Application Note

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Peptide Mapping and Quantitation of Oxidation and Deamidation in Monoclonal Antibodies

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Abstract

This application note demonstrates an integrated workflow of peptide mapping and quantitation of chemical-induced oxidation and deamidation in monoclonal antibodies (mAbs). The mAbs identity were verified with high-sequence coverage. Oxidation and deamidation at various sites in the mAbs were quantified intuitively by the optimized MassHunter BioConfirm algorithm. The results demonstrated excellent reproducibility highlighting the benefits of automated sample preparation using the AssayMAP Bravo system.

Introduction

Monoclonal antibodies (mAbs) are engineered to function as substitute antibodies. They can restore, enhance, adjust, or replicate the immune system's response to harmful cells.

The ICH Guideline Q6B[1] outlines the use of peptide mapping specifications as one of acceptance criteria in biological product evaluation. The product identity is verified through its amino acid sequence from peptide mapping. In addition, peptide mapping is a valuable tool for evaluating product stability by monitoring changes in post-translational modification (PTM) sites. High-resolution LC/MS has been widely accepted as a platform method for peptide mapping.

Oxidation and deamidation are the most common chemical degradation processes of mAbs.[2] They can cause changes in physical properties such as hydrophobicity, charge, and high-order structure. These changes can alter the binding affinity, half-life, and efficacy of the product. Therefore, understanding oxidation and deamidation processes and developing strategies to mitigate their impact is crucial for the successful development and application of mAb-based therapies.

Automated sample preparation in mAb digestion offers several advantages over manual digestion, including consistent and reproducible results, time efficiency, low risk of contamination, high precision, and high-throughput analysis.

In this study, a peptide mapping workflow was executed to verify the sequences of two mAb samples and quantify induced deamidation and oxidation. The integrated workflow includes an Agilent AssayMAP Bravo platform, an Agilent 1290 Infinity II Bio LC system with an Agilent AdvanceBio Peptide Mapping column, an Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software (Figure 1).

Experimental

Materials

Guanidine hydrochloride (GdnHCl), Trizma base, tris(2-carboxyethyl)phosphine (TCEP), 2-iodoacetamide (IAA), trypsin, trifluoroacetic acid (TFA), 30% (v:v) hydrogen peroxide (H_2O_2), and acetonitrile (LC/MS grade) were purchased from MilliporeSigma (Burlington, MA, USA). Formic acid (LC/MS grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA). The NISTmAb reference material was from Agilent Technologies (Santa Clara, CA, USA). The rituximab sample was purchased from Alliance Pharm (Singapore, Singapore). Ultrapure water was collected from an in-house MilliporeSigma Milli-Q system (Burlington, MA, USA).

