High Resolution and Rapid Peptide Mapping of Monoclonal Antibody Using an Agilent 1290 Infinity UHPLC and an Agilent 6550 iFunnel Q-TOF LC/MS System

Application Note

Authors

Ning Tang
Agilent Technologies, Inc.
Santa Clara, CA, USA

James Martosella and Alex Zhu
Agilent Technologies, Inc.
Wilmington, DE, USA

Introduction

Peptide mapping is the common analytical technique for characterizing biologic therapeutic proteins. The protein is first digested into smaller peptides enzymatically using one or two proteases. The peptide mixture is typically separated by reversed-phase chromatography and detected by UV or mass spectrometry. Mass spectrometry provides the masses of the peptides, which greatly enhances the information content for peptide mapping. With the additional dimension of separation by mass, coeluting peptides can be easily identified and the gradient length significantly reduced. In addition, tandem mass spectrometry can fragment the peptides into smaller pieces and provide evidence for the amino acid sequence of the peptide and the post-translational modifications (PTM) such as glycosylation, phosphorylation, oxidation, and deamidation.

This Application Note describes how peptide mapping of an IgG (1) therapeutic monoclonal antibody (mAb) was performed using a novel superficially porous peptide mapping column coupled to an Agilent 6550 iFunnel Q-TOF LC/MS System. The unique resolving power of the peptide column to maximize resolution and efficiency in combination with the MS/MS analysis ensured confident and sensitive identification of peptides and resulted in high sequence coverage during a rapid runtime of 15 minutes. The 6550 iFunnel Q-TOF LC/MS System delivered excellent mass accuracy and sensitivity for peptide identification, and the MS/MS capability helped to confirm modified peptide identifications with high confidence. Additionally, optimization of the IgG (1) peptide gradient and LC/MS/MS parameters were explored for achieving robust, rapid, and reliable mAb peptide maps.

Agilent Technologies

Experimental

Sample preparation

Twenty microliters of mAb IgG1 (30 mg/mL) was first mixed with 20 µL of 100 mM ammonium bicarbonate (pH 8). Then, 50 µL of trifluorolethanol (TFE) and 5 µL of 200 mM dithiothreitol (DTT) were added to the protein sample and heated at 65 °C for 30 minutes to denature and reduce the protein. After the protein was cooled to room temperature, 20 µL of 200 mM iodoacetamide (IAM) was added to the sample. The sample was kept at room temperature in the dark for 1 hour. Subsequently, 5 µL of DTT was added in the sample to react with the excess IAM for 1 hour. The sample was diluted with 200 µL of 100 mM ammonium bicarbonate and 600 µL of water. 40 µL of trypsin was added to the sample and incubated at 37 °C with shaking (300 rpm) overnight. After overnight incubation, 5 µL of neat formic acid was added to quench the digest.

Instrumentation

LC system

Agilent 1290 Infinity LC System including:

- Agilent 1290 Infinity Binary Pump G4220A

- Agilent 1290 Infinity TCC G1316C

- Agilent 1290 Infinity Sampler G4226A

- Agilent 1290 Infinity FC/ALS Therm G1330B

MS system

Agilent 6550 iFunnel Q-TOF LC/MS System with Agilent JetStream

LC/MS Parameters

Parameter	Agilent 1290 Infinity LC System		
Column	Agilent AdvanceBio Peptide Mapping Column 2.1 × 250 mm, 2.7 µm (p/n 651750-902)		
Column temperature	60 °C		
Sample thermostat	5 °C		
Mobile phase A	0.1 % formic acid in water		
Mobile phase B	90 % acetonitrile in water with 0.1 % formic acid		
Gradient	0 minutes 3 % B 10 minutes 35 % B 12 minutes 90 % B 14 minutes 90 % B 15 minutes 3 % B		
Stop time	15 minutes		
Post time	10 minutes		
Flow rate	0.5 mL/min		

