Enhancing The Quality Of Peptide Mapping Separation For The Analysis Of PTM

Application Note

Biotherapeutics and Biosimilars

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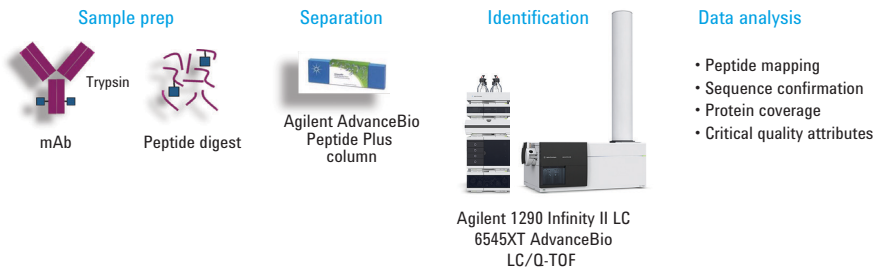
Introduction

Post-translational modifications (PTMs) on proteins such as monoclonal antibodies (mAbs) are critical quality attributes (CQAs) that define the drug efficacy and safety. Characterization of PTMs is a difficult task that has been accelerated by advancements in analytical techniques. In the biopharma industry, peptide mapping is a routinely used method to confirm protein identity. It involves enzymatic digestion to produce peptide fragments, followed by liquid chromatography (LC) separation, detection, and identification of peptides. Peptide mapping can be used to identify single amino acid changes as well as PTMs when coupled with mass spectrometry (MS) detection. LC/MS is the preferred technique for mapping PTMs. However, due to the complexity of protein digest, the separation of peptide maps is a challenging task. High-resolution and reliable peptide mapping separations are essential for confident identification of PTMs. The current LC/MS methods with MS-friendly formic acid (FA) ion-pairing agent provides increased signal intensity compared to other modifiers. The drawback with FA is broader, tailing peaks with many C18 stationary phases leading to coelution of peptide pairs. Characterization of PTMs requires the high-resolution LC separation of modified and unmodified peptides from a complex tryptic digest. The peptide retention and MS signals can be affected by the choice of reversed-phase LC columns and mobile phase ion-pairing agents, respectively. Thus, correct LC column choice is critical for enhanced separation of PTM-containing peptides.



Agilent Technologies

Previously, we demonstrated improved peptide separation using an Agilent AdvanceBio Peptide Plus column [1]. The column features a positively charged surface and a C18 stationary phase, providing high-efficiency separation of peptides with narrow peak shapes. In this study, we evaluated the performance of the AdvanceBio Peptide Plus column for the separation of PTM-containing peptides. Peptide mapping of a therapeutic mAb was performed using this novel charge hybrid/C18 hybrid superficially porous column coupled to an Agilent 6545XT AdvanceBio LC/Q-TOF system. The superior performance of the AdvanceBio Peptide Plus column delivered high-efficiency peptide map separation. This high-efficiency separation with high mass accuracy, generated reliable mAb peptide maps with high sequence coverage.

Materials

Trastuzumab was bought from a local pharmacy, and stored according to manufacturer's instructions. DL-Dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), and LC/MS grade solvents were bought from Sigma-Aldrich. High-quality sequence grade trypsin was obtained from Agilent Technologies, Inc.

Trypsin digestion

Before the digestion of the mAb with trypsin, the disulfide bonds were reduced (DTT) and alkylated (IAA) under denaturing conditions (guanidine-HCl). This pretreatment ensured complete mAb denaturation, solubilization, and efficient access of the protease to the target substrate. After reduction and the alkylation step, the pH of the solution was adjusted to pH 7-8, and trypsin digestion (20:1, protein to protease w/w) was performed overnight, incubating at 37 °C. The samples were either immediately analyzed through LC/MS, or stored at –80 °C until use. Relative percentage modification was calculated using Equation 1.

