Comprehensive Peptide Mapping and PTM Analysis of Monoclonal Antibodies using High-Resolution Mass Spectrometry

Objective

This technical note highlights the peptide mapping analysis of monoclonal antibodies (mAbs), enabling confident identification and characterization of potential post-translational modifications (PTMs) while providing comprehensive sequence coverage.

Introduction

Biotherapeutics are complex, heterogeneous molecules produced in living cells, often from hamster, mouse, or bacterial systems. These systems are highly sensitive to variations in growth conditions, making the physical environment a critical factor in product consistency. Even minor changes can significantly impact the physicochemical properties of the final purified drug. Consequently, large-scale manufacturing processes for biotherapeutics require extensive optimization during development to ensure consistent product quality and efficacy.

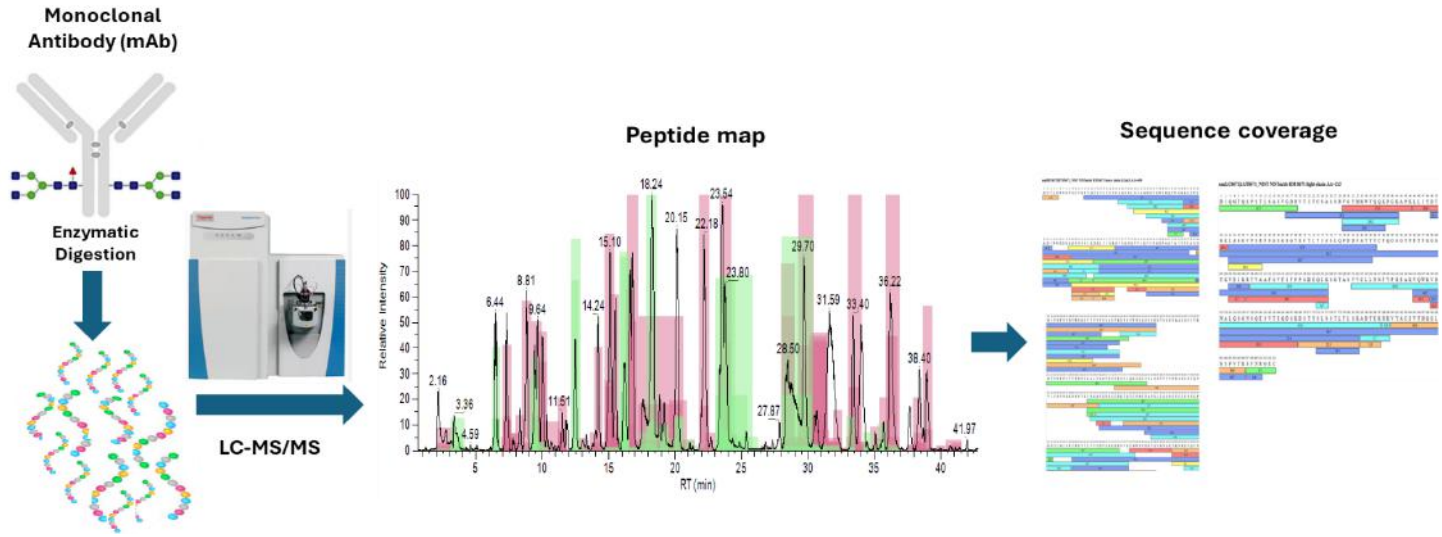

Figure 1. Comprehensive characterization of monoclonal antibody using peptide mapping workflow. mAb was reduced, digested with protease, and the enzymatic digests are further analyzed using liquid chromatography/tandem mass spectrometry. The base peak chromatogram of peptide mapping showed excellent separation and sequence coverage.

Introduction (Cont.) Due to their structural complexity, mAbs are susceptible to various PTMs, some of which may impact product safety, stability, and efficacy. Modifications with potential clinical relevance are classified as Critical Quality Attributes (CQAs). Therefore, sensitive and validated analytical methods are essential to monitor these PTMs throughout development and in the final product to ensure quality and regulatory compliance. UHPLC-HRMS is a powerful tool for characterization of complex proteins like mAbs, with peptide mapping playing a key role in identifying and tracking CQAs.

Key Highlights

- Demonstrating high-resolution rapid peptide mapping analysis using high-resolution mass spectrometry.
- Peptide mapping plays a critical role in protein characterization, offering detailed insights into molecular structure and integrity. Its flexibility makes it valuable throughout all phases of biologic development, from early research to final product release.
- Monitoring of PTMs including glycosylation, aspargine deamidation, methionine oxidation, isomerization, etc.