Parameter	Agilent 6550 Q-TOF LC/MS System		
Ion mode	Positive ion mode,		
Source	Agilent Dual JetStream		
Drying gas temperature	250 °C		
Drying gas flow	14 L/min		
Sheath gas temperature	250 °C		
Sheath gas flow	11 L/min		
Nebulizer	35 psi		
Capillary voltage	3,500 V		
Nozzle	0 V		
MS range (m/z)	300–1,700		
MS/MS range (m/z)	50–1,700		
MS scan rate (spectra/second)	8		
MS/MS scan rate (spectra/second)	3		
Ramped collision energy	**Charge state**	**Slope**	**Offset**
	2	3.1	1
	3 and > 3	3.6	−4.8
	1	3.5	6
Data analysis	The data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis Software B.06 and Agilent MassHunter BioConfirm Software B.06		

Results and Discussion

A peptide mapping experiment may contain hundreds of peptides with various modifications. Automatic data processing improves the efficiency for data mining. The peptide mapping workflow provided by Agilent MassHunter BioConfirm is depicted in Figure 1. After data acquisition, compounds were extracted automatically based on the chromatography characteristics. The table of compounds was then matched to the list of theoretical peptide digests with potential modification groups. The MS/MS spectrum of each peptide was interpreted by the b/y fragments of the peptides. After the peptides were identified, a sequence coverage map was compiled and the peptide mapping report was automatically generated at the end of the workflow.

Traditional peptide mapping methods typically use 90-minute gradients or longer to achieve the desired resolution. With the advancement in UHPLC and related column technologies, gradient time for peptide mapping has been greatly reduced, while coupling LC to MS detection offers an additional dimension of separation in the mass domain. In this work, peptide mapping of an IgG (1) therapeutic monoclonal antibody (mAb) was performed using a novel superficially porous peptide mapping column coupled to a 6550 iFunnel Q-TOF LC/MS System. The unique resolving power of the peptide column to maximize resolution and efficiency, in combination with the MS/MS analysis, ensured confident and sensitive identification of peptides.

For mAb IgG1, sequence coverage of 92.1 % was achieved using the 15 minutes gradient. A total of 77 peptides including various PTMs, such as glycosylation, oxidation, and deamidation were identified by MS/MS.

Employing a 60-minute gradient, 95.1 % sequence coverage was achieved with 82 peptides identified by MS/MS. No additional PTMs were identified with the 60-minute gradient. Using the Agilent AdvanceBio Peptide Mapping column and a steep 15-minute gradient, a high resolution and high peak capacity peptide map was achieved with high sequence coverage. Figure 2 shows the overlaid extracted compound chromatograms for (A) 15-minute gradient and (B) 60-minute gradient.
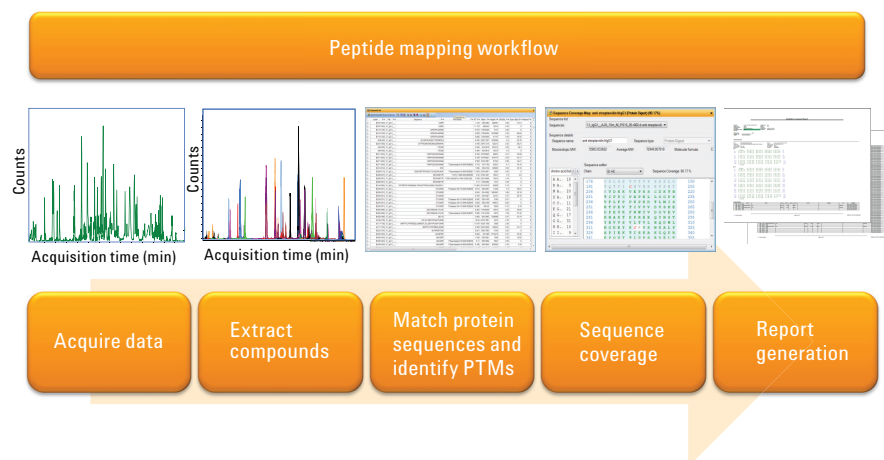

