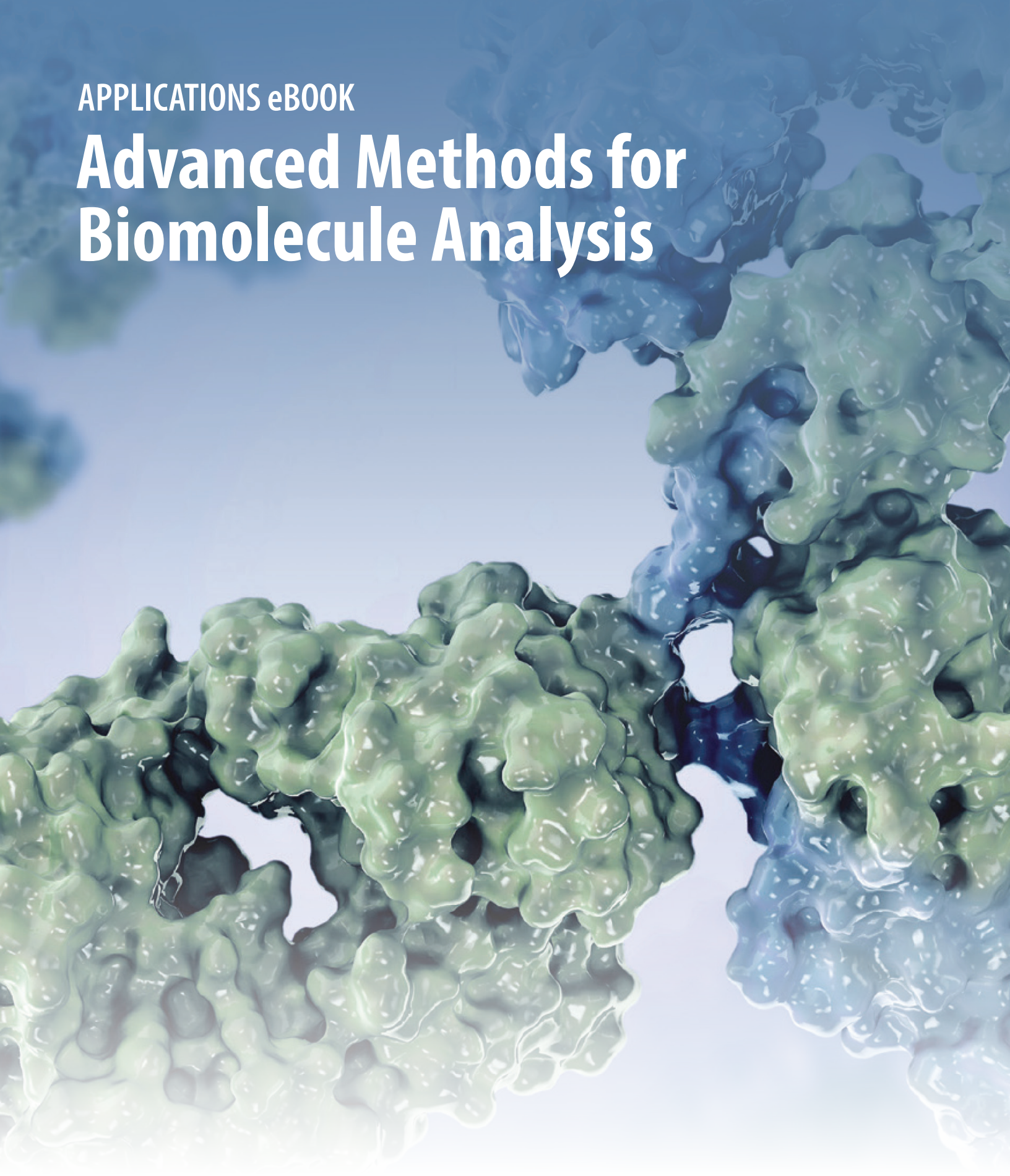
APPLICATIONS eBOOK

Advanced Methods for Biomolecule Analysis



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Advanced Methods for Biomolecule Analysis

Sensitive Native Mass Spectrometry of Macromolecules Using Standard Flow LC/MS
Describes a robust and sensitive LC/MS method using standard LC flow for the analysis of native protein analysis. The workflow comprised the Agilent 1290 Infinity II LC, the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software.

High-Resolution, High-Throughput Size Exclusion Chromatography Analysis of Monoclonal Antibodies
Demonstrates the use of the Agilent AdvanceBio SEC 200 Å 1.9 µm column for high-resolution and high-throughput size exclusion chromatography (SEC) analysis of a monoclonal antibody (mAb).

High Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins
Analysis of recombinant human growth hormone (hGH), granulocyte colony-stimulating factor (hG-CSF), and interferon α-2b (INF α-2b) proteins demonstrate the superior performance of the AdvanceBio column for small protein therapeutic applications.

Analysis of Camelid Single-Domain Antibodies Using Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns
Describes the use of size exclusion chromatography and hydrophobic interaction chromatography for the analysis of camelid single-domain antibodies (nanobodies). Nanobodies are a growing class of single-domain antibody fragments used for therapeutic purposes.

Monitoring Product Quality Attributes of Biotherapeutics at the Peptide Level Using the Agilent InfinityLab LC/MSD XT System
This study serves as a proof of concept for monitoring multiple product quality attributes (PQAs) using an SQ LC/MS system with software that is recommended for laboratories requiring regulatory compliance.

Critical Quality Attribute Monitoring of mAbs at the Intact and Subunit Levels Using Cost-Effective, Simple and Robust LC/MS
Describes use of the Agilent LC/MSD XT mass selective detector, a single quadrupole LC/MS system with a mass range of 10 to 3,000 *m/z*, for mass determination of mAbs at intact and subunit levels.

Charge Variant and Aggregation Analysis of Innovator and Biosimilars of Rituximab
This study compares two rituximab biosimilars from different manufacturers to the innovator for their aggregation and charge variant profiles by following two analytical workflows using Agilent 1260 Infinity II bio-inert LC and Agilent AdvancedBio columns.

Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS
Describes the preparation and analysis of released N-glycans from biotherapeutic glycoproteins using two labels, InstantPC and 2-aminobenzamide (2-AB).

A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis
This study demonstrates how to increase sample throughput for glycan characterization workflows using the Agilent AssayMAP Bravo liquid handling platform.

Profiling Glycosylation of Monoclonal Antibodies at Three Levels Using the Agilent 6545XT AdvanceBio LC/Q-TOF
A complete workflow solution for antibody glycoforms characterization by integrating the Agilent AssayMAP Bravo liquid handling platform, UHPLC technologies, the Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software.

Glycopeptide Characterization for Various Monoclonal Antibodies Using the Agilent 6545XT AdvanceBio LC/Q-TOF
An optimized LC/MS workflow for mAb glycopeptide characterization using the Agilent AssayMAP Bravo liquid-handling robot, the 1290 Infinity II LC system, the 6545XT AdvanceBio LC/Q-TOF, and automatic data analysis using MassHunter BioConfirm software.

Application Note

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Sensitive Native Mass Spectrometry of Macromolecules Using Standard Flow LC/MS

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Abstract

Native mass spectrometry can be used for a variety of protein-based applications, such as protein-protein interaction, protein-ligand binding, protein complex structures, protein folding and antibody-drug conjugates. Most of the native MS analyses are using a nano-electrospray approach which faces significant challenges.

This application note describes a robust and sensitive LC/MS method using standard LC flow for the analysis of native protein analysis. The workflow comprised the Agilent 1290 Infinity II LC, the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software.

Introduction

Native mass spectrometry (MS) has emerged as a widely used technique for the characterization of intact proteins and noncovalent protein complexes. Various sizes of protein complex structures (protein-ligand binding or protein-protein interaction) ranging from a few kDa to more than 1 MDa have successfully been analyzed and studied by this technique despite its tremendous analytical challenges.[1-4] Without organic solvent and acid to enhance sample desolvation and ionization, native MS analysis of protein samples at neutral pH conditions tends to have fewer charges per molecule and much lower abundance MS signals at higher *m/z* ranges. In the past decade, the nano-electrospray ionization (nESI) approach has become a crucial method used in native protein analysis. The nESI forms fine charged droplets, which can dramatically increase the sample desolvation and ionization efficiency while preserving the noncovalent protein-protein complexes. However, it has commonly been observed that the neutral aqueous protein samples tend to aggregate easily under the unstable nanoflow rate condition and cause the nanospray emitter to clog. Also, well trained or experienced researchers are needed to produce good-quality MS data using the nESI technique.

In this study, we demonstrate a highly sensitive analytical flow LC/MS methodology for the analysis of native proteins and protein complexes. This workflow uses the AdvanceBio size exclusion chromatography (SEC) column for online sample separation. The 6545XT AdvanceBio LC/Q-TOF, featuring large molecule SWARM autotune and 30,000 *m/z* extended mass range, was used for rapid and reproducible native protein analysis (Figure 1).

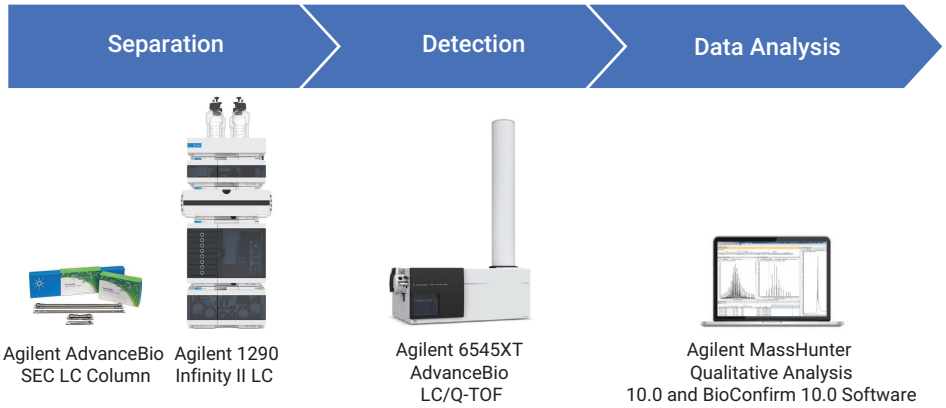

Figure 1. Analytical components of the native protein analysis workflow.

Experimental

Materials and methods

Monoclonal antibody standard, RM 8671, was purchased from the National Institute of Standards and Technology (NIST), often referred to as NIST-mAb. The formulated Herceptin (trastuzumab) was obtained from Genentech (South San Francisco, California, USA). The formulated trastuzumab emtansine (TDM1, ADC) was also from Genentech. All other protein samples and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

To perform native MS analysis, it is crucial to preserve the protein samples at neutral pH and volatile aqueous solutions, such as ammonium acetate or ammonium formate. Therefore, sample desalting and buffer exchange are usually needed prior to the MS analysis. Briefly, protein stock solutions (1 to 10 mg/mL) were desalted and solvent exchanged into 100 mM ammonium acetate using Bio-Rad Bio-Spin P-6 (6,000 MW limit) or P-30 (40,000 MW limit) cartridges. The cartridge was first fully equilibrated with 100 mM ammonium acetate buffer. Protein sample was then pipetted to the top of the column and centrifuged for 5 min at 1000 × g. The protein was then buffer exchanged into the 100 mM ammonium acetate and was ready for MS analysis. This desalting protocol caused minimal sample loss and much less structural alteration of the protein molecule.

Instrumentation

- Agilent 1290 Infinity II LC including:
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B)
  - Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC coupled with a 6545XT AdvanceBio LC/Q-TOF system equipped with an Agilent Jet Stream source. Agilent MassHunter Acquisition (B.09.00) workstation software with the large molecule SWARM autotune feature was used.

LC separation was obtained with an Agilent AdvanceBio SEC guard column (4.6 × 30 mm, 200 Å, 1.9 µm).

Tables 1 and 2 list the LC/MS parameters used.

Data processing

All MS data of the native intact mAbs or protein complexes were processed using Agilent MassHunter Qualitative Analysis 10.0 and BioConfirm 10.0 software.

