Application Note

Biotherapeutics and Biosimilars

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Peptide Mapping on Monoclonal Antibodies Using Agilent 6545XT AdvanceBio LC/Q-TOF and Protein Metrics Byos Software

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Introduction

Liquid chromatography/mass spectrometry (LC/MS) plays an increasingly critical role in the characterization and quantitation of biotherapeutics. Peptide mapping methods, based on proteolytic digestion followed by LC/MS analysis, have been widely used in the biopharmaceutical industry for therapeutic protein characterization and process development and product quality monitoring, among other uses. Recently, there has been an increasing interest in relying on peptide mapping methods to characterize and monitor biotherapeutic quality, called the multi-attribute method (MAM).[1] Peptide mapping analysis requires a workflow solution for reproducible sample preparation, high-quality LC/MS data generation and streamlined data processing. Previous application notes demonstrated an integrated workflow for peptide mapping using Agilent-only instruments and software, including the Agilent AssayMAP Bravo platform, the Agilent 1290 Infinity II LC system, the Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software.[2,3] In biopharma industry labs, analytical instruments from multiple vendors often co-exist, and therefore users sometimes prefer a unified data processing platform for all acquired data. In this scenario, vendor-neutral software could be beneficial. Protein Metrics Byos is software that meets this need. It provides multiple streamlined data processing workflows for various applications, e.g., peptide post-translational modification (PTM) identification and quantification, Host Cell Protein (HCP) analysis, MAM applications, released glycan analysis, intact protein analysis, ADC analysis, *de novo* sequencing, and other analyses.

This application note demonstrates an LC/MS-based peptide mapping method for protein characterization and quantification in control and chemical-stressed NISTmAb samples using a workflow solution including the AssayMAP Bravo, the 1290 Infinity II LC system, the 6545XT AdvanceBio LC/Q-TOF, and Protein Metrics Byos software (Figure 1). The results demonstrate the compatibility between Agilent instruments and Protein Metrics software.

Experimental

Materials

NISTmAb was purchased from the National Institute of Standards and Technology (NIST).

Instrumentation

- Agilent AssayMAP Bravo Protein Sample Prep platform (G5571AA)
- Agilent 1290 Infinity II LC system including:
- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B) with sample cooler (Option 100)
- Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549A)
- Agilent Dual Jet Stream ESI source (G1958-65268)

Chemical induction and sample preparation

To induce significant levels of deamidation, an aliquot of NISTmAb samples were exposed to an elevated temperature (37 °C) in a Tris-HCl buffer at pH 8.7 for six days. To induce oxidation, another aliquot of NISTmAb samples were incubated in Tris-HCl buffers containing 0.002% of oxidizing agent H_2O_2 overnight at room temperature. All untreated and chemical-induced samples were lyophilized and stored in –80 °C before sample digestion.

All samples were subjected to reduction, iodoacetamide alkylation, and trypsin digestion, followed by desalting using the Agilent AssayMAP Bravo before LC/MS analysis.

