Application Note

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Assessing Multiple Critical Quality Attributes of Monoclonal Antibody and Comparability Assessments

Author

Suresh Babu C.V. and
Brian Liau
Agilent Technologies, Inc.

Abstract

Multi-attribute method (MAM) based LC/MS peptide mapping is gaining much attention in the field of biopharmaceutical analysis. This application note demonstrates the LC/MS peptide mapping approach to characterize and quantify different monoclonal antibody (mAb) critical quality attributes (CQAs). The peptide mapping results were compared with conventional methods and found to yield highly similar estimates of the extent of lysine truncation, oxidation, deamidation, N-terminal cyclization, and glycosylation.

Introduction

Monoclonal antibodies (mAbs) are derived from living systems that are experiencing rapid growth. Due to their biological origin, these complex molecules undergo various post-translational modifications (PTMs) during production, leading to a significant degree of heterogeneity. It is important that these unintended modifications be characterized and monitored as CQAs during development and production of mAbs. Due to the chemical dissimilarity of each CQA, several analytical testing methods have traditionally been used for this purpose, with each technique having various strengths and weaknesses. Recently, the LC/MS-based multi-attribute method (MAM)[1] approach has gained popularity as it promises a single method for comprehensive characterization of mAb CQAs, representing significant savings in analysis time and cost over traditional methods. However, before the MAM approach can be applied to highly regulated quality control environments, a necessary first step is to verify that traditional analytical methods correspond accurately with peptide mapping results. It is important to ensure that the peptide mapping workflow provides comparable results to traditional assays.

This study compares the performance of the peptide mapping method to the traditional assays including ion-exchange chromatography, hydrophilic interaction chromatography (HILIC), and sub-unit level reversed-phase chromatography. Peptide mapping of mAbs was performed using an integrated workflow consisting of an Agilent AssayMAP Bravo liquid-handling platform, an Agilent 1290 Infinity II LC system, an Agilent 6545XT AdvanceBio LC/Q-TOF, and data analysis through Agilent MassHunter BioConfirm software. The relative % quantification of each CQA was compared between the peptide mapping method and the traditional assays.

Experimental

Materials

mAb1, mAb2, and mAb3 were purchased from a local distributor (Singapore), and stored according to the manufacturers' instructions. Trizma base, Tris-HCl, guanidine hydrochloride, *tris*(2carboxyethyl)phosphine (TCEP), iodoacetamide (IAA), formic acid, trifluoroacetic acid, sodium phosphate monobasic, sodium phosphate dibasic, sodium chloride, and LC/MS-grade solvents were purchased from Sigma-Aldrich. IdeS protease was purchased from Genovis. High-quality sequencing grade trypsin was obtained from Agilent Technologies, Inc.

Instrumentation, column, and software

Automation	
Agilent AssayMAP Bravo liquid handling platform (G5542A)	
LC Systems	
Agilent 1260 Infinity II Bio-inert LC	**Agilent 1290 Infinity II LC System**
• Agilent 1260 Infinity II Bio-inert Pump (G5654A) • Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler • Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger • Agilent 1260 Infinity Bio-inert • Analytical-scale Fraction Collector (G5664A)	• Agilent 1290 Infinity II High Speed Pump (G7120A) • Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) • Agilent 1290 Infinity II Multisampler (G7167B)
Detection	
• Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert flow cell • Agilent 1260 Infinity Fluorescence Detector (G1321B) • Agilent 6545XT AdvanceBio LC/Q-TOF	
Columns	
• Agilent Bio MAb, nonporous, 4.6 × 250 mm, 5 µm HPLC, PEEK • Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 100 mm, 1.8 µm • Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm • Agilent AdvanceBio Peptide Mapping 2.1 × 150 mm, 2.7 µm, 120Å	
Software	
• Agilent OpenLab CDS Version 2.3 • Agilent Buffer Advisor A.01.01 [009] • Agilent OpenLAB CDS ChemStation Edition for LC Systems, Rev. C.01.07 • Agilent MassHunter Acquisition (B.10.00) • Agilent MassHunter BioConfirm 10.0 • Agilent VWorks Automation Control 13.1.1.1383	

Experimental methods

Charge variants: Samples were directly injected without dilution (10 mg/mL). Tables 1 and 2 show the chromatographic parameters used for weak cation exchange (WCX) chromatography of mAb1 and mAb2. Collected WCX fractions were subjected to trypsin digestion and peptide mapping analysis.

Oxidation: Five-microliter mAb samples (2 mg/mL in Tris-HCl buffer) were added to 5 µL of IdeS protease and incubated at 30 °C for 30 minutes, then allowed to cool to room temperature before analysis.

Released glycan: Labeled N-glycan samples were prepared using the AssayMAP Bravo liquid handling system with the GlykoPrep Rapid N-Glycan kit (GPPNG-PC).[2] Labeled N-glycans were separated by HILIC.

Trypsin digestion

Fraction collected WCX samples and mAb samples were reduced/alkylated and trypsin-digested followed by desalting using the Agilent AssayMAP Bravo liquid-handling platform.[3] Briefly, sample plates containing mAbs were reconstituted in denaturation buffer (8 M guanidine-HCl with 5 mM TCEP and 150 mM Tris pH 8) and incubated at 60 °C for 60 minutes. After denaturation and reduction of disulfide bonds, alkylation of free cysteines was carried out by adding 133 mM iodoacetamide (40 minutes at room temperature). The sample was then acidified and diluted with 1.75% TFA. Next, samples were subjected to Protein Cleanup application using RP-W cartridges and trypsin digestion (20:1, protein to protease w:w) was performed overnight, at 37 °C. The samples were later acidified with 0.1% formic acid to stop the trypsin activity using the AssayMAP reagent transfer utility. AssayMAP Bravo Peptide Cleanup protocol (desalting) was performed using C18 reversed-phase cartridges.

Table 1. LC conditions.