Figure 1. Agilent MassHunter BioConfirm offers automated peptide mapping data acquisition and analysis workflow.
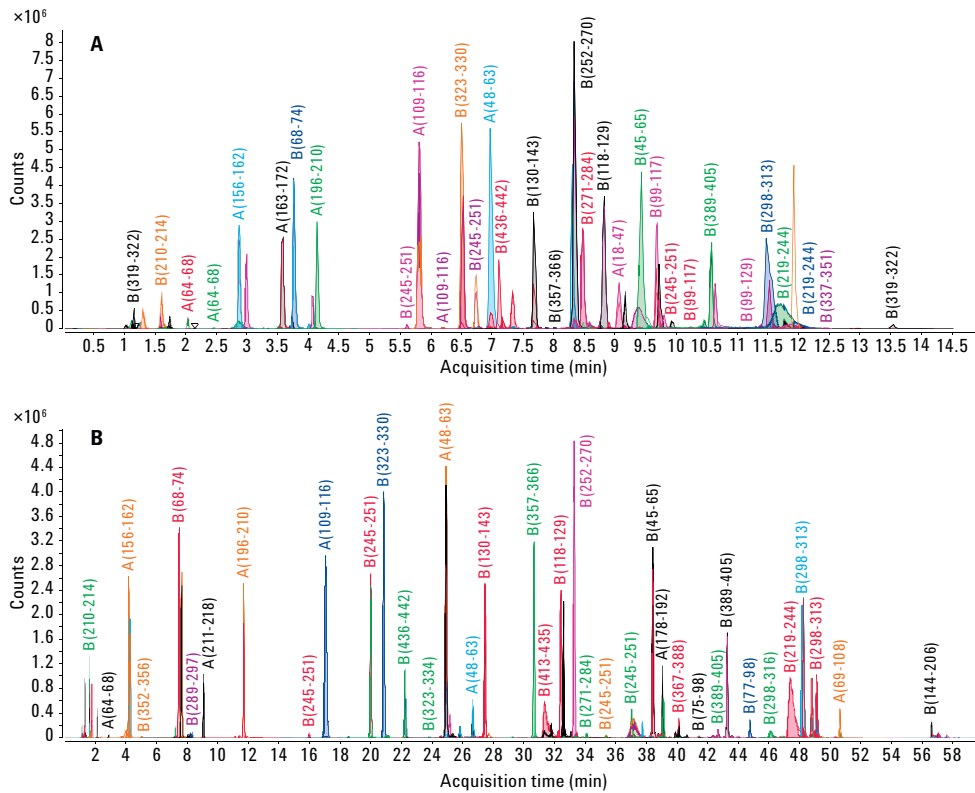
Figure 2. Identified peptide chromatograms with peptides identified for (A) 15-minute run and (B) 60-minute run.

Deamidation is an important PTM to monitor in peptide mapping. Deamidation can occur during storage, purification, and sample manipulation. Deamidation can be identified by an LC/MS/MS peptide mapping experiment. By adding a negative charge, deamidation can cause structural and functional changes. In the rapid peptide map generated in 15 minutes, light chain peptide 48-63, which contains Asn 53, was identified. Its native form and two deamidated forms, isoaspartic acid and aspartic acid, were separated into three peaks (Figure 3A). The precursor MS of the three peaks are shown in Figure 3B, which clearly shows the addition of 0.98 Da of peaks 2 and 3. Isoaspartic acid and aspartic acid are isobaric. The MS/MS spectra of the three peptides are shown in Figure 3C, wherein, the y-series fragments are the predominant ions.
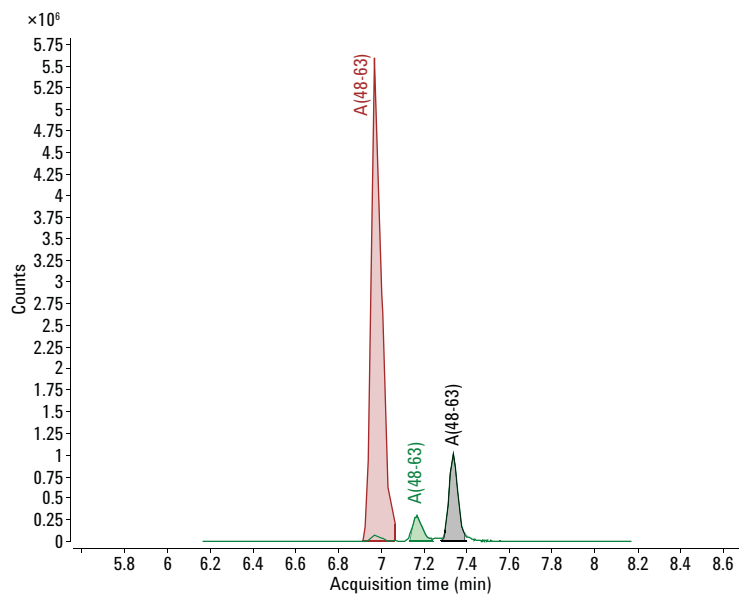