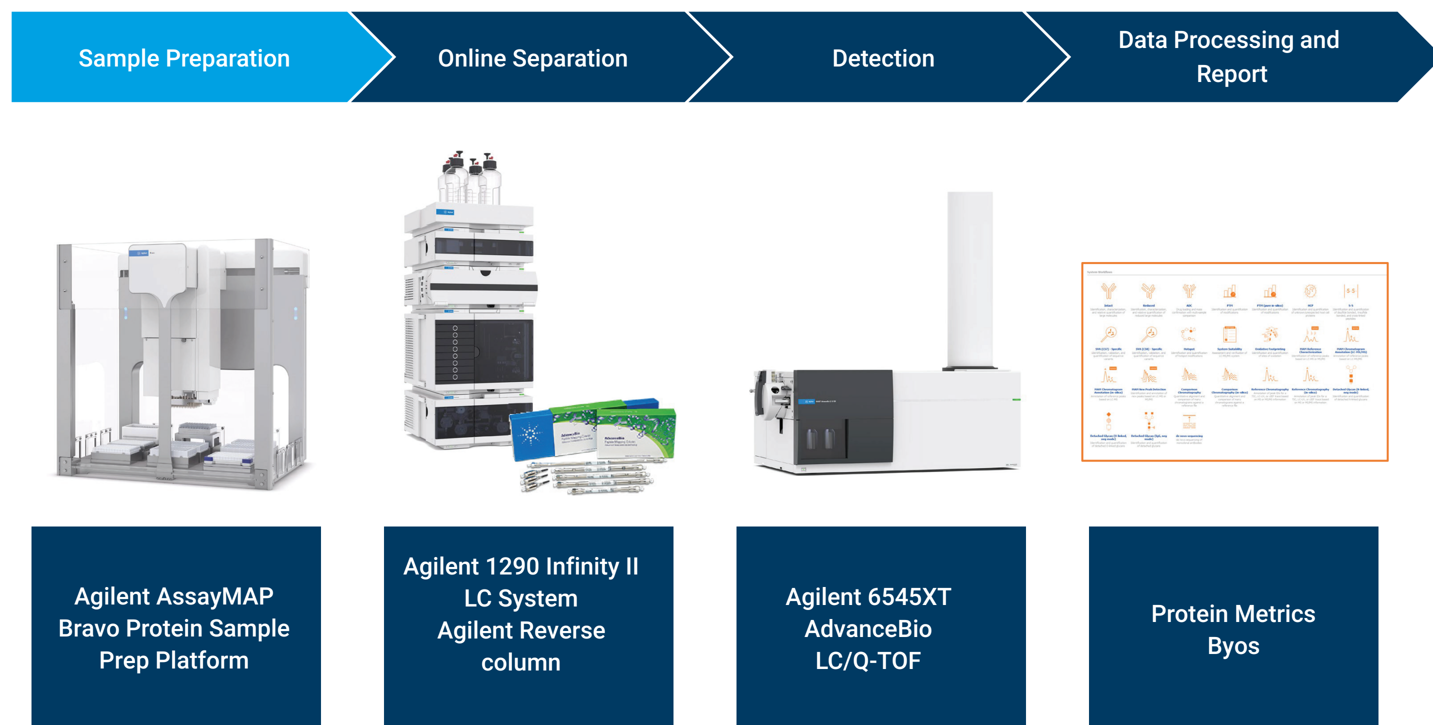

Figure 1. An overview of the peptide mapping workflow using the Agilent AssayMAP Bravo Protein Sample Prep platform, Agilent 1290 Infinity II LC system, the Agilent 6545XT AdvanceBio LC/Q-TOF system and Protein Metrics Byos software.

LC/MS analysis

Peptide separation was performed on an Agilent 1290 Infinity II LC system using an Agilent ZORBAX RRHD 300Å StableBond C18 column (2.1 × 150 mm, 1.8 µm, part number 863750-902) (Table 1). The MS data were acquired using an Agilent 6545XT AdvanceBio LC/Q-TOF (Table 2).

Data processing

All the raw data were processed using the Byos software (v3.10-52, Protein Metrics Inc.). Default parameters in the Byos preconfigured system workflows were used for the NISTmAb peptide identification and chromatogram annotation in the reference file, and quantitative comparison in the batch analysis.

Results and discussion

Peptide mapping for protein characterization

Peptide mapping is widely used in the biopharmaceutical industry for protein product characterization, e.g., protein sequence identification and PTM. This requires an integrated workflow including sample preparation, peptide identification, data processing, and reporting. This study prepared three trypsinized samples using control, high pH-induced deamidated and H_2O_2-induced oxidated NISTmAbs, respectively. All samples were prepared using the AssayMAP Bravo platform, and then analyzed on the 6545XT AdvanceBio LC/Q-TOF system followed by data processing using the PMI-Byos software for sequence and PTM identification using the MAM reference characterization workflow.