Results and discussion

Method optimization for native protein and protein complex analysis

To overcome the challenges of native protein analysis, some key method developments and optimizations were made:

- The use of offline desalting cartridges (Bio-Rad Bio-Spin P-30) for sample preparation (desalting and buffer exchange) prior to the MS analysis and online SEC column further separated the target protein from background salts, which led to higher MS sensitivity and improved MS data quality.
- The use of a conventional flow rate (0.2 mL/min) of 100 mM ammonium acetate buffer not only eliminated the sample aggregation but also improved LC/MS analytical reproducibility for well-preserved native protein samples.
- The 6545XT AdvanceBio LC/Q-TOF system was equipped with large molecule SWARM autotune for optimizing macromolecular ions transmission, and the extended mass range of up to *m/z* 30,000 for the native protein complex analysis with high sensitivity.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC	
Column	AdvanceBio SEC (200 Å, 4.6 × 30 mm, 1.9 µm) (p/n: PL1580-1201)
Thermostat	4 °C
Solvent (A)	100 mM NH4OAc (pH 7)
Isocratic Elution	0–5 min, 100% A
Column Temperature	Room temperature
Flow Rate	0.2 mL/min
Injection Volume	1–5 µL

Table 2. Native MS data acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF			
Sample Type	Myoglobin	Intact mAbs	Intact Macroprotein Complexes
Source	Agilent Jet Stream	Agilent Jet Stream	Agilent Jet Stream
Dry Gas Temperature	150 °C	365 °C	365 °C
Dry Gas Flow	10 L/min	12 L/min	12 L/min
Nebulizer	30 psig	35 psig	35 psig
Sheath Gas Temperature	150 °C	300 °C	300 °C
Sheath Gas Flow	10 L/min	12 L/min	12 L/min
VCap	5000 V	5500 V	5500 V
Nozzle Voltage	2000 V	2000 V	2000 V
Fragmentor	250 V	300 V	300 V
Skimmer	100 V	220 V	220 V
Quad AMU	*m/z* 500	*m/z* 1000	*m/z* 3000
Mass Range	*m/z* 300–7000	*m/z* 3000–10,000	*m/z* 5000–25,000
Acquisition Rate	1.0 spectrum/s	1.0 spectrum/s	1.0 spectrum/s
Acquisition Mode	Positive, extended (*m/z* 10,000) mass range	Positive, extended (*m/z* 10,000) mass range	Positive, extended (*m/z* 25,000) mass range

Native MS analysis of intact myoglobin (with heme)

Native MS analysis of noncovalent interactions of myoglobin has been well-studied.[4] In myoglobin, heme is noncovalently attached to the globin through hydrogen bonds and hydrophobic interactions. When the heme is attached to the globin, the protein is referred to as holomyoglobin (the native conformation). Monitoring of the charge state distributions of myoglobin ions in mass spectra of ESI-MS has been used in protein folding/unfolding studies.[4] The apomyoglobin (with no heme) with high charge states indicated the disruption of the native heme-protein interaction, which led to a considerable degree of protein unfolding. As shown in Figure 2A, myoglobin was denatured in the organic and acid solvent, and under harsh MS source conditions. The charge envelope of the denatured myoglobin ranged from *m/z* 1,000 (17+) to 3,500 (5+) while the most intense charged ion was 12+. Most of the native holomyoglobin was denatured into apomyoglobin and heme (inset in Figure 2A). Our optimized native MS analysis of myoglobin clearly demonstrated that the native conformation of myoglobin was retained (Figure 2B). Only trace amounts of apomyoglobin and heme could be detected. The charge envelope of holomyoglobin was from 9+ to 5+ and the charge state of 8+ was the most abundant ion. The overall MS signal intensities of the native MS ions were about 1/10 of those in the denatured MS spectrum.

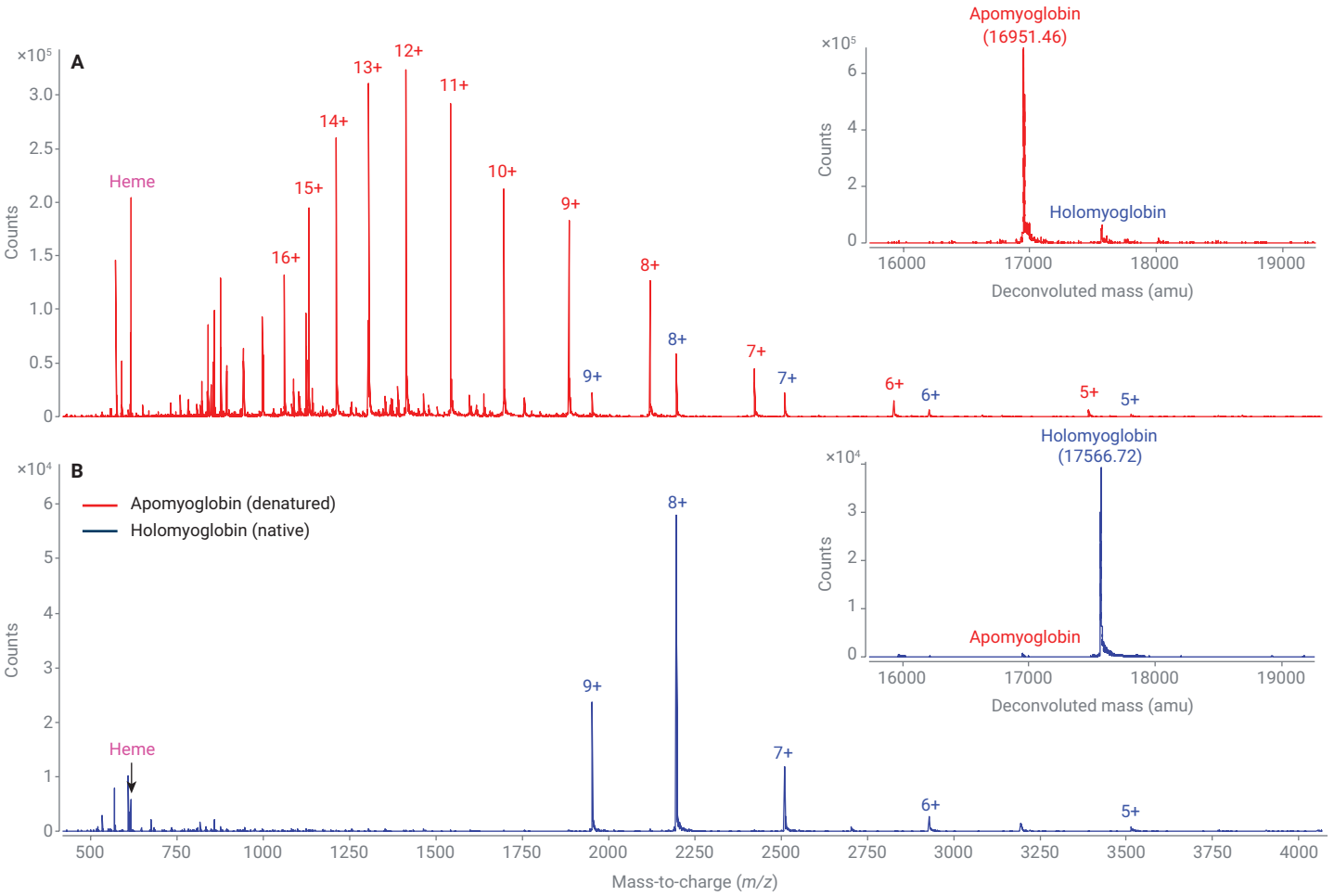

Figure 2. LC/MS analysis of intact myoglobin sample. A) Myoglobin sample was analyzed under denatured LC/MS conditions (previous studies). The heme group was dissociated from the protein complex and the majority of the protein was apomyoglobin (inset figure). B) Native MS analysis of myoglobin. The holomyoglobin (with heme) structure was preserved and only trace amount of heme was detected.

The native MS analysis results confirmed that ionic strength of the SEC column mobile phase also played a key role in maintaining the protein native conformation.[2] Figure 3 demonstrates the native MS analysis of alcohol dehydrogenase (ADH, tetramer) under two mobile phase conditions. Even though both mobile phases were at neutral pH, protein dissociation products (dimer) were observed when ammonium formate was substituted for ammonium acetate in the mobile phase (Figure 3A and 3B). Also, the charge state envelope of the intact native ADH in the 50 mM ammonium formate was shifted to a lower *m/z* range compared to that in the 100 mM ammonium acetate (Figure 3C). The results indicate that use of ammonium formate in the mobile phase increases the number and extent of multiply charged ions (max at 26+ compared to 23+ using ammonium acetate, Figure 3), although the ADH species may still be considered an intact protein tetrameric complex. Therefore, we believe that the 100 mM ammonium acetate solution offered better structural protection to protein complexes during the native MS analysis.

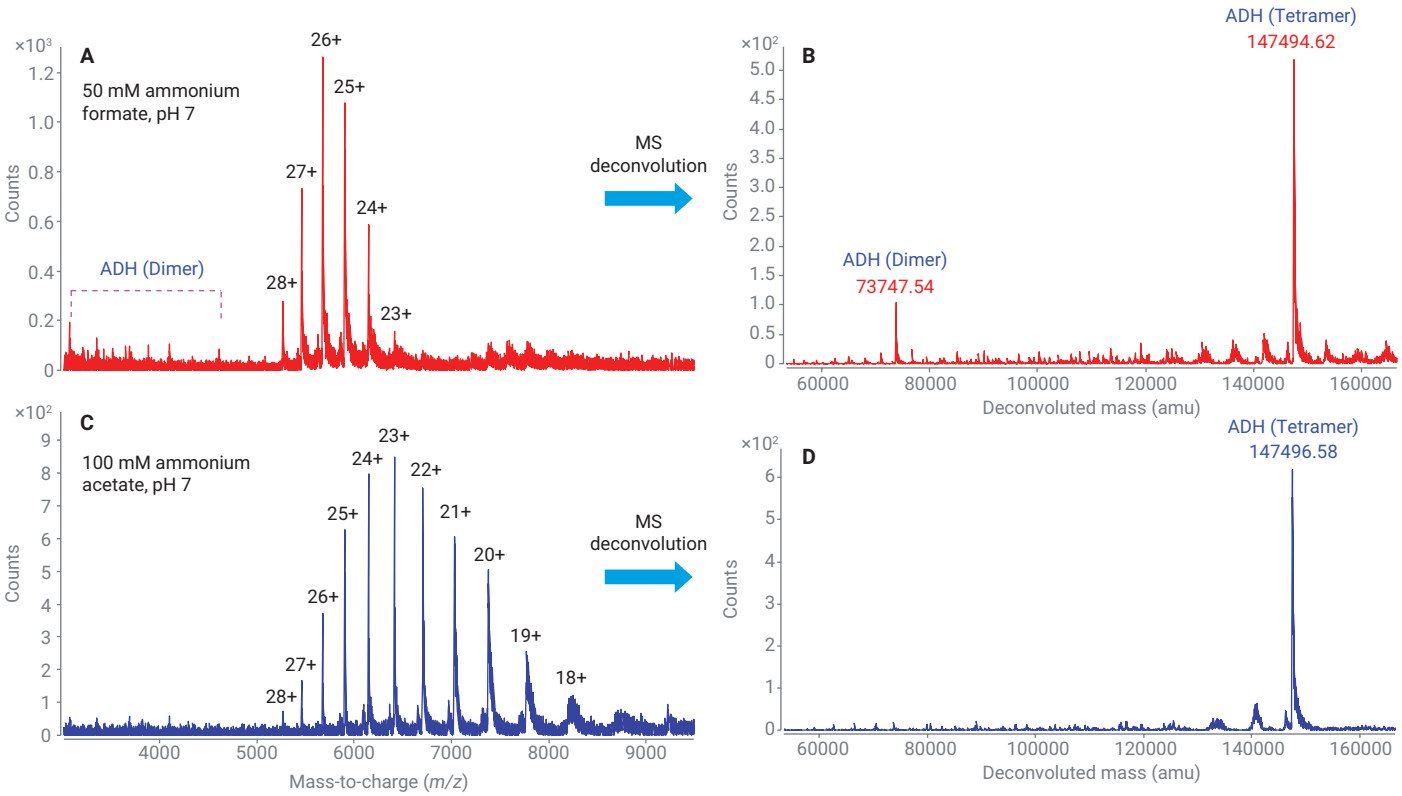

Figure 3. Native alcohol dehydrogenase (ADH, tetramer) analysis under various solvent conditions. A) Native ADH in 50 mM ammonium formate, pH 7. C) Native ADH in 100 mM ammonium acetate, pH 7. B) and D) deconvoluted spectrum of both samples.

Native MS analysis of intact mAbs

Monoclonal antibodies (mAbs) and their derivative products have quickly become an important class of biopharmaceutical molecules with a wide range of therapeutic applications. Native MS analysis of mAbs can provide valuable information, such as: protein folding, mAb aggregation (mAb dimer or trimer), antibody drug conjugates (ADCs), bispecific mAbs, etc.

In this study, we applied the online SEC method for rapid and robust native mAbs MS analysis. Approximately 0.5 to 1.0 µg of mAb was injected onto an AdvanceBio SEC guard column using a 5 min isocratic flow at 0.2 mL/min of 100 mM ammonium acetate solvent. The Q-TOF source conditions were optimized for excellent quality of native MS spectra over the mass range from *m/z* 5,000 to 10,000. Figure 4 demonstrates the LC/MS analysis of intact NIST mAb standard under the denaturing MS conditions (Figure 4A and 4B) as well as the native MS conditions (Figure 4C and 4D). In both conditions, all major glycoforms of the NIST mAb were well resolved (Figure 4A and 4C, inset). The charge state distribution of denatured NIST mAb spanned the mass range of *m/z* 2,000 to 5,000 (30+ to 75+), while the native NIST mAb had a charge envelope in the range of *m/z* 5,000 to 10,000 (15+ to 30+). As shown in the MS deconvoluted spectra (Figure 4B and 4D), low ppm in mass errors were obtained for all major glycoforms. We also achieved very good agreement with the data for the intact NIST mAb analysis under both MS conditions.

