Application Note

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Monitoring Product Quality Attributes of Biotherapeutics at the Peptide Level Using the Agilent InfinityLab LC/MSD XT System

Authors

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Abstract

Single quadrupole (SQ) LC/MS has been adopted in the biopharmaceutical QC labs for its low-cost, robustness, and simple operation. This Application Note describes a simple, generic method for routine biotherapeutic peptide map analysis using the Agilent InfinityLab liquid chromatography/mass selective detector XT (LC/MSD XT), an SQ system with an extended mass range up to *m/z* 3,000, in combination with an Agilent 1290 Infinity II LC System and Agilent OpenLab ChemStation software. Streamlined data processing and reporting were demonstrated for pre-identified peptides of a recombinant monoclonal antibody (mAb), including complementary-determining regions (CDR) peptides, deamidated peptides, oxidized peptides, and glycopeptides using OpenLab ChemStation. This study serves as a proof of concept for monitoring multiple product quality attributes (PQAs) using an SQ LC/MS system with software that is recommended for laboratories requiring regulatory compliance.

Introduction

In the biotherapeutic industry, optically based chromatographic methods have widely been used for quality control (QC). However, protein-based biotherapeutics are generally very complex, making an orthogonal detection method (for example, mass spectrometry) very attractive or necessary to assess product quality attributes at a molecular level. Therefore, SQ-based LC/MS has been adopted in the QC environment. Due to the product complexity, comprehensive analysis of protein-based therapeutics often requires running a panel of analytical methods. The concept of using a single LC/MS analytical method to monitor multiple PQAs has gained momentum in the biopharmaceutical industry. Therefore, it is valuable to develop an SQ-based LC/MS assay for monitoring multiple PQAs.

In the QC environment, an important need is to support regulatory compliance. OpenLab ChemStation in combination with central data storage (OpenLab ECM or OpenLAB Server) provides functionality that labs need to achieve compliance: controls for managing system access, audit trail, versioning of data, electronic signature, secured records and data archival.[1,2]

This Application Note develops a simple, untargeted, generic LC/MS method for routine biotherapeutic peptide map analysis using the InfinityLab LC/MSD XT system, coupled with a 1290 Infinity II LC and OpenLab ChemStation software. In a stress study using NIST monoclonal antibody (NISTmAb), we demonstrate that this compliance-ready system allows streamlined data processing and reporting for multiple PQAs in a single analysis, such as product identification confirmation, post translation modification (PTM) analysis, and glycopeptide analysis.

Experimental

Materials

All reagents and solvents were LC/MS grade. The NISTmAb reference material was purchased from National Institute of Standards and Technology.

Sample preparation

To induce asparagine deamidation, NISTmAb was exposed to elevated temperature (37 °C) in a Tris-HCl buffer system at pH 8.7 for six days. To induce methionine oxidation, NISTmAb was incubated in Tris-HCl buffers containing 0.002% (v/v) oxidizing agent H_2O_2 overnight at room temperature. Both reference and stress-induced NISTmAb were denatured, reduced, alkylated, and trypsin-digested followed by desalting using the Agilent AssayMAP Bravo platform.[3] Digested samples were injected at a concentration of approximately 0.5 µg/µL onto the LC/MS system.

LC/MS analysis

LC separation was carried out using an Agilent 1290 Infinity II LC, consisting of an Agilent 1290 Infinity II High-Speed Pump (G7120A), an Agilent 1290 Infinity II Multisampler (G7167B) with sample cooler (option 100), and an Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with an Agilent ZORBAX 300StableBond C18 column (2.1 × 150 mm, 300 Å, 1.8 µm, p/n 863750-902) (Table 1). The MS system used was the Agilent InfinityLab LC/MSD XT system (G6135BA) with the Agilent Jet Stream source (G1958-65138). Agilent OpenLab ChemStation (version C 01.09) was used for data acquisition, processing, and reporting. The data were acquired in positive scan mode ranging from *m/z* 360 to 1,400 (Table 2).