LC conditions

Parameter	Agilent 1290 Infinity II LC
Column:	Agilent AdvanceBio Peptide Plus 2.1 × 150 mm, 2.7 µm, 120 Å (675950-902)
Injection volume:	1 µL (1 µg/µL)
Sample thermostat:	5 °C
Mobile phase A:	0.1% FA in water
Mobile phase B:	0.1% FA in ACN
Gradient:	At 0 minutes → 3% B
	At 1 minutes → 3% B
	At 31 minutes → 40% B
	At 33 minutes → 95% B
	At 34 minutes → 95% B
	At 34.1 minutes → 3% B
Stop time:	34.1 minutes
Post time:	5 minutes
Column temperature:	55 °C
Flow rate:	0.5 mL/min

MS conditions

Parameter	Agilent 6545XT AdvanceBio LC/Q-TOF		
Ion mode:	Positive ion mode, dual AJS ESI (profile)		
Drying gas temperature:	325 °C		
Drying gas flow:	13 L/min		
Sheath gas temperature:	275 °C		
Sheath gas flow:	12 L/min		
Nebulizer:	35 psi		
Capillary voltage:	4,000 V		
Fragmentor voltage:	175 V		
Skimmer voltage:	65 V		
Oct RF Vpp:	750 V		
Acquisition parameters MS mode			
Data were acquired in extended dynamic range			
MS mass range:	100–1,700 *m/z*		
MS/MS mass range:	50–1,700 *m/z*		
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
Data analysis	Agilent BioConfirm software B.08.00		

$$\text{Relative \% modification} = \frac{(\sum\text{Area of modified peptide ions})}{(\sum\text{Area of modified peptide ions}) + (\sum\text{Area of unmodified peptide ions})} \times 100$$

Equation 1.

Instrumentation

LC system

Agilent 1290 Infinity II LC system including:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1290 Infinity II multisampler (G7167B)

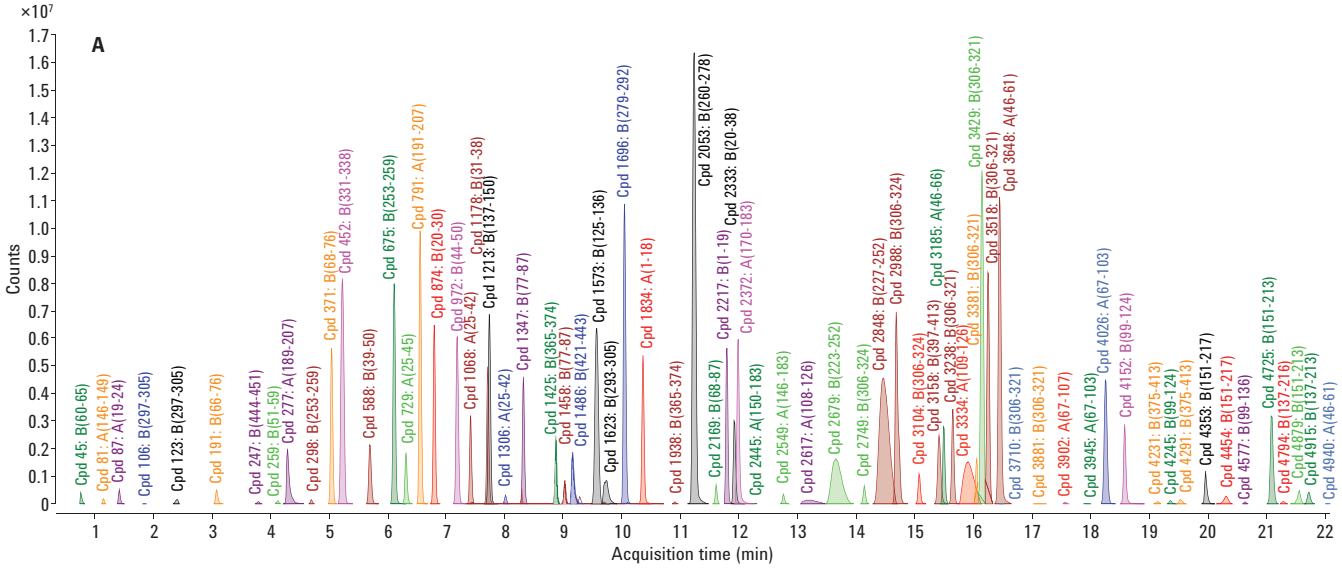
MS system

Agilent 6545XT AdvanceBio LC/Q-TOF

Results and Discussion

Peptide mapping

The tryptic digest of the mAb was analyzed by LC/MS. Figure 1A shows the identified peptide chromatogram obtained with AdvanceBio Peptide Plus column. All peptides were detected before 23 minutes with a good resolution between the peaks. Using Molecular Feature Extraction (MFE) and the BioConfirm Sequence editor, peptide masses from the LC/MS run were matched with the theoretical digest at a 5 ppm error with preferred modifications included in the antibody sequence. Results of BioConfirm analysis showed 99.26% sequence coverage. The enhanced performance of