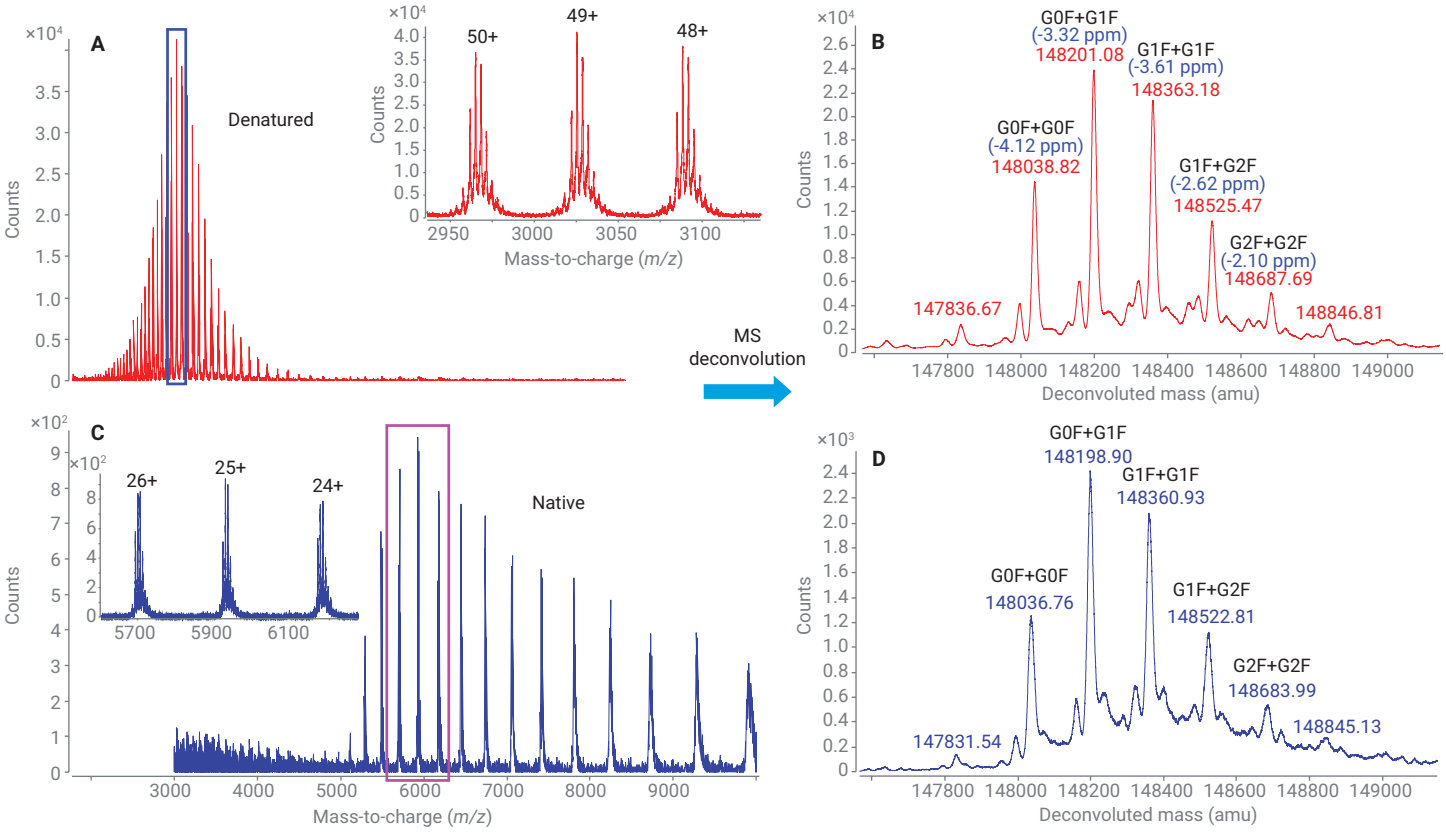

Figure 4. LC/MS analysis of NISTmAb under: A) denaturing MS conditions (acetonitrile and 0.1% formic acid) (previous work, Ref. 5) and C) native MS conditions (in 100 mM ammonium acetate, pH 7). The deconvoluted MS spectra of both samples are shown in B) and D), respectively.

Similarly, native MS analysis of a biotherapeutic drug (trastuzumab, brand name: Herceptin) and its ADC (trastuzumab emtansine, T-DM1) was performed and compared. Figure 5A illustrates the native mass spectrum of intact Herceptin, showing a nicely distributed charge envelope from *m/z* 5,000 to 10,000 with charge states between 15+ and 28+. The most prominent charge state was at 24+ which indicated the intact Herceptin was in its native/folded conformation. High mass accuracies for the major glycoforms were achieved as shown in the inset deconvoluted spectrum.

Native MS analysis enables probing of protein molecules while preserving their native structural conformation. As this method minimizes the interferences from organic solvent and acid in the mobile phase, it is an ideal analytical tool for noncovalent protein complexes or acid labile protein conjugates, such as some ADCs. Figure 5 shows the native raw and deconvoluted (inset) MS spectrum of T-DM1. The average DAR value calculated using the BioConfirm DAR Calculator was 3.5 (Figure 5B, inset), which is consistent with the DAR values of the intact ADC reported by Genentech (the manufacturer).

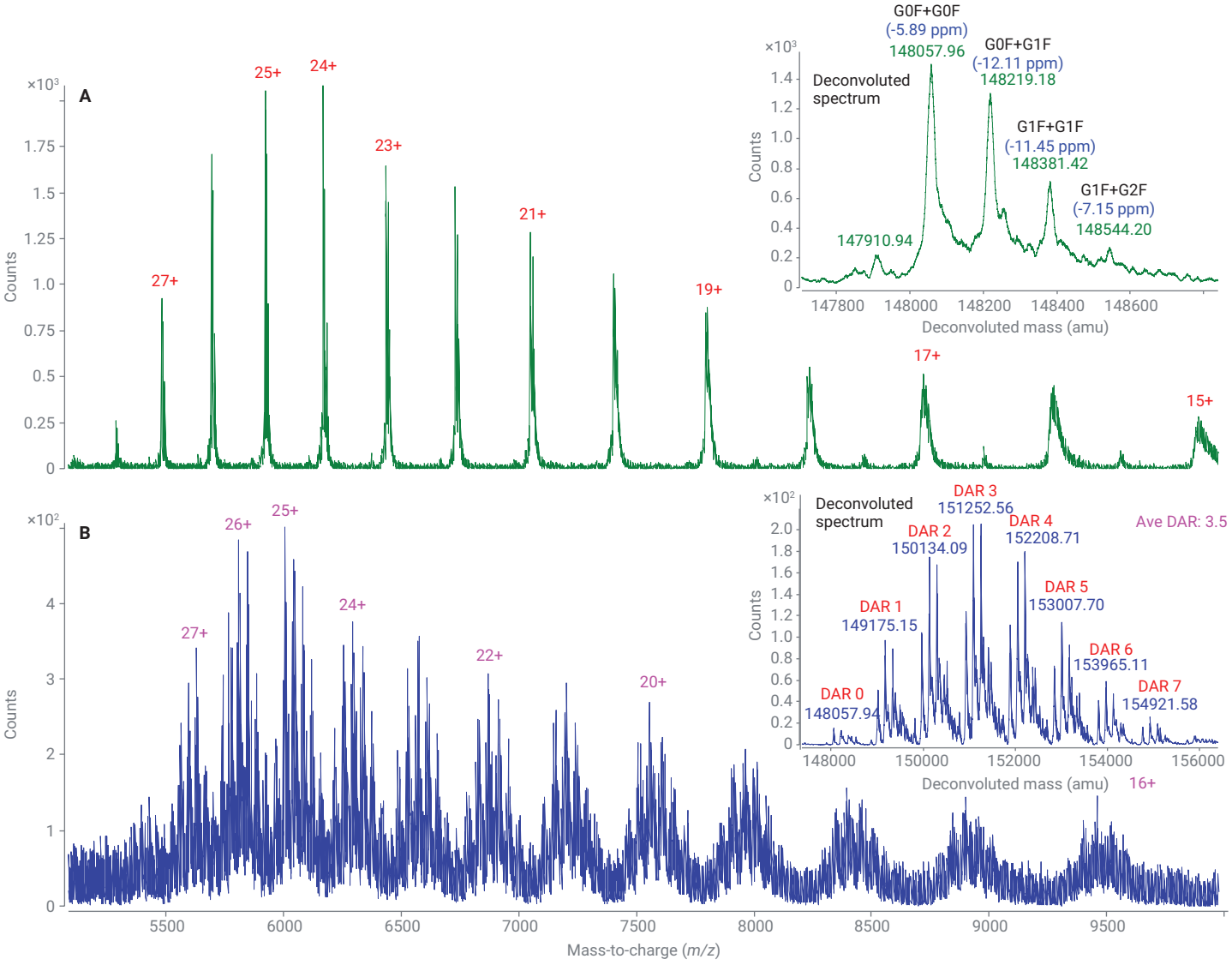

Figure 5. Native LC/MS analysis of mAb and its antibody drug conjugate (ADC): A) Herceptin and B) T-DM1. The deconvoluted MS spectra of both samples are shown in the inset figures.

Native MS analysis of intact protein complexes

The optimized native MS analysis method was also evaluated by three protein complex samples. They were: tetrameric pyruvate kinase (PK, 232 kDa), hexameric glutamate dehydrogenase (GDH, 335 kDa), and tetrameric β-galactosidase (466 kDa). The 6545XT AdvanceBio LC/Q-TOF system offers large molecule SWARM autotune for optimizing macromolecular ions transmission in the extended mass range up to *m/z* 30,000. It is an ideal LC/MS system for native protein complex analysis. Figure 6A shows the native mass spectrum of the tetrameric pyruvate kinase. Two major charge envelopes ranging from *m/z* 6,000 to 10,000 with charge state of 24+ to 37+ were detected. The deconvoluted spectrum revealed that there were two multi-proteoform complexes of PK tetramers in the sample: full-length pyruvate kinase and truncated PK tetramer (three intact subunits plus one PK proteoform with N-terminal cleavage).[3]

The 6545XT system also demonstrated excellent detection sensitivity for protein complexes at higher *m/z* (>*m/z* 10,000). Figure 6B and 6C show the native MS spectrum of GDH and β-galactosidase. Both of their protein charge envelopes were greater than *m/z* 8,000, whereas the most abundant ions were at *m/z* 9,566 (35+) for GDH and *m/z* 10,832 (43+) for β-galactosidase, respectively. The molecular mass of the intact hexameric GDH was determined to be 334,754 and 465,788 Da for the tetrameric β-galactosidase with 1 to 10 µg sample injections.