Parameters	WCX (C-Terminal Lys, N-Terminal Cyclization Variants, Deamidation) Agilent 1260 Infinity II Bio-inert LC	RP (Oxidation) Agilent 1290 Infinity II LC	HILIC (Released Glycans) Agilent 1290 Infinity II LC	RP (Peptide Mapping) Agilent 1290 Infinity II LC
Column	Agilent Bio MAb NP5, 4.6 × 250 mm, 5 µm PEEK	Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 100 mm, 1.8 µm	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm	Agilent AdvanceBio Peptide Mapping 2.1 × 150 mm, 2.7 µm, 120 Å
Injection Volume	1 to 10 µL	4 µL	2 µL	2 to 8 µL
Sample Thermostat	5 °C	5 °C	5 °C	5 °C
Mobile Phase	A) Water B) NaCl (1,000 mM) C) NaH_2PO_4 (55 mM) D) Na_2HPO_4 (50 mM)	A) 0.1% FA, 0.1% TFA in water B) 0.1% FA, 0.1% TFA in 80% IPA, 10% ACN, 10% Water	A) 50 mM Ammonium formate, pH 4.5 B) ACN	A) 0.1% formic acid in water B) 0.1% formic acid in ACN
Gradient	**mAb1 and mAb2** Time (min) %A %B %C %D 0.0 30.3 0 59.6 10.1 2.0 26.0 5.0 56.9 12.1 8.0 21.5 10.0 54.9 13.6 20.0 13.3 19.0 51.9 15.8 21.0 30.3 0 59.6 10.1 **mAb3** Time (min) %A %B %C %D 0.0 44.5 0 44.4 11.1 20.0 27.7 16.2 38.7 17.4 21.0 44.5 0 44.4 11.1	At 0 min → 20% B At 2 min → 20% B At 42 min → 35% B At 42.5 min → 80% B At 45 min → 80% B At 45,1 min → 20% B	At 0 min → 25% B At 2 min → 30% B At 15.5 min → 37% B At 17 min → 80% B At 20 min → 80% B At 21 min → 25% B At 30 min → 25% B	At 0 min → 3% B At 30 min → 40% B At 33 min → 90% B At 35 min → 90% B At 37 min → 3% B At 40 min → 3% B
Column Temperature	25 °C	74 °C	40 °C	60 °C
Flow Rate	0.75 mL/min	0.4 mL/min	15.5 to 20 min: 0.25 mL/min, otherwise 0.4 mL/min	0.4 mL/min
UV	220/280 nm	–		–
FLD	–	–	Ex/Em = 285 nm/345 nm	–
Fraction Collection Mode	Time-based fraction trigger	–	–	–

Table 2. LC/MS conditions.

Parameters	WCX (C-Terminal Lys, N-Terminal Cyclization Variants, Deamidation)	RP (Oxidation)	HILIC (Released Glycans)	RP (Peptide Mapping)
Agilent 6545XT AdvanceBio LC/Q-TOF				
Ion Mode	–	Positive ion mode, dual AJS ESI	Positive ion mode, dual AJS ESI	Positive ion mode, dual AJS ESI
Drying Gas Temp	–	350 °C	150 °C	325 °C
Drying Gas Flow	–	12 L/min	9 L/min	13 L/min
Sheath Gas Temp	–	350 °C	300 °C	275 °C
Sheath Gas Flow	–	11 L/min	10 L/min	12 L/min
Nebulizer	–	35 psi	35 psi	35 psi
Capillary Voltage	–	4,000 V	3,000 V	4,000 V
Nozzle Voltage	–	2,000 V	500 V	0 V
Fragmentor Voltage	–	180 V	180 V	175 V
Skimmer Voltage	–	65 V	65 V	65 V
Reference Mass	–	922.009798	922.009798	121.050873, 922.009798
Acquisition Mode	–	Extended (10,000 *m/z*) mass range	Low mass, Hi Resolution (4Ghz)	Extended Dynamic Range (2 GHz)
MS Mass Range	–	800 to 5,000 *m/z*	300 to 1,700 *m/z*	110 to 1,700 *m/z*
Acquisition Rate	–	1 spectrum/sec	2 spectra/sec	8 spectra/sec
Auto MS/MS Range	–	–	–	50 to 1,700 *m/z*
MS/MS Acquisition Rate	–	–	–	3 spectra/sec
Isolation Width	–	–	–	Narrow (~1.3 *m/z*)
Precursors/Cycle	–	–	–	Top 10
Collision Energy	–	–	–	Charge state Slope Offset 2 3.1 1 3 and >3 3.6 −4.8
Threshold for MS/MS	–	–	–	1,000 counts and 0.001%
Dynamic Exclusion On	–	–	–	One repeat, exclude for 0.1 or 0.2 min
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

