Application Note

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Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies

Using Agilent 6545XT AdvanceBio LC/Q-TOF and Agilent MassHunter BioConfirm Software

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Introduction

Modifications such as asparagine (Asn) deamidation, aspartate (Asp) isomerization, and methionine (Met) oxidation are typical degradation products for recombinant antibodies. Previous studies have shown that degradation of Asn, Asp, and Met residues in mAbs can affect protein activity[1-4]. Therefore, those modifications in a protein drug candidate, for example, a mAb, are critical quality attributes (CQAs), and are closely monitored under storage and formulation conditions. They are often the focus of stress and forced degradation studies conducted during drug development. To assess these CQAs, simultaneous identification and quantification are needed.

This Application Note demonstrates simultaneous identification and quantification of chemical-induced deamidation and oxidation on recombinant mAbs with a peptide-mapping method using an integrated workflow including an Agilent AssayMAP Bravo platform, an Agilent 1290 Infinity II LC, an Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software (Figure 1).

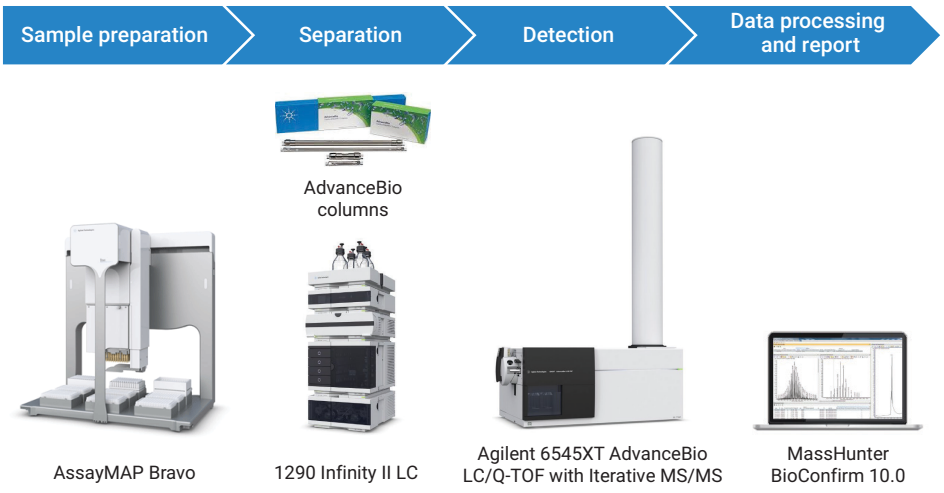


Figure 1. Integrated workflow for peptide mapping.

Experimental

Materials

The mAb1 sample was a recombinant CHO-cultured IgG1 mAb, which was produced and purified by a third-party partner. NISTmAb was purchased from the National Institute of Standards and Technology (NIST).

Instrumentation

- Agilent AssayMAP Bravo system (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)
- Dual Agilent Jet Stream ESI source (G1958-65268)

Chemical induction and sample preparation

To induce significant levels of Asn deamidation, mAb1 samples were exposed to elevated temperature (37 °C) in a Tris-HCl buffer system at pH 8.7 for 0, 3, 6, and 13 days, respectively. To induce Met oxidation, mAb1 and NISTmAb samples were incubated in Tris-HCl buffers containing different concentrations of oxidizing agent H_2O_2 (ranging from 0 to 0.2 % v/v) overnight at room temperature. All samples were lyophilized and stored in –80 °C before sample digestion.

All samples were reduced, alkylated, trypsin-digested, and desalted using the AssayMAP Bravo platform[5]. Digested samples were subjected to LC/MS analysis.

LC/MS analysis

LC separation was performed on an Agilent reversed-phase C18 column with a charged surface (2.1 × 150 mm, 2.7 µm) using a 30-minute gradient (Table 1). The raw data were acquired by a 6545XT AdvanceBio LC/Q-TOF (Table 2).

Table 1. Liquid chromatography parameters.

LC parameters	
Analytical column	Agilent reversed-phase C18 column with a charged surface
Mobile phase A	H_2O, 0.1 % formic acid
Mobile phase B	90 % acetonitrile and 0.1 % formic acid in H_2O
Column temperature	60 °C
Flow rate	0.4 mL/min
Gradient	0.0 minutes → 3 %B 30.0 minutes → 22 %B 32.0 minutes → 90 %B 35.0 minutes → 90 %B 37.0 minutes → 3 %B
Stop time	40 minutes

Table 2. MS parameters.