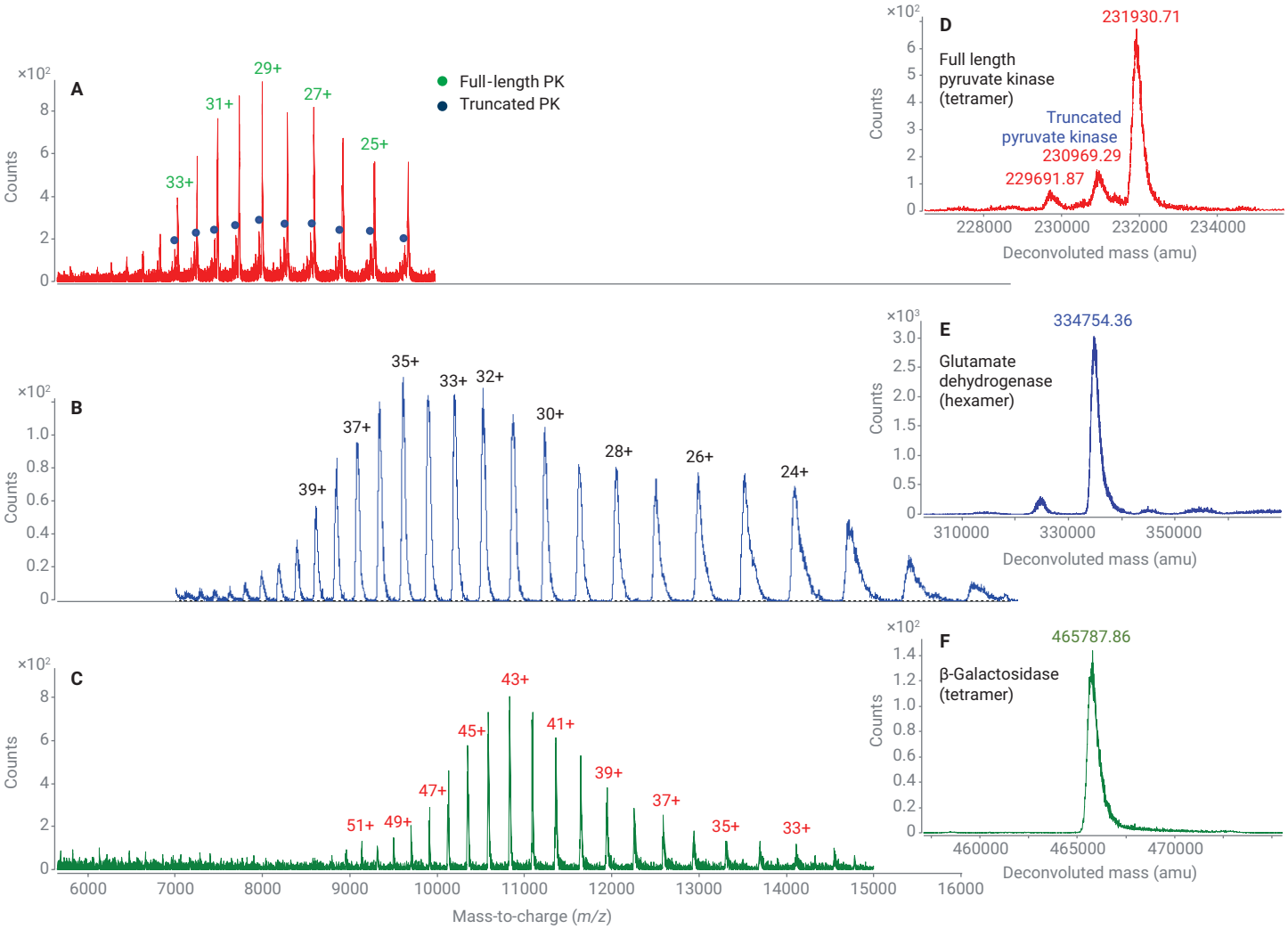

Figure 6. Native LC/MS analysis of various intact protein complexes. A) Pyruvate kinase (PK, tetramer, 232 kDa), B) glutamate dehydrogenase (GDH, hexamer, 335 kDa) and C) β-galactosidase (tetramer, 466 kDa). The deconvoluted spectra are shown in D) to F), respectively. The raw MS spectrum in Figure 6B was smoothed using the mMass open-source MS software tool.

Conclusion

We have developed a highly sensitive and robust LC/MS workflow methodology for native protein analysis. This optimized workflow utilizes the 1290 Infinity II LC with the AdvanceBio SEC column, the 6545XT AdvanceBio LC/Q-TOF with extended mass range up to *m/z* 30,000, and MassHunter BioConfirm software. The following benefits have been demonstrated by this native MS analysis method:

- Use of an online SEC column at typical analytical LC flow rates eliminates the challenging issues (protein aggregation and instable spraying flow) associated with nanoESI analysis.
- The optimized native MS conditions provide high confidence in ADC characterization, with accurate determined DAR values.
- The large molecule SWARM autotune feature, along with the extended mass range of the 6545XT AdvanceBio LC/Q-TOF, enables the sensitive detection and characterization of native intact macroprotein complexes.

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Application Note

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High-Resolution, High-Throughput Size Exclusion Chromatography Analysis of Monoclonal Antibodies

Using an Agilent AdvanceBio SEC 200 Å 1.9 µm column

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Abstract

This Application Note demonstrates the use of the Agilent AdvanceBio SEC 200 Å 1.9 µm column for high-resolution and high-throughput size exclusion chromatography (SEC) analysis of a monoclonal antibody (mAb). The optimized sub-2 µm particle enables faster separations and uncompromised high resolution for accurate quantitation.

Introduction

Aggregates and fragments are critical quality attributes of biotherapeutic proteins that need to be well characterized. Size exclusion chromatography (SEC) is commonly used to analyze these size variants. There are cases where high-throughput SEC analysis is in high demand. Examples are in the early stage of drug development during clone selection, or process development, where large numbers of samples need to be analyzed daily. The AdvanceBio SEC 200 Å 1.9 µm column, with its unique, durable sub-2 µm particles, offers fast analysis with high resolution. These features significantly improve sample throughput, while delivering robust and accurate results.

Experimental

Materials

SILu Lite SigmaMAb universal antibody standard was purchased from MilliporeSigma and reconstituted with water to 1 mg/mL. Monobasic and dibasic sodium hydrogen phosphate and sodium chloride were purchased from MilliporeSigma. All chemicals used were ≥99.5 % pure. Water was purified from a Milli-Q A10 water purification system (Millipore). Solutions were prepared fresh daily, and filtered through a 0.22 µm membrane filter prior to use.

Instrumentation

LC system

An Agilent 1260 Infinity LC with the following configuration was used:

- Agilent 1260 Infinity II bio-inert quaternary pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II variable wavelength detector (G7114A)

Instrument conditions

Parameter	1260 Infinity II LC
Column Temperature	25 °C
Mobile Phase	50 mM sodium phosphate, 200 mM NaCl, pH 7.0
Flow Rate	0.3 to 0.7 mL/min
Injection Volume	1 µL
Detection	UV at 220 nm

Columns

- Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 300 mm (p/n PL1580-5201)
- Agilent AdvanceBio SEC 200 Å 1.9 µm, 4.6 × 150 mm (p/n PL1580-3201)

Software

Agilent OpenLab 2.2 CDS.

Results and discussion

Figure 1 shows SEC chromatograms of mAb with aggregates and fragments using 300 mm columns with flow rates at 0.35, 0.4, and 0.5 mL/min. Excellent resolution of both dimer/monomer and monomer/fragment1 was achieved even at 0.5 mL/min (Table 1) by saving 28 % of run time versus 0.35 mL/min flow rate without compromising resolution.

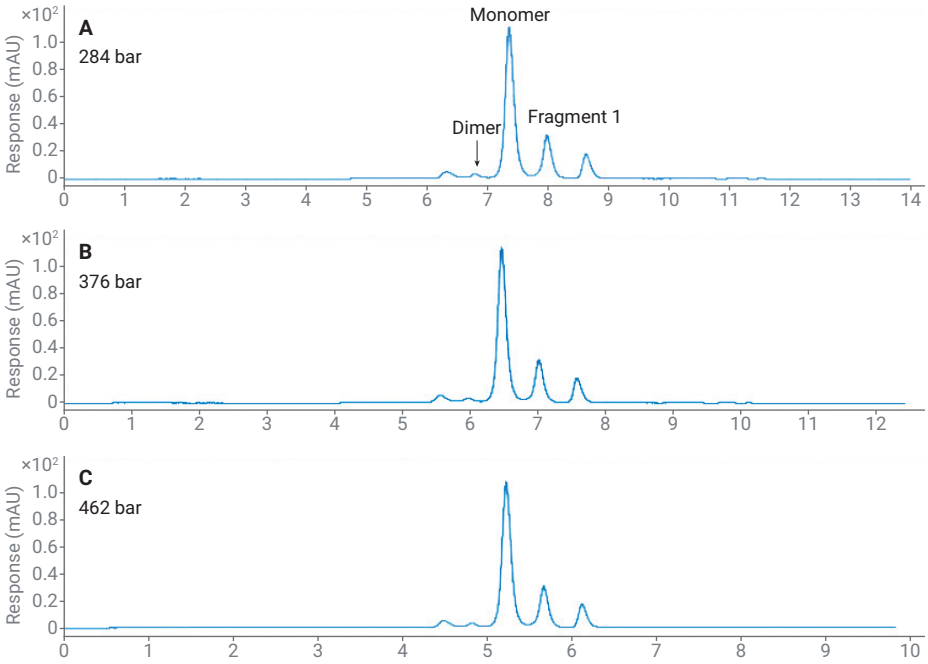

Figure 1. Size exclusion chromatograms of SigmaMAb (mixed with its $F(ab')_2$ and Fc fragments) using 4.6 × 300 mm SEC columns running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.35 mL/min; B) 0.4 mL/min; C) 0.5 mL/min.

Shorter 150 mm columns offer a solution for fast, high-throughput SEC analysis, which is commonly used in the early stage of biotherapeutic development or where rapid analysis times are essential (for instance, during process monitoring). Figure 2 shows that different flow rates up to 0.7 mL/min were tested on a 150 mm AdvanceBio SEC 200 Å 1.9 µm column.

Table 1. Peak tailing factor and resolution under different flow rates.

Flow Rate (mL/min)	Tailing Factor (Monomer)	Resolution (Dimer/Monomer)	Resolution (Monomer/Fragment 1)
0.35	1.18	1.98	2.37
0.4	1.16	1.96	2.36
0.5	1.14	1.91	2.29

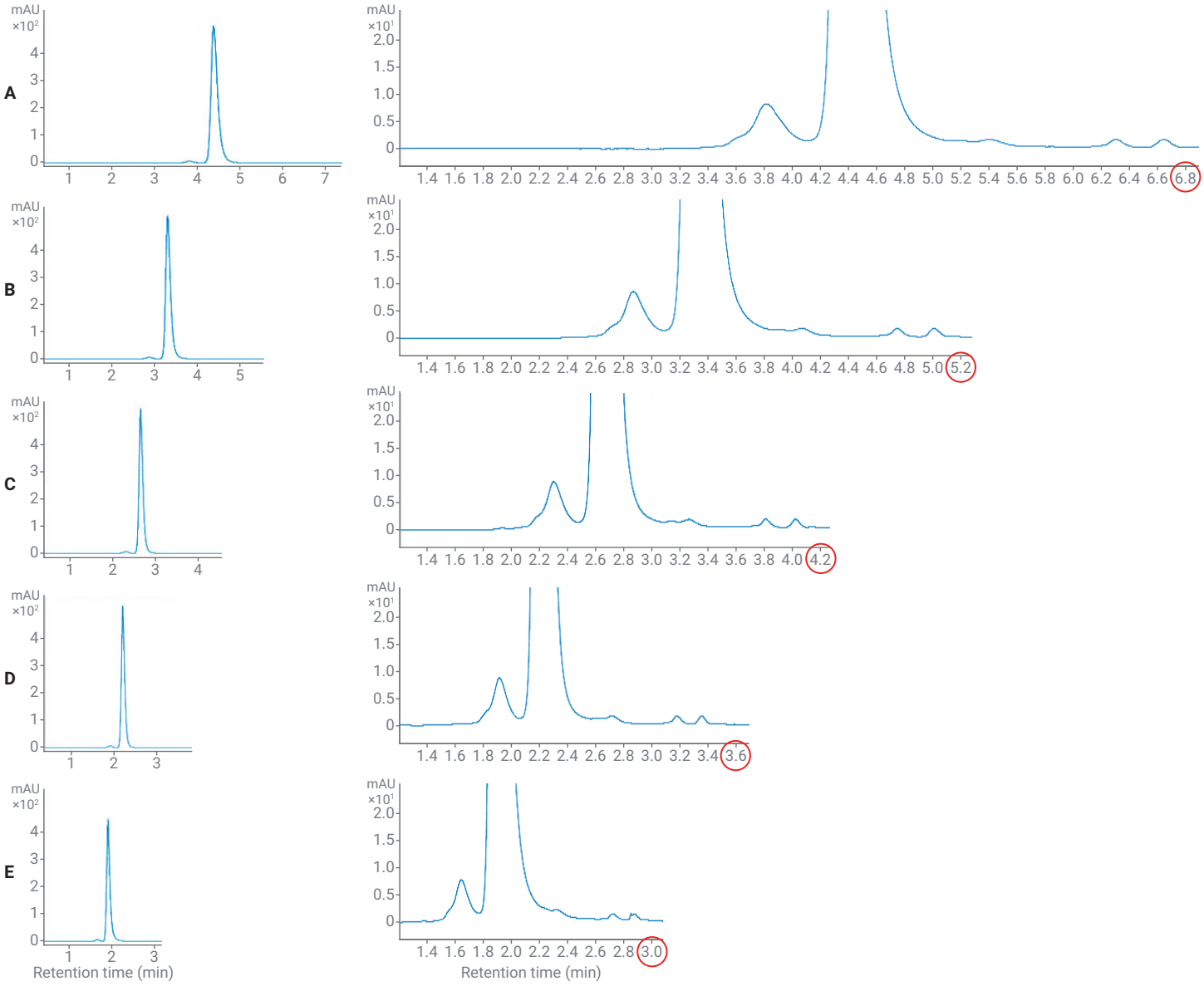

Figure 2. Size exclusion chromatograms of SigmaMAb using 4.6 × 150 mm SEC columns running with 50 mM sodium phosphate, 200 mM NaCl, pH 7.0 at A) 0.3 mL/min; B) 0.4 mL/min; C) 0.5 mL/min; D) 0.6 mL/min; E) 0.7 mL/min.