Figure 3A. Overlaid extracted compound chromatograms of regular peptide and deamindated peptides.

4

All three peptides have the same y10 fragment, however from the y11 fragment and beyond, the ions are 0.98 Da higher for peaks 2 and 3, thus the corresponding sequences for the three peaks are labeled in Figure 3C. Automatic compound extraction (Molecular Feature Extraction) also adds all the ion intensities (isotopes, charge states, adducts, and so forth) belonging to the peptide together (labeled as Volume in the peptide table, Figure 3D). The percentage of modified peptides can easily be derived from the data. In this sample, the two deamidated forms were 3.7 % and 13 % respectively.
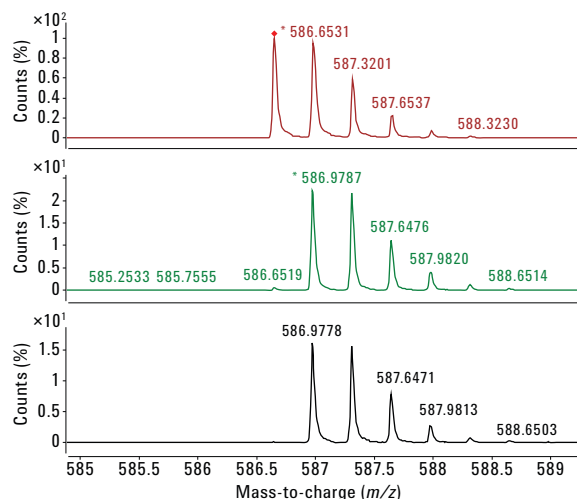