Table 1. LC conditions.

LC Parameters	
Analytical Column	Agilent ZORBAX RRHD 300Å StableBond C18, 2.1 × 150 mm, 1.8 µm (p/n 863750-902)
Mobile Phase A	H_2O with 0.1% (v/v) formic acid
Mobile Phase B	Acetonitrile with 0.1% (v/v) formic acid
Flow Rate	0.25 mL/min
Injection Volume	5 µL
Gradient	Time (min) %B 0 1 5 1 6 10 70 35 72 90 77 90 79 1 81 1
Column Temperature	50 °C

Results and discussion

Monitoring multiple PQAs in a single analysis

To evaluate the InfinityLab LC/MSD XT system for monitoring multiple attributes of biomolecules, NISTmAb was stressed under two conditions to induce deamidation and oxidation, respectively. The LC/MS method using MS positive scan mode described above was applied to collect the full peptide map for each sample. Figure 1 shows the total ion chromatogram of the peptide map data with 2.5 µg of NISTmAb digest loaded on-column, showing the sample complexity, as well as the high sensitivity and ultrafast scan speeds of the MSD within the InfinityLab LC/MSD XT system. The full scan of the NISTmAb peptide map allows monitoring of multiple attributes of interest using customized data processing methods. The scan also avoids re-acquiring data if additional attributes are of interest in the future.

Table 2. MS conditions.

Agilent MSD XT Parameters	
Drying Gas Flow	11 L/min
Drying Gas Temperature	325 °C
Sheath Gas Flow	10 L/min
Sheath Gas Temperature	325 °C
Nebulizer Pressure	35 psi
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Peak Width	0.07 minutes
Scan	360 to 1,400 *m/z* in positive mode from 5 to 80 minutes, step size 0.1
Fragmentor Ramp	Mass Value 300 125 V 2,000 200 V
Cycle Time	0.62 sec/cycle

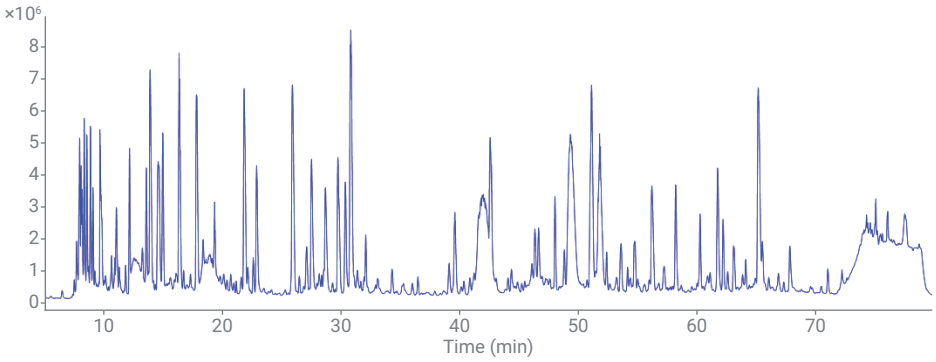

Figure 1. Total ion chromatogram of peptide map detection by Agilent LC/MSD XT with positive scan.

OpenLab ChemStation software supports automated data processing and reporting. To avoid manual extraction and integration of each peptide, a processing method can be created for extracted ion chromatograms (EICs) of multiple peptides of interest. Figure 2 shows screen captures of the EIC method setup for multiple peptides by the following steps:

1. MS chromatograms for the peptides of interest are defined with targeted *m/z*, then the targeted MS chromatograms are extracted accordingly (Figure 2A).
2. These targeted EICs are added to the processing method with adjustable retention time windows for automatic signal extraction and loading (Figure 2B).
3. The compound names, associated retention times, and EIC signals are linked through the Calibration Table setup (Figure 2C).