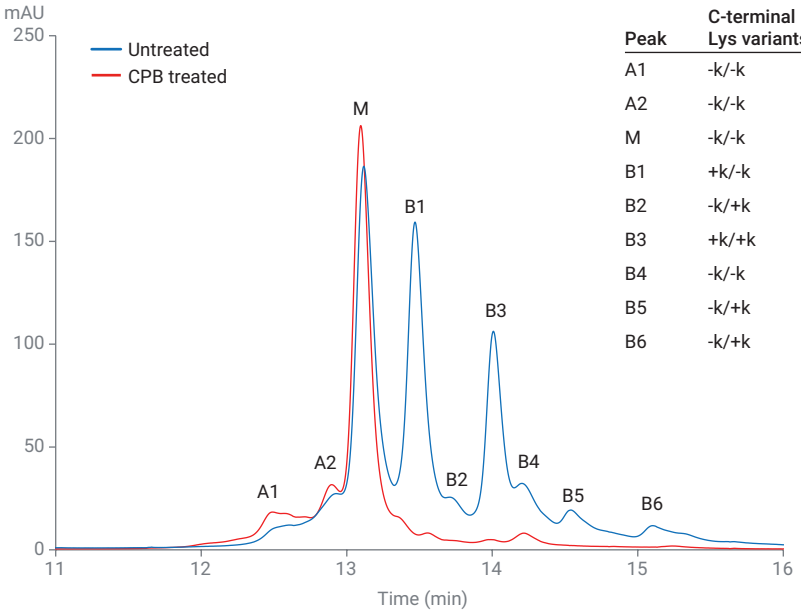
Results and discussion

C-terminal Lysine truncation

Truncation of the C-terminal lysine on the heavy chain is a commonly observed PTM due to carboxypeptidase activity. Loss of the lysine residue decreases net positive charge on mAbs, resulting in charge heterogeneity. The C-terminal lysine basic variants (with Lys and without Lys) can be well separated by cation exchange chromatography (CEX). Figure 1A shows the high-resolution separation of charge variant profiles of mAb1 on an Agilent Bio MAb PEEK column with three distinct peaks. The peak at 13.11 minutes was designated as the main peak (M). Early and late-eluting peaks were called acidic (A1, A2) and basic variants (B1, B2, B3, B4, B5, B6), respectively. The peak area percent of main, acidic, and basic charge variants are tabulated in Figure 1A. Charge variant peaks were collected as individual fractions and pooled over several injections before being subjected to LC/MS peptide mapping analysis to characterize C-terminal lysine truncation. Based on the peptide mapping analysis, CEX peaks are annotated with C-terminal lysine truncated variants. Further confirmation of C-terminal lysine heterogeneity was confirmed by carboxypeptidase B (CPB) treatment. The relative content of C-terminal lysine truncation was calculated from the area percent of charge variant peaks and found to be 58.28% (Table 3).

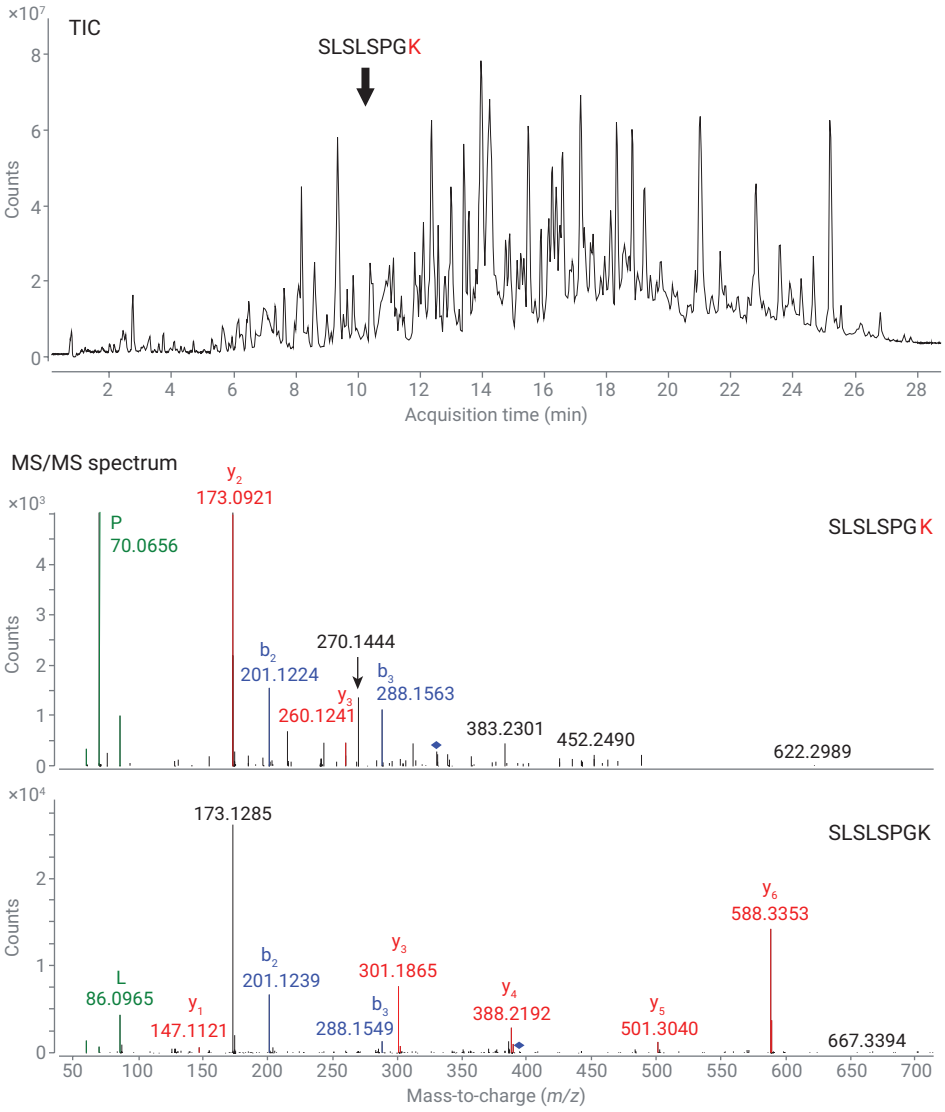
For peptide mapping based quantification of C-terminal lysine variant profile, mAb1 was subjected to the LC/MS peptide mapping method. Figure 1B shows the results of the LC/MS peptide mapping analysis for mAb1. MS/MS analysis confirmed the identity of modified and unmodified heavy chain C-terminal lysine truncated peptides, and the relative content was found to be 55.30%.

Comparison of C-terminal lysine truncated variants quantification between conventional CEX and peptide mapping methods are shown in Table 3. Both methods estimated similar relative content of C-terminal lysine truncation.



Peak	C-terminal Lys variants	Area %
A1	-k/-k	3.05
A2	-k/-k	5.48
M	-k/-k	30.42
B1	+k/-k	24.69
B2	-k/+k	3.42
B3	+k/+k	15.41
B4	-k/-k	6.97
B5	-k/+k	6.25
B6	-k/+k	4.31

Figure 1A. Charge variant profiles of mAb1 on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column. Overlay of CPB treated (red) and untreated (blue) of mAb1 profiles.



Sequence	*m/z*	Targeted mass	Modification	Relative level
SLSLSPGK	394.7289	659.349	–	44.70%
SLSLSPGK	330.6815	787.444	Lys loss	55.30%

Figure 1B. LC/MS peptide mapping of mAb1 using an Agilent Peptide Mapping, 2.1 × 150 mm, 2.7 µm column.

Table 3. Relative % quantitation comparison (Conventional versus peptide mapping methods).

