Application Note

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Glycopeptide Characterization for Various Monoclonal Antibodies Using the Agilent 6545XT AdvanceBio LC/Q-TOF

Author

David L. Wong
Agilent Technologies, Inc.

Introduction

Monoclonal antibodies (mAbs) and their derivatives represent a very complex but important class of biopharmaceutical molecules with a wide range of applications. As mAbs are heterogeneous molecules by nature, comprehensive analytical characterization is required. The full range of biotherapeutics characterization usually includes confirmation of the protein sequences, protein post-translational modification (PTM) locations, and their relative quantitative information. Protein glycosylation is one of the major PTMs of an mAb, and is involved in many biological regulatory processes as well as therapeutic efficacy and immunogenicity[1]. Therefore, it is important to understand the various glycans' distribution and composition for pharmaceutical bioprocessing.

Quadrupole time-of-flight (Q-TOF) liquid chromatography/mass spectrometer (LC/MS) systems are often used to analyze intact mAbs and mAb subunits, perform mAb peptide sequence mapping, and characterize PTMs due to their excellent mass accuracy and resolution in the high-mass range[2,3].

Typically, four levels of LC/MS workflows for glycoform/glycan characterization are used:

- Levels 1 and 2 focus on the analysis of glycoforms on intact and reduced mAb molecules. The intact mAb workflow provides rapid assessment of the major glycoforms of the intact mAb, while the mAb subunit workflow offers detailed quantitative information about individual glycans such as G0F, G1F, and G2F.
- Level 3 is the analysis of glycopeptides generated from the proteolytical digestion of mAbs, commonly part of the peptide sequence mapping workflow[4]. This workflow shows results not only in glycan-relative quantitation, but also N-glycosylation site(s) information.
- Level 4 is the characterization of glycans that have been released by enzymatic cleavage or other mechanisms. It provides high analytical sensitivity and the best quantitation for glycan analysis (Figure 1)[5].

Peptide mapping of mAbs has widely been used as an analytical technique for the comprehensive characterization of protein biotherapeutics. This technique provides not only the complete amino acid sequences of mAbs and their variants, but also information on PTMs and locations. In routine analysis, peptides resulting from proteolytic digestion are typically separated by reversed-phase (RP) chromatography. RP-C18 or C8 columns are the most commonly used due to their excellent chromatographic separation power for regular peptides as well as peptides with PTMs such as oxidation and deamidation.

However, some protein modifications are not so easy to resolve through RP-type separation. Glycopeptides, which post relatively higher hydrophilicity, demonstrate very low retention and poor resolution on RP columns. In this case, hydrophilic interaction liquid chromatography (HILIC) with an amide-bonded stationary phase is often used as it can provide significantly more retention for glycosylated peptides.

This work demonstrates an optimized LC/MS workflow for mAb glycopeptide characterization (level 3) using the Agilent AssayMAP Bravo liquid-handling robot, the Agilent 1290 Infinity II LC system, the Agilent 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using Agilent MassHunter BioConfirm software for various glycopeptide identification and their relative quantitation (Figure 2). HPLC separation of glycopeptides from three different mAbs (NISTmAb, Trastuzumab, and A CHO cell cultured human IgG1 mAb) were compared on both the Agilent Peptide Mapping (RP-C18) column and the Agilent AdvanceBio Glycan Mapping (HILIC) column.