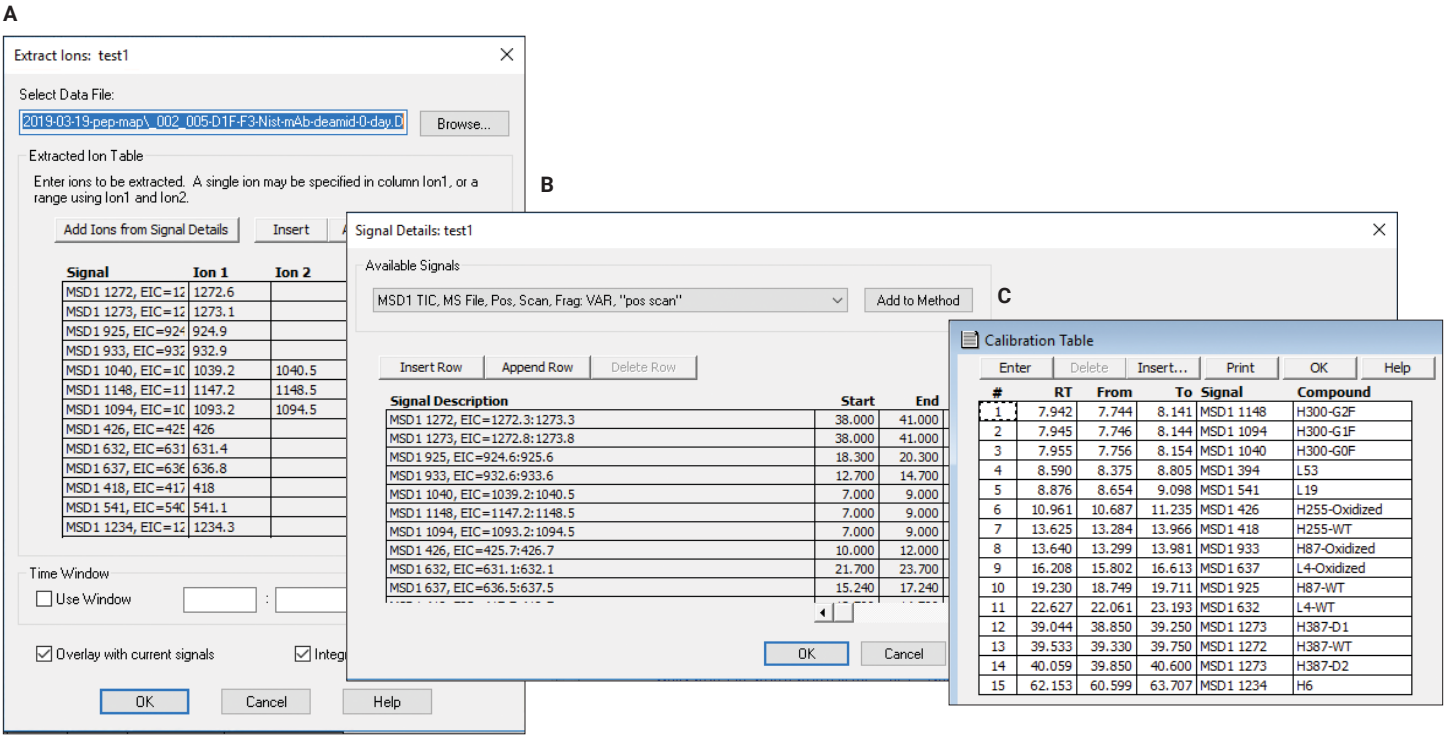

Figure 2. ChemStation screen captures of EIC method setup for multiple peptide attributes.

EICs for monitoring product attributes

To evaluate the performance using the InfinityLab LC/MSD XT, 15 precharacterized peptides were selected for identification and quantification analysis for the NISTmAb stress study (Table 3).[4,5] The identity and retention time of these peptides was predetermined using a high-resolution LC/Q-TOF system with the same LC gradient as in Table 1. A processing method, including all 15 peptides, was created using the steps described earlier, and a single dominant charge state was used to identify each of the peptides. If desired, the user could sum up additional charge states for each peptide.