Parameter	Value
Instrument	6545XT AdvanceBio LC/Q-TOF
Gas temperature	325 °C
Drying gas flow	13 L/min
Nebulizer	35 psig
Sheath gas temperature	275 °C
Sheath gas flow	12 L/min
VCap	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
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ *m/z* 1.3)
Precursors/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 from LC/MS/MS analysis were processed using MassHunter BioConfirm 10.0 software. For the chemical-induced deamidation study, searching parameters were set up as trypsin digest allowing semitryptic peptides and maximum two missed cleavages, fixed modifications containing cysteine (C) alkylation and N-terminal pyroGlu (E/Q), variable modifications containing asparagine (N) or glutamine (Q) deamidation, and methionine (M) oxidation. Mass tolerance allows 10 ppm for MS1 and 20 ppm for MS2. Peptide length was limited to 5 to 60 amino acids (AAs). Peptide-spectrum matches required MS/MS features, and were filtered by a 0.1 % false discovery rate (FDR). For the chemical-induced oxidation study, the searching parameters were the same as the induced deamidation study. An exception was that tryptophan (W) oxidation (+4 Da, +16 Da, or +32 Da) was added[6].

Results and discussion

Separation and identification of deamidated peptides

Asn deamidation is one of the most frequently observed modifications in proteins and peptides. It is a major source of instability in formulation and storage conditions during biopharmaceutics production. The Asn residue can be deamidated either through hydrolysis to form Asp or through a cyclic succinimide intermediate to form both aspartate or iso-aspartate. Since there is only a 0.984 Da shift for deamidation, it could be difficult to accurately identify and quantify deamidation and aspartate isomerization when the different forms coelute.

The deamidation of Asn in the conserved CH_3 region has been identified as being solvent-accessible and sensitive to chemical degradation in previous reports[3,4]. The PENNY peptide matched to this region (heavy chain sequence location 376–397, GFYPSDIAVEWESN$_{389}$GQPEN$_{394}$N$_{395}$YK) contains three Asn residues, each with a different susceptibility to deamidation under exposure to high pH conditions. We used this peptide to demonstrate the simultaneous identification and quantification of Asn deamidation and Asp isomerization using the Agilent peptide-mapping workflow.

Figure 2 shows the extracted compound chromatograms (ECCs) for the various PTM forms of the PENNY peptide using a 30-minute LC gradient. Seven different forms of the PENNY peptide are shown, including the unmodified wildtype (WT) peptide, the N$_{389}$ Asn deamidation/Asp isomerization, and the double deamidation/Asp isomerization forms. It shows that all the forms were dispersed within a six-minute time window, and the modified forms were well separated from the unmodified form.