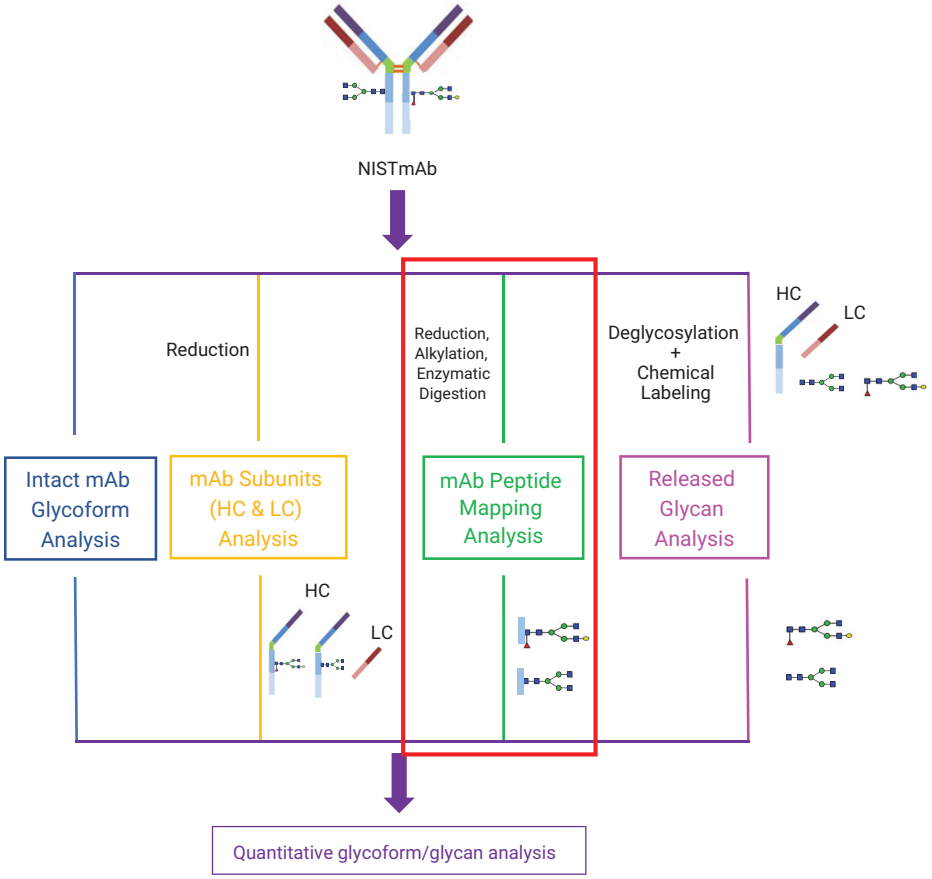


Figure 1. Various glycoform/glycan quantitative analysis workflows. The glycopeptide workflow is highlighted in the red box.

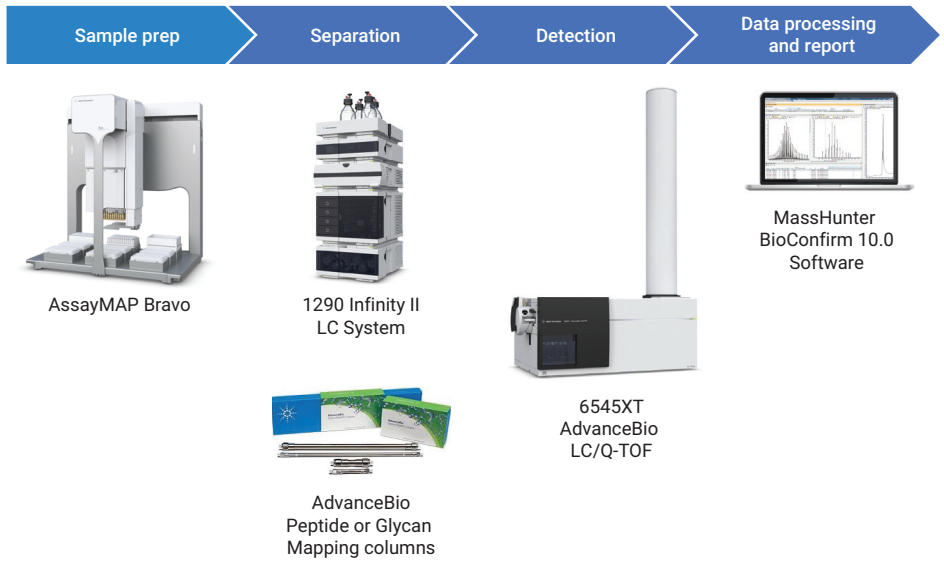


Figure 2. Analytical components of the mAb glycopeptide characterization workflow.

Experimental

Materials and methods

Three mAb samples were used in this study:

- The mAb standard, RM 8671, was from National Institute of Standards & Technology (NIST), aka NISTmAb.
- Formulated Herceptin (Trastuzumab) was from Genentech (So. San Francisco, California, USA).
- CHO mAb1 (A-mAb) was obtained from a collaborator.