Sample and materials

Commercial monoclonal NIST monoclonal antibody (NIST RM #8671) (10 mg/ml) was purchased from NIST research laboratories. 500 mM Iodoacetamide (IAM), 500 mM dithiothreitol (DTT) and 8 Mm Guanidine Hydrichloride (GnHCl), Tris-HCl and Trifluoroacetic acid (TFA).

Preparation of protein digests

NISTmAb was diluted to 10 µg/µL with deionized water. The diluted samples were denatured using 7.6 M GnHCL/ 50 mMm Tris-HCl (pH7.4). After denaturation reduction and alkylation steps were performed followed by 5 Hrs. digestion with trypsin.

Table 1: LC-Gradient Parameters

Column	Waters Peptide BEH C18, 4 µm, 2.1 × 50 mm
Flow rate	0.3 µL/min
Time	%B
0.0	2
1.0	2
45.0	40
46.0	80
50.0	80
50.5	2
65.0	2

Chromatography

Reversed-phase chromatography for all samples was performed on a Thermo Scientific™ Vanquish™ Flex UHPLC system. Waters Peptide BEH C18, 4 µm, 2.1 × 50 mm column was used for the separation, employing a gradient elution with solvent A (water containing 0.1% formic acid) and solvent B (acetonitrile containing 0.1% formic acid). The column temperature was maintained at 60 °C. Details of the gradient and mass spectrometry conditions are provided in Table 1 & 2.

Data Analysis

The raw MS files were subjected to BioPharma Finder™ 3.0 (Thermo Fisher Scientific) for peptide mapping. The peptide identifications were performed by searching the processed data against the NISTmAb sequence-based accurate mass of a full mass scan and assignments of product ions in MS/MS spectra. The data was filtered to report only the peptides with a mass tolerance of ±10 ppm.

Table 2: Mass Spectrometry Scan Parameters

Parameter	Value
Polarity	Positive
Scan Type	Full MS-ddMS2
Scan range	200-2000 m/z
Fragmentation	NA
Resolution	60000
Microscans	1
AGC target	3.00E+06
Maximum injection time	100
dd-MS2 / dd-SIM	Top 5
window	2.0 m/z
Fixed first mass	-
(N) CE/ stepped (N) CE	30
dd settings	
Minimum AGC target	8.00 e3
Intensity threshold	4.0 e4
Apex trigger	NA
Charge exclusion	NA
Peptide match	preferred
Exclude isotopes	on
Dynamic exclusion	7.0 s
Sheath gas flow rate	40
Aux gas flow rate	10
Sweep gas flow rate	0
Spray Voltage (kV)	3.8
Spray current (uA)	NA
Capillary temp (°C)	325
Funnel RF level	50
Aux gas heater temp (°C)	400

Results

Peptide mapping is a routine analytical technique in biopharmaceutical development, primarily used to evaluate PTMs in therapeutic proteins. Monitoring PTMs is essential during bioprocessing and batch release to ensure drug product safety, and peptide mapping is increasingly being adopted in quality control workflows.

LC-MS–based peptide mapping is the preferred method for both identification and quantification of PTMs. Sample preparation for LC-MS typically involves three key steps: denaturation/reduction, alkylation, and enzymatic digestion. Digestion of different mAbs yields a diverse set of peptide fragments, ranging in size from single amino acids to long polypeptides. Due to the wide variation in peptide hydrophobicity, reversed-phase chromatography—typically using a C18 column—is the method of choice for peptide separation in mapping workflows.

Figure 2 shows the base peak chromatogram of a NISTmAb digest, with 30 pmol injected on-column. The chromatogram displays over 100 detected peaks. To investigate potential PTMs in the antibody, the data were searched against the NISTmAb light and heavy chain sequences. The database search yielded 100% tryptic peptide sequence coverage for the light chain and 97.9% for the heavy chain, confirming the high sequence coverage, and indicating that no other proteins were present in the sample. The different colored bars on the sequence coverage map illustrate the peptide recovery.