B A: LC Monoisotopic mass: 23713.6312 Average mass: 23728.549

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  1  N-term  DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLI  48
 49  YSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEI  106
107  KRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVT  164
165  EQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC  214
     C-term
```

B: HC Monoisotopic mass: 49945.8483 Average mass: 49977.6986

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  1  N-term  EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWV  48
 49  ARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYA  106
107  MDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG  164
165  ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPPK  222
223  SCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFN  280
281  WYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK  338
339  TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYK  396
397  TTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK  451
     C-term
```

Figure 1. A) Overlaid extracted compound chromatograms for the identified peptides using an Agilent AdvanceBio Peptide Plus column. B) Sequence coverage (99.26%) map of the heavy and light chains by Agilent Bioconfirm analysis. Matched peptides are marked in green.

the AdvanceBio Peptide Plus column with high resolution and efficiency separation power along with high accuracy MS enabled the identification of most of the mAb tryptic peptides. Figure 1B shows a snapshot of the BioConfirm window for the detailed sequence coverages of heavy and light chains. The robustness of the method was evaluated by five consecutive repeated injections of the mAb tryptic digest (1 µg/injection). The sequence coverage map for each run is shown in Table 1. The results demonstrate highly reproducible sequence coverage for each run.

Table 1. Sequence coverage map reproducibility.

Replicates	Sequence coverage (%)
Replicate 1	99.26
Replicate 2	98.89
Replicate 3	98.35
Replicate 4	99.10
Replicate 5	97.29

The greater challenge of tryptic mAb digest analysis is the complexity of the sample. PTM-containing peptides represent the minority of the tryptic mixture and are often masked by more dominant peptide signals. Thus, separation of this complex digest is crucial for the identification of all the PTM-containing peptides. In the following sections, we evaluated the performance of the AdvanceBio Peptide Plus column for the separation and identification of critical PTMs such as oxidation, deamidation, and glycosylation.

Oxidation

Methionine (Met, M) oxidation is a common PTM of mAbs. The rate and extent of oxidation depends on the process and storage conditions. The DLTMISR peptide in the heavy chain region is most susceptible to oxidation. Figure 2A shows the separation of oxidized and nonoxidized DLTMISR peptide. Met-oxidation reduces the hydrophobicity of the peptide, and results in earlier elution. The peak at 16 minutes corresponds to the oxidized form, completely resolved from the native peptide. The peak at 19 minutes suggests that the DLTMISR peptide was not completely oxidized. The separation power of the column obtained between both forms suffices for calculating the relative % of oxidation, which is 1.6%. Figure 2B shows representative MS/MS spectra for DLTMISR peptide in both modified and unmodified forms. The comparison of y_4 and y_5 ion masses shows an increase in mass of approximately 16 Da in modified peptide, suggesting that oxidation of Met occurred.

