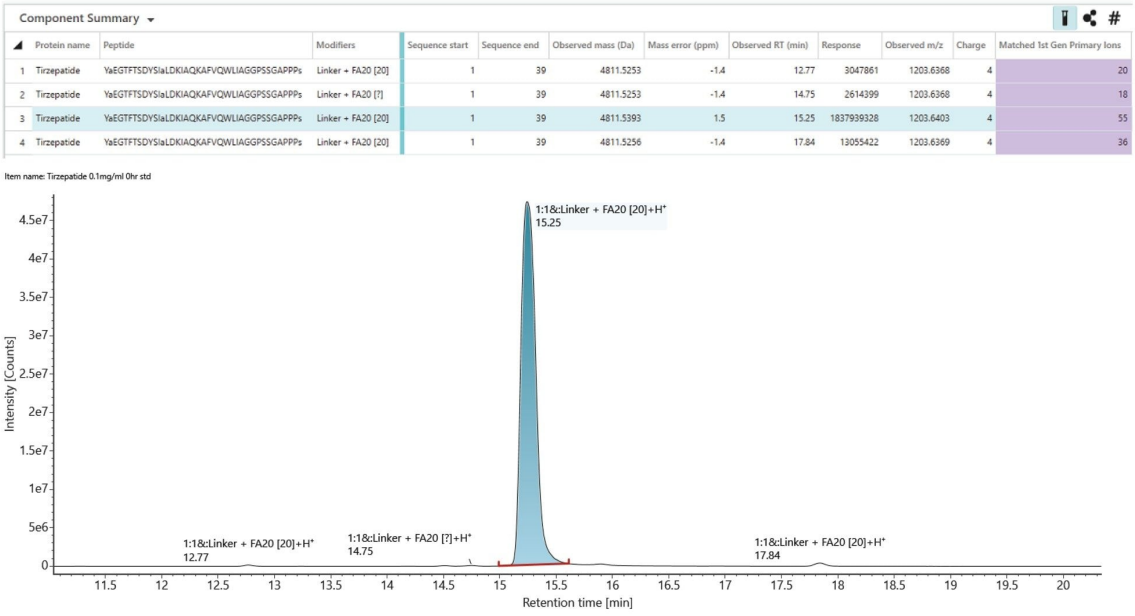


Figure 4. Component summary and chromatogram overview of the four identified isomers of tirzepatide.

Oxidation Products

Oxidation is a primary degradation pathway for tirzepatide which can significantly compromise its stability, structural integrity and therapeutic efficacy. Under accelerated conditions tirzepatide undergoes oxidation at the tryptophan residue leading to the formation of impurities. Using the Xevo G3 QTof with the UNIFI Application peptide mapping workflow, the characterization of these degradants and putative location of the sites of oxidation are demonstrated.

Within the UNIFI Application, the Summary Plot function allows the monitoring of component features across a sample set. Here, the trend plot demonstrates how degradation of tirzepatide coincides with increasing impurities formed as a result of oxidative and heat stress (Figure 5A-D). Using the 9 hour timepoint as an example, the single oxidation species demonstrated a ppm mass error of -0.8 with 50 primary fragment ions. The double oxidation species returned a mass error of -2.0 ppm and 40 primary fragments ions, and the triple oxidation -2.1 ppm mass error and 44 matched primary fragments.