This study highlights the analytical evaluation of oxidation as a CQA in monoclonal antibody therapeutics. Oxidation most commonly affects methionine and tryptophan residues, though cysteine and histidine can also be modified under certain conditions. These oxidative modifications can compromise protein integrity, leading to structural changes and altered biological activity. For instance, oxidation at key methionine sites can reduce the antibody's affinity for the neonatal Fc receptor (FcRn), potentially diminishing its pharmacokinetic performance. Because oxidation can occur during various stages of production and storage, routine monitoring is essential for ensuring product stability, guiding process development, and meeting regulatory expectations for biopharmaceutical quality.

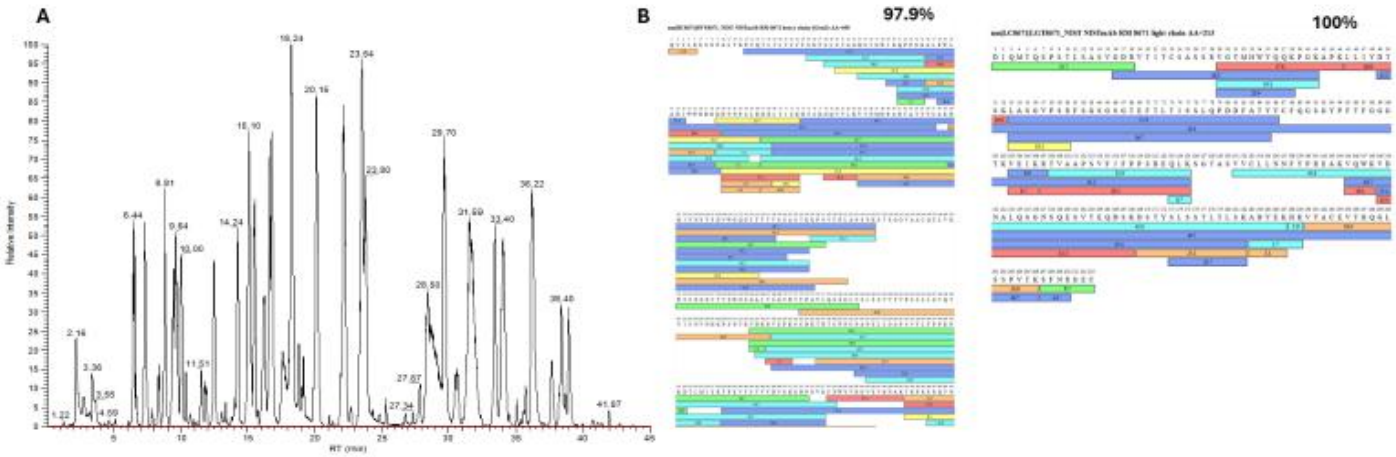

Figure 2. (A) Base peak chromatogram of the NISTmAb tryptic digest, showing tryptic peptides derived from both the heavy and light chains. (B) Sequence coverage map of NISTmAb obtained from peptide mapping. Peptides are color-coded based on recovery levels. Complete (100%) sequence coverage was achieved for the light chain, and 97.9% coverage was obtained for the heavy chain.