The peptides listed in Table 3 can be separated into three categories according to the different monitoring purposes. The first category is the CDR peptides including peptides L4, L19, L53, H6, and H87. During product monitoring, an important need is to confirm the identity of a given biomolecule product. The sequences of CDR peptides are variable among different mAbs and can be used to confirm the product identity. Figure 3 shows the EIC of the CDR peptides that can be used to confirm protein identity.

Table 3. Peptide information for monitored attributes.

Peptide	Peptide sequence	Modification	Calculated *m/z*	Charge state (z)	Expected retention time (min)	mAb region
L4	DIQMTQSPSTLSASVGDR	Oxidation	637.0	3	16.24	CDR
L4	DIQMTQSPSTLSASVGDR	WT	631.6	3	22.68	CDR
L19	VTITCSASSR	WT	541.3	2	8.966	CDR
L53	LASGVPSR	WT	393.7	2	8.353	CDR
H6	ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR	WT	1234.3	3	62.105	CDR
H87	VTNMDPADTATYYCAR	WT	924.9	2	13.64	CDR
H87	VTNMDPADTATYYCAR	Oxidation	932.9	2	19.23	CDR
H255	DTLMISR	Oxidation	426.2	2	11.01	CH2
H255	DTLMISR	WT	418.2	2	13.71	CH2
H300	TKPREEQYNSTYR	G0F	1039.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G1F	1093.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G2F	1147.5	3	7.98	CH2
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	39.07	CH3
H387	GFYPSDIAVEWESNGQPENNYK	WT	1272.6	2	39.56	CH3
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	40.06	CH3

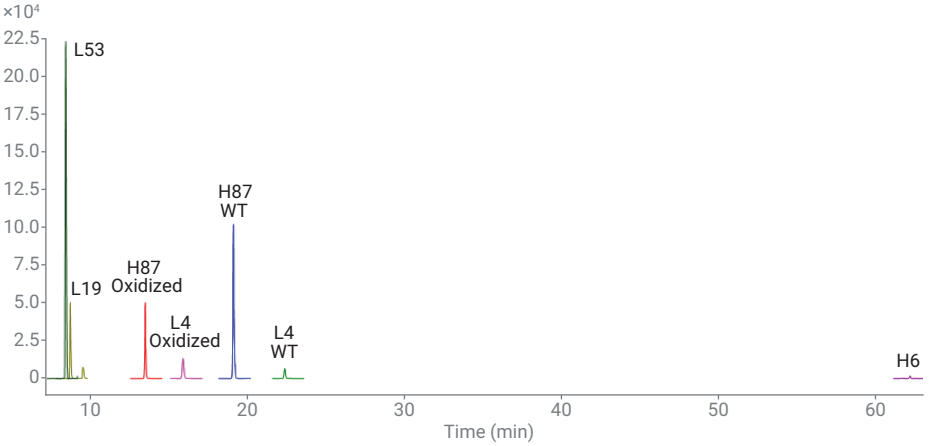

Figure 3. EICs of the CDR peptides.

The second category is peptides with variable modification sites, which are responsive for chemically induced deamidation and oxidation (L4, H87, H255, and H387).[6,7] PTMs such as asparagine deamidation, aspartate isomerization, and methionine oxidation lead to degradation products typical for recombinant antibodies. Process changes during manufacturing or storage conditions can affect the rate and extent of these modifications, which could potentially impact the stability and function of the protein drug. Therefore, these PTMs are closely monitored during process development and drug production. Figure 4A shows EICs of the wild type H387 peptide and its