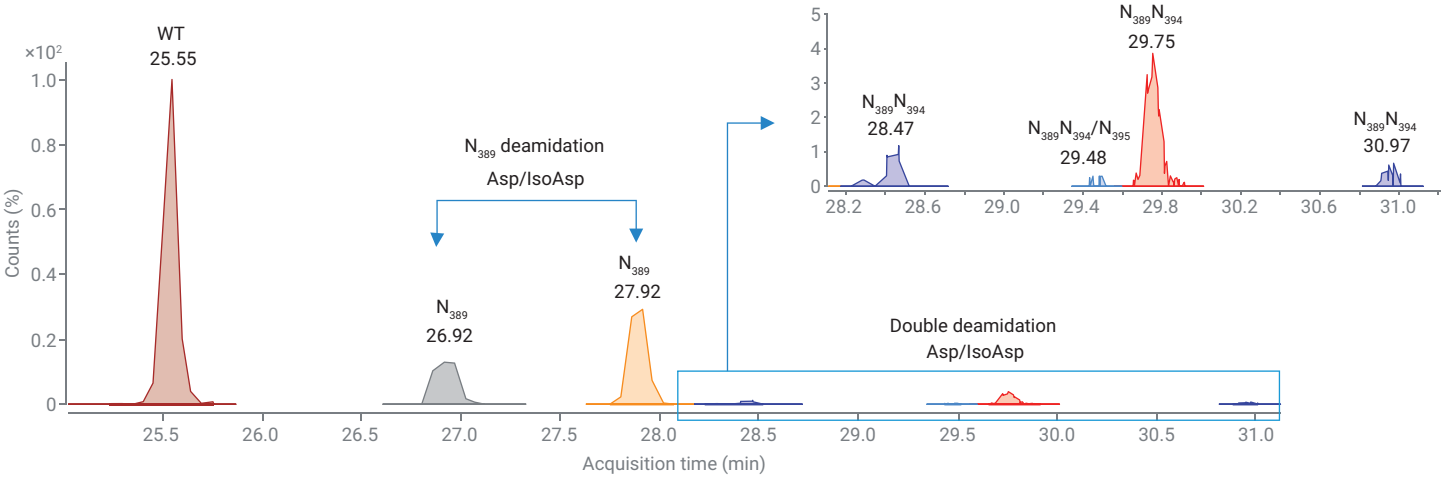


Figure 2. ECC of Asn deamidation, Asp isomerization, and wild type forms of the GFYPSDIAVEWESN$_{389}$GQPEN$_{394}$N$_{395}$YK peptide on a reversed-phase C18 column with charged surface using a 30-minute LC gradient .

MassHunter BioConfirm software provides a user-friendly interface for PTM analysis (Figure 3). One of the software features allows an analyst to select the peptides of interest in the biomolecules table for side-by-side comparison of their MS/MS spectra. Figure 4 shows the MS/MS spectrum comparison of the wild type (WT, RT = 25.55 minutes) and the double deamidated form (N_{389}, N_{394}, RT = 30.97 minutes) of the PENNY peptide using BioConfirm software. The spectra can be zoomed synchronously for detailed fragment ion comparison. The y_3 product ions show the same *m/z* on both WT and deamidated forms, confirming that there is no deamidation on N_{395}. The y_6 product ion has +1 Da mass shift, and the y_{10} product ions has +2 Da mass shift on deamidated peptides, showing that the double-deamidated sites are at N_{389} and N_{394}.

All the MS/MS spectra for the peptide forms shown in Figure 2 have been inspected to verify the assignment of deamidation localization. Almost all the deamidated forms were unambiguously identified with their deamidation localization. An exception was that the double deamidated forms (N_{389}, N_{394}/N_{395}) with a retention time of 29.48 minutes contain an ambiguous Asn deamidation assignment on N_{394} or N_{395}.