Figure 2 shows screenshots of the Byos user interface after data processing by the MAM reference characterization workflow. As shown in the protein coverage view tab, a protein coverage of 98.44% for heavy chain and 94.84% for light chain were achieved. One of the identified Asn-deamidated peptides (FNWYVDGVEVHnAK) were selected in the Peptides tab. To have a comprehensive analysis, users often need to inspect the LC/MS data quality thoroughly, including protein identification coverage, extracted ion chromatogram, MS and MS/MS mass accuracy and spectral quality for the selected peptide. Especially for low-abundant PTM peptides, comparisons between PTM peptides and their corresponding wild-type peptides are often executed. As shown in Figure 2A, the wild-type peptide matching to the selected PTM peptide was automatically displayed, and the side-by-side comparison between the PTM and wild-type peptides were automatically displayed in XIC plots, the MS2 spectrum plot, MS2 mass error plots, and MS1 isotope plots for easy inspection. Automated reporting is another essential feature for a

Table 1. Liquid chromatography 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

Table 2. Mass spectrometer and source conditions.

6545XT Q-TOF 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
Fragmentor Voltage	175 V
Acquisition Mode	AutoMS2 or MS1 as specified
MS1 Acquisition Rate	5 spectra/second

A

B

Protein name ↑	Sequence (unformatted) ↑	Mod. Names ↓	Mod. AAs ↑	Var. Pos. Protein ↑	MS Alias name ←	Control (%)	Deamidated (%)	Oxidated (%)
NISTmAb_HC NISTmAb heavy chain	ALEWLADIWWDDK	Oxidation/15.9949	W	54			5.7	1.1
	ALEWLADIWWDDKK	Oxidation/15.9949	W	49		2.6	1.3	2
				54		3	5.4	2.5
				55		0.31	3.2	1.1
	DMIFNFYFDVWGQGTTVTVSSASTKGPSVFPLAPSSK	Oxidation/15.9949	M	101		3.6	5.1	100
	DTLMISR	Oxidation/15.9949	M	255		15	21	99
	EPQVYTLPPSREEMTK	Oxidation/15.9949	M	361		3.5	18	100
	ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIRQPPGK	Oxidation/15.9949	M	34				97
	FNWYVDGVEVHNAK	Oxidation/15.9949	W	280		0.44	0.72	1.6
		Deamidated/0.9840	N	279		0.085		0.094
				289		1.4	2	1.2
	GFYPSDIAVEWESNGQPENNYK	Oxidation/15.9949	W	384			0.53	1.4
		Deamidated/0.9840	N	387		20	28	15
				392		6.8	18	15
	GQPREPQVYTLPPSREEMTK	Oxidation/15.9949	M	361		6.2	9.8	98
	NQVVLK	Deamidated/0.9840	N	78		3.4	5.1	4.3
	SRWQQGNVFSCSVMHEALHNHYTQK	Oxidation/15.9949	M	431				100
	VTNMDPADTATYYCARDMIFNFYFDVWGQGTTVTVSSASTKGPSVFPLAPSSK	Oxidation/15.9949	M	87			5.1	
	VVSVLTVLHQDWLNGK	Oxidation/15.9949	W	316		0.89	1.8	1.5
		Deamidated/0.9840	N	318		23	34	15
			Q	314		4.9		3.6

Figure 2. Screenshots of Byos software user interface after data processing by the MAM reference characterization workflow showing (A) an easy view for data inspection, and (B) automated reporting to highlight CQAs.

streamlined workflow. Figure 2B shows an example of the Byos reporting table, which automatically highlights changes in critical quality attributes (CQAs). In summary, Byos provides convenient tools and an easy view for protein characterization, and LC/MS/MS data acquired from Agilent instruments are completely compatible with this software.