2,2,2-Trifluoroethanol (TFE), DL-dithiothreitol (DTT), iodoacetamide (IAA), and *tris*(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. High-quality mass spec grade Trypsin/Lys-C enzyme mix was obtained from Promega. AssayMAP C18 cartridges were from Agilent Technologies.

All mAb samples were diluted with DI water to 1.0 µg/µL prior to sample preparation using the AssayMAP Bravo liquid handling system.

Instrumentation

- Agilent AssayMAP Bravo system (G5542A)
- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II high speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

Sample preparation

The AssayMAP Bravo liquid handling system was used to dilute, digest, and desalt the mAb samples[6]. Samples were then dried down and resuspended with 0.1 % formic acid (FA) in DI water for analysis on the Peptide Mapping column. The digested samples that needed to be analyzed by the Glycan Mapping (HILIC) column were resuspended with 80 % acetonitrile solution, which allowed effective sample loading and better chromatographic separation.

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system coupled with a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. LC separation was obtained with either an AdvanceBio Peptide Mapping column (2.1 × 150 mm, 2.7 µm) or an AdvanceBio Glycan Mapping column (2.1 × 150 mm, 2.7 µm). Tables 1–3 list the LC/MS parameters used. Approximately 2 µg of protein digest was injected onto the Peptide Mapping column, and 5 µg of protein digest was used on the Glycan Mapping column for the glycopeptide analyses.

Two separate sample data acquisitions were run for glycopeptide quantitative analysis: one with MS/MS data acquisition mode (using the shaded parameters in Table 3) for peptide identification; the other, with MS-only acquisition mode, was for glycopeptide quantitation.

Table 1. Liquid chromatography parameters.

1290 Infinity II LC System		
Column	AdvanceBio Peptide Mapping, 2.1 × 150 mm, 2.7 µm, (p/n 653750902)	AdvanceBio Glycan Mapping, 2.1 × 150 mm, 2.7 µm, (p/n 683775913)
Thermostat	4 °C	4 °C
Solvent A	0.1 % Formic acid in water	0.1 % Formic acid in acetonitrile
Solvent B	0.1 % Formic acid in acetonitrile	0.1 % Formic acid in water
Gradient	0–15 minutes, 0–10 %B 15–45 minutes, 10–40 %B 45–55 minutes, 40–90 %B	0–30 minutes, 5–40 %B 30–40 minutes, 40–60 %B 40–55 minutes, 60–90 %B
Column temperature	60 °C	50 °C
Flow rate	0.4 mL/min	0.4 mL/min
Injection volume	8.0 µL	20 µL
Amount on column	2 µg	5 µg

Table 2. MS acquisition parameters.

6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	250 °C
Drying gas	10 L/min
Nebulizer	25 psig
Sheath gas temperature	250 °C
Sheath gas flow	12 L/min
VCap	3,500 V
Nozzle voltage	0 V
Fragmentor	170 V
Skimmer	65
Quad AMU	95
Reference mass	121.0509 922.0098

Data processing

Raw data acquired from LC/MS/MS were processed using MassHunter BioConfirm 10.0 software. This software simplifies data analysis, enabling automatic identification and relative quantitation of targeted biomolecules for all major biopharma workflows.

Results and discussion

mAb glycoform profiling through the routine peptide mapping approach has been a widely used method. To demonstrate the effectiveness of glycopeptide separation by the HILIC column compared to the conventional RP-C18 column, three humanized IgG-1 type of mAbs were selected in this study. All mAbs were reduced, alkylated, and digested with a Trypsin + Lys-C enzymes mix using the same protocol in the AssayMAP Bravo liquid handling system. The digested mAb samples were then injected and separated by both the RP-C18 and the HILIC columns with the same HPLC run time (60 minutes). Figures 3 and 4 illustrate the chromatographic retention differences between the regular peptides and glycopeptides in the RP-C18 and the HILIC conditions.