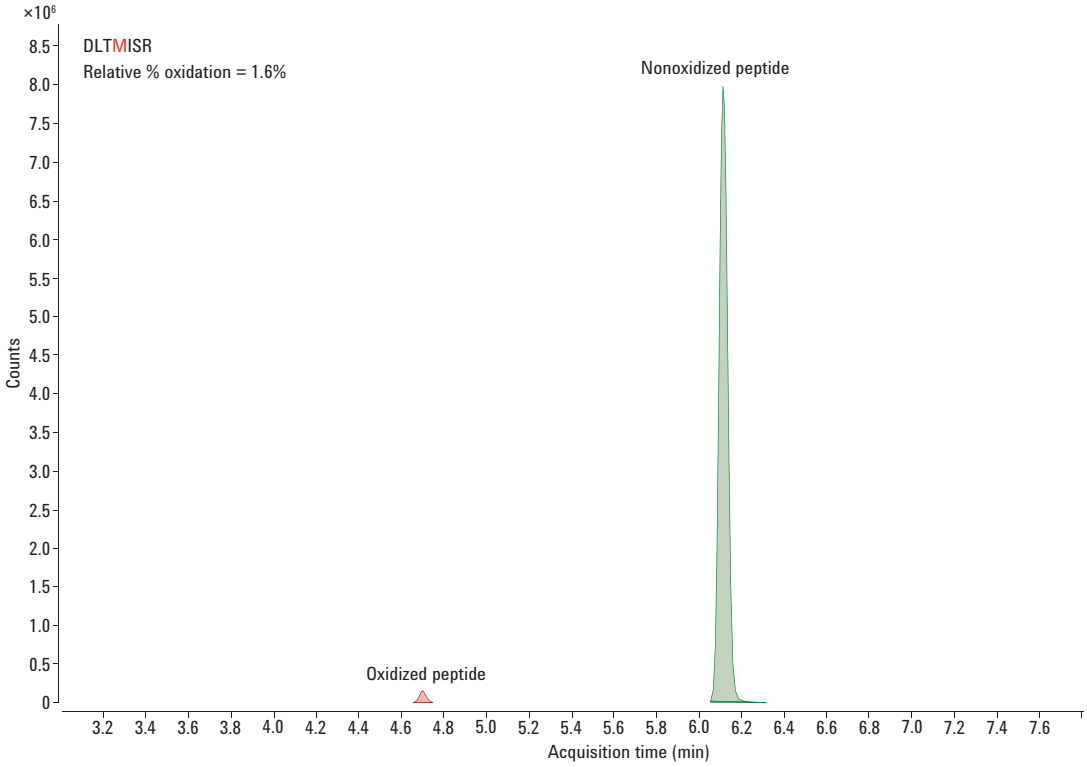

Figure 2A. Overlaid extracted compound chromatograms showing the separation of nonoxidized and oxidized DLTMISR peptide using an Agilent AdvanceBio Peptide Plus column.