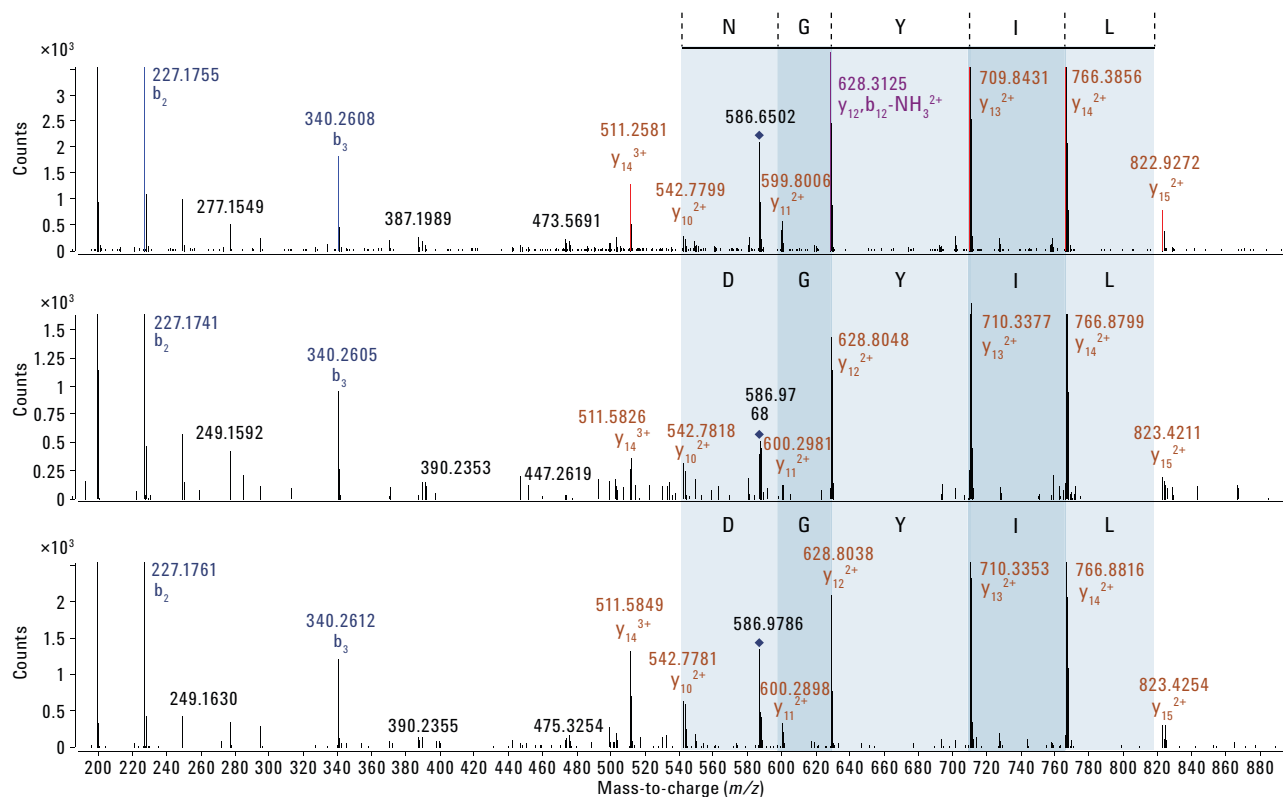Figure 3B. MS spectra of three peptides showing delta mass of 0.98 Da.

Figure 3C. MS/MS spectra of three peptides.

	File	Label	Sequence	Pred Mods	RT	Mass	Vol
⊞	21_IgG1__AJS_15m_M_PS25_05-r002.d	A(48-63)	LLIYGNSNRPSGVPDR		6.973	1756.9294	19378806
⊞	21_IgG1__AJS_15m_M_PS25_05-r002.d	A(48-63)	LLIYGNSNRPSGVPDR	1*Deamidation(+0.984016)A53	7.168	1757.917	862153
⊞	21_IgG1__AJS_15m_M_PS25_05-r002.d	A(48-63)	LLIYGNSNRPSGVPDR	1*Deamidation(+0.984016)A53	7.34	1757.9128	3006008

Figure 3D. Peptide table that showed the native peptide A(48-63) and two deamindated forms with their sequence, modification, retention time, mass and volume.

Conclusions

Peptide mapping of a monoclonal antibody using an Agilent 1290 Infinity LC System coupled to an Agilent 6550 iFunnel Q-TOF LC/MS System has been demonstrated. LC/MS/MS ensured confident and sensitive identification of peptides.

- Rapid and efficient HPLC peptide mapping of a monoclonal antibody (IgG1) was demonstrated using an Agilent AdvanceBio Peptide Mapping Column. The separation speed and efficiency are comparable to UHPLC sub-2 µm performance but at a fraction of the system back pressure.

- The 1290 Infinity LC System provided fast and superior separation power, and 6550 iFunnel Q-TOF LC/MS System delivered excellent mass accuracy and sensitivity for peptide identification. MS/MS capability helped confirm the peptide ID, especially for modified peptide. A 92 % sequence coverage was achieved with a rapid 15-minute run.

- Agilent MassHunter BioConfirm software provided automated data extraction, sequence matching, PTM identification, and sequence coverage calculation.

www.agilent.com/chem

Agilent Technologies