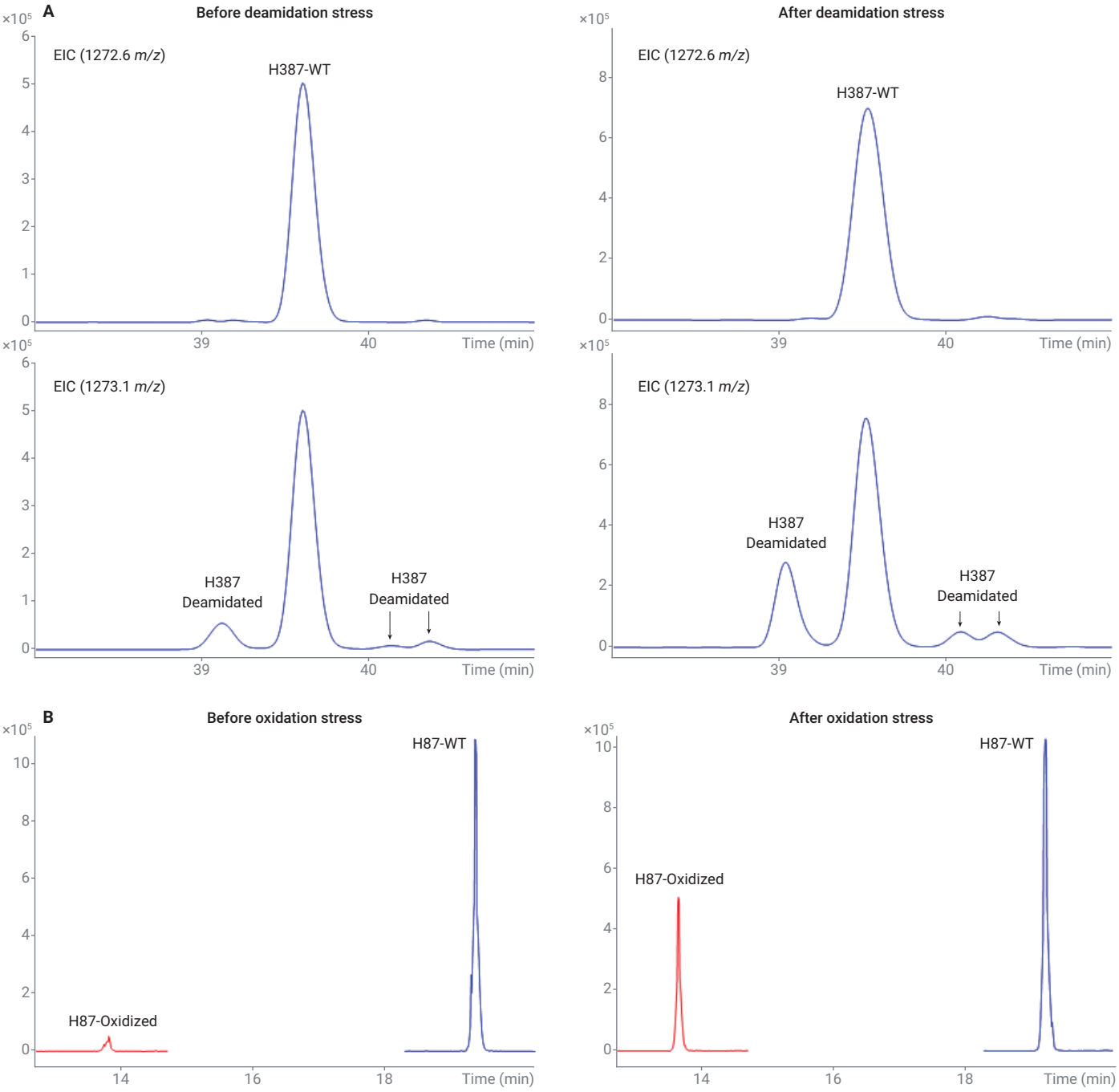

Figure 4. EICs of the peptides with variable PTMs. A) EIC comparison of the WT and deamidated H387 peptides before and after deamidation stress induction. B) EIC comparison of the WT and oxidized H87 peptides before and after oxidation stress induction.

deamidation forms, which is also called the PENNY peptide, in the reference and deamidated samples. The deamidated forms of H387 are elevated after deamidation induction. Figure 4B shows the overlaid EICs of the wild type peptide and its oxidized form from peptide H87 in both NISTmAb reference and oxidized samples. As expected, the extent of oxidation of H87 peptide was increased after oxidation induction.