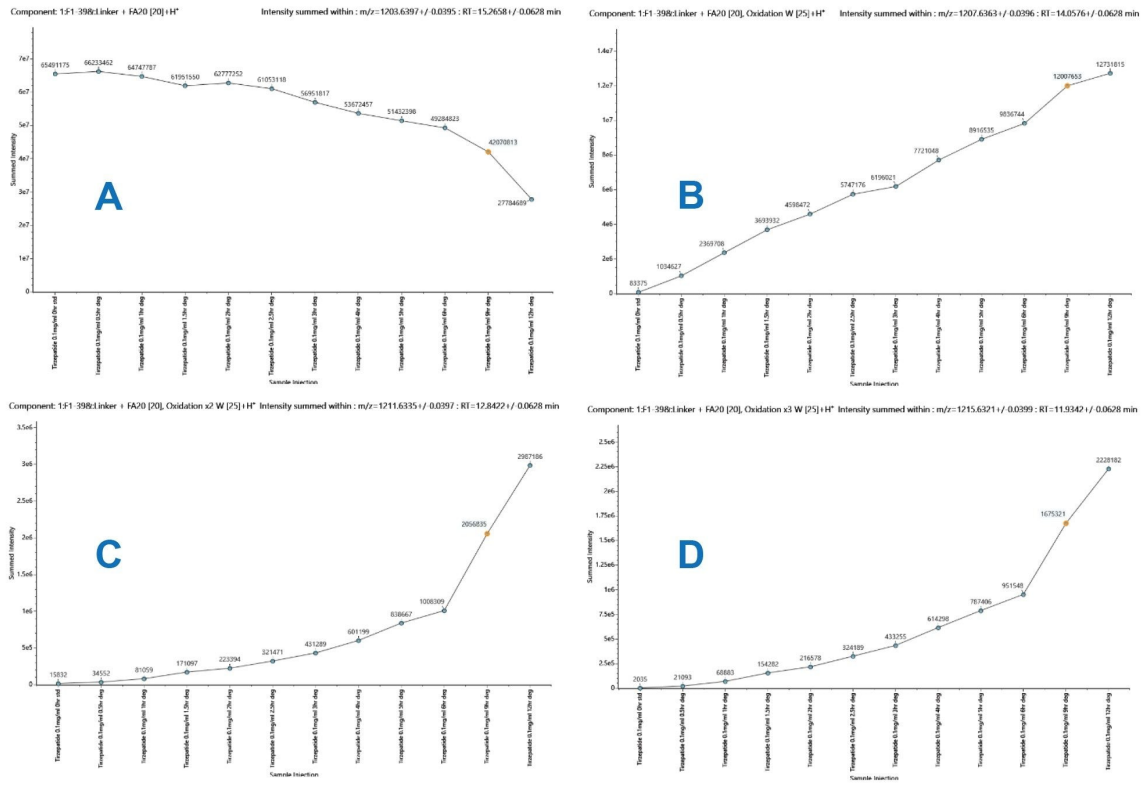
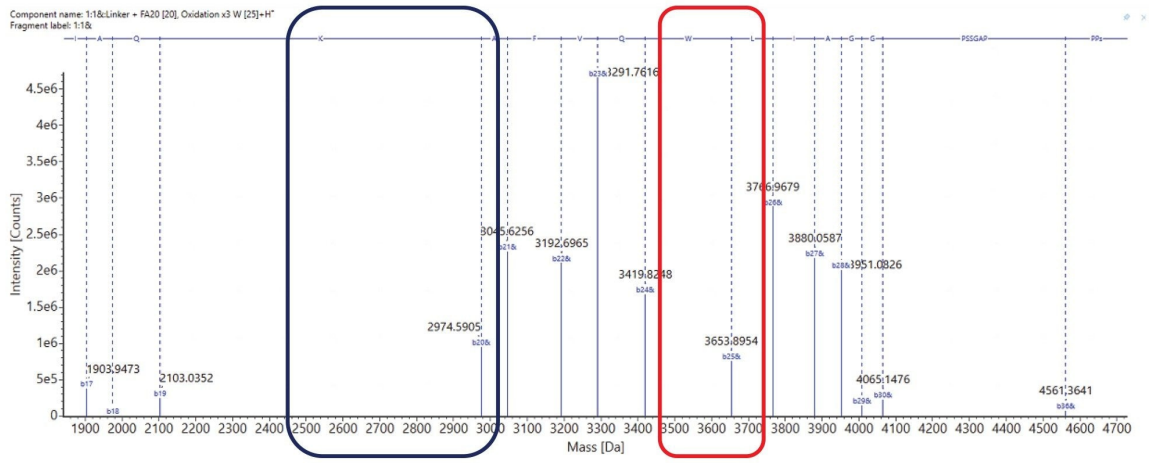


Figure 5A-D. Temporal monitoring trend plot of tirzepatide (A) drug and selected oxidation degradant products i.e. single oxidation (B), double oxidation (C) and triple oxidation (D).

To localize the specific residues which have been modified, established knowledge of potential peptide modifications combined with fragmentation data for oxidized species can be used. The fragmentation data produces a comprehensive series of *b*- and *y*-ions which contain the N- and C- terminus, respectively, visualized within the UNIFI Application Fragmentation Viewer. The associated Fragment Ion Table highlights the modifications to specific fragment ions.

As an example, the N-terminal fragment series from the t = 12 hour incubation is displayed in (Figure 6), confirming the triple oxidation of tryptophan (b25). This demonstrates how the fragment labeling pinpoints sites of modification, and the ion table (Table 1) provides confirmation when multiple amino acid modifications have been identified.