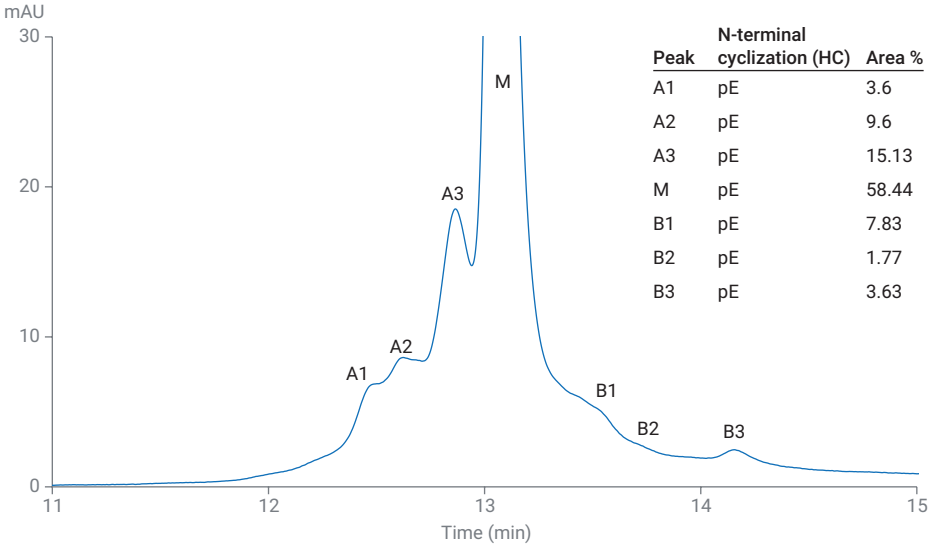
Technique	Relative Content %	
C-terminal Lys variant		
CEX	58.28	
Peptide Mapping	55.30	
N-terminal cyclization variant (HC)		
CEX	100	
Peptide Mapping	99.87	
Asp55 deamidation		
CEX	3.86	
Peptide Mapping	4.79	
Oxidation (M256+M432)		
RP-LC/MS (Subunit)	2.38	
Peptide Mapping	2.33	
Glycans		
	HILIC	Peptide mapping
Man5	10.9	12.43
G0	6.48	8.35
G0F	49.13	50.41
G1F	28.80	24.57
G2F	4.69	4.24

N-terminal cyclization

N-terminal cyclization is caused by rearrangement of N-terminal Glu or Gln to pyroglutamate (pyro-Glu). This results in a net decrease in positive charge, making the mAb more acidic. As in C-terminal lysine truncation, the charge heterogeneity resulting from this PTM can be observed using CEX. Figure 2A shows the high-resolution separation of charge variants of mAb2 on an Agilent Bio MAb PEEK column. The peak at 13.1 minutes was designated as the main peak (M). Early and late-eluting peaks were called acidic (A1, A2) and basic variants (B1, B2, B3), respectively. Figure 2A summarizes the area percent of main, acidic, and basic charge variants. Charge variant peaks were collected as individual fractions and pooled over several injections before being subjected to LC/MS peptide mapping analysis to characterize N-terminal cyclization variant. Based on the peptide mapping analysis, CEX peaks are annotated with the N-terminal cyclization variant. The relative content of heavy chain N-terminal cyclization was calculated from the area percent of charge variant peaks and found to be 100% (Table 3).

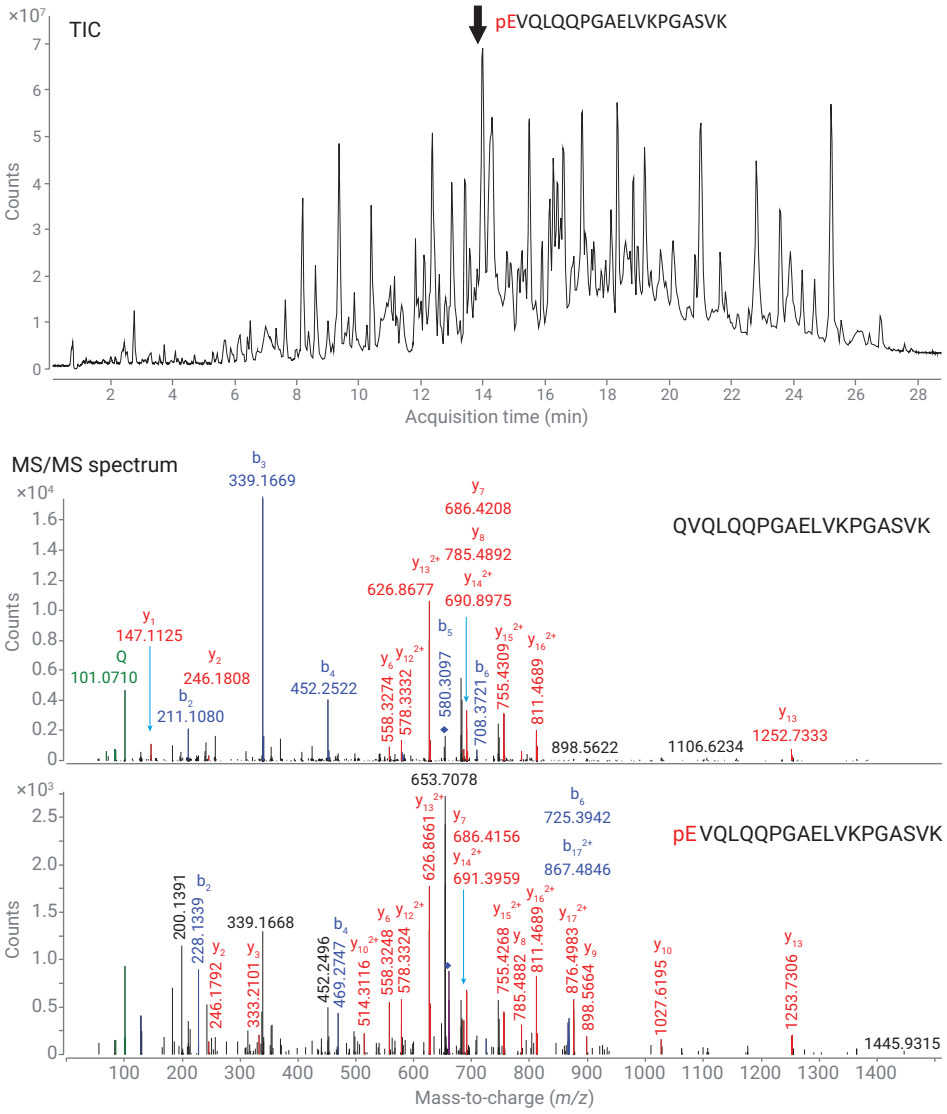
For peptide mapping based quantification of heavy chain N-terminal cyclization, mAb2 was subjected to the LC/MS peptide mapping method. Figure 2B shows the results of the LC/MS peptide mapping analysis for mAb2. MS/MS analysis confirmed N-terminal cyclization in heavy chain peptides, and the percentage of heavy chain molecules showing N-terminal cyclization was found to be 99.98%.