The third category is glycopeptide (H300). Relative abundance of each glycopeptide can provide valuable information about the abundance of protein glycoforms. According to a previous publication on glycoanalysis in the NISTmAb tryptic digest using high-resolution LC/MS/MS, the glycopeptide located at heavy chain 292–304 (TKPREEQYNSTYR) was chosen as the dominant tryptic form[5]. Figure 5 shows the overlaid EICs of three glycopeptides (G0F, G1F, and G2F) used for determining their relative abundance. This result is consistent with a previous report on the relative abundance of these NISTmAb glycopeptides obtained using high-resolution LC/MS/MS.[5]

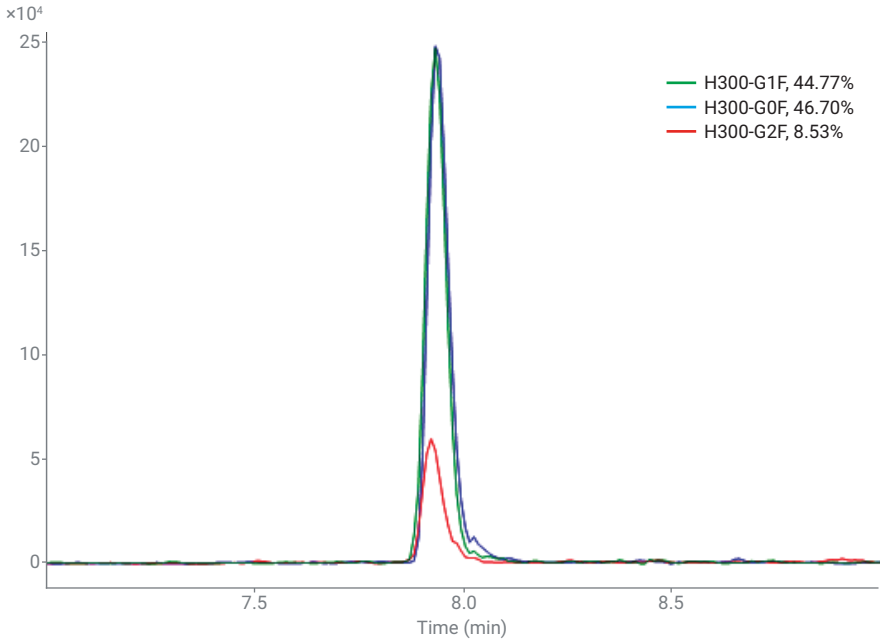

Figure 5. EICs of the three glycopeptides for determining relative abundance.

Intelligent reporting

OpenLab ChemStation software enables automated intelligent reporting. Intelligent reporting provides superior flexibility and allows the user to customize their report templates as desired. Figures 6A and 6B show examples of intelligent reports generated for monitoring multiple attributes.

Single Injection Report

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Data file:
Sample name:
Description:
Sample amount:

Instrument:
Injection date:
Acq. method:
Analysis method:

Location:
Injection:
Injection volume:
Acq. operator:

Analyst: _____

Date: _____

Pass/Fail: _____

Peak Summary Table: Glycopeptides

Name	RT [min]	Area	Area Percent
H300-G0F	7.955	1207180	46.70%
H300-G1F	7.945	1157171	44.77%
H300-G2F	7.942	220399	8.53%
	AreaSum	2584749	

Peak Summary Table: Deamidation

Name	RT [min]	Area	Area Percent	P/F
H387-D1	39.112	146688	2.86%	Pass
H387-WT	39.592	4914364	95.93%	Pass
H387-D2	40.123	61809	1.21%	Pass
	AreaSum	5122861		