Component name: 1:1&Linker + FA20 [20], Oxidation x3 W [25]+H+
Fragment label: 1:1&
I A Q K A F V Q W L I A G G PSSGAP PP1
4.5e6
4e6
3.5e6
3e6
2.5e6
2e6
1.5e6
1e6
5e5
0
Intensity [Counts]
1903.9473
2103.0352
2974.5905
3045.6256
3192.6965
3291.7616
3419.8248
3653.8954
3766.9679
3880.0587
3951.0826
4065.1476
4561.3641
1900 2000 2100 2200 2300 2400 2500 2600 2700 2800 2900 3000 3100 3200 3300 3400 3500 3600 3700 3800 3900 4000 4100 4200 4300 4400 4500 4600 4700
Mass [Da]

Fragment Ion	Fragment Ion Mass (Da)	Intensity (Counts)	Modifiers
b17	1903.932	447863	
b18	1974.969	70883	
b19	2103.028	321909	
b20&	2974.580	1022346	
b21&	3045.617	2335220	Linker + FA20 (1)
b22&	3192.685	2179868	Linker + FA20 (1)
b23&	3291.754	4727105	Linker + FA20 (1)
b24&	3419.812	1745874	Linker + FA20 (1)
b25&	3653.876	826225	Linker + FA20 (1), Oxidation x3 W (1)
b26&	3766.961	2954913	Linker + FA20 (1), Oxidation x3 W (1)
b27&	3880.045	2245080	Linker + FA20 (1), Oxidation x3 W (1)
b28&	3951.082	2080652	Linker + FA20 (1), Oxidation x3 W (1)
b29&	4008.103	216999	Linker + FA20 (1), Oxidation x3 W (1)
b30&	4065.125	301722	Linker + FA20 (1), Oxidation x3 W (1)
b36&	4561.353	145404	Linker + FA20 (1), Oxidation x3 W (1)

Figure 6/Table 1. High-quality fragmentation data highlighting the comprehensive series of N-terminal ions which identify the position of the linker and fatty acid chain, as well as identifying a triple oxidation of Tryptophan at b25. The visual Fragmentation Viewer tool is paired with the Fragment Ion Table on UNIFI Application for detailed peptide mapping and data reporting.

Deamidation

Deamidation of GLP-1 is a degradation pathway often occurring during production and storage of GLP-1 drugs, resulting in the conversion of an amide group to a carboxylic acid by the removal of an ammonia molecule from an amide-containing amino acid i.e. glutamine (Q19).

In this experiment, the deamidation impurity at retention time 15.9 minutes is separated and identified – as highlighted in red (Figure 7A). The summary plot (Figure 7B) shows detectable levels of this impurity in the first timepoint, suggesting it has formed during storage and not as a result of the incubation. A gradual decline in intensity across the incubation timepoints is observed in part due to accelerated oxidative conditions and suggests that this environment is not a significant driver in the generation of deamidated species in tirzepatide.

Figure 8 displays the associated fragmentation data, indicating how this tool can localize specific residue modification, even with subtle *m/z* changes.