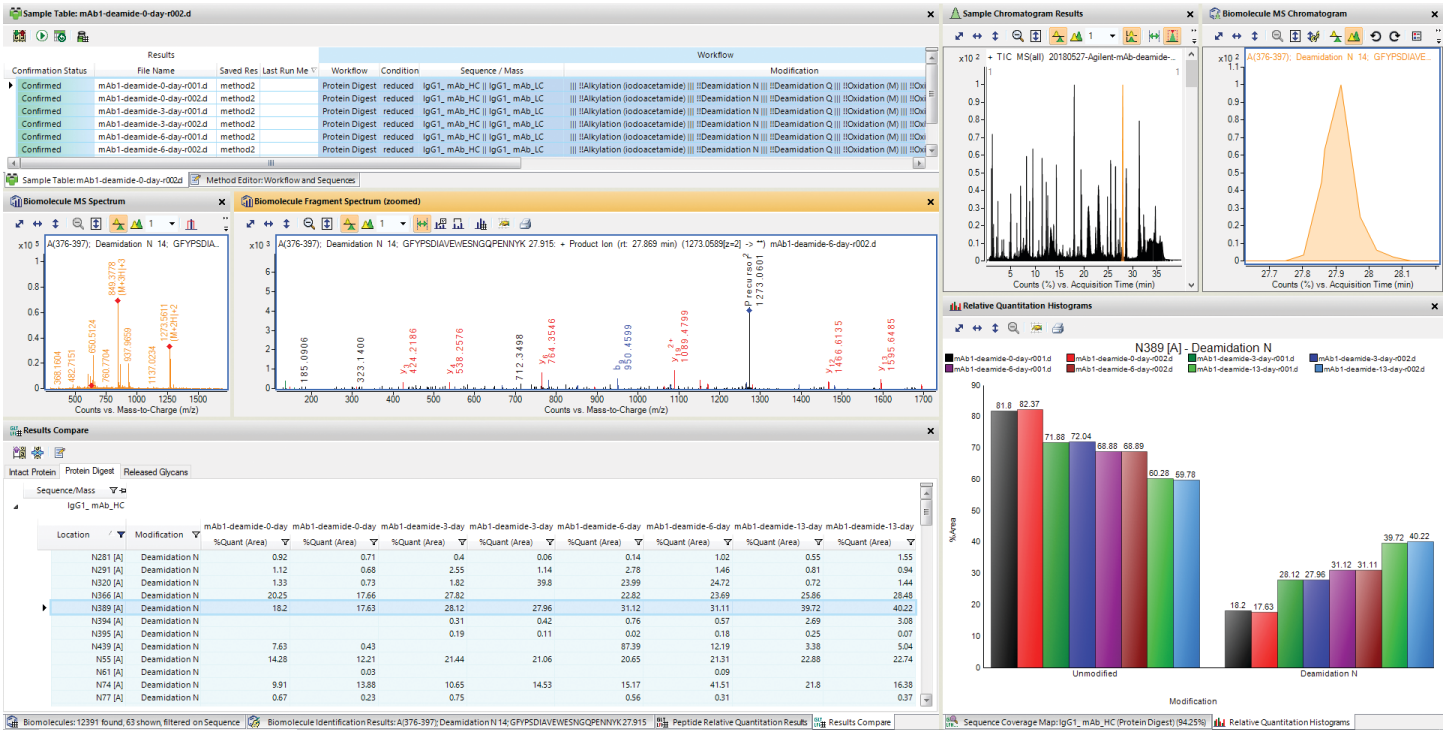


Figure 3. Screenshot of MassHunter BioConfirm 10.0 software for PTM identification and quantitation.