Peak Summary Table: Oxidation

Name	RT [min]	Area	Area Percent	P/F
H87-Oxidized	13.811	113448	3.47%	Pass
H87-WT	19.367	3160505	96.53%	Pass
	AreaSum	3273952		

Name	RT [min]	Area	Area Percent	P/F
L4-Oxidized	16.208	105370	9.00%	Pass
L4-WT	22.627	1065867	91.00%	Pass
	AreaSum	1171237		

Name	RT [min]	Area	Area Percent	P/F
H255-Oxidized	10.961	1967116	16.80%	Fail
H255-WT	13.625	9738910	83.20%	Fail
	AreaSum	11706026		

Figure 6A. Intelligent reporting by Agilent OpenLab ChemStation. Example of a single injection report. Peak tables summarize the relative abundance of WT and PTM forms for each peptide sequence by custom calculation.

Sequence Summary Report

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Sequence name:

Acquisition date:

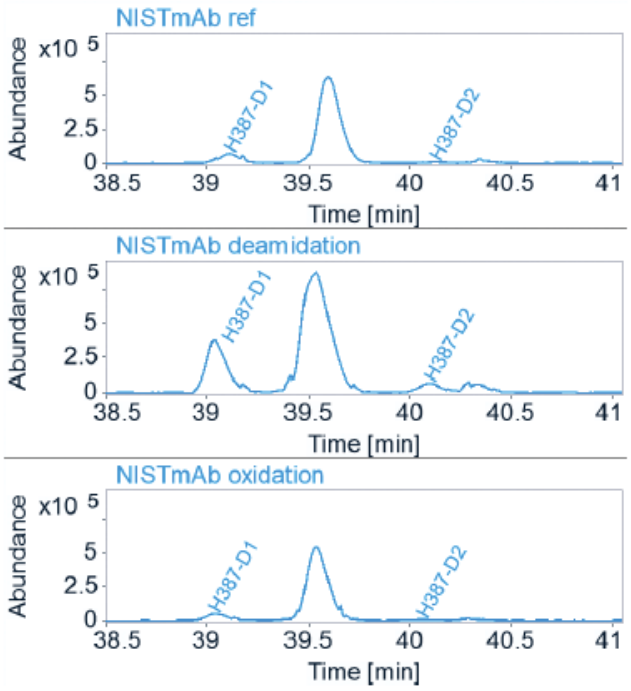
Acquired by:

Approved by: _____

Signature: _____

Date: _____

Extracted Ion Chromatogram **EIC=1272.8 :1273.8**



			H387-D1	H387-WT	H387-D2
			RT (min)	RT (min)	RT (min)
1	NISTmAb ref	_001_005-D1F-F3-Nist-mAb-deamid-0-day.D	39.11	39.59	40.12
2	NISTmAb deamidation	_002_006-D1F-F4-Nist-mAb-deamid-6-day.D	39.04	39.53	40.10
3	NISTmAb oxidation	_003_003-D1F-F2-Nist-mAb-H2O2-002pct.D	39.04	39.53	40.06

Figure 6B. Intelligent reporting by Agilent OpenLab ChemStation. Example of a sequence summary report comparing a NISTmAb reference sample, deamidated sample, and oxidized sample for the EIC and retention time of H387 peptide.

Conclusion

The Agilent InfinityLab LC/MSD XT system provides a simple and cost-effective solution for monitoring multiple PQAs in a development and quality control environment, assuming those attributes that have been precharacterized using a high-resolution MS instrument. This Application Note demonstrates that the InfinityLab LC/MSD XT system can deliver quantitative analysis for monitoring multiple attributes of biotherapeutics at the peptide level, including CDR peptides, oxidized and deamidated peptides, and glycopeptides in a single analysis. Automated data processing and reporting through Agilent OpenLab ChemStation software avoid manual interrogation and allow high-throughput analysis. OpenLab ChemStation in combination with central data storage provides a compliance solution for chromatography and mass spectrometry data collected in compliant environments.

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