Under routine RP HPLC conditions, peptides are separated by their hydrophobicity. The less hydrophobic peptides elute earlier than the more hydrophobic peptides. Since our HPLC gradient was optimized for the mAb tryptic digested samples, most of the peptides were separated nicely in the HPLC run. The glycopeptides are more hydrophilic and, thus, had shorter retention on the RP column. Figure 3 shows that all glycopeptides were eluted in the early gradient, with an approximately one-minute retention time window.

Table 3. MS/MS acquisition parameters.

Parameter	Value
Acquisition mode	Extended Dynamic Range (2 GHz)
Mass range	m/z 150–1,700
Acquisition rate	8 spectra/sec
Auto MS/MS range	*m/z* 50–1,700
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ 1.3 *m/z*)
Precursors/cycle	Top 10
Collision energy	3.6*(*m/z*)/100–4.8
Threshold for MS/MS	2,000 counts and 0.001%
Dynamic exclusion	On; 3 repeat then exclude for 0.2 minutes
Precursor abundance based scan speed	Yes
Target	25,000
Use MS/MS accumulation time limit	Yes
Purity	100 % stringency, 30 % cutoff
Isotope model	Peptides
Sort precursors	By abundance only; +2, +3, >+3

Data acquired for glycopeptide quantitation analysis used the MS-only acquisition mode. Highlighted parameters were used for peptide identification.

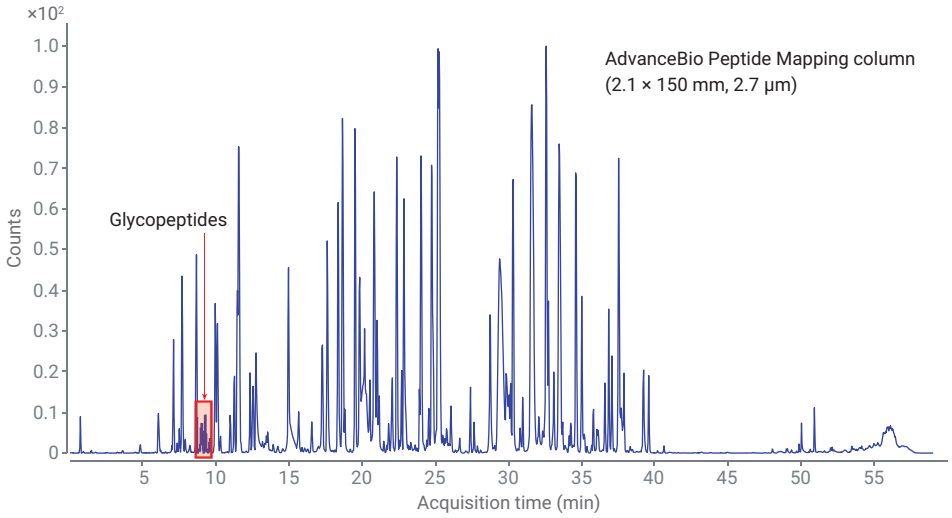


Figure 3. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Peptide Mapping (RP-C18, 2.1 × 150 mm, 2.7 µm) column.

The HILIC separation is an orthogonal method to the RP, where the HPLC gradient is reversed. The lyophilized mAb digests should be dissolved in a high organic content solution to have better sample loading retention. High resolution in separation was achieved, and all major glycopeptide peaks were eluted between 28–34 minutes, as shown in Figure 4.

For LC/MS data analysis, the Peptide Digest Workflow in MassHunter BioConfirm 10.0 software was used. This software program enables the quick setup for batch sample analysis. A modification file of most major PTMs, including oxidation, deamidation, and many glycans imported from a personal compound database (PCD), can be generated easily. The Agilent proprietary Peptide Feature Extraction (PFE) algorithm[7] was used for the identification of biomolecules, which were then confirmed by matching the measured masses with theoretical masses based on the known mAb sequences in the protein database. The relative quantitation on all identified peptides (including the glycopeptides) was also automatically calculated using either peak heights or peak areas of the mass spectra. Figure 5 is a screen capture of the BioConfirm 10.0 software layout showing the compound list of matched glycopeptides of NISTmAb. This program allows quick review of detailed peptide information including mass, retention