Chromatogram annotation

Protein characterization and quantification of mAbs in different types of stress conditions allow researchers to identify relevant chemical degradation sites in mAbs. Monitoring of these CQAs is required throughout the development and manufacturing process of therapeutic proteins. In addition to peptide identification, Byos also provides an MAM chromatogram annotation workflow to annotate selected peaks in the whole chromatogram. Figure 3 shows the full chromatogram from the control NISTmAb digest, with peaks labeled with peak ID, peptide sequence, and modification name. The selected chromatographic peaks can be annotated in a user-desired format and serve as a reference for quantitative comparison.

Chromatography comparison

In peptide mapping-based multiple attribute monitoring, another important task is to quantitatively align and compare many chromatograms against a reference file. Byos software provides tools to streamline this analysis, such as chromatogram alignment, tracking and quantification of individual peaks and automated report generation. The raw data can be acquired either by precursor scan mode or by data-dependent acquisition (DDA) mode. For example, the precursor scan data files from the chemical stressed NISTmAb samples were compared against the control reference file. From the automated reporting, it is easy to filter and locate changed peaks or new peaks. Figure 4 shows an increase of oxidation on the peak matching to the DTLmISRTPEVTCVVVDVSHEDPEVK peptide, which was flagged in red while unchanged peaks remained grey. The *Warp on* tool was applied to align chromatograms. This function is capable of adjusting minor drifting on LC retention time among various LC/MS runs.

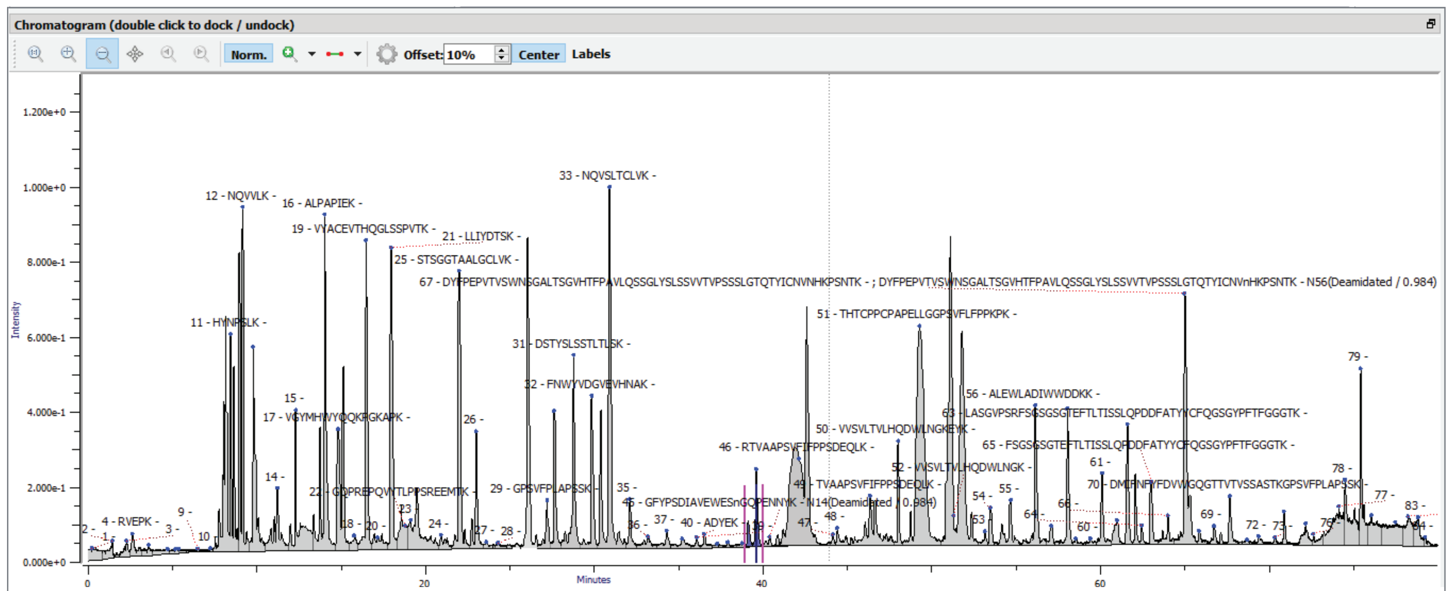

Figure 3. Annotated chromatogram from control NISTmAb digest.