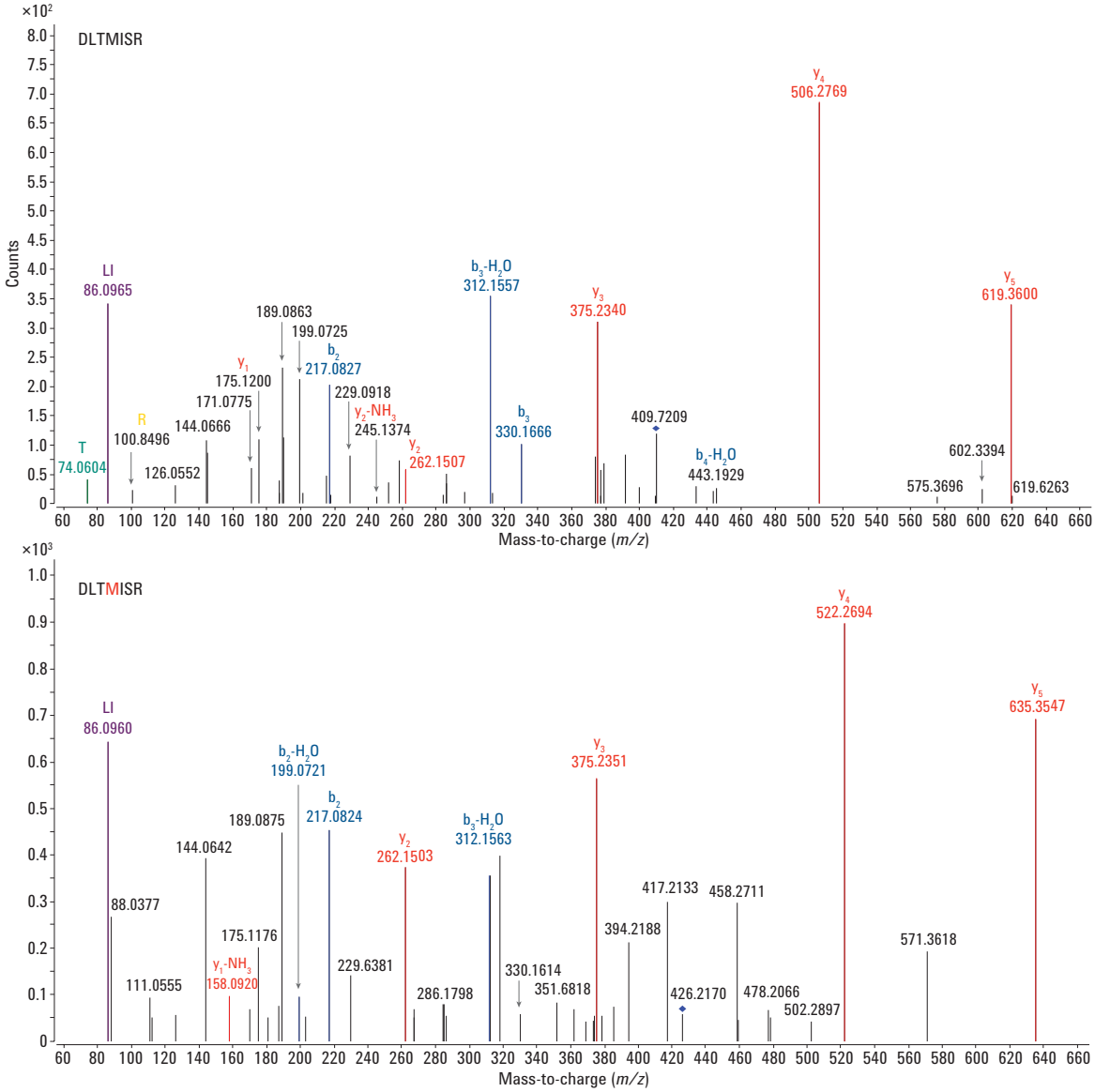

Figure 2B. MS/MS spectra between nonoxidized (upper panel) and oxidized (bottom panel) DLTMISR peptide.

Deamidation

Deamidation is the most frequent modification of proteins, and is a significant concern in manufacturing mAb biopharmaceuticals. Deamidation of asparagine (Asn, N) residues introduces a negative charge onto the protein resulting in the conversion of Asn into acidic isomers aspartic acid (Asp, D) and isoaspartic acid (isoAsp). Glutamine (Gln, Q) residues can also undergo deamidation to yield glutamic acid (Glu, E) but at a much slower rate than Asn. Deamidation results in a +0.98 Da shift, which can be identified using MS. However, it is a significant challenge for MS to distinguish the isomeric deamidation products. The structural difference between isoAsp and Asp residues in peptides changes the retention time in reversed-phase separation, and this retention time shift can be used to assign the peptide modifications.

As an example, Figure 3 shows the separation of deamidated and nondeamidated forms of two representative peptide pairs. The NTAYLQMNSLR peptide exhibited a varying degree of deamidation at multiple sites. The AdvanceBio Peptide Plus column was able to separate all of the deamidated forms from the native form, demonstrating the high resolving power of the column. In the case of NTAYLQMNSLR peptide, the relative % of deamidation varied from 15.9 to 0.21%. Figure 4B shows the representative MS/MS spectra for deamidated and nondeamidated NTAYLQMNSLR peptide.