The unique particles in this column enable excellent stability at much higher flow rates with high resolution of dimer/monomer for accurate quantitation of the dimer peak area (Table 2).

Table 2 calculates the effect of flow rate on sample throughput. When increasing the flow rate from 0.3 to 0.7 mL/min, 480 samples can be analyzed per day, which is a 2.3-fold improvement in throughput. Compared to running the 300 mm column at 0.3 mL/min, which can only analyze 105 samples per day, the throughput increases 4.6-fold.

Table 2. Effect of flow rate on resolution, monomer area percentage, and sample throughput.

Flow Rate (mL/min)	Run time (min)	Backpressure (bar)	Resolution (Dimer/Monomer)	Dimer Area %	Samples Per Hour	Samples Per Day (24 hours)
0.3	6.8	164	1.81	2.33	8-9	211
0.4	5.2	218	1.79	2.35	11-12	276
0.5	4.2	272	1.78	2.35	14	342
0.6	3.6	324	1.77	2.39	16-17	400
0.7	3.0	380	1.58	2.30	20	480

Conclusion

This study demonstrates the ability of the AdvanceBio SEC 200 Å 1.9 µm column to be used for fast analysis of mAb aggregates. The durable particles enable running at a higher flow rate without loss of high resolution. By reducing column length from 300 to 150 mm, and by increasing flow rate from 0.3 to 0.7 mL/min, we can enhance sample throughput 4.6-fold.

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High Resolution Size Exclusion Chromatography Analysis of Small Therapeutic Proteins

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Abstract

Protein denaturation processes involving aggregation are among the factors impeding the development of stable protein drug formulations. The use of size exclusion chromatography (SEC) HPLC for determining purity and aggregates of these proteins is a relatively straightforward technique. Regular calibration of SEC methods ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems with samples and batches. Agilent AdvanceBio SEC 120 Å 1.9 µm columns are compared to columns with sub-2 µm particle technology from other vendors. Analysis of recombinant human growth hormone (hGH), granulocyte colony-stimulating factor (hG-CSF), and interferon α-2b (INF α-2b) proteins demonstrate the superior performance of the AdvanceBio column for small protein therapeutic applications

Introduction

In recent years, there has been a large increase in the development of biologically derived therapeutics, known as biologics, to treat a myriad of diseases. Some of the biologic drugs include small protein therapeutic agents such as growth factors and cytokines because of their key roles in regulating the production, maturation and activity of blood, muscle and bone cells. For example, human growth hormone (hGH) is used to stimulate growth in children and adults exhibiting slow or subnormal growth due to hormonal deficiencies.[1] Granulocyte colony-stimulating factor (hG-CSF) is employed to treat cancer patients undergoing chemotherapy, to help raise white blood cell levels that have been reduced by cytotoxic therapeutic agents.[2] Interferons are a class of glycoproteins that have multiple therapeutic uses but are known to form partially unfolded species as well as aggregates particularly when exposed to pH or thermal degradation.[3]

Protein denaturation processes involving aggregation are among the prime factors impeding the development of stable protein drug formulations. The United States Pharmacopeia monograph method recommends size exclusion chromatography (SEC) HPLC for determining purity and aggregates of these proteins. SEC is a relatively straightforward technique. SEC relies on simple diffusion into the pore structure of the stationary phase; larger molecules cannot permeate the particles, and elute first, while smaller molecules diffuse readily into the pores, and elute later. Agilent AdvanceBio SEC 120 Å 1.9 µm columns are designed for aqueous size exclusion chromatography (SEC) of biomolecules. The particles have been manufactured using proprietary technology to combine optimum pore size and pore volume for separating molecules such as smaller proteins and peptides.

Experimental

Equipment and materials

All chemicals and reagents were HPLC grade or higher and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Water was purified using a Milli-Q A10 (Millipore).

Instrumentation

Agilent 1260 Infinity II Bio-inert LC instrument comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option #100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option #019)
- Agilent 1260 Infinity II Diode Array Detector WR (G7115A) with bio-inert flow cell (option #028)

Software

OpenLab 2.2 CDS

Method conditions

HPLC Conditions	
Column	AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	150 mM Sodium phosphate, pH 7.0
Flow Rate	0.30 or 0.35 mL/min (as shown in text)
Column Temperature	25 °C
Injection Volume	2 µL, 1 mg/mL
Samples	Low molecular weight protein standard mix Human growth hormone, rhGH Human granulocyte colony stimulating factor, rG-CSH
Total Run Time	15 or 20 minutes (depending on flow rate)

Results and discussion

Proteins are complex molecules containing numerous side chain functionalities: acidic, basic, neutral, and hydrophobic. Finding the optimum conditions to avoid secondary interactions can be challenging, however the AdvanceBio SEC product range has a polymeric surface coating applied to the silica particle that overcomes many of these issues. The mechanism of separation relies on differences in size of molecules in solution (hydrodynamic radius). Protein structures are often compact and globular in nature, and proteins often aggregate under stress conditions such as extremes of temperature, pH, or salt composition and for dimers and larger units. This is a particular issue for protein molecules, where the presence of aggregated proteins can lead to adverse effects if administered as a therapeutic molecule. SEC provides the ideal tool for quantifying and monitoring protein aggregation. Figure 1 represents the SEC separation of low molecular weight protein and peptide standards. The calibration curve of these standards based on their retention time is shown in Figure 2. One can estimate the optimal molecular range for this column to be 1 to 80 kDa.

Peak	Protein/Peptide	Molecular Weight (Da)
1	Ovalbumin	44,000
2	Myoglobin	17,000
3	Aprotinin	6,700
4	Neurotensin	1,700
5	Uridine	244

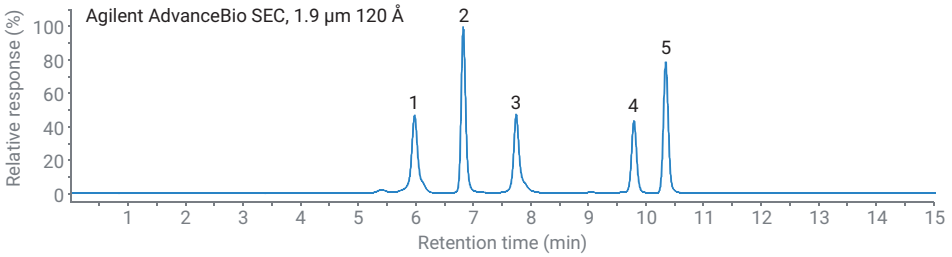

Figure 1. Size-exclusion chromatogram of low molecular weight protein and peptide mix at 0.35 mL/min.

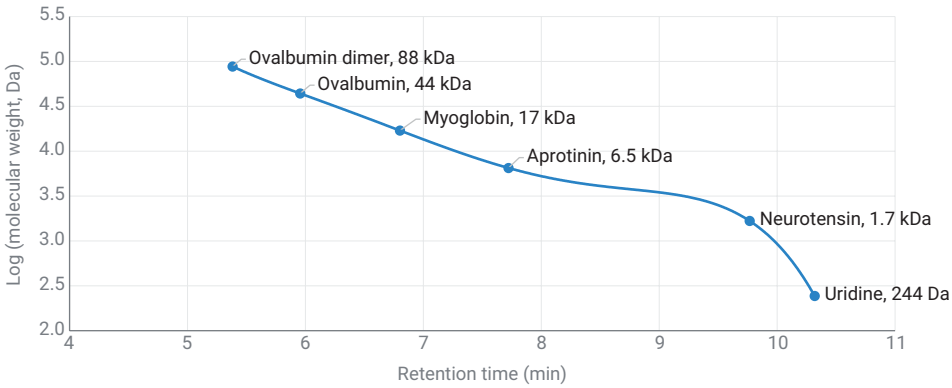

Figure 2. AdvanceBio SEC 1.9 µm 120 Å calibration curve of low molecular weight protein and peptide standards.

Even if the intention is to use the AdvanceBio SEC column for quantification of monomer and dimer content, it is still good practice to regularly perform a calibration using appropriate molecular weight standards. Regular calibration ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems, reducing system downtime and troubleshooting. For protein separations, the standards should be a range of well-characterized proteins covering the entire operating range of the column. The proper choice of standards provide two key aspects for the successful use of SEC: There should be minimal, secondary interactions between the analyte and the stationary phase. The pore size should be chosen to match the size of molecules being analyzed.

This application note demonstrates high resolution separation with an Agilent AdvanceBio SEC 120 Å 1.9 µm column for size-exclusion chromatography (SEC) analysis of the recombinant hGH and hG-CSF therapeutic proteins compared to current competition with sub-2 µm particle technology. By further optimizing the mobile phase conditions, the SEC separation of nondegraded and thermally degraded interferon alpha-2b (IFN α-2b) is also compared.

By comparing the retention time of the analyte of interest with the calibration curve, it is possible to determine if there are any signs of secondary interactions. Peaks that elute earlier or later than expected or have poor shape are signs that the mobile phase conditions may not be sufficiently optimized. Figure 3 shows the size-exclusion chromatogram of hG-CSF on the AdvanceBio SEC 1.9 µm 120 Å column where the retention time corresponds well to that of a protein of around 20 kDa.

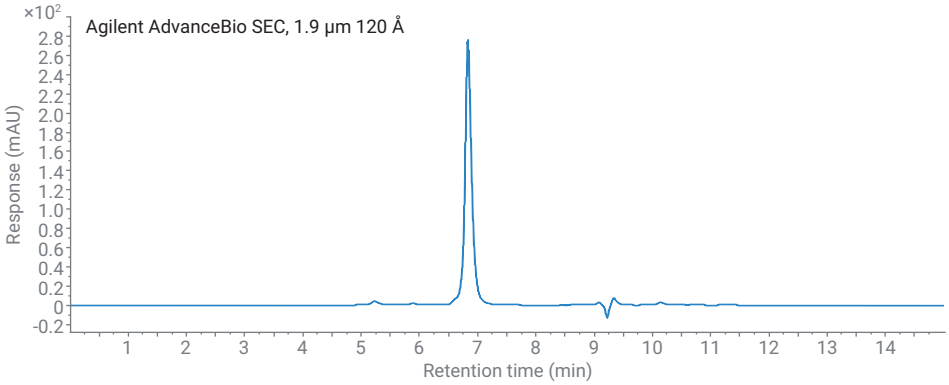

Figure 3. Size-exclusion chromatogram of hG-CSF on an Agilent AdvanceBio SEC 1.9 µm 120 Å 4.6 × 300 mm column at 0.35 mL/min.

Figure 4 shows the close up of the baseline of hG-CSF run on the AdvanceBio SEC 1.9 µm 120 Å column as well as other sub-2 µm columns from other vendors. The chromatogram at the bottom of the diagram is indicative of problems associated with secondary interactions (later than expected elution time and tailing peak).

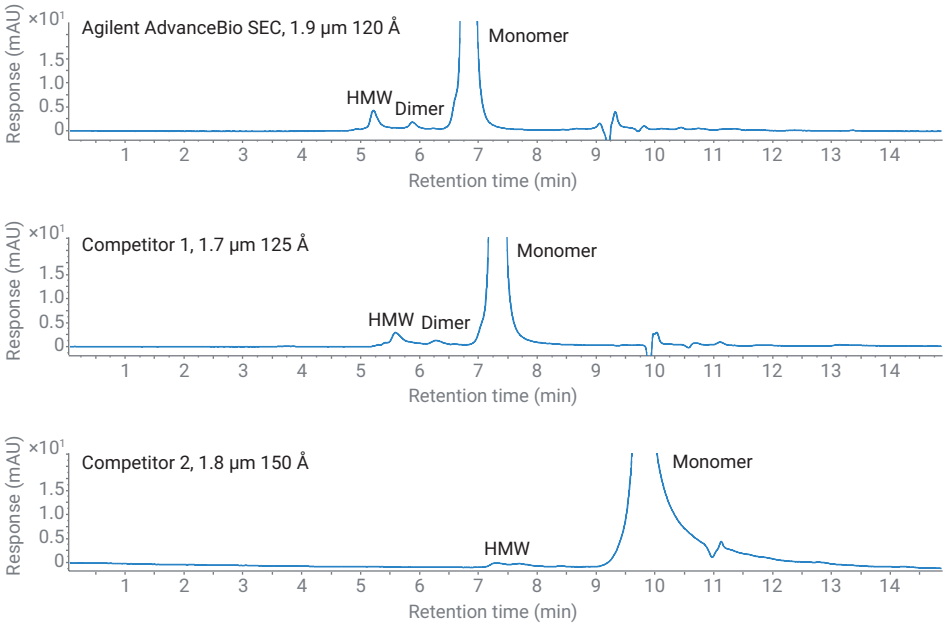

Figure 4. Close up of size-exclusion chromatograms of hG-CSF at 0.35 mL/min.