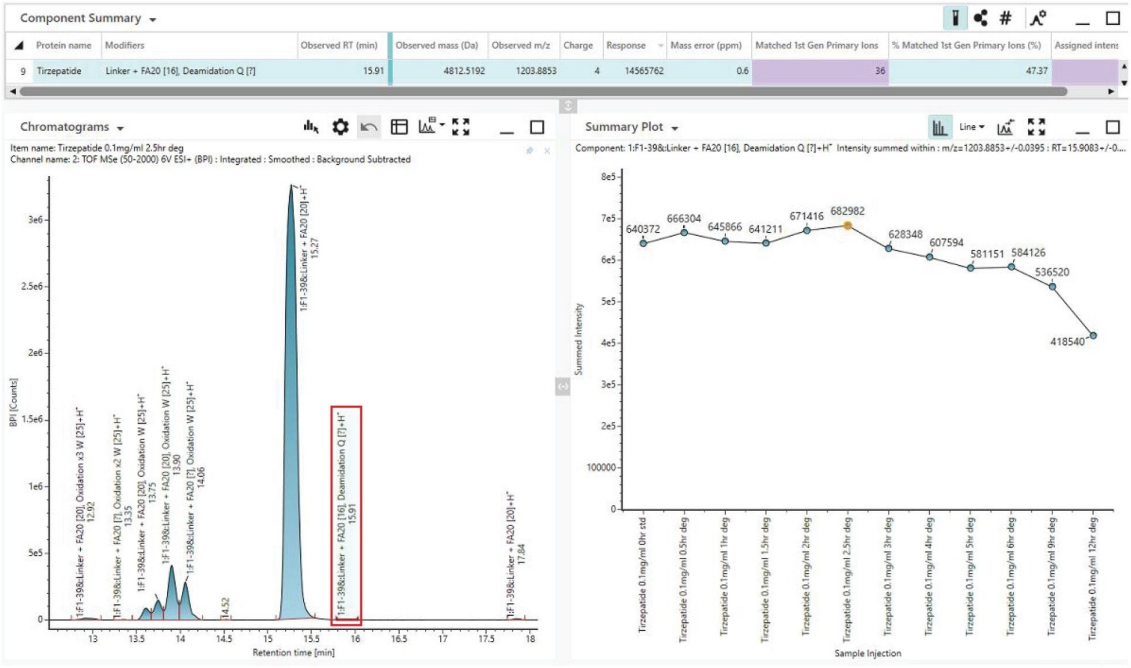


Figure 7A-B. Summary of identified impurities, using the peptide mapping workflow to view a final results list. Figure A shows the extracted mass chromatogram for all identified components with Figure 7B showing a summary plot visualizing the intensity trend across the degradation timeline for the selected component i.e. tirzepatide main compound. Customizable filters can be applied to view specific types of data, allowing a comprehensive and targeted assessment of degradation data.

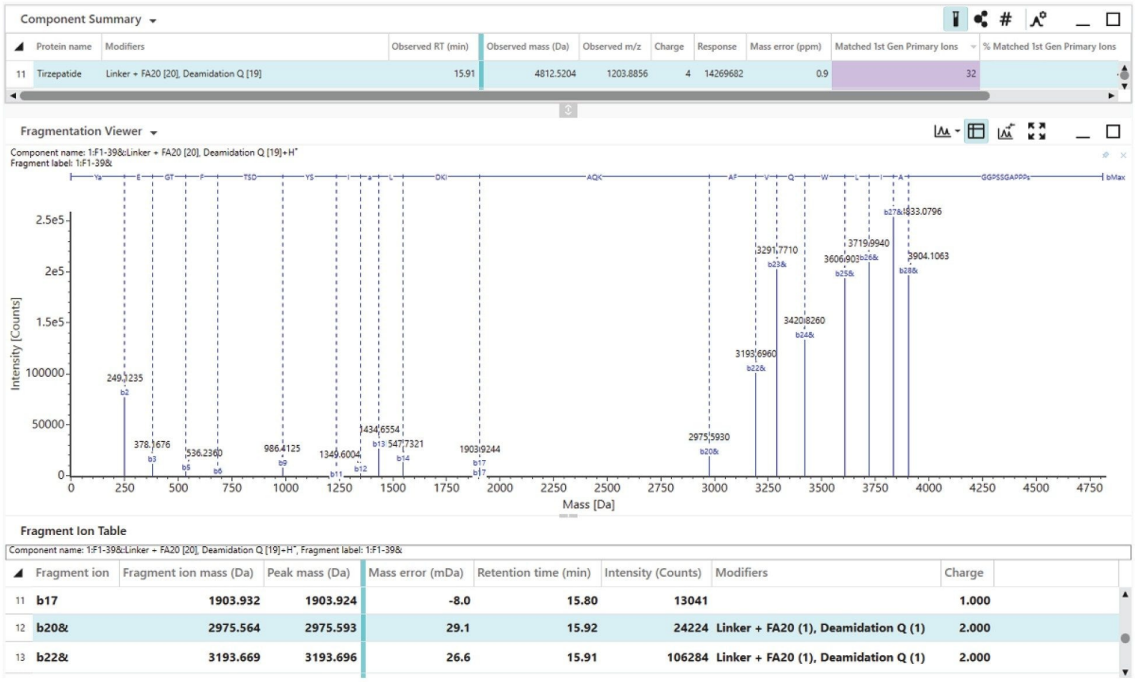
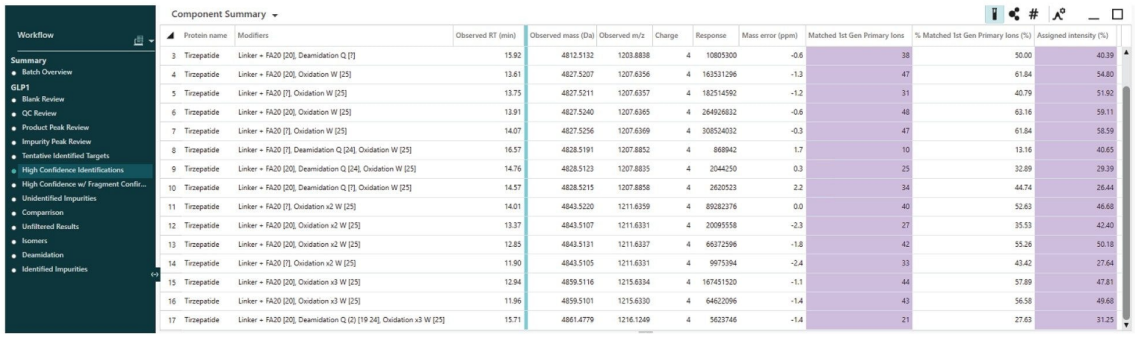