Comparison of N-terminal cyclization quantification between conventional CEX and peptide mapping methods were shown in Table 3. Both methods estimated similar relative content of N-terminal cyclization.



Peak	N-terminal cyclization (HC)	Area %
A1	pE	3.6
A2	pE	9.6
A3	pE	15.13
M	pE	58.44
B1	pE	7.83
B2	pE	1.77
B3	pE	3.63

Figure 2A. Charge variant profiles of mAb2 on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column.



Sequence	*m/z*	Targeted mass	Modification	Relative level
QVQLQQPGAELVKPGASVK	659.7089	1976.1159	–	0.13%
pEVQLQQPGAELVKPGASVK	654.0339	1959.0219	N-terminal cyclization	99.87%

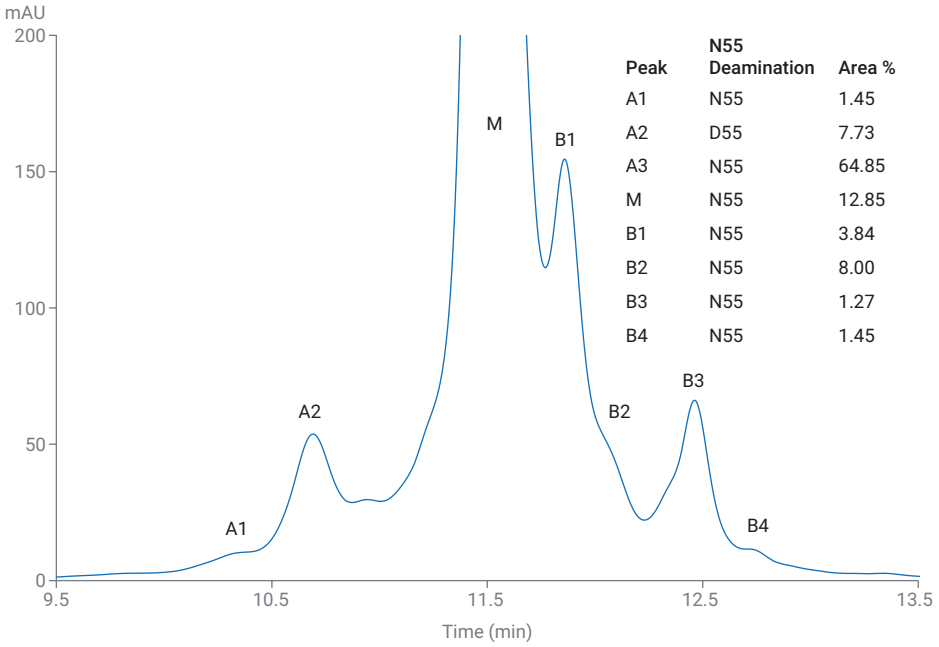
Figure 2B. LC/MS peptide mapping of mAb2 using an Agilent Peptide Mapping, 2.1 × 150 mm, 2.7 µm column.

Deamidation

Deamidation is the most frequent PTM in mAbs and is associated with the protein degradation pathway. Deamidation of asparagine (Asn, N) residues converts Asn into the acidic isomers aspartic acid (asp, D) and isoaspartic acid (isoAsp), resulting in a net decrease in positive charge. Asn deamidation in the CDR region results in acidic charge variants. Figure 3A shows the high-resolution separation of charge variant profiles of mAb3 on an Agilent Bio MAb PEEK column. The peak at 11.4 minutes was designated as the main peak (M). Early and late-eluting peaks were called acidic (A1, A2) and basic variants (B1, B2, B3, B4) respectively. Figure 3A summarizes the area percent of main, acidic, and basic charge variants. Charge variant peaks were collected as individual fractions and pooled over several injections before being subjected to LC/MS peptide mapping analysis to characterize deamidation. Peptide mapping analysis identified Asn55 deamidation in fraction A2. The relative content of N55 deamidation was calculated from the area percent of charge variant peaks and found to be 3.86% (Table 3).