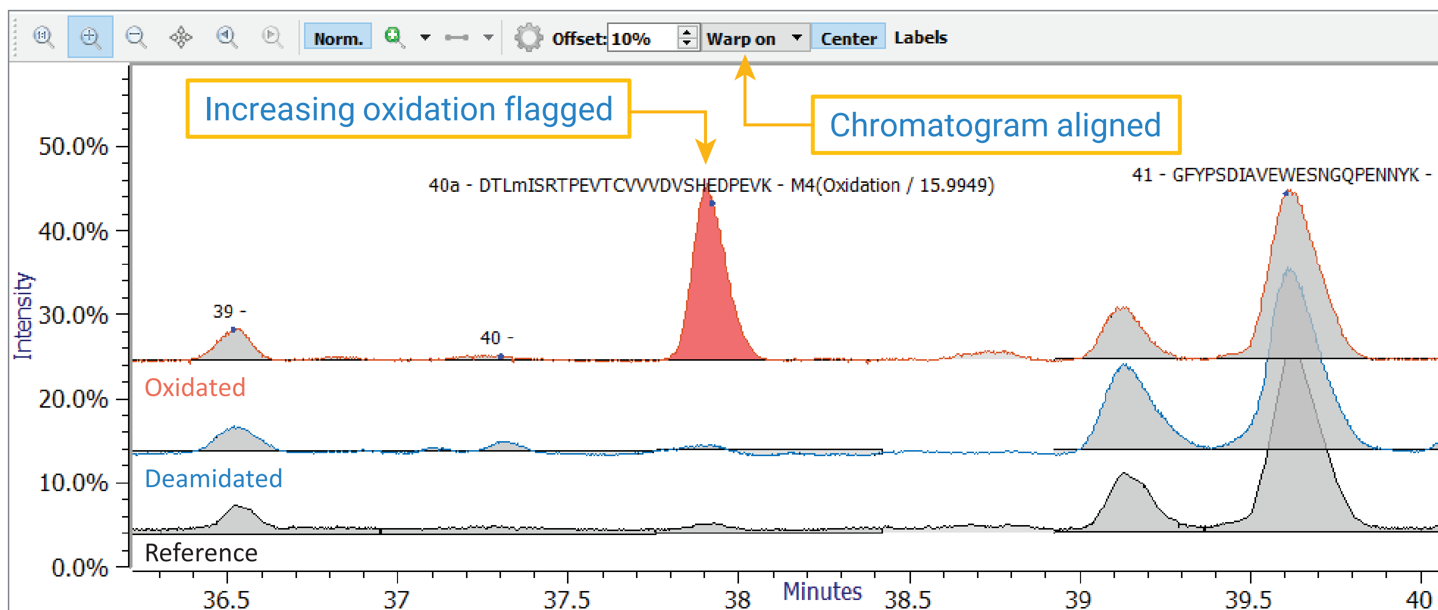

Figure 4. An intuitive view shows chromatogram comparison in the control, deamidated and oxidated NISTmAb sample digests. Chromatograms were aligned by applying the *Warp on* tool. Peptide attributes with changed peak area were automatically flagged.

Conclusion

A complete peptide mapping workflow has been demonstrated for in-depth NISTmAb characterization and quantitative comparison using the Agilent AssayMAP Bravo for automated sample preparation, Agilent 1290 Infinity II LC system for peptide separation, Agilent 6545XT AdvanceBio LC/Q-TOF for data acquisition and Protein Metrics Byos software for data processing. The results demonstrate that the raw data acquired by Agilent LC/Q-TOF are directly compatible with Protein Metrics software. The preconfigured system workflows in Byos can be directly used as a reasonable starting point.

References

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