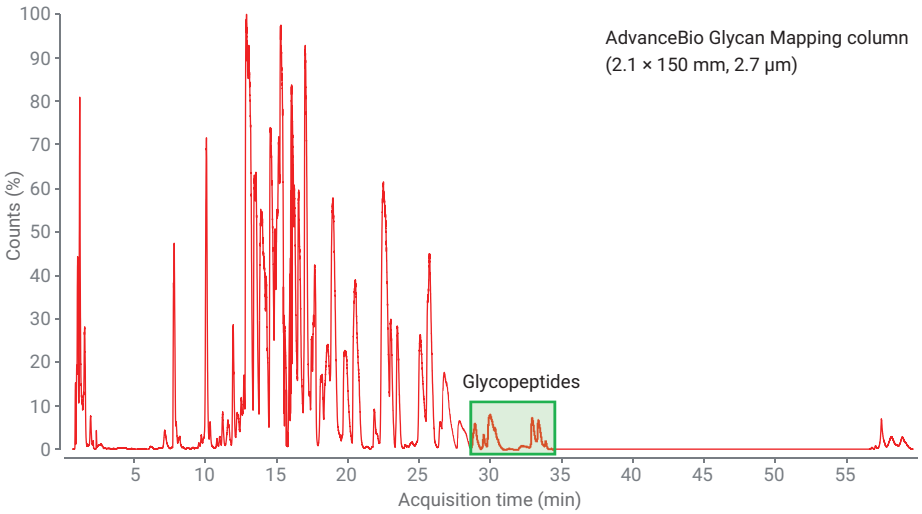


Figure 4. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Glycan Mapping (HILIC, 2.1 × 150 mm, 2.7 µm) column.