Figure 8. Fragmentation spectra at t = 4 hour suggesting the deamidation is present on Q19. The Fragment Ion Table explicitly identifies both the linker and fatty acid moiety and the deamidation of Q on b20 and all subsequent N-terminal ions, indicating the deamidation of Q19.

Summary of Impurities



	Protein name	Modifiers	Observed RT (min)	Observed mass (Da)	Observed m/z	Charge	Response	Mass error (ppm)	Matched 1st Gen Primary Ions	% Matched 1st Gen Primary Ions (%)	Assigned intensity (%)
3	Tirzepatide	Linker + FA20 [20], Deamidation Q [7]	15.92	4812.5132	1203.8838	4	10805300	-0.6	38	50.00	40.39
4	Tirzepatide	Linker + FA20 [20], Oxidation W [25]	13.61	4827.5207	1207.6356	4	163531296	-1.3	47	61.84	54.80
5	Tirzepatide	Linker + FA20 [7], Oxidation W [25]	13.75	4827.5211	1207.6357	4	182514592	-1.2	31	40.79	51.92
6	Tirzepatide	Linker + FA20 [20], Oxidation W [25]	13.91	4827.5240	1207.6365	4	264926832	-0.6	48	63.16	59.11
7	Tirzepatide	Linker + FA20 [7], Oxidation W [25]	14.07	4827.5256	1207.6369	4	308524032	-0.3	47	61.84	58.59
8	Tirzepatide	Linker + FA20 [7], Deamidation Q [24], Oxidation W [25]	16.57	4828.5191	1207.8852	4	868942	1.7	10	13.16	40.65
9	Tirzepatide	Linker + FA20 [20], Deamidation Q [24], Oxidation W [25]	14.76	4828.5123	1207.8835	4	2044250	0.3	25	32.89	29.39
10	Tirzepatide	Linker + FA20 [20], Deamidation Q [7], Oxidation W [25]	14.57	4828.5215	1207.8858	4	2620523	2.2	34	44.74	26.44
11	Tirzepatide	Linker + FA20 [7], Oxidation x2 W [25]	14.01	4843.5220	1211.6359	4	89282376	0.0	40	52.63	46.68
12	Tirzepatide	Linker + FA20 [20], Oxidation x2 W [25]	13.37	4843.5107	1211.6331	4	20095558	-2.3	27	35.53	42.40
13	Tirzepatide	Linker + FA20 [20], Oxidation x2 W [25]	12.85	4843.5131	1211.6337	4	66372596	-1.8	42	55.26	50.18
14	Tirzepatide	Linker + FA20 [7], Oxidation x2 W [25]	11.90	4843.5105	1211.6331	4	9975394	-2.4	33	43.42	27.64
15	Tirzepatide	Linker + FA20 [20], Oxidation x3 W [25]	12.94	4859.5116	1215.6334	4	167451520	-1.1	44	57.89	47.81
16	Tirzepatide	Linker + FA20 [20], Oxidation x3 W [25]	11.96	4859.5101	1215.6330	4	64622096	-1.4	43	56.58	49.68
17	Tirzepatide	Linker + FA20 [20], Deamidation Q [2] [19 24], Oxidation x3 W [25]	15.71	4861.4779	1216.1249	4	5623746	-1.4	21	27.63	31.25

Figure 9. Summary of identified impurities, using the peptide mapping workflow detailing the final results list. Customizable filters can be applied to view specific types of data, allowing a comprehensive and targeted assessment of the degradation profile.

Conclusion

In this application note, it was demonstrated that the Xevo G3 QTof Instrument combined with the UNIFI Application peptide mapping workflow can be utilized to carry out comprehensive characterization of tirzepatide and extensively profile drug-related impurities presenting as a result of a forced-degradation study. This is essential to drive effective drug development and adequate supporting analytical methodologies. The high resolution and mass accuracy of the Xevo G3 QTof Instrument delivers excellent mass confirmation and species identification, as well as rigorous impurity assignment and characterization to ensure purity and stability criteria are met for regulatory compliance.

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