Many other biotherapeutic proteins have similar molecular weights and are therefore also suitable for analysis on the same AdvanceBio SEC 1.9 µm 120 Å column. The recombinant form of hGH, somatropin, may contain some impurities due to post-translational modification or as a result of downstream processing. Figure 5 shows the size-exclusion chromatogram of somatropin carried out under the same conditions as described previously. The inset shows the zoomed baseline region where dimer and higher molecular weight aggregates are evident.

Other proteins may require further method development to obtain the optimum peak shape and resolution. A series of experiments with different mobile phase conditions was used to determine the optimum composition for peak shape and protein recovery of IFN α-2b as shown in Table 2.

Table 1. Peak area data for high molecular weight (HMW), dimer, and monomer peaks for hG-CSF.

	AdvanceBio SEC 1.9 µm 120 Å				Competitor 1, 1.7 µm 125 Å				Competitor 2, 1.8 µm 150 Å			
	RT (min)	%Area	Rs USP	Peak Tailing	RT (min)	%Area	Rs USP	Peak Tailing	RT (min)	%Area	Rs USP	Peak Tailing
HMW	5.22	2.61		1.16	5.59	2.49		1.28	7.40	2.01		1.37
Dimer	5.88	1.02	2.41	1.11	6.27	0.83	1.68	1.26	N.D.			
Monomer	6.82	96.37	3.77	1.13	7.31	96.68	3.04	1.11	9.74	97.99		2.13

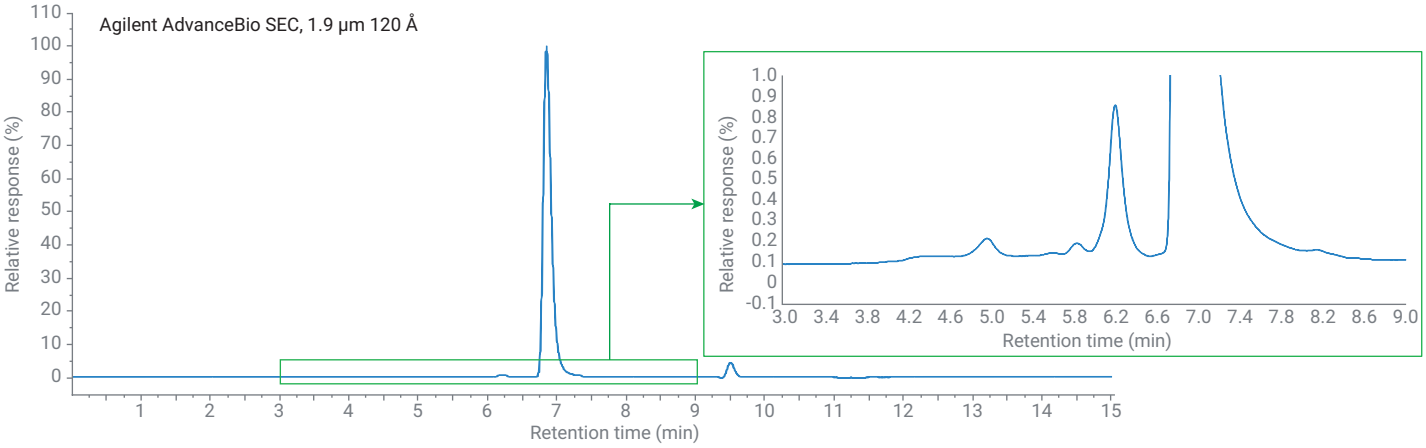

Figure 5. Size-exclusion chromatogram of somatropin (rhGH).

Table 2. Peak shape data during method optimization for IFN α-2b.

NaCl (mM)	Peak Width (min)	Tailing	Resolution HMW-Monomer	Resolution Monomer-LMW
100	0.20	2.88	1.94	1.98
150	0.18	2.65	2.25	2.31
200	0.16	2.52	2.26	2.66
250	0.15	2.39	2.84	2.86
400	0.14	2.08	3.32	3.59

The size-exclusion chromatograms of interferon alpha-2b reference material run on three different sub-2 µm SEC columns is shown in Figure 6, along with the retention time and peak tailing data. The difference in column performance may lead to a difference in resolution when separating IFN α-2b impurities by SEC therefore the experiment was repeated using a degraded sample.

In the case of interferon alpha-2b, it has been suggested that the partial unfolding of the molecule is involved in the formation of aggregates, but that the partially unfolded species are somewhat stable.[3] Furthermore, the presence of O-glycosylation can also reduce the thermal stability of these molecules4. The choice of cell line for recombinant protein manufacture is a critical parameter since *E. coli* cell lines do not introduce glycosylated variants.

Optimized HPLC Conditions for INF α-2b	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	200 mM Sodium phosphate + 250 mM NaCl, pH 6.5
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	2 µL, 1 mg/mL
Samples	Interferon alpha-2b (INF α-2b) Heat stressed interferon alpha-2b (INF α-2b): 60 °C for 30 min
Total Run Time	15 min

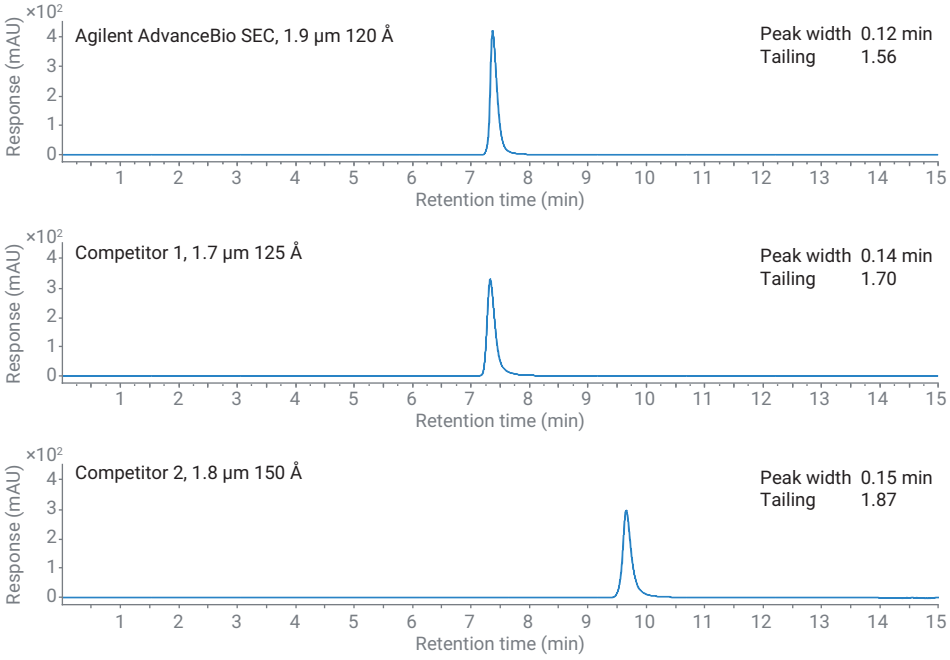

Figure 6. Size-exclusion chromatograms of interferon α-2b.

By exposing the interferon alpha-2b sample to thermal degradation (heated to 60 °C for 30 minutes), it was possible to introduce various impurities. The impurities include both early eluting high molecular weight species (HMW) as well as later eluting low molecular weight species (LMW) as seen in Figure 7. As expected, the resolution of both the HMW to monomer and monomer to LMW species is greatest on the AdvanceBio SEC 1.9 µm 120 Å column. This column had the narrowest peaks and the least amount of peak tailing in the previous separation of the nondegraded sample.

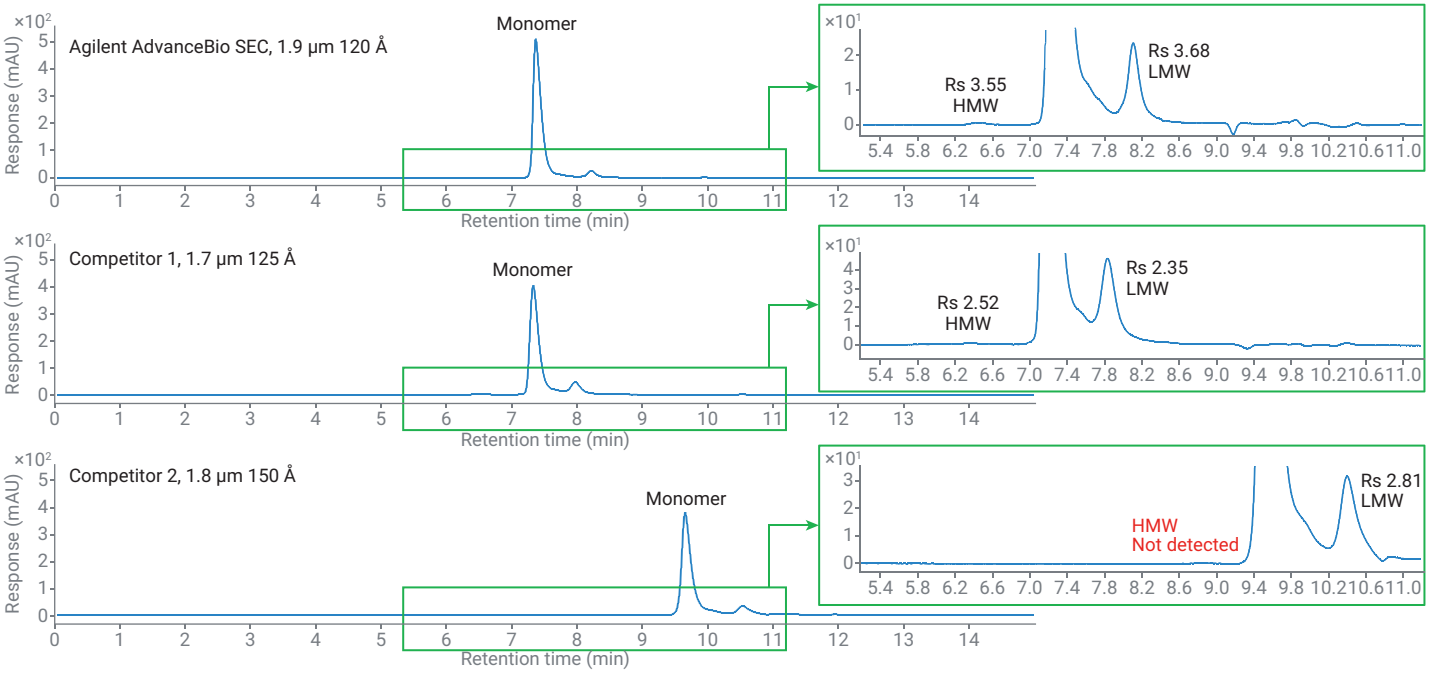

Figure 7. Size-exclusion chromatograms of heat stressed interferon α-2b.

Conclusion

Agilent AdvanceBio SEC offers a range of column dimensions and different pore sizes suitable for differently sized molecules. The featured AdvanceBio SEC 120 Å 1.9 µm column demonstrates superior performance with high resolution SEC analysis of small protein therapeutic applications when compared to columns of similar particle size and pore size characteristics from other vendors.

Calibrating your AdvanceBio SEC size exclusion column with appropriate standards ensures you understand the correct working range. These standards allow you to use calibration curves to estimate the molecular size of unknown molecules. However, regular calibration with a selection of standards is beneficial, and can be used to monitor column performance over time, allowing early detection of potential problems. In turn, corrective action can be taken, ultimately reducing system downtime and improving productivity.

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Analysis of Camelid Single-Domain Antibodies Using Agilent AdvanceBio SEC 120 Å 1.9 µm and AdvanceBio HIC Columns

Authors

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Agilent Technologies, Inc.

Abstract

This application note describes the use of size exclusion chromatography (SEC) and hydrophobic interaction chromatography (HIC) for the analysis of camelid single-domain antibodies (nanobodies). Nanobodies are a growing class of single-domain antibody fragments used for therapeutic purposes. The Agilent AdvanceBio SEC 120 Å 1.9 µm column provides a unique advantage over other SEC columns for high-resolution separation of nanobody aggregates and fragments. The Agilent AdvanceBio HIC column enables analysis of nanobody post-translational modifications (PTMs) such as glutamine/pyroglutamate conversion. An SEC-based native LC/MS method is also demonstrated for greater understanding of PTM and impurity characterization.