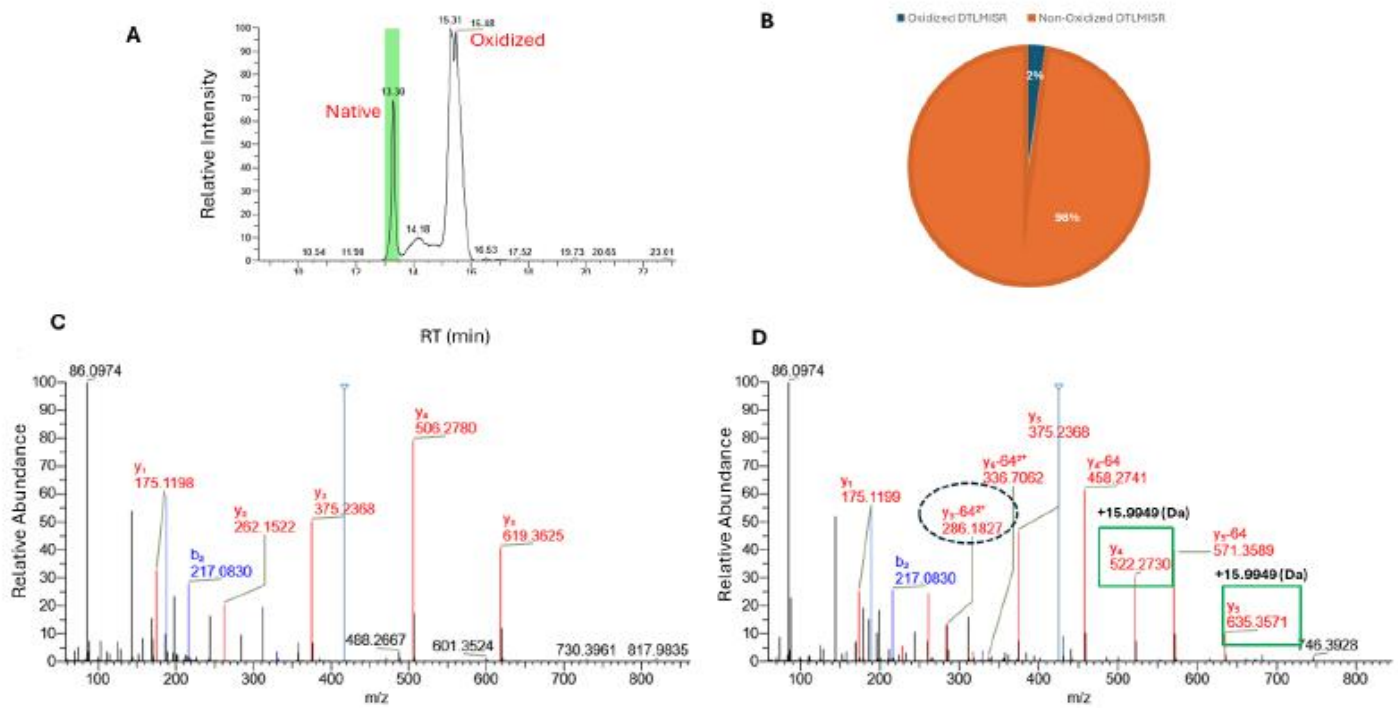

Figure 3. (A) Extracted ion chromatogram (XIC) for the peptide DTLMISR, with colored shading distinguishing the native and oxidized forms. (B) Venn diagram illustrating the relative abundance of oxidized versus unmodified peptide, indicating ~2% methionine oxidation. (C–D) MS² HCD fragmentation spectra of the native and oxidized forms of DTLMISR. A mass shift of +15.9952 Da, corresponding to methionine oxidation, was observed in the y4 and y5 fragment ions. Additionally, a diagnostic neutral loss of approximately 64 Da—attributed to the loss of methane sulfenic acid (CH_3SOH)—was detected from fragment ions containing the oxidized methionine residue, further confirming the presence of methionine sulfoxide.

Results (Cont.)

Oxidation of NISTmAb using the Orbitrap QExactive mass spectrometer was assessed and quantified to be 2% which is within the acceptable range of monoclonal antibodies. This ensured that the oxidized peptides were adequately detected by LC-MS analysis. Methoionine 261 (M261) was determined as 2% detected with a 100% confidence level. The clear chromatographic separation between the unmodified and the oxidized peptide shown in Figure 3 A provides a clear distinction between the two species. High-resolution MS/MS spectra were acquired with excellent mass accuracy (<1 ppm), and the observed mass shift between the oxidized and unmodified forms of the peptide closely matched the theoretical value of +15.9949 Da, as shown in Figure 3D. The presence of methionine sulfoxide was further confirmed through HCD MS² fragmentation, with methionine-containing fragment ions (y_4 and y_5) displaying the expected mass shift. Additionally, a characteristic neutral loss of methane sulfenic acid was observed in these fragment ions, further supporting the identification. These high-quality MS/MS data underscore the capability for in-depth characterization of the NISTmAb reference standard.

Conclusion

- The analysis of NISTmAb RM 8671 yielded high-quality LC-MS/MS data, providing confident identification and characterization of the molecule. Exceptional sequence coverage was achieved —100% for the light chain and 97.9% for the heavy chain—demonstrating the robustness of the workflow.
- As expected for a well-characterized reference standard, the levels of PTMs were low. Nevertheless, the workflow successfully identified key PTMs such as methionine oxidation and N-terminal pyroglutamate formation, highlighting the sensitivity and specificity of the method.
- The chromatographic conditions employed provided excellent peak resolution and reproducibility, while the MS/MS data enabled detailed structural characterization at the peptide level.
- Overall, this workflow supports in-depth structural analysis of therapeutic monoclonal antibodies and is readily applicable for routine characterization, comparability studies, and quality control in biopharmaceutical development and manufacturing.

References

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