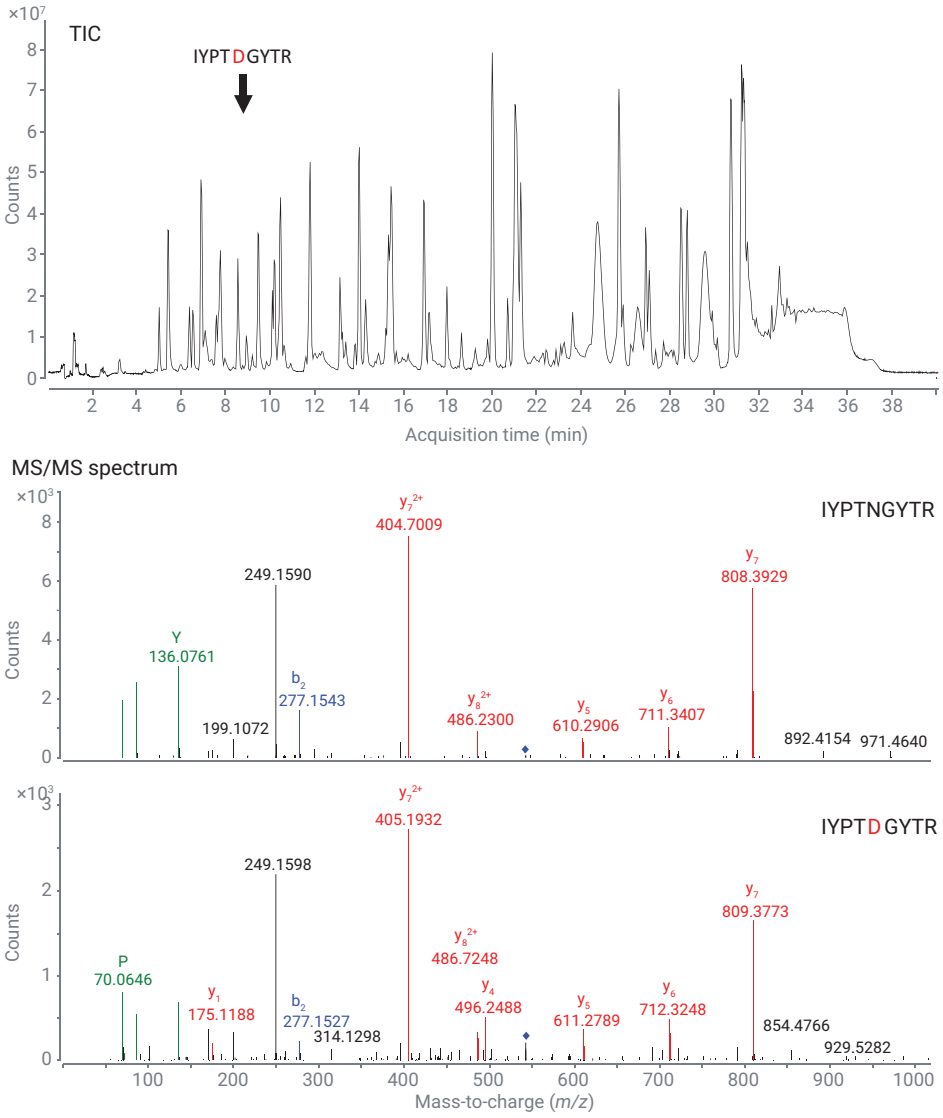
For peptide mapping based quantification of deamidation, mAb3 was subjected to LC/MS peptide mapping method. Figure 3B shows the results of LC/MS peptide mapping analysis for mAb3. MS/MS analysis confirmed the identity of modified and unmodified IYPTN^{55}GYTR peptides and the relative content of Asn55 deamidation was found to be 4.79%.

Comparison of deamidation quantification between conventional CEX and peptide mapping methods were shown in Table 3. Both methods estimated similar relative content of Asn55 deamidation levels.



Peak	N55 Deamination	Area %
A1	N55	1.45
A2	D55	7.73
A3	N55	64.85
M	N55	12.85
B1	N55	3.84
B2	N55	8.00
B3	N55	1.27
B4	N55	1.45

Figure 3A. Charge variant profiles of mAb3 on an Agilent 1260 Infinity Bio-inert Quaternary LC using an Agilent Bio Mab, 4.6 × 250 mm, 5 µm, PEEK column.



Sequence	*m/z*	Targeted mass	Modification	Relative level
IYPTNGYTR	1084.4541	1083.5349	–	95.21%
IYPTDGYTR	543.2567	1084.5139	Deamidation	4.79%

Figure 3B. LC/MS peptide mapping of mAb3 using an Agilent Peptide Mapping, 2.1 × 150 mm, 2.7 µm column.

Oxidation

Oxidation of Methionine (Met) is common in mAbs, and results in protein conformational changes which can alter function. The Fc region contains two Methionine (Met) residues (M256 and M432), which are more susceptible to oxidation. Oxidation results in a +16 Da mass increase, which can be measured in both peptides and intact mAbs.

Figure 4A shows the LC/MS profiles of Ides digested mAb2 Fc subunits separated using an Agilent ZORBAX RRHD 300 SB-C18 column. The dominant peak in the deconvoluted mass spectra corresponds to two oxidation species, which are mostly attributable to M256 and M432. The quantification levels of Fc oxidation were calculated using the peak areas of the deconvoluted mass spectra and found to be 2.38%.

For peptide mapping based quantification of oxidation, mAb2 was subjected to LC/MS peptide mapping method. Figure 4B shows the LC/MS profiles of tryptic mAb2 peptides. MS/MS analysis confirmed the identities of oxidized and unmodified peptide variants (DTLM^{256}ISR and WQQGNVFSCSVM^{432}HEALHNHYTQK) and the relative oxidation content (M256 + M432) was found to be 2.33%.

Comparison of oxidation quantification using either subunit analysis or peptide mapping methods is shown in Table 3, with both methods yielding similar estimates.