Introduction

Despite the success of biotherapeutics such as monoclonal antibodies (mAbs), many significant drawbacks still exist for this class of drugs. For example, IgG mAbs, the most widely used biologic drugs, have a complex structure and rather large size (150 kDa). The large size hampers their efficient *in vivo* delivery to diseased cells such as those found in tumors. Alternatively, single-domain antibodies, also known as nanobodies, provide tremendous opportunity in terms of reaching their intended targets.[1]

Nanobodies are small (~15 kDa), natural single-domain proteins derived from the camelid heavy chain antibody (Figure 1). They are recombinantly produced antigen-binding V_{HH} fragments with binding affinity equivalent to that of conventional IgG mAbs. Due to their small size, nanobodies can bind to antigen motifs that are frequently inaccessible to conventional mAbs, providing access to presently "undruggable" targets. In addition, the relatively simple protein conformation offers many advantages to drug developers such as ease of manufacturing and different administrative routes.[1] These promising features make nanobodies and V_{HH} fragment-derived biologics the rising stars in the biopharma research and development pipeline.[2]

This application note presents thorough characterization of two V_{HH} fragments (anti-PD1 and anti-PDL1 single-domain antibodies) using SEC and HIC, both with UV detection. Furthermore, we demonstrate SEC-based native LC/MS analysis of the two samples. The Agilent AdvanceBio SEC 120 Å 1.9 µm column is designed for aqueous SEC separation of small biomolecules such as proteins in the molecular weight range of 1 to 80 kDa. The column is perfectly suitable for nanobody analysis. The proprietary

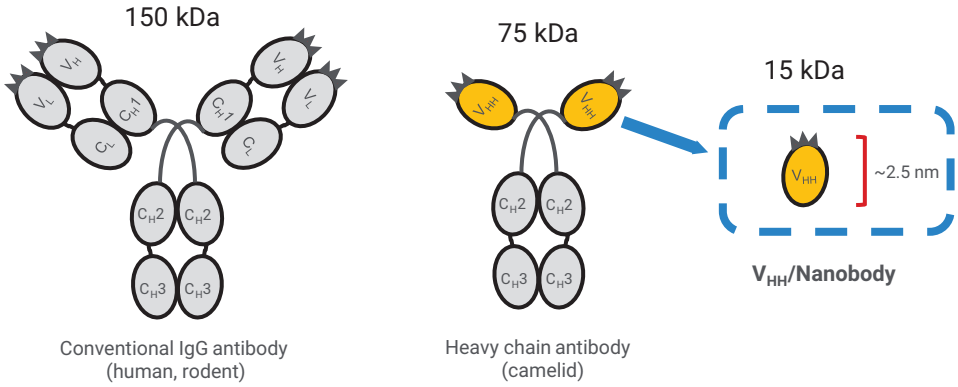

Figure 1. Schematic of a V_{HH} single-domain antibody (nanobody) compared to a conventional mAb.

sub-2 µm hydrophilic polymer-coated silica packing technology enables higher resolving separation of aggregates and fragments compared to SEC columns from other vendors. HIC analysis allows reserved-phase-like separation of protein variants in a native condition. The Agilent AdvanceBio HIC column exhibits optimal hydrophobicity and selectivity for nanobody PTM characterization. Using a generic HIC method (without organic solvent modifier), a common PTM, glutamine/pyroglutamate conversion is revealed. The combination of these approaches offers a complete solution for in-depth analysis of nanobody purity and critical quality attribute assessment.

Experimental

Samples and chemicals

- Llama anti-PD1 single-domain antibody [F12A8]; purchased from ProSci Inc (Poway, CA)
- Llama anti-PDL1 single-domain antibody [F2G2]; purchased from ProSci Inc (Poway, CA)
- Glutaminyl-peptide cyclotransferase; purchased from R&D Systems (Minneapolis, MN)
- Human anti-IL8 IgG monoclonal antibody; produced in house from CHO cells

All chemicals and solvents used were HPLC grade or higher. Sodium phosphate monobasic and dibasic, sodium chloride, and ammonium sulfate were from Sigma-Aldrich. Water was purified using a Milli-Q Integral system equipped with LC-Pak Polisher and a 0.22-µm membrane point-of-use cartridge (Millipore).

Columns

- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
- Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
- Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)

Instrumentation

For HPLC experiments, an Agilent 1260 Infinity II Bio-inert LC system was used comprising:

- Agilent 1260 Infinity II Bio-inert Pump (G5654A)
- Agilent 1260 Infinity II Bio-inert Multisampler (G5668A) with sample cooler (option 100)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) with bio-inert heat exchanger (option 019)
- Agilent 1260 Infinity II Variable Wavelength Detector (G7114A)

For LC/MS experiments, an Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS and 1290 Infinity II LC were used comprising:

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Thermostatted Column Compartment (G7116B)
- Dual ESI Agilent 6224 accurate-mass time-of-flight (TOF) LC/MS

Results and discussion

SEC is a gold standard technique for characterizing aggregation of biologics. The chromatographic separation mechanism is unique in that analytes are not retained by the stationary phase; instead, they are separated based on accessibility to available particle pore volume. Therefore, careful selection of column pore size based on the protein analyte's size in solution (hydrodynamic radius) is important. Nanobodies are small proteins with molecular weight of approximately 15 kDa, translating to a hydrodynamic radius of approximately 25 Å. SEC columns packed with narrow pore (120 to 130 Å) particles offer linear separation in the range suitable for such an application.[3,4] Figure 2 shows an SEC separation of anti-PD1 single-domain antibody (sdAb) using a standard method with sodium phosphate pH 7 as the mobile phase (to maintain the native state of the protein). Excellent separation of sdAb monomer from impurities, i.e., high-molecular weight (HMW) and low molecular weight (LMW) species, can be

HPLC Conditions (SEC Analysis)	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	150 mM sodium phosphate, pH 7.0
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	5 µL, 1 mg/mL
Total Run Time	15 min
Detection	UV at 214 nm

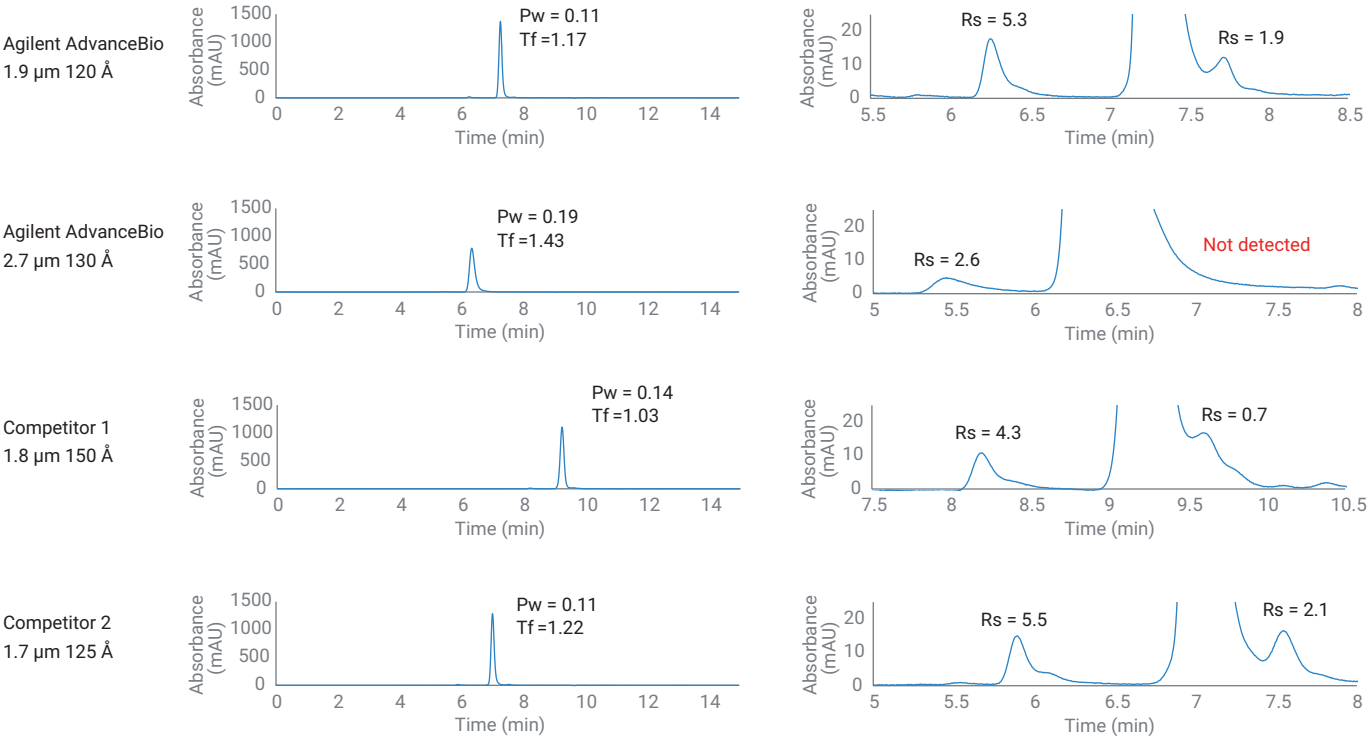

Figure 2. SEC of anti-PD1 single-domain antibody. Right panels show the magnified baseline of the same run on the left. Resolution of the HMW aggregate peak (eluted earlier) or the LMW fragment peak (eluted later) compared to the monomer peak are shown.

seen with the AdvanceBio SEC 1.9 µm 120 Å column. The resolution of HMW and LMW species as well as the peak width and tailing factor of the monomer peak are significantly better compared to columns packed with larger (2.7 µm) particles. Compared to other vendors' offering of sub-2 µm SEC technology, the AdvanceBio SEC 1.9 µm 120 Å column exhibits best-in-class performance.

This is due to careful design of particle surface bonding coverage to eliminate undesirable secondary interactions.[3,4]

SEC is a relatively straightforward chromatographic method where the column is run in isocratic mode. Method development and optimization involve the selection of mobile phase parameters (pH, salt concentration, etc.) to minimize potential secondary interactions such as ionic or hydrophobic interaction. Figure 3 shows SEC salt plot studies for method optimization of anti-PD1 and anti-PDL1 sdAb analysis. A shorter column of 15 cm length was selected for this experiment to achieve higher throughput and speed up the method optimization process. Results showed that anti-PD1 sdAb had minimal or no secondary interaction with the column at the range of mobile phase

HPLC Conditions (SEC Salt Plot Study)	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 150 mm (p/n PL1580-3250)
Mobile Phase	20 mM sodium phosphate, pH 7.0 with concentration of sodium chloride indicated in Figure 3
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	2 µL, 1 mg/mL
Total Run Time	7 min
Detection	UV at 214 nm

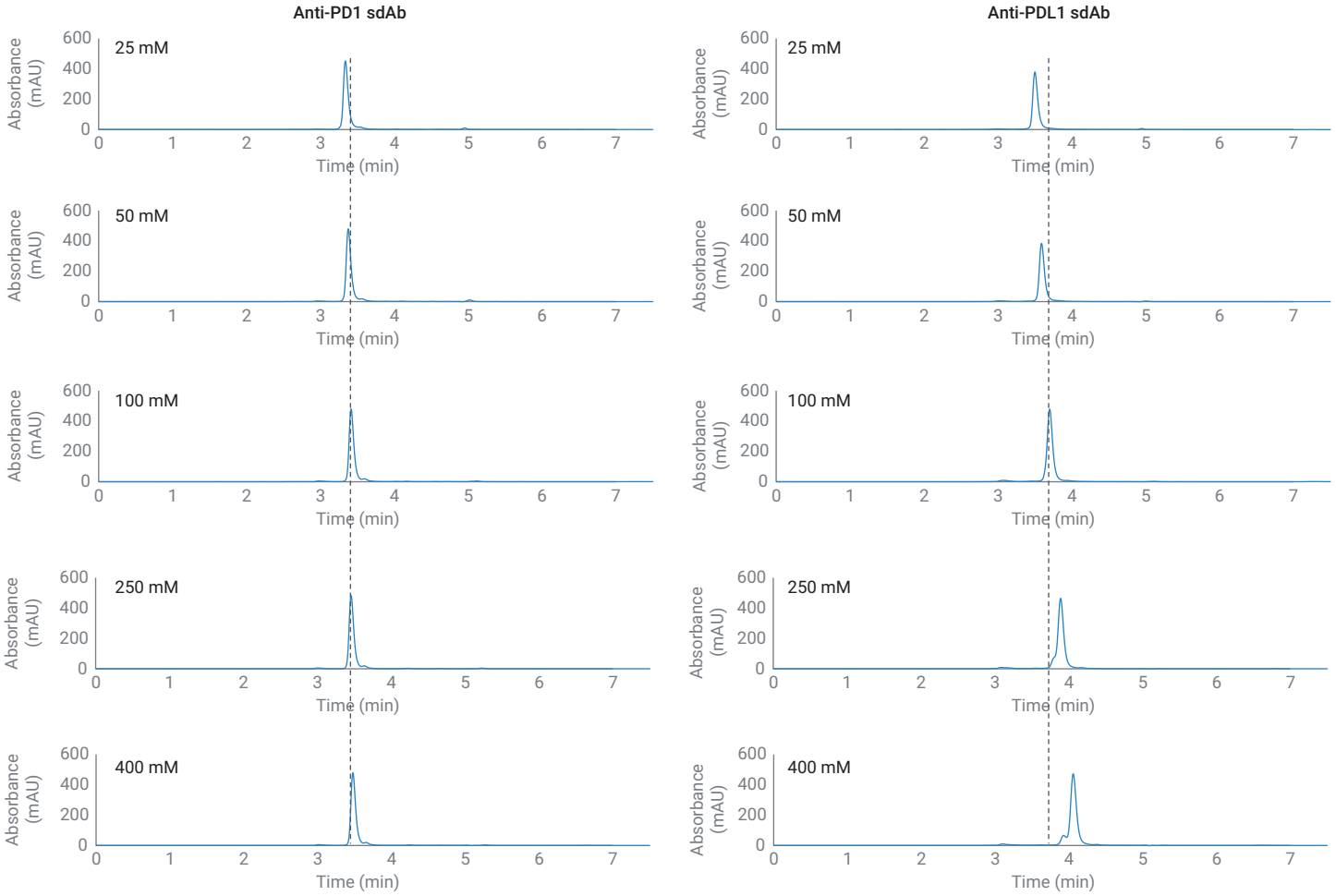

Figure 3. Salt plot studies of anti-PD1 (left) and anti-PDL1 (right) single-domain antibodies using the Agilent AdvanceBio SEC 1.9 µm 120 Å column.