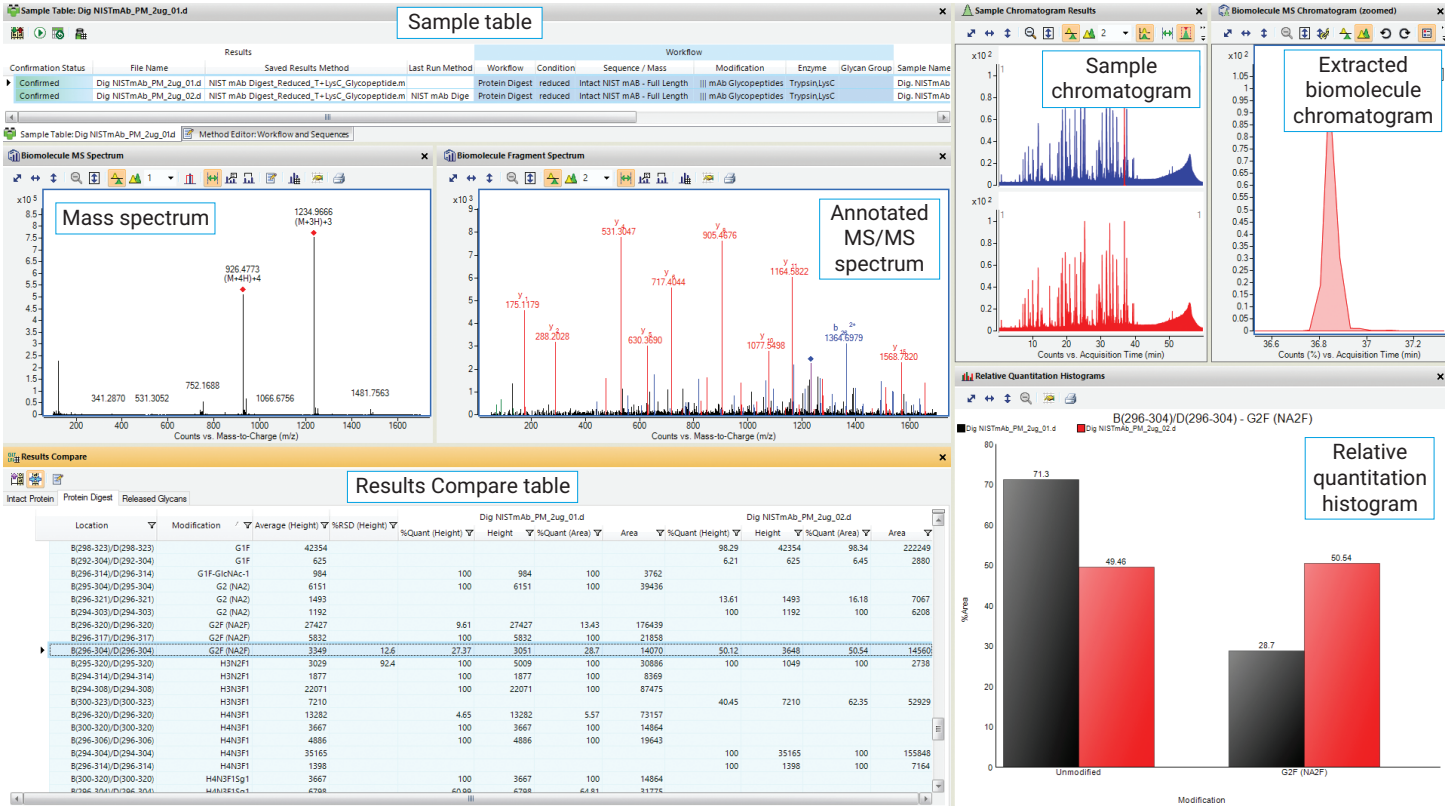
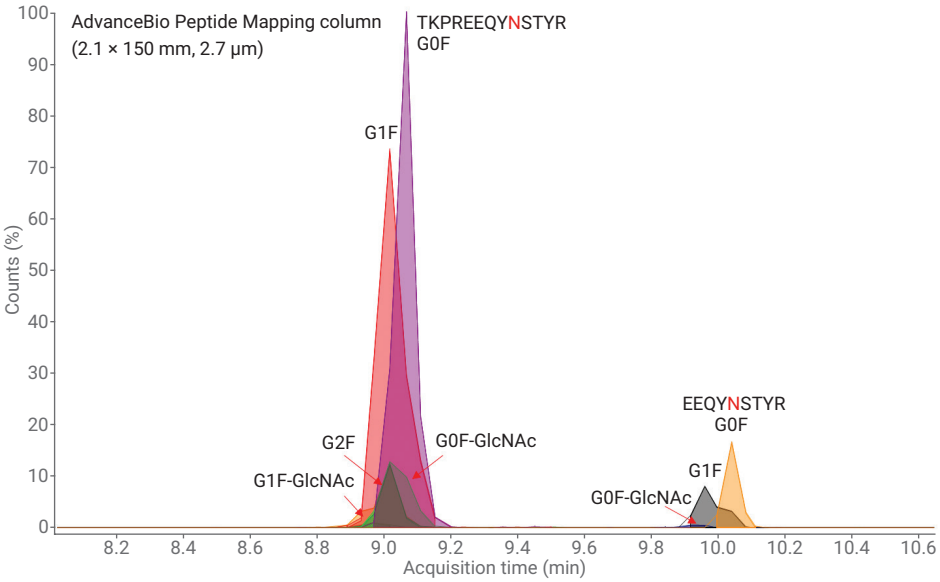


Figure 5. Screen capture of MassHunter BioConfirm 10.0 software with representative glycopeptide profiling results and histogram of relative quantitation on glycopeptides.