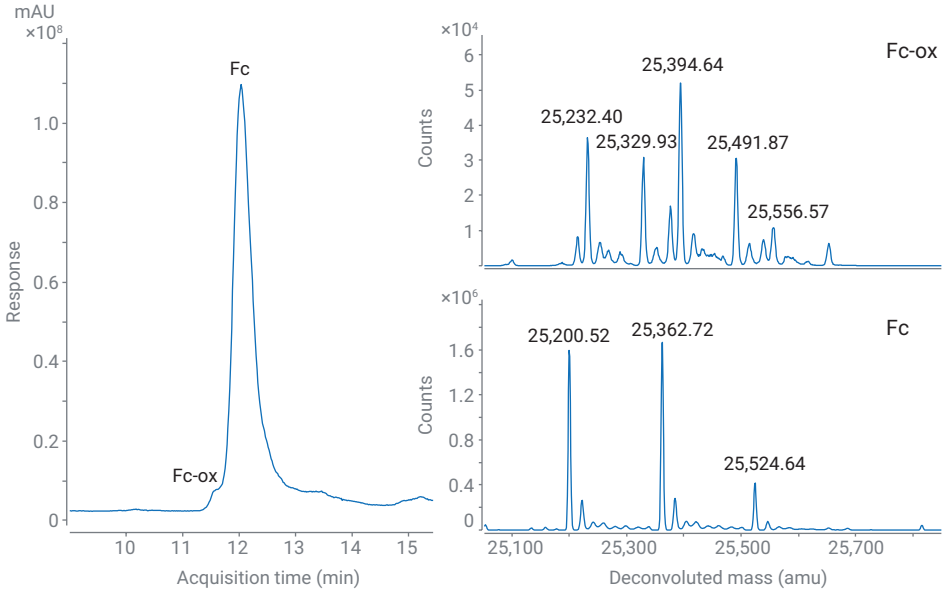
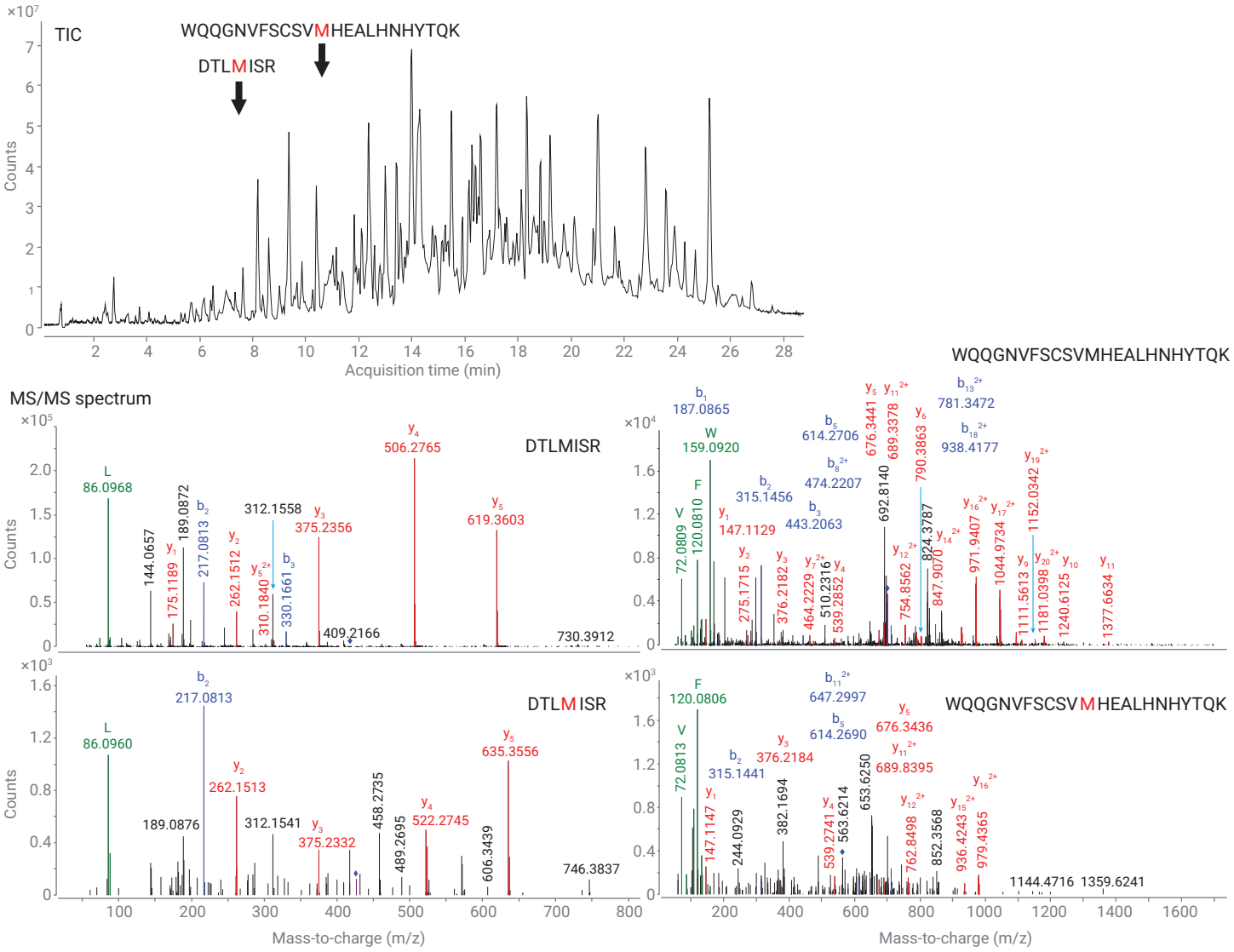


Figure 4A. LC/MS analysis of Ide digested mAb2 on an Agilent ZORBAX RRHD 300 SB-C18, 2.1 × 100 mm, 1.8 µm, PEEK column. Deconvoluted mass spectra of Fc fragments shows a 32 Da shift (corresponding to two oxygen addition).



Sequence	*m/z*	Targeted mass	Modification	Relative level
DTLMISR	418.2216	834.4269	–	98.24
DTLMISR	418.2200	850.4219	Oxidation	1.76
WQQGNVFSCSVMHEALHNHYTQK	1401.1368	2800.2598	–	99.43
WQQGNVFSCSVMHEALHNHYTQK	705.0704	2816.2548	Oxidation	0.57

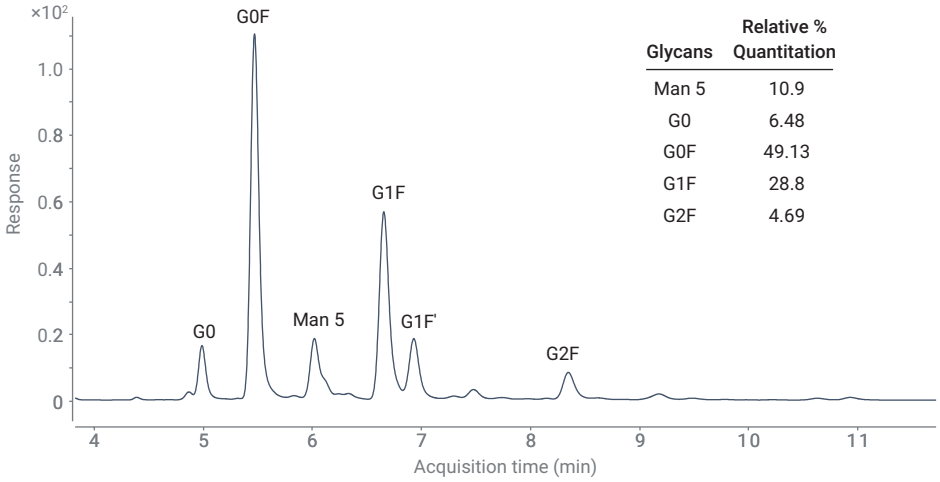
Figure 4B. LC/MS peptide mapping of mAb2 using an Agilent Peptide Mapping, 2.1 × 150 mm, 2.7 µm column.