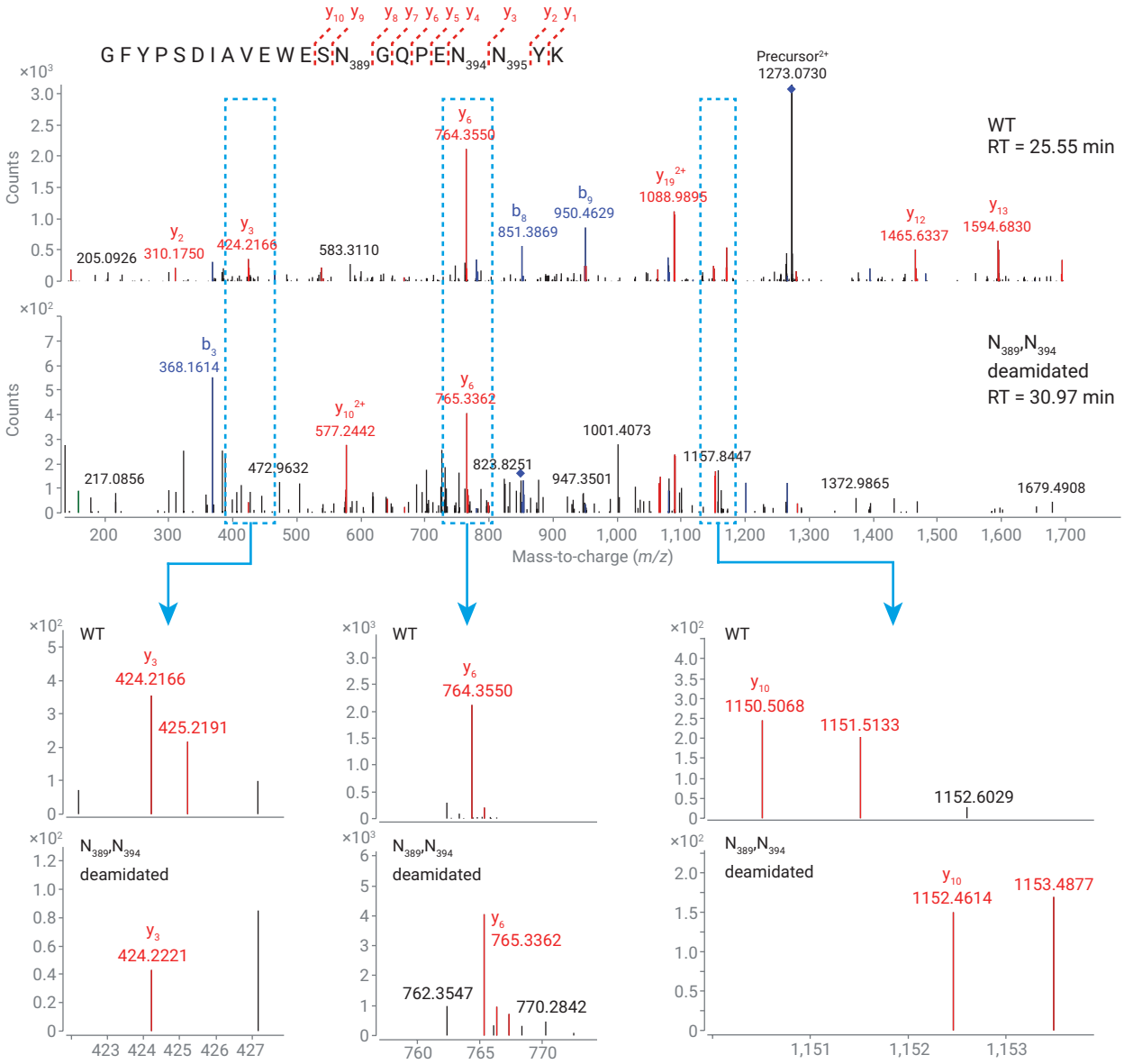


Figure 4. Comparison of MS/MS spectra between wild type and the double-deamidated form (N_{389}, N_{394}, RT = 30.97 minutes) of the peptide GFYPSDIAVEWESNGQPENNYK using MassHunter BioConfirm software. The fragment ions with a differential feature for PTM localization assignment were zoomed in for detailed comparison.

Quantitation of deamidation

MassHunter BioConfirm software enables an analyst to quantify the extent of PTMs on all the residues with a modification across a series of samples. It calculates the %peak area (or %peak height as an option) of the modified peptide relative to the sum of modified and unmodified forms. It also summarizes the comparison results in a table (Figure 5). This Results Compare table enables an analyst to have an overview of the PTM quantitation results for all the modified residues across a series of samples. In addition to the Results Compare table, BioConfirm also allows visualization of the quantitation result with a histogram. To display the histogram for each modified residue, the user simply clicks the row containing the residue of interest in the Results Compare table.

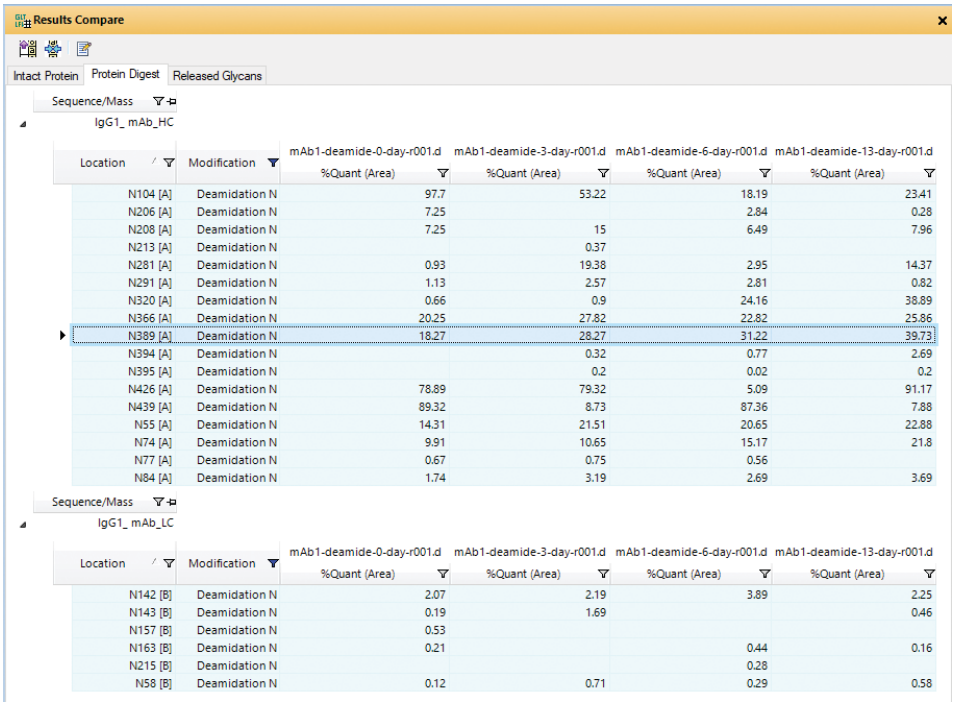


Figure 5. Results Compare table for PTM quantitation, showing a quantitative comparison of Asn deamidation in mAb1 heavy chain and light chain in high-pH conditions over a time course.