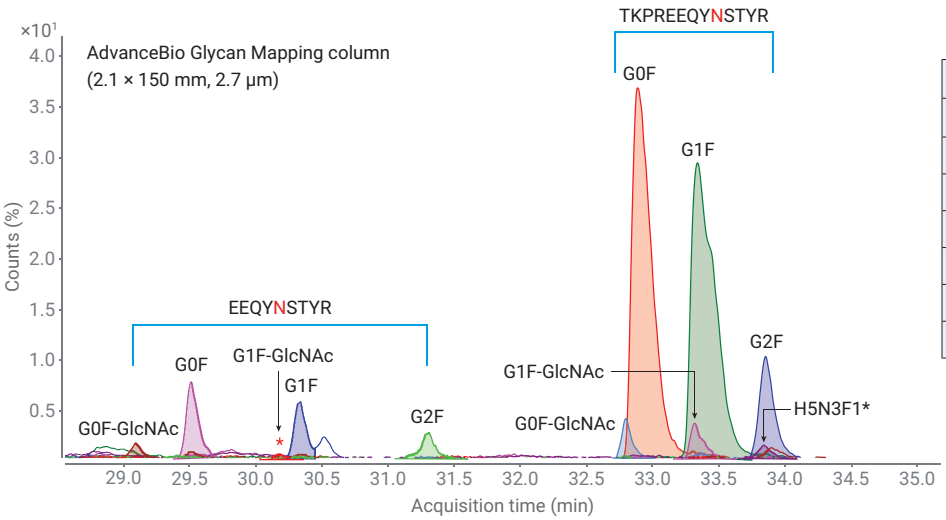
times, sequences, modifications, scores, and quantitative results by either peak heights or peak areas. One feature of the BioConfirm 10.0 software is that users have the ability to select or deselect certain peptides for grouping in relative quantitation analysis, with the results shown in histogram format.

Detailed inspection of raw MS data from Figures 3 and 4 reveals that there were two major group of glycopeptides (EEQYNSTYR and TKPREEQYNSTYR) with various glycans attached at the asparagine (N300 of heavy chain) position. In the RP separation, three glycopeptides with sequence of EEQYNSTYR, and six glycopeptides in TKPREEQYNSTYR were identified (Figure 6). However, the same group of glycopeptides were coeluted, and poor chromatographic resolution was observed. Conversely, the HILIC column demonstrated great resolution for the separation of the same sets of glycopeptides (Figure 7).



TKPREEQYNSTYR (292-304)			
Glycan	Mass (Da)	RT (min)	Quant (%)
G0F	3115.3386	9.066	41.82
G1F	3277.3918	9.016	41.70
G2F	3439.4406	9.016	9.08
G0F-GlcNAc	2912.2556	9.016	2.87
G1F-GlcNAc	3074.3058	9.016	3.35
H5N3F1*	3236.3534	8.930	1.18

Figure 6. MS extracted compound chromatograms (ECCs) and relative % quantitation of the identified glycopeptides from RP LC separation. H5N3F1* may be denoted as FM4A1G1 or FA1G1Ga1 in other publications.



TKPREEQYNSTYR (292-304)			
Glycan	Mass (Da)	RT (min)	Quant (%)
G0F	3115.3386	32.923	43.07
G1F	3277.3918	33.334	39.89
G2F	3439.4406	33.877	9.31
G0F-GlcNAc	2912.2556	32.797	3.10
G1F-GlcNAc	3074.3058	33.321	3.37
H5N3F1*	3236.3534	33.864	1.25

Figure 7. MS ECCs and relative % quantitation of the identified glycopeptides from HILIC separation.

Although more than nine glycopeptides (Figures 6 and 7) were detected and identified in different LC conditions, a set of six major abundant glycopeptides with the sequence of TKPREEQYNSTYR were selected for relative quantitation analysis (tables in Figures 6 and 7) to have fair comparison results.

Figure 8 summarizes the relative quantitation and reproducibility results of the six major glycopeptides of the NISTmAb from three replicate sample injections of 2 µg (RP-C18) and 5 µg (HILIC) on-column, respectively. The quantitative results from the peak area of the RP method were similar to those from the HILIC method. However, due to the better glycopeptide separation, the HILIC results represented higher quantitation accuracy and smaller average standard deviations (SDs) for all glycopeptides (<0.2 %); the average SDs of the RP method results were approximately 0.56 %.

We used the same HILIC method for glycopeptide relative quantitative comparison among three mAbs (NISTmAb, Herceptin, and A-mAb). Figure 9 shows the relative % quant of the top six most abundant glycopeptides. Unlike the NISTmAb that posted similar abundances of G0F and G1F (43 % and 40 %), the Herceptin sample contained a very high level of G0F (>65 %) and low level of G2F (~2 %). In addition, no H5N3F1 could be detected in either Herceptin or A-mAb samples. Two degraded glycan molecules (G0F-GlcNAc and G1F-GlcNAc) were found at trace levels (<0.5 %) as well in the A-mAb sample.