NaCl concentration used. This is evident from the similar chromatographic peak shapes and retention times at each mobile phase condition. In contrast, anti-PDL1 sdAb had slightly deteriorated peak shape with delayed retention time at higher salt concentration mobile phase, indicative of potential hydrophobic interaction between analytes and the column. Interestingly, at 250 mM NaCl and above, a secondary (shoulder) peak became evident. This phenomenon is commonly encountered with challenging proteins that contain highly hydrophobic motifs or which have extreme isoelectric points. As nanobodies contain only the variable domain of an antibody, the amino acid sequence varies significantly when comparing one nanobody to another, potentially causing large differences in protein physicochemical characteristics. The method demonstrated here is useful for fast screening of SEC mobile phase conditions to determine the optimum conditions for analyzing different samples. The data inform the use of lower salt concentration (50 mM or below) in the mobile phase for anti-PDL1 sdAb.

To further characterize the two nanobodies, native SEC-LC/MS experiments were conducted (Figure 4). Results showed that the AdvanceBio SEC 1.9 µm 120 Å column was suitable for SEC-MS, where low concentrations of volatile aqueous buffer (i.e., 50 mM ammonium acetate) are used. Excellent ion chromatograms can be seen for both sdAb samples, together with high-resolution mass spectra. The deconvoluted MS results showed accurate molecular weight measurement for both samples: anti-PD1 sdAb (16,528 Da)

Native SEC-LC/MS Conditions	
Column	Agilent AdvanceBio SEC 1.9 µm 120 Å, 4.6 × 300 mm (p/n PL1580-5250)
Mobile Phase	50 mM ammonium acetate, pH 7.0
Flow Rate	0.35 mL/min
Column Temperature	25 °C
Injection Volume	20 µL, 1 mg/mL
Total Run Time	15 min
MS Detection	Min range: 300 *m/z* Max range: 7,000 *m/z* Ion polarity: Positive
MS Source Parameters	Gas temperature: 325 °C Gas flow: 5 L/min Nebulizer: 30 psi Vcap: 5,500 V Fragmentor: 250 V Skimmer: 65 V Octopole RF peak: 750 V

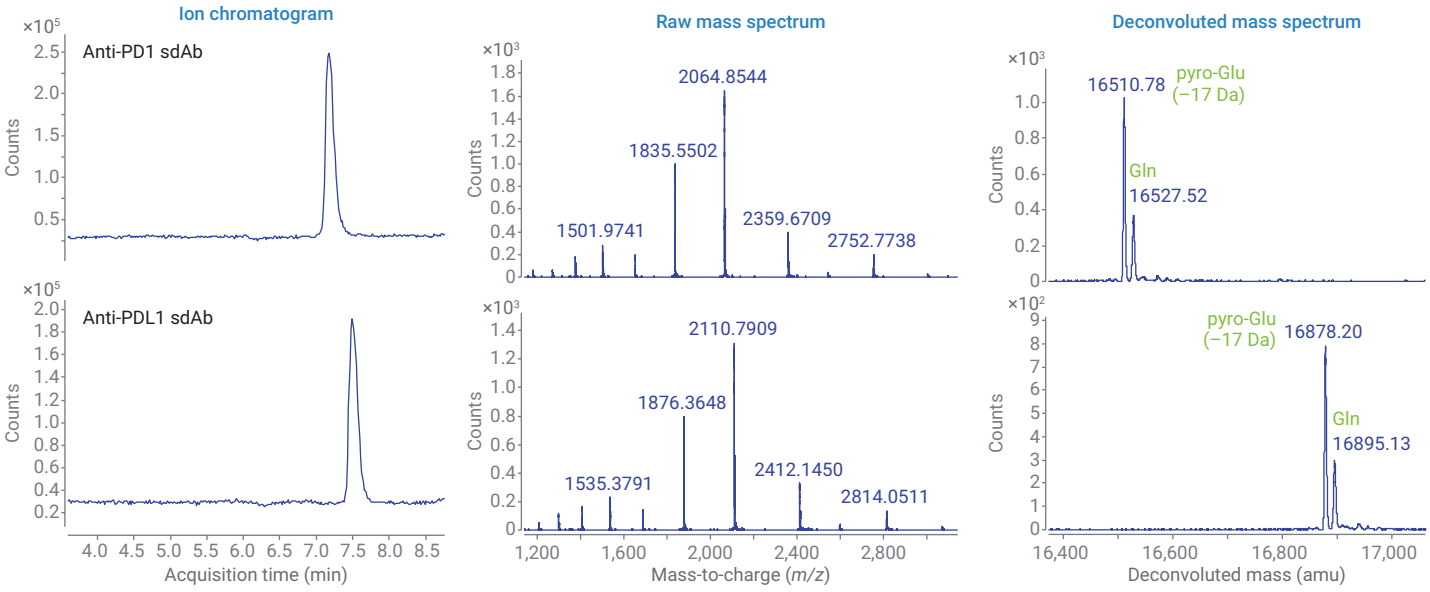

Figure 4. Native SEC-LC/MS analysis of anti-PD1 (top) and anti-PDL1 (bottom) single-domain antibodies.

and anti-PDL1 sdAb (16,895 Da). Interestingly, MS results revealed a large amount of a –17 Da modification on both samples. This is a typical mass shift associated with cyclization of N-terminal glutamine (Gln) to form pyro-glutamic acid (pyro-Glu).[5] The Gln/pyro-Glu conversion commonly occurs at the heavy chain variable domain of the antibody; thus, it is not surprising to observe this PTM in nanobodies (V_{HH} fragment). Both sdAb samples analyzed here were produced in bacteria (*E. coli*). It has been well documented that production in prokaryotic systems may result in proteins being recovered as inclusion bodies, thus leading to unusual PTMs.[5] Because the N-terminal Gln residues of V_{HH} are near the complementarity-determining region (CDR), pyro-Glu formation can potentially have significant impact on target binding.[1] Therefore, careful characterization and documentation of this PTM is typically required.[5]

To characterize protein Gln/pyro-Glu conversion, chromatographic methods such as ion-exchange chromatography and HIC can be used. Here, HIC was chosen because, in addition to PTM analysis, it also provided an assessment of hydrophobicity for the two sdAb samples. HIC uses a salting-out mechanism to separate intact, native proteins based on hydrophobicity under near physiological conditions. Figure 5 shows the HIC separation of anti-PD1 and anti-PDL1 sdAbs and an IgG mAb using a generic ammonium sulfate gradient method. For both Nbs, two chromatographic peaks were well resolved, indicating that both samples contained two species of different hydrophobicity. This result was consistent with the LC/MS data showing the Gln/pyro-Glu conversion. In addition, HIC data suggested that the anti-PDL1 sdAb was very hydrophobic. The anti-PDL1 sdAb retention time was much longer than anti-PD1 sdAb

HPLC Conditions (HIC analysis)	
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
Mobile Phase	A) 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0 B) 50 mM sodium phosphate, pH 7
Gradient	0 min: 50% B 2 min: 50% B 17 min: 100% B 20 min: 100% B 22 min: 50% B 32 min: 50% B
Flow Rate	0.4 mL/min
Column Temperature	30 °C
Injection Volume	5 µL, 0.8 mg/mL (mAb) 5 µL, 1.0 mg/mL (sdAb)
Detection	UV at 214 nm

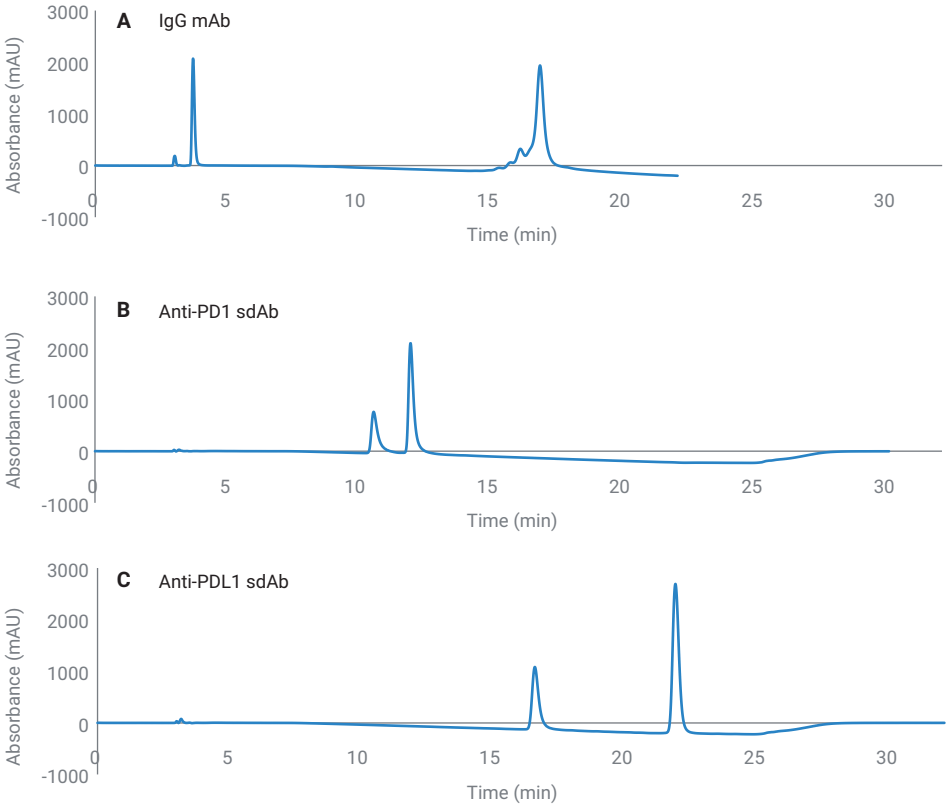

Figure 5. Hydrophobic interaction chromatograms of (A) an IgG mAb, (B) anti-PD1 single-domain antibody, and (C) anti-PDL1 single-domain antibody.

and the IgG mAb (with a molecular weight that is 10 times larger). These results help explain the observations from the SEC salt plot study (Figure 3), suggesting major physicochemical property differences between the two single-domain antibodies.

To further confirm whether the two peaks separated using HIC were indeed sdAb species that contained Gln or pyro-Glu, a biochemical approach was developed using glutaminyl-peptide cyclotransferase (QPCT) (Figure 6). The enzyme is known to catalyze the conversion of N-terminal glutaminyl residues of proteins to pyroglutamyl groups.[6] Indeed, treatment of both sdAb samples with QPCT resulted in chromatograms containing only the pyro-Glu peak. The experiments presented here using the AdvanceBio HIC column demonstrated excellent selectivity for nanobody PTM analysis.

Conclusion

Nanobodies are revolutionary, new biotherapeutic modalities that offer many advantages over conventional mAb therapy. For research and development of this novel class of biologic, it is of utmost importance to characterize and document quality attributes that can be formed or changed during the process of production and storage. These attributes have been shown to impact drug potency, pharmacokinetics, immunogenicity, and safety. Reliable and robust analytical tools and methods are needed. This application note presents SEC, HIC, and SEC-LC/MS techniques that can successfully be applied to nanobody characterization. Important critical quality attributes (CQAs) such as HMW aggregates and LMW fragments can be characterized using SEC in a high-resolution and high-throughput manner. Subtle changes on the molecules such as post-translational Gln/pyro-Glu conversion can be detected using HIC and SEC-MS approaches. The methods described here offer guidance for careful analysis of nanobodies in native, non-denaturing modes.