Figure 6 shows the histograms of quantitation for the three Asn deamidations (N_{389}, N_{394}, and N_{395}) on mAb1 heavy chain in high-pH conditions during a time course using BioConfirm software. Two technical replicates were performed for each time point. As observed, N_{389} has the highest deamidation level among the three residues during the stress study. This observation is consistent with the work of other groups, which shows that Gly on the C-terminus of Asn has a higher deamidation rate compared to other residues. A large hydrophobic residue containing aromatic rings on its C-terminus also generally correlates with very slow deamidation[7,8]. Since the assignment of deamidation localization on N_{394} or N_{395} is sometimes ambiguous, it is reasonable to consider combining the deamidation quantitation on these two sites for data analysis.

In a peptide-mapping workflow, the therapeutic protein is first digested to produce peptide fragments, which sometimes generate incomplete digestion. Due to the complexity of the sample digest, an analyst should inspect the peptide forms used for PTM quantitation. BioConfirm software generates a table titled *Peptide Relative Quantitation Results*, listing all the modified residues and their corresponding peptides from each data file (Figure 7). Figure 7 shows the quantification of N_{389} deamidation on mAb heavy chain in the eight data files during the stress study. To show all the corresponding peptides identified in this data file, the subtable for the last data file is expanded. To automatically determine the peptide forms used for PTM quantitation, BioConfirm applies a series of rules related to protein digestion and PTM analysis. It also allows a user to adjust the peptide selection by clicking the **Use for %Quant** check box. The calculated %Quant, the Results Compare Table, and the histogram are immediately synchronized with updated quantitation results.