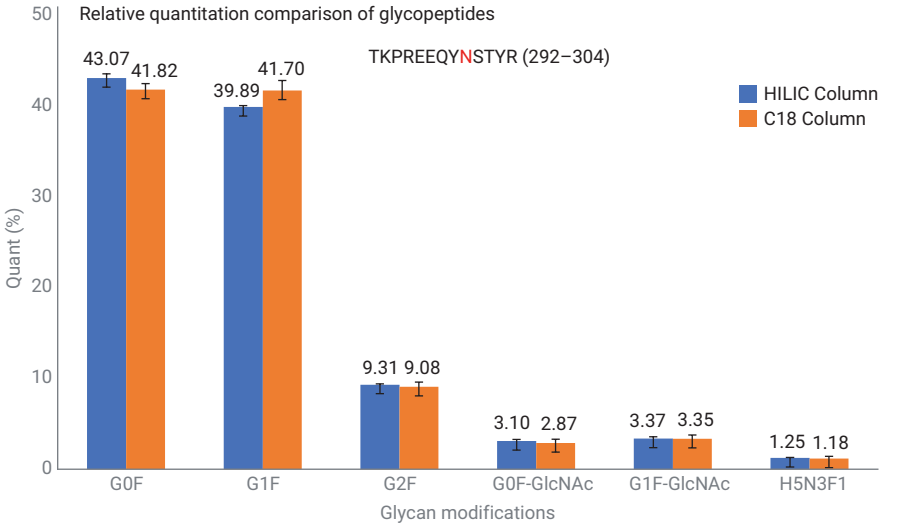


Figure 8. Relative quantitative comparison of NISTmAb glycopeptides analysis (RP-C18 versus HILIC, three replicates).

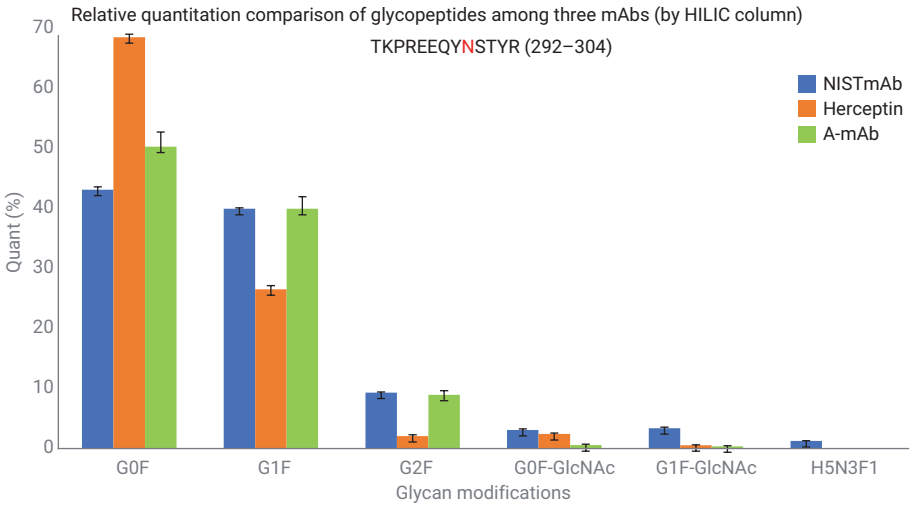


Figure 9. Relative % quantitation of the top six glycopeptides in each of the three mAb samples. All digested mAb samples were separated by the HILIC column (three replicates).

Conclusion

A complete workflow solution for mAb glycopeptide characterization by integrating the AssayMAP Bravo liquid handling platform, 1290 Infinity II LC, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software has been developed. The major benefits of this new workflow include:

- The AdvanceBio Glycan Mapping (HILIC) column demonstrated strong retention and increased resolution for hydrophilic glycopeptides. Various glycoforms of the same peptide were well resolved.
- The glycopeptide analysis through peptide mapping workflow resulted in not only glycan relative quantitation, but also N-glycosylation site(s) information.
- The automated data processing capability of BioConfirm 10.0 resulted in accurate glycopeptide profiling—identification and relative quantitation. A batch of samples or different mAb digests can easily be analyzed and compared.

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