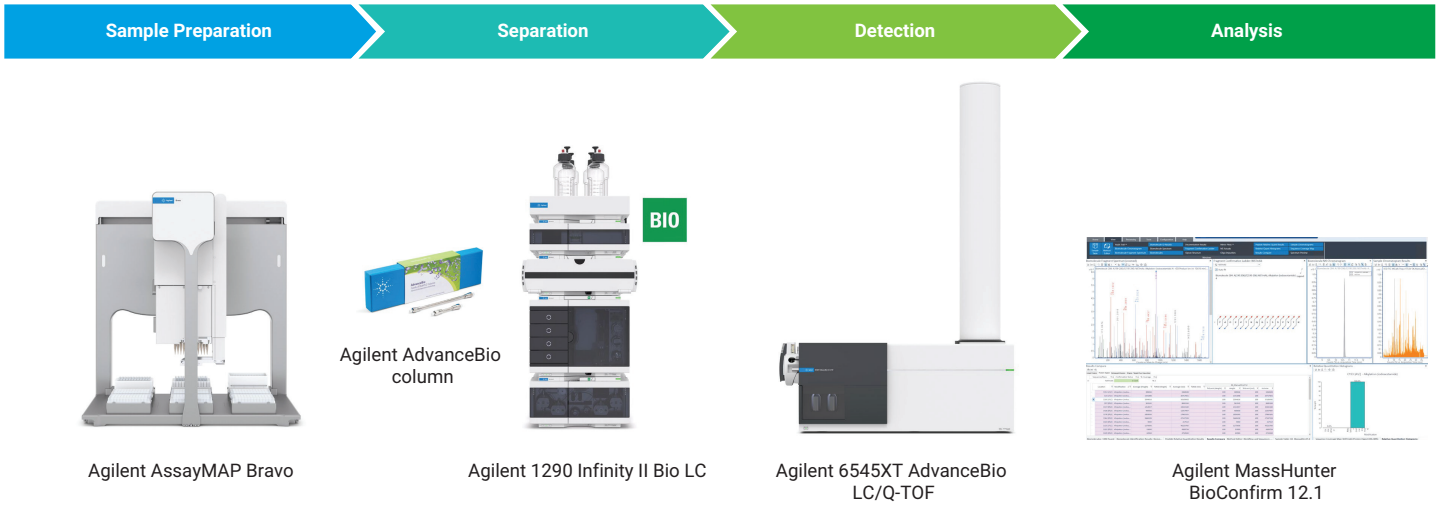

Figure 1. Integrated peptide mapping workflow with Agilent equipment.

Chemical induction and sample preparation

To induce oxidation, samples were incubated at different concentrations of the oxidizing agent H_2O_2 (0, 0.002%, 0.02%, 0.2% v:v) for 24 hours at room temperature. To induce deamidation, samples were exposed to 37 °C in a Tris buffer pH 8.9 for three days.

All samples were reduced, alkylated, trypsin-digested, and cleaned using the AssayMAP Bravo platform.[3] Table 1 lists the reagents and cartridge used in the sample preparation. Digested samples were subjected to LC/MS analysis.

Table 1. Sample preparation reagents.

On-Deck Reagents	
Parameter	**Value**
In-Solution Digestion Single Plate v2.0	
Reduction	8 M GdnHCl (4.8 M final) 450 mM Tris base (270 mM final) 16.7 mM TCEP (10 mM final)
Alkylant	132 mM IAA (12 mM final)
Diluent	Water (GdnHCl 0.9 M final)
Protease	Trypsin, 0.125 µg/µl (1:20 final)
Acidification	10% TFA (1% final)
Peptide Cleanup v2.0	
Cartridges	5 µL C18 cartridges
Priming and Syringe Wash	0.1% TFA, 50% acetonitrile
Equilibration and Cartridge Wash Buffer	0.1% TFA
Elution Buffer	0.1% TFA, 50% acetonitrile
Eluate Collection Plate	0.1% TFA

Instrumentation

- Agilent AssayMAP Bravo (G5571AA)
- Agilent 1290 Infinity II Bio LC system including:
  - Agilent 1290 Infinity II Bio high-speed pumps (G7132A) with Agilent Bio Jet Weaver, 35 µL volume (G7132-68135)
  - Agilent 1290 Infinity II Bio multisampler (G7137A) with Agilent InfinityLab Sample Thermostat (option #101, G4761A)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B) equipped with Agilent bio-inert Quick-Connect heat exchanger, standard flow (option #065, G7116-60071)
- Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent Dual Jet Stream ESI source

Software

- Agilent VWorks automation control software 14.1
- Agilent MassHunter data acquisition software 11.0
- Agilent MassHunter BioConfirm software 12.1

2D-LC/MS analysis

Tables 2 and 3 list the parameters for LC and MS data acquisition used in the workflow.

Table 2. Liquid chromatography parameters.

Parameter	Value
Column	Agilent AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm, 120 Å
Thermostat	4 °C
Solvent A	0.1% Formic acid
Solvent B	90% Acetonitrile and 0.1% formic acid in H_2O
Flow Rate	0.4 mL/min
Gradient	Time (min) %B 0.0 3 30.0 40 33.0 90 35.0 90 37.0 3
Stop Time	40 minutes
Injection Volume	10 µL or 6 µg on column
Column Temperature	60 °C

Table 3. MS data acquisition parameters.

Parameter	Value
Source	Agilent Dual Jet Stream ESI
Polarity	Positive
Drying Gas Temperature	325 °C
Drying Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temperature	275 °C
Sheath Gas Flow	12 L/min
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Acquisition Mode	Extended dynamic range (2 GHz)
Mass Range	*m/z* 300 to 1,700
Acquisition Rate	8 spectra/sec
Auto MS/MS range	*m/z* 50 to 1,700
Minimum MS/MS Acquisition Rate	3 spectra/sec
Isolation Width	Narrow (~ *m/z* 1.3)
Precursor/Cycle	Top 10
Collision Energy	3.1*(*m/z*)/100 + 1 for charge 2; 3.6*(*m/z*)/100 – 4.8 for charge 3 or greater than charge 3
Threshold for MS/MS	1,000 Counts and 0.001%
Dynamic Exclusion On	1 Repeat, then exclude for 0.2 minutes
Precursor Abundance-Based Scan Speed	Yes
Target	25,000 counts/spectrum
Use MS/MS Accumulation Time Limit	Yes
Purity	100% Stringency, 30% cutoff
Isotope Model	Peptides
Sort Precursors	By charge state, then abundance; +2, +3, > +3

Data processing

Data acquired were processed following the Protein Digest workflow in MassHunter BioConfirm 12.1 software. Key method parameters are listed in Table 4.