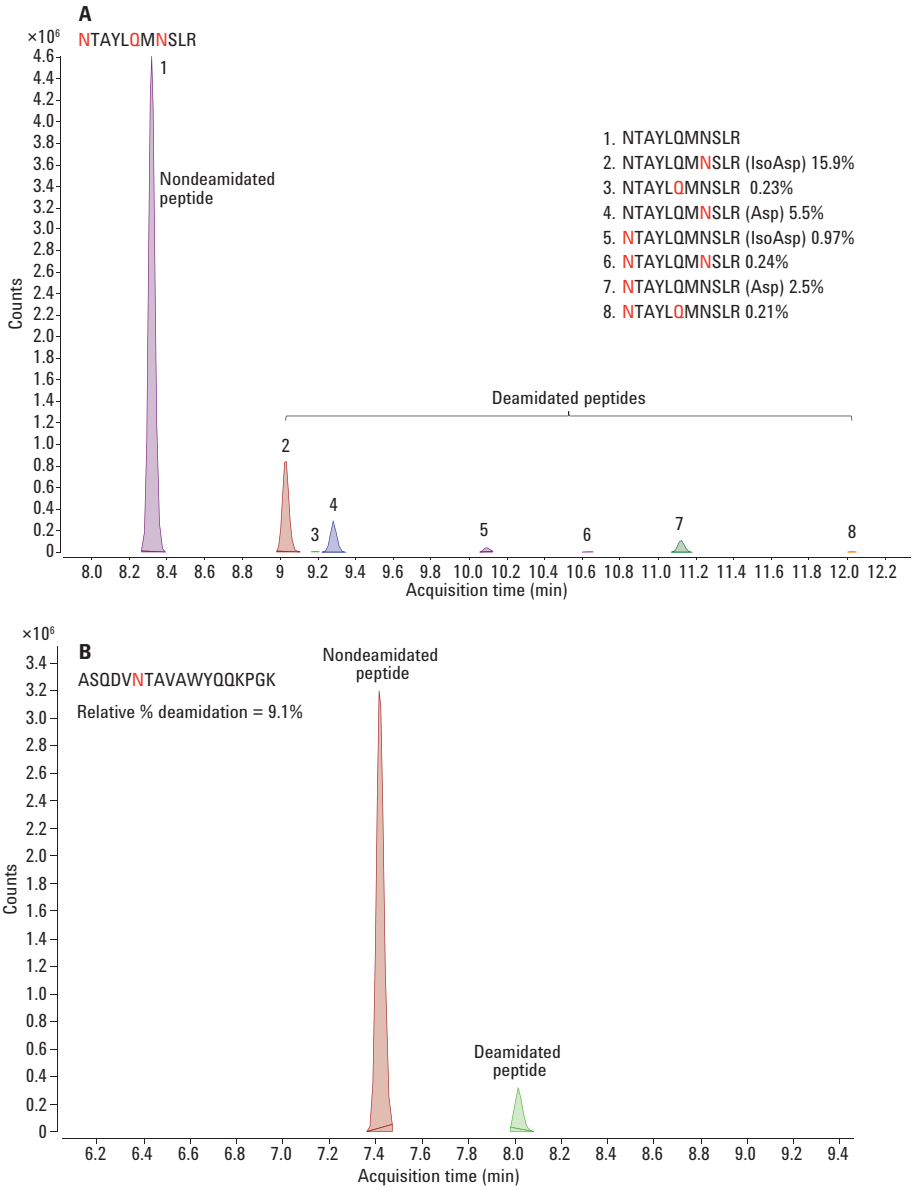

Figure 3A. Overlaid extracted compound chromatograms showing the separation of nondeamidated and deamidated peptides using an Agilent AdvanceBio Peptide Plus column. Modified sites are marked in red.