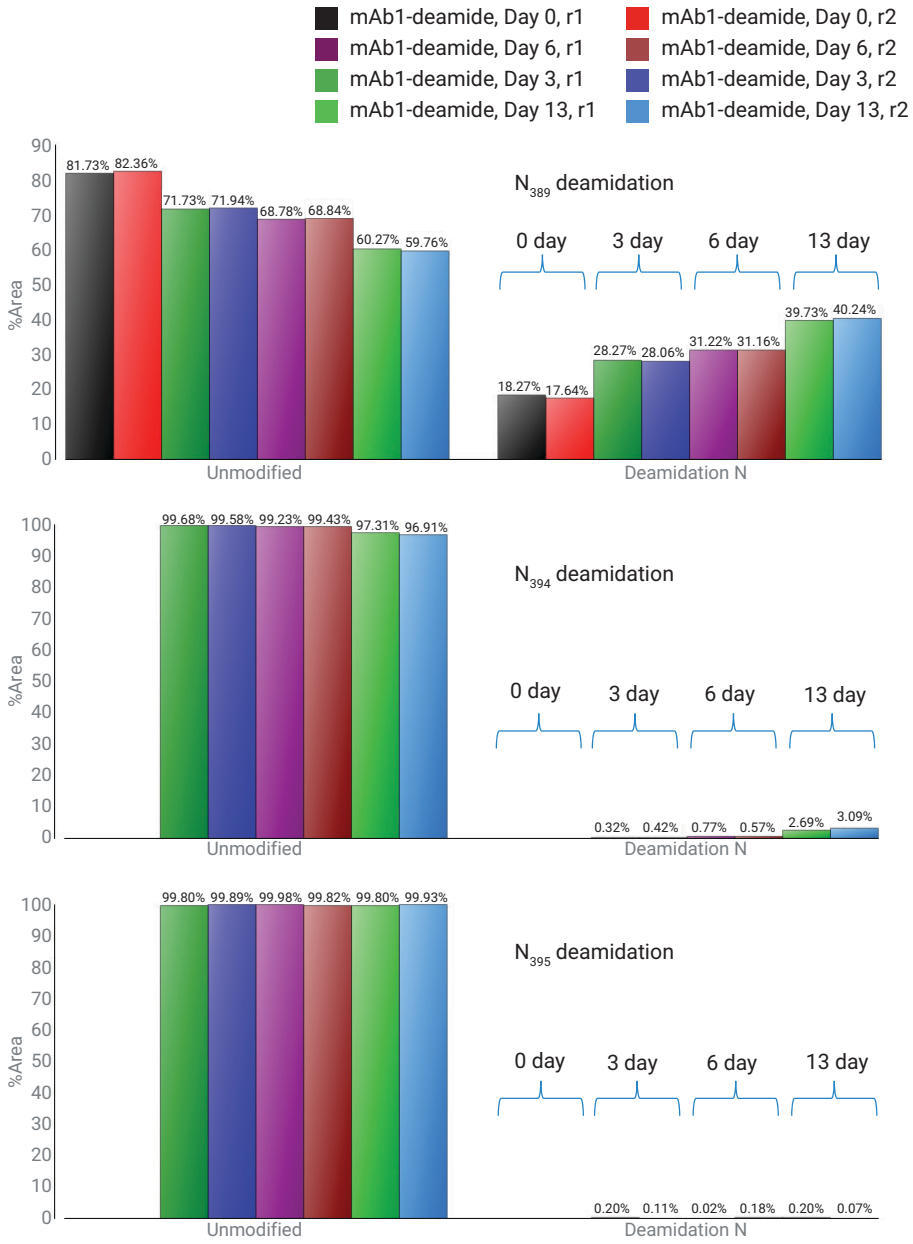
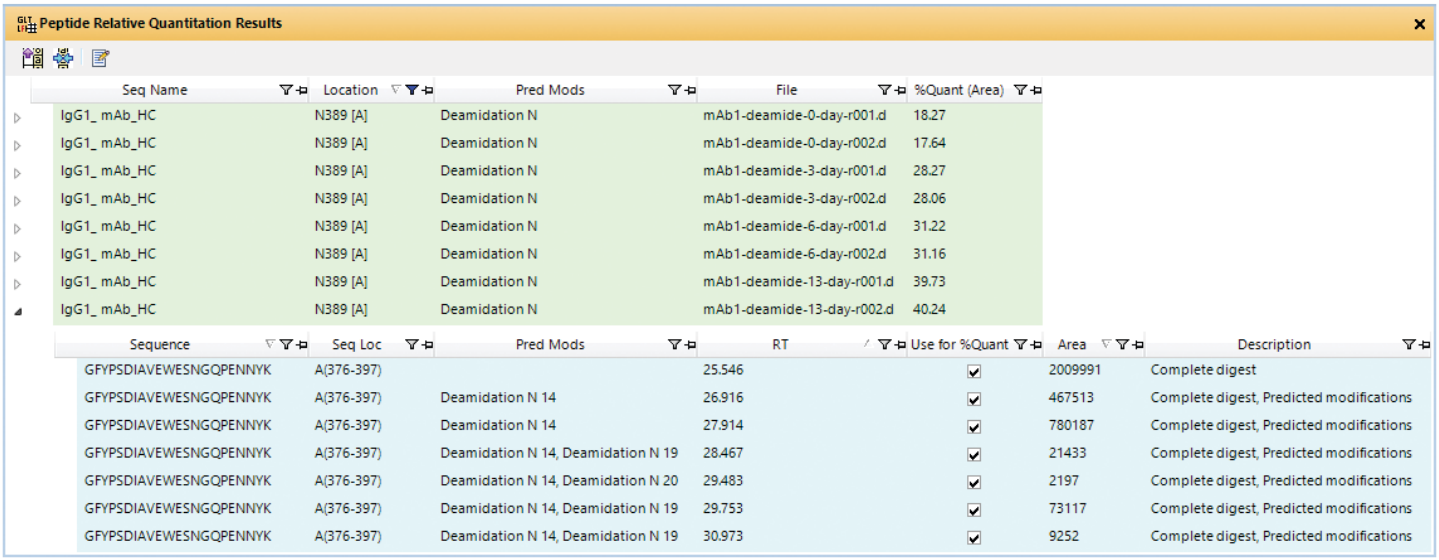


Figure 6. Histograms of quantitation for the three Asn deamidations (N_{389}, N_{394}, and N_{395}) on mAb1 heavy chain in high-pH conditions during a time course using MassHunter BioConfirm software. Two technical replicates were performed for each time point.



Seq Name	Location	Pred Mods	File	%Quant (Area)
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-0-day-r001.d	18.27
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-0-day-r002.d	17.64
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-3-day-r001.d	28.27
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-3-day-r002.d	28.06
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-6-day-r001.d	31.22
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-6-day-r002.d	31.16
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-13-day-r001.d	39.73
IgG1_ mAb_HC	N389 [A]	Deamidation N	mAb1-deamide-13-day-r002.d	40.24

Sequence	Seq Loc	Pred Mods	RT	Use for %Quant	Area	Description
GFYPSDIAVEWESNGQPENNYK	A(376-397)		25.546	☑	2009991	Complete digest
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14	26.916	☑	467513	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14	27.914	☑	780187	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 19	28.467	☑	21433	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 20	29.483	☑	2197	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 19	29.753	☑	73117	Complete digest, Predicted modifications
GFYPSDIAVEWESNGQPENNYK	A(376-397)	Deamidation N 14, Deamidation N 19	30.973	☑	9252	Complete digest, Predicted modifications

Figure 7. Screenshot of a portion of the Peptide Relative Quantitation Results table from MassHunter BioConfirm.

Quantitation of oxidation

The extent of Met oxidation in two mAbs (mAb1 and NISTmAb) was also evaluated under accelerated oxidation conditions. As an example, Figure 8 shows the quantitative histograms of light chain Met 4 (M4) oxidation for mAb1 and NISTmAb in response to accelerated H_2O_2 exposure using BioConfirm software. As expected, both mAbs show increasing extent of oxidation at M4 with a different oxidation rate in response to accelerated H_2O_2 exposure.

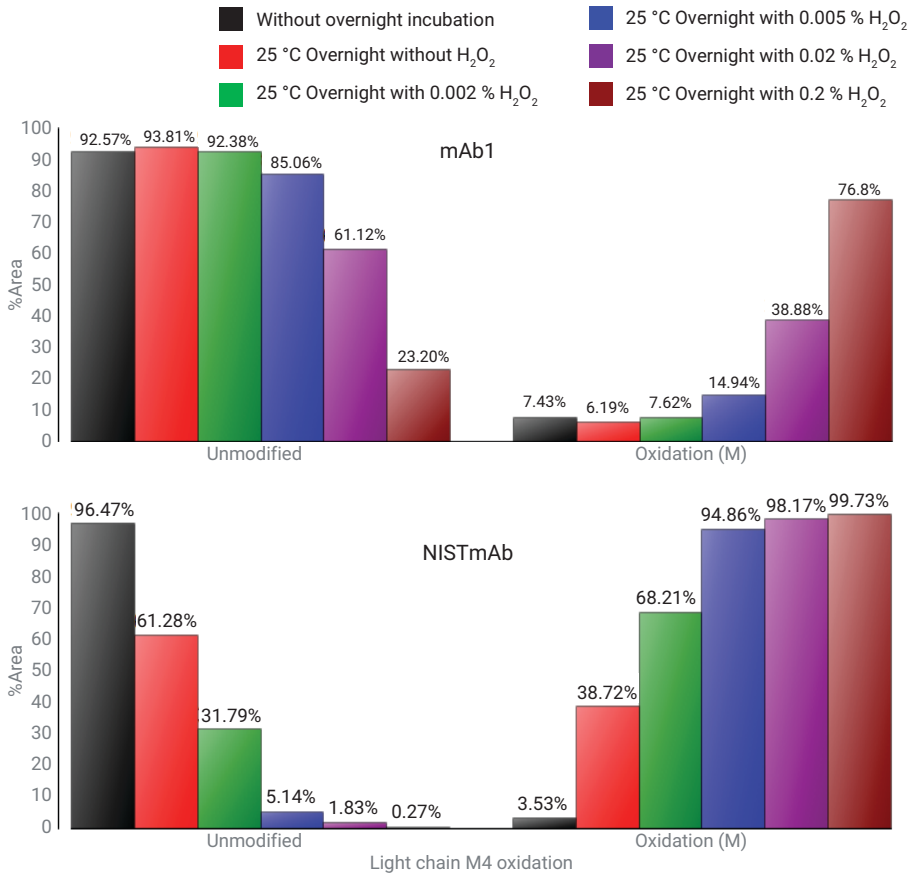


Figure 8. Quantitative histograms of light chain M4 oxidation for mAb1 and NISTmAb under accelerated oxidation conditions using MassHunter BioConfirm software.

Conclusion

A complete workflow including automated sample preparation using an AssayMAP Bravo platform, LC separation with a 1290 Infinity II LC, data acquisition using 6545XT AdvanceBio LC/Q-TOF, and data analysis using MassHunter BioConfirm software has been demonstrated for the simultaneous identification and quantification of chemical-induced deamidation and oxidation on mAbs. The MassHunter BioConfirm 10.0 software is capable of:

- Automated data batch processing
- Peptide-spectrum matching with statistical score and FDR
- Linked navigation through the results table to the mass spectra and chromatograms
- Quantitation analysis of PTMs

A combination of these features enhances the workflow for peptide mapping and PTM quantitation during the development and manufacture of protein biotherapeutic drugs.

References

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