Glycosylation

Asparagine-linked N-Linked glycosylation is one of the most important PTMs and contributes to mAb heterogeneity. Glycan characterization is of crucial importance during biopharmaceutical manufacturing. Figure 5A shows the fluorescence chromatogram of InstantPC-labeled N-Glycans from mAb1 separated on an Agilent AdvanceBio Glycan Mapping column, showing the typical major glycans G0F, G1F, and G2F. Glycan species were sufficiently well resolved to calculate relative content, with the table inset listing the relative quantities of each glycan. To confirm glycan annotations, MS signals from each peak were matched with an isotopic model of multiply charged H^+ and Na^+ adduct ions.

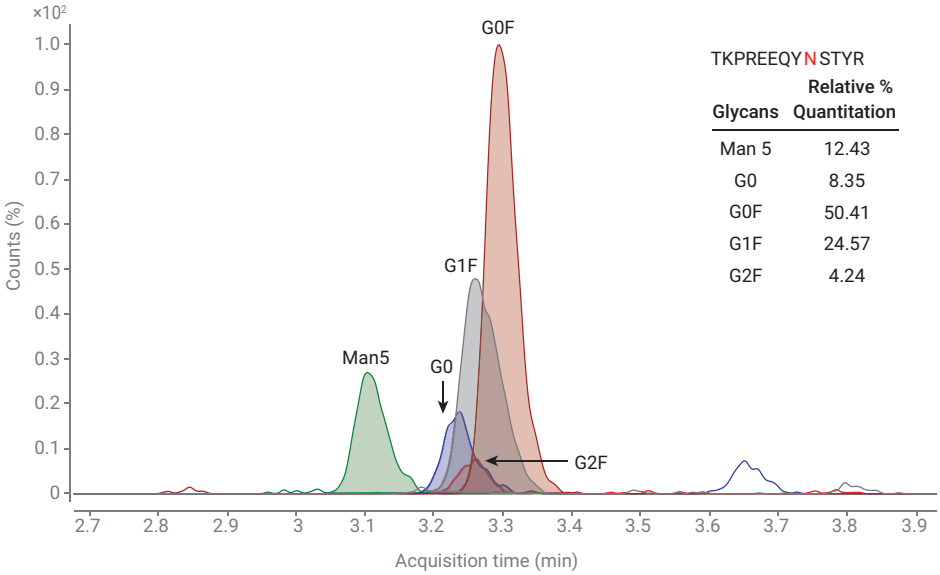
For peptide mapping based quantification of glycan species, tryptic peptides of mAb1 were subjected to LC/MS. Figure 5B shows the extracted ion chromatograms (EIC) overlay of different glycosylated TKPREEQYNSTYR peptides separated using the AdvanceBio Peptide Mapping column. MS analysis identified masses consistent with the major glycoforms (G0, G1F, G0F, and G2F) of the TKPREEQYNSTYR peptide, with the most abundant glycoforms corresponding to G0F and G1F.

Comparison of glycan quantification between released glycan (HILIC) and peptide mapping methods is shown in Figure 5C and Table 3, with both methods yielding similar estimates. The correlation coefficient 0.98 indicates a strong positive correlation between HILIC and RP peptide mapping methods for the major glycans.



Glycans	Relative % Quantitation
Man 5	10.9
G0	6.48
G0F	49.13
G1F	28.8
G2F	4.69

Figure 5A. Fluorescence chromatogram of InstantPC-labeled glycans from mAb1 and quantitation results.



TKPREEQYNSTYR

Glycans	Relative % Quantitation
Man 5	12.43
G0	8.35
G0F	50.41
G1F	24.57
G2F	4.24

Figure 5B. MS extracted compound chromatograms of *m/z* ratio matching to glycosylated TKPREEQYNSTYR peptide and quantitation results.

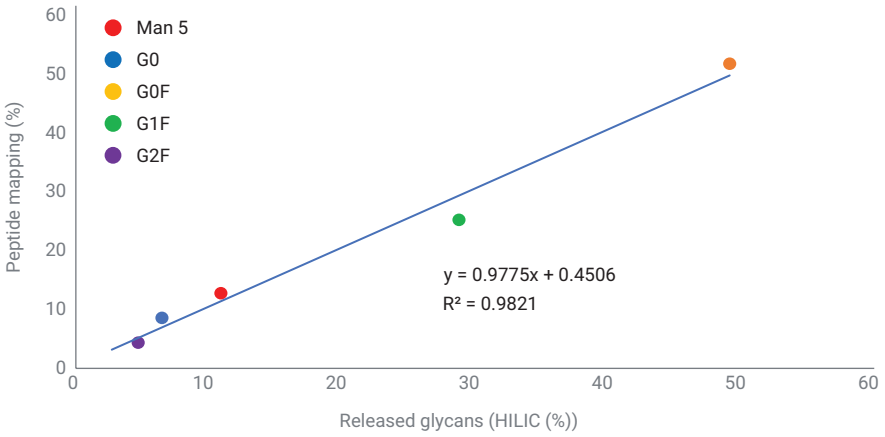


Figure 5C. Correlation graph. Comparison of glycan quantitation by HILIC and peptide mapping methods.

Conclusion

The present study demonstrates the Agilent peptide mapping workflow for monitoring multiple CQAs of mAbs. Comparison of relative % quantification of multiple attributes between peptide mapping and traditional assays showed a very good correlation. The results convey that the peptide mapping approach can replace traditional assays to monitor mAb quality attributes thereby reducing analysis time and cost.

References

1. Rogers, R. S. *et al.* A View on the Importance of "Multi-Attribute Method" for Measuring Purity of Biopharmaceuticals and Improving Overall Control Strategy. *The AAPS Journal* **2018**, *20*, 7.
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