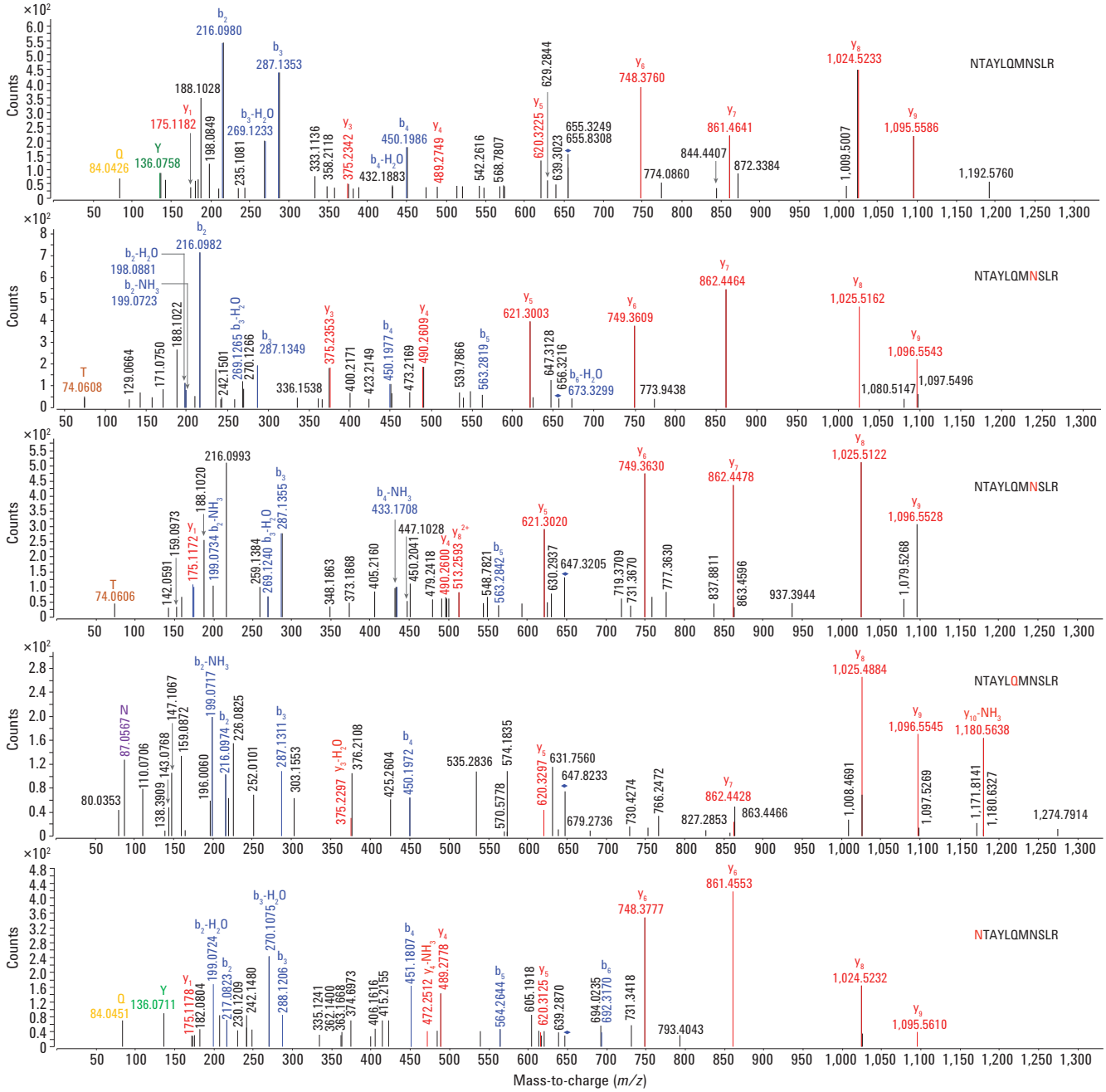

Figure 3B. MS/MS spectra between nondeamidated peptide and deamidated NTAYLQMNSLR peptide.

Glycosylation

Glycosylation is an important quality control parameter for the characterization of mAbs. Glycosylation yields a mixture of glycoforms sharing the same peptide backbone with different glycan structures. N-linked glycosylation occurs predominantly at the NXT/S motif of the peptide sequence. A combination of different a-fucosylated or fucosylated complex glycans represents the complexity of glycosylation heterogeneity. It is important to isolate these glycoforms to understand the structure/function relationship. Figure 4A shows the separation of glycosylated and nonglycosylated EEEQYNSTR peptides using the AdvanceBio Peptide Plus column. MS analysis identified multiple glycoforms (G0, G1F, G0F, and G2F) of the EEEQYNSTR peptide that were well resolved from the native peptide. As expected, the glycopeptides usually have little retention under reversed-phase conditions, and eluted close together. The quantification was performed using a relative sum percentage based on EIC peak area. The overall distribution of each glycosylated species was found to be: G0 (6.1%), G1F (47%), G0F (39%), and G2F (7.3%). Figure 4B shows the BioConfirm results for the matched glycopeptide with <5 ppm mass accuracy.