Table 4. Protein Digest processing method parameters.

Parameter	Value
Condition	Reduced
Mods and Profiles	Alkylation (iodoacetamide), deamidation, oxidation
Enzyme	Trypsin
Find Peptides	Display biomolecules containing MS/MS scans
Match Tolerances	MS match tolerance: ± 10 ppm MS/MS match tolerance: ± 20 ppm Warn if score is < 7.00 Do not match if score is < 5.00 Allow missed cleavages up to 2 Peptide length range: 2 to 60 Allow terminal truncation Maximum number of modifications: 4

Results and discussion

MassHunter BioConfirm 12.1

Beginning with version 12.1, MassHunter BioConfirm has been upgraded with a contemporary user interface that aligns with other Agilent software programs, such as Agilent OpenLab CDS. This enhancement fosters a consistent user experience across most MassHunter and OpenLab applications.

Figure 2 presents a user-defined layout of the Protein Digest workflow. The fragment confirmation ladder marks fragment ions based on their occurrence in the MS/MS spectra. Amino acids lacking coverage are displayed in gray. This feature facilitates a rapid assessment of the quality of the MS/MS spectra for the biomolecule.

Sequence coverage

Peptide sequences of both NISTmAb and rituximab were confirmed with sequence coverage above 95%. The biomolecule MS chromatogram of digested NISTmAb is shown in Figure 3.

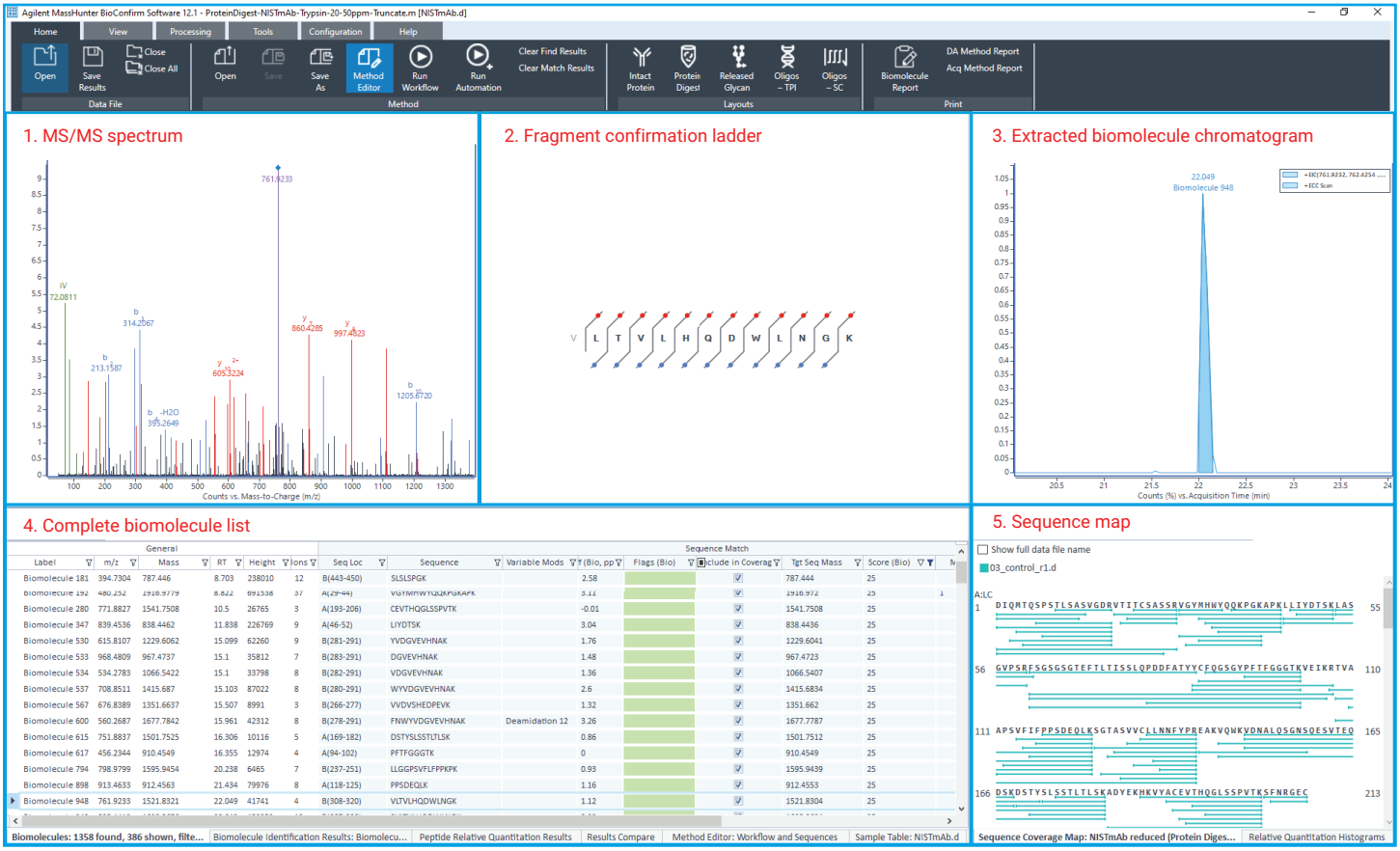

Figure 2. Agilent MassHunter BioConfirm 12.1 Protein Digest workflow layout.

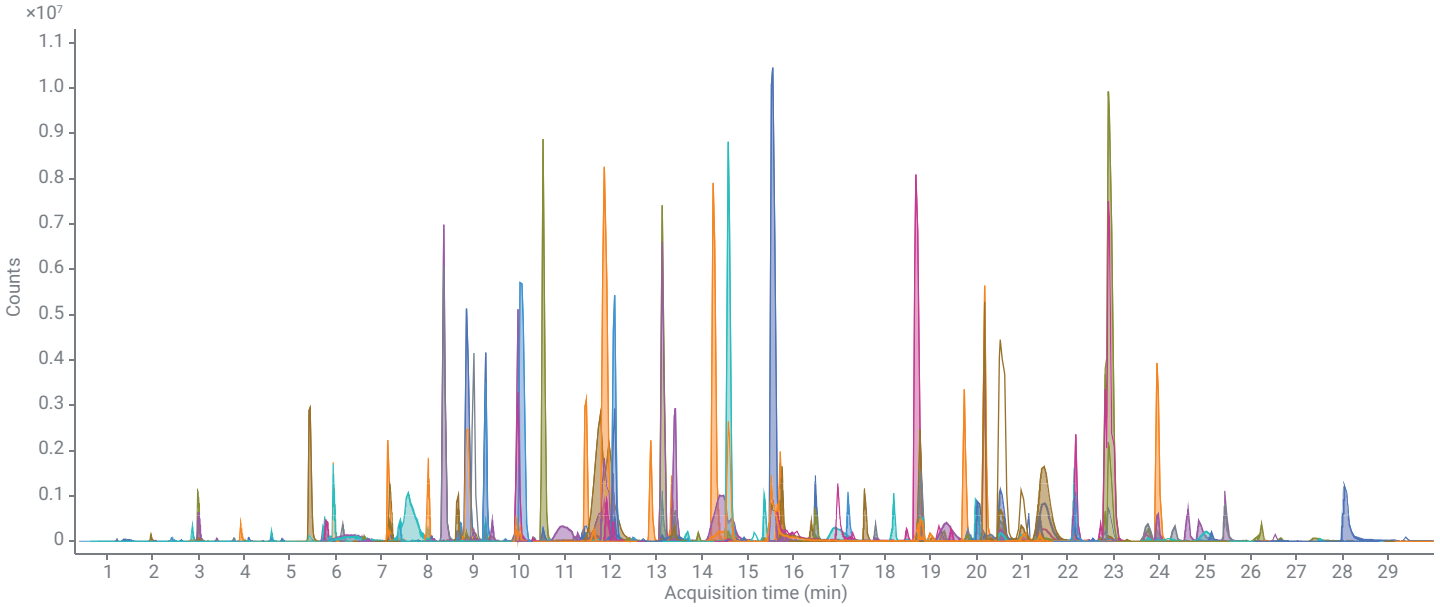

Figure 3. Biomolecule MS chromatogram of peptides from trypsin-digested NISTmAb.

Identification and quantitation of oxidation

Methionine (Met) oxidation is the most commonly observed PTM in mAbs. When the oxidized methionine is in the complementarity-determining region (CDR), it can affect antigen binding and reduce biological activity.[4] The level of Met oxidation in both NISTmAb and rituximab was assessed after being exposed to different concentrations of H_2O_2 solution for 24 hours at room temperature. The mass of oxidized peptide shifts by 16 Da, as compared to a wild type (WT) unmodified one.

Figure 4 shows the quantitative histograms of Met oxidation at M255 and M4 residues of NISTmAb and M256 of rituximab. For all the three sites, the oxidation level increased in proportion to the concentration of H_2O_2. The quantitation results showed excellent reproducibility with standard deviation less than 2.5% across four technical replicates (n = 4) for all the samples.

In the NISTmAb, M255 was more susceptible to oxidative stress than M4 when exposed to 0.002% and 0.02% H_2O_2. However, both sites underwent almost complete oxidation at 0.2% H_2O_2. M255 is located in the heavy chain Fc domain, while M4 is in the light chain N-terminus. The site-specific oxidation could be due to the accessibility of methionine to the oxidizing reagent or its exposure to the solvent.

As for rituximab, its sequence was engineered to eliminate some methionines, particularly in the CDR, to enhance product stability. The M256 in rituximab is in similar location to M255 in NISTmAb; therefore, it exhibited a similar response to varying concentrations of H_2O_2-induced oxidation.

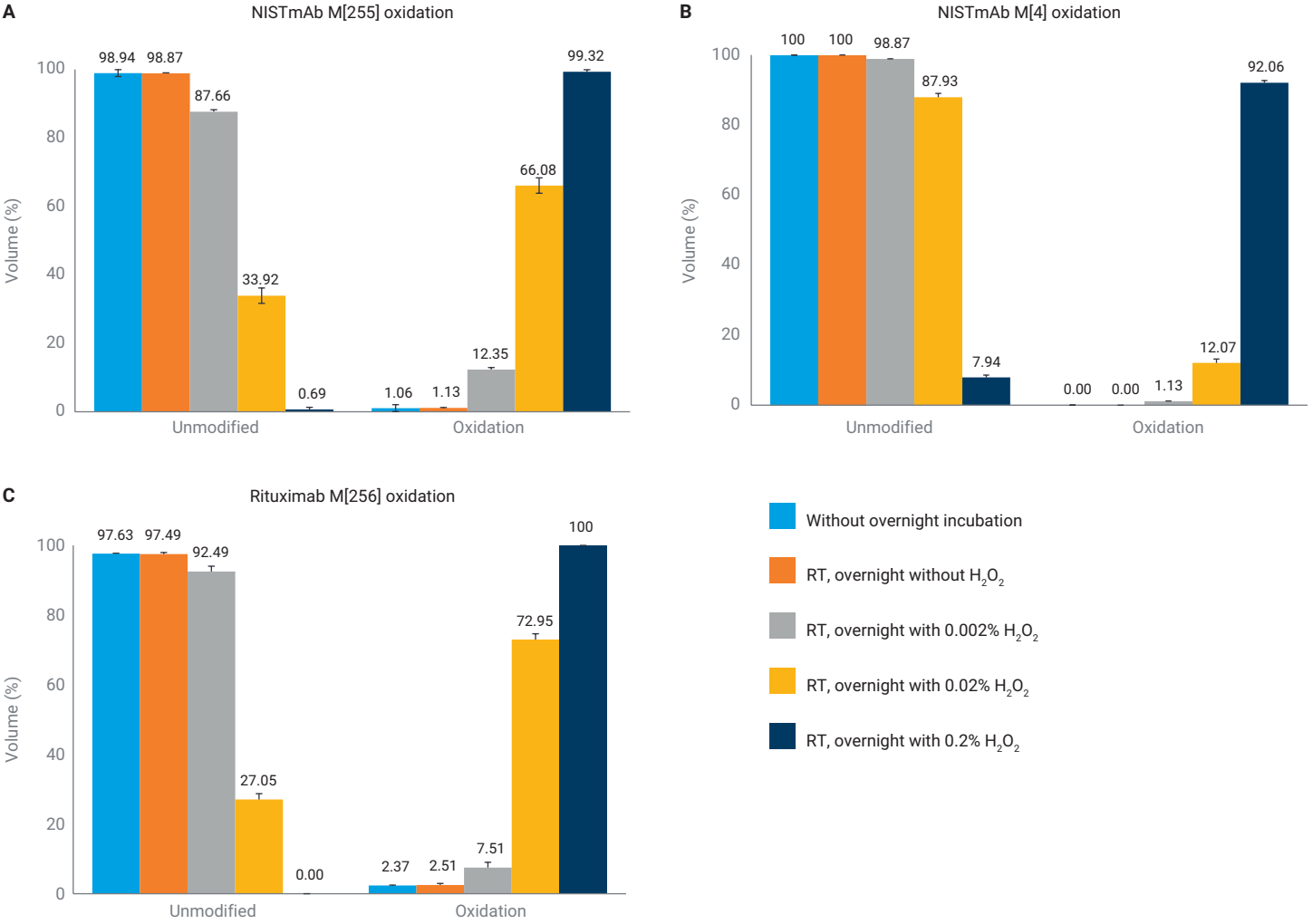

Figure 4. Quantitative histograms of Met oxidation at (A) M255 of NISTmAb, (B) M4 of NISTmAb, and (C) M256 of rituximab.

Identification and quantitation of deamidation

Deamidation is another common PTM in monoclonal antibodies, which can affect the stability, efficacy, and immunogenicity of the antibody. In deamidation, asparagine or glutamine residues convert to (iso)aspartic acid or glutamic acid, respectively. The change leads to a mass shift of 0.984 Da on the deamidated peptide. Deamidation can be a result of exposure to high pH environments and elevated temperatures.

Figure 5 shows that after incubation, three deamidated forms were observed in the peptide NQVSLTCLVK of NISTmAb, including glutamine deamidation, asparagine deamidation, and aspartic acid isomerization. All the forms including WT eluted within a 2-minute time window, and the modified forms were well separated from the WT. The relative amount of deamidated Q365 was 1.38% and deamidated N364 was 3.09%.

MassHunter BioConfirm software is designed to intelligently determine the most suitable PTM localization for biomolecules based on the MS/MS score (Figure 6). In this case, a score above 7 was considered a good match. However, the software also provides flexibility by allowing users to choose other biomolecules from the list as they see fit.

By comparing the MS/MS spectra of WT and deamidated biomolecules (Figure 7), the correct localization assignment by the software was confirmed. The b_2 product ion in the middle and lower spectra shifted by 1 Da compared to WT spectra, indicating deamidation at either site of N or Q in the peptide. The zoomed-in spectra (Figure 7 inset) shows the b_1 ion in the middle spectra remained same as WT. This confirms the deamidation happens on the second amino acid Q in the sequence. The N364 deamidation was further confirmed with an unshifted y_9^{2+} ion.

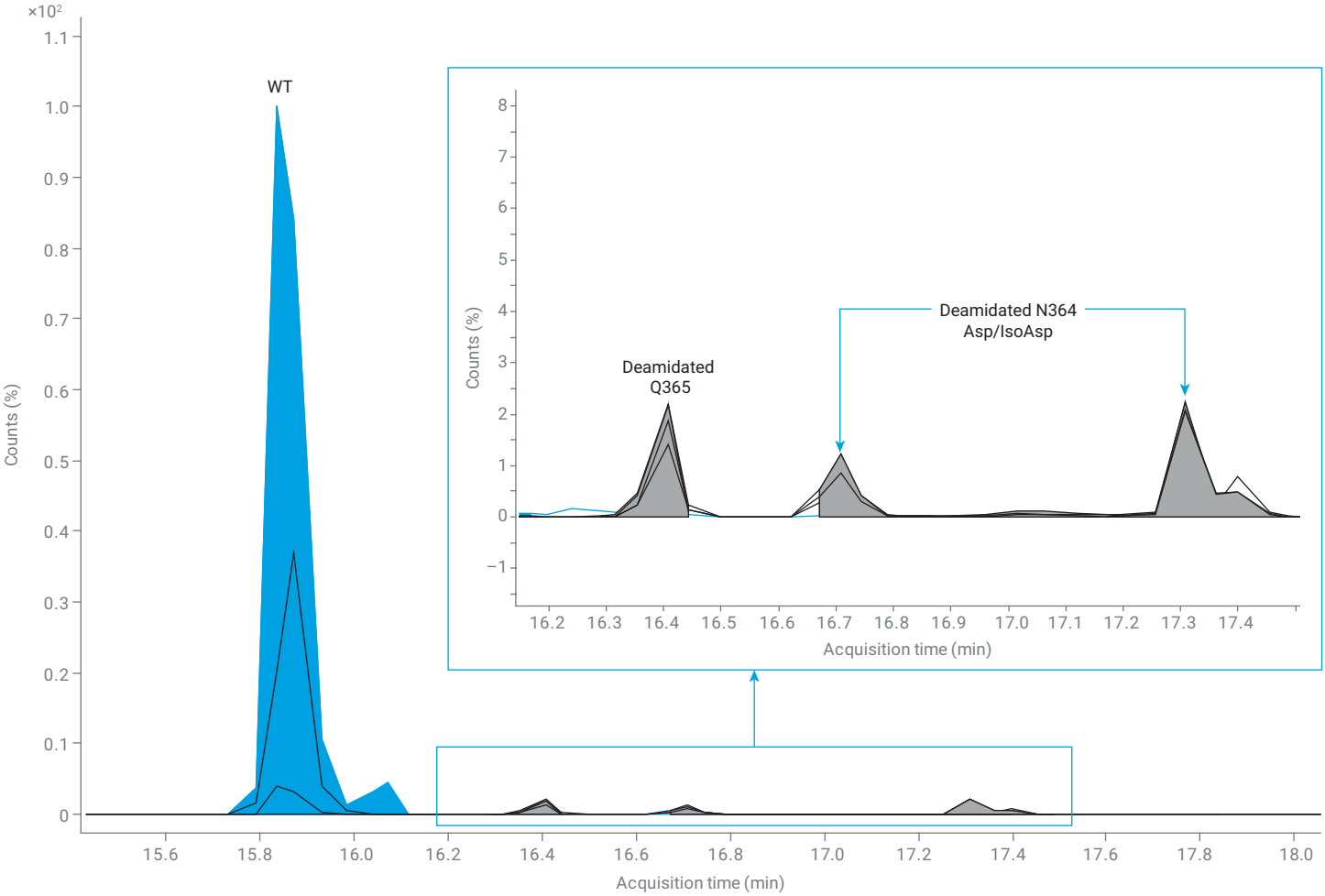

Figure 5. Biomolecule MS chromatogram of WT (15.8 minutes) and three deamidated forms of the peptide NQVSLTCLVK in NISTmAb including deamidated Q365 (16.4 minutes) and deamidated N364 (16.7 and 17.3 minutes).

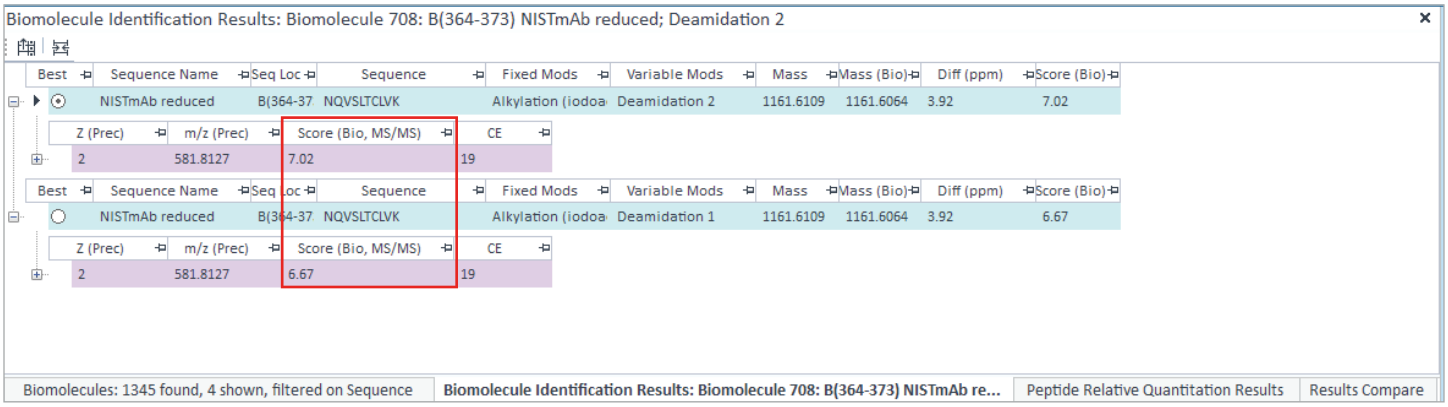

Figure 6. Snapshot of the biomolecule identification results panel in Agilent MassHunter BioConfirm software for deamidation site assignment of the peptide NQVSLTCLVK.

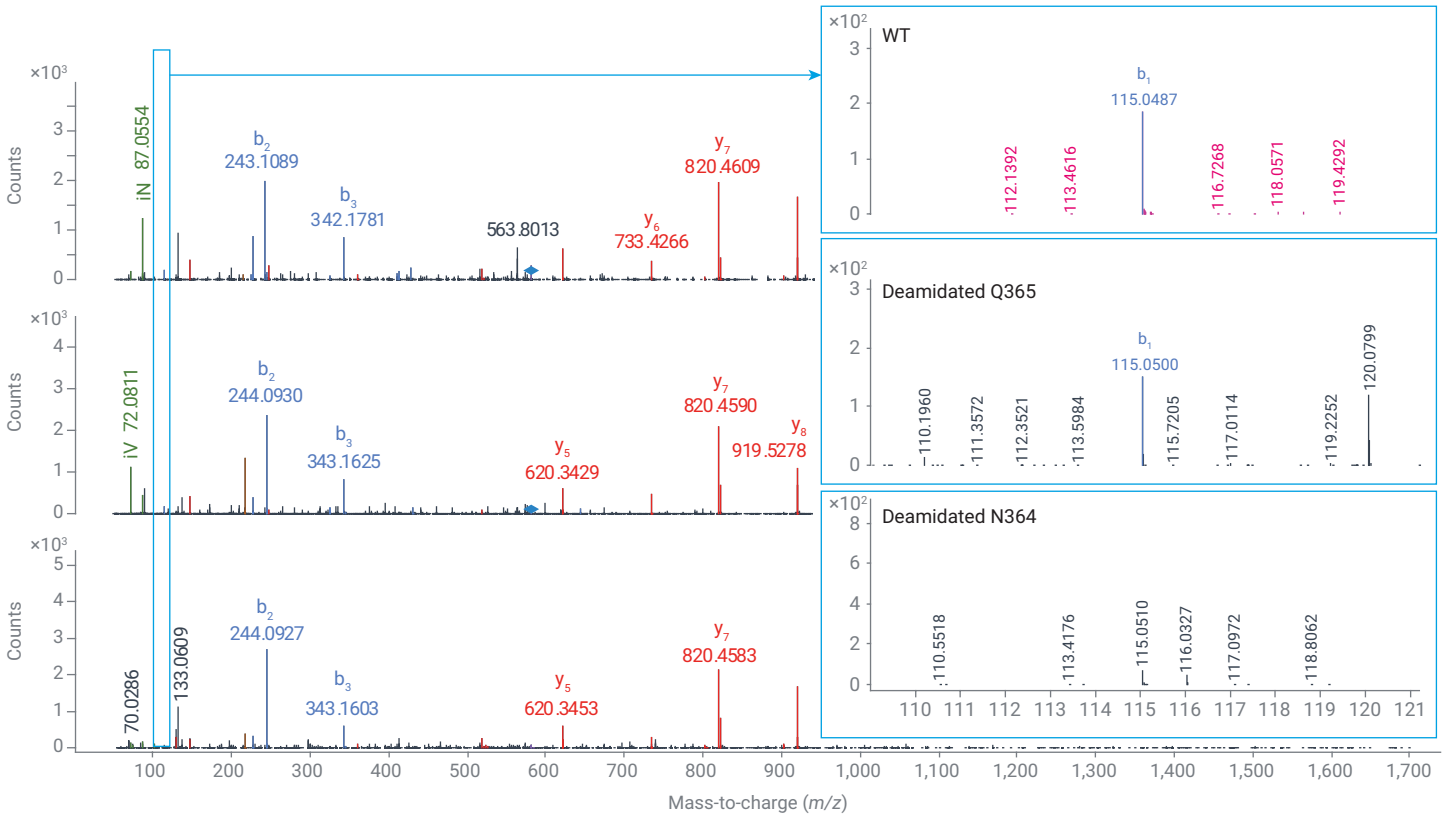

Figure 7. Comparison of MS/MS spectra between WT and the Q365 and N364 deamidated forms of the peptide NQVSLTCLVK in Agilent MassHunter BioConfirm software.

Conclusion

A complete workflow including automated sample preparation was demonstrated for mAb peptide mapping and oxidation and deamidation quantitation. The workflow involved an Agilent AssayMAP Bravo platform, LC separation with an Agilent 1290 Infinity II Bio LC system, data acquisition with the Agilent 6545XT AdvanceBio LC/Q-TOF, and data analysis with Agilent MassHunter BioConfirm 12.1 software.

Protein sequences were confirmed with high sequence coverage. The fragment confirmation ladder feature enables simple visualization of MS/MS fragment ions identified in matched peptides. The software streamlines the identification and quantitation of oxidation and deamidation in mAbs with its enhanced algorithm. Good quantitation reproducibility was achieved with AssayMAP protein digestion automation.

References

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