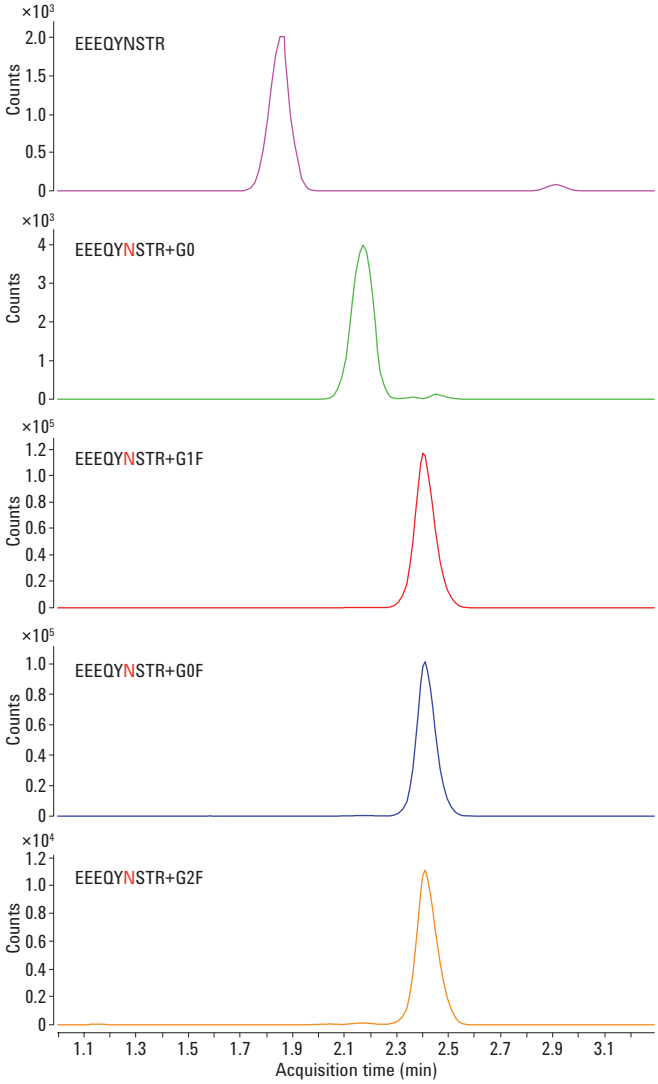

Figure 4A. Extracted ion chromatograms of *m/z* ratio matching to glycosylated and nonglycosylated EEEQYNSTR peptide.

General				Sequence Match				Molecular Feature
Label	m/z	RT	Height	Tgt Seq Mass	Sequence	Pred Mods	Diff (Bio, mDa)	Vol
Biomolecule 158: B(297-305)	595.2595	1.859	1873	1188.5047	EEQYNSTYR		-0.03	12727
Biomolecule 165: B(297-305)	829.9992	2.172	2624	2486.9807	EEQYNSTYR	1*G0 (NGA2)(+1298.475971)B301	-6.73	52349
Biomolecule 192: B(297-305)	932.7057	2.403	23019	2795.0914	EEQYNSTYR	1*G1F(+1606.586706)B301	4.02	401877
Biomolecule 205: B(297-305)	878.6872	2.408	20598	2633.0386	EEQYNSTYR	1*G0F (NGA2F)(+1444.533881)B301	3.48	332787
Biomolecule 214: B(297-305)	986.7226	2.409	5146	2957.1443	EEQYNSTYR	1*G2F (NA2F)(+1768.639531)B301	0.24	62854

Figure 4B. Agilent BioConfirm window showing the glycosylated and nonglycosylated EEEQYNSTR peptide details.

Conclusions

- This application note demonstrates robust and reliable peptide mapping using an Agilent AdvanceBio Peptide Plus column.
- High-resolution and high-efficiency separation of mAb peptide map provided >99% sequence coverage.
- The AdvanceBio Peptide Plus column generated well resolved peptide peaks, enabling significant improvement for peptide mapping separation.
- Precise characterization of PTMs was achieved using an AdvanceBio Peptide Plus column and the Agilent Accurate Mass 6545XT AdvanceBio LC/Q-TOF.

Reference

1. Suresh Babu C. V. *LC/MS analysis of peptide mapping with formic acid ion-pairing agent*; Application note, Agilent Technologies, Inc. Publication number 5991-7979EN, **2017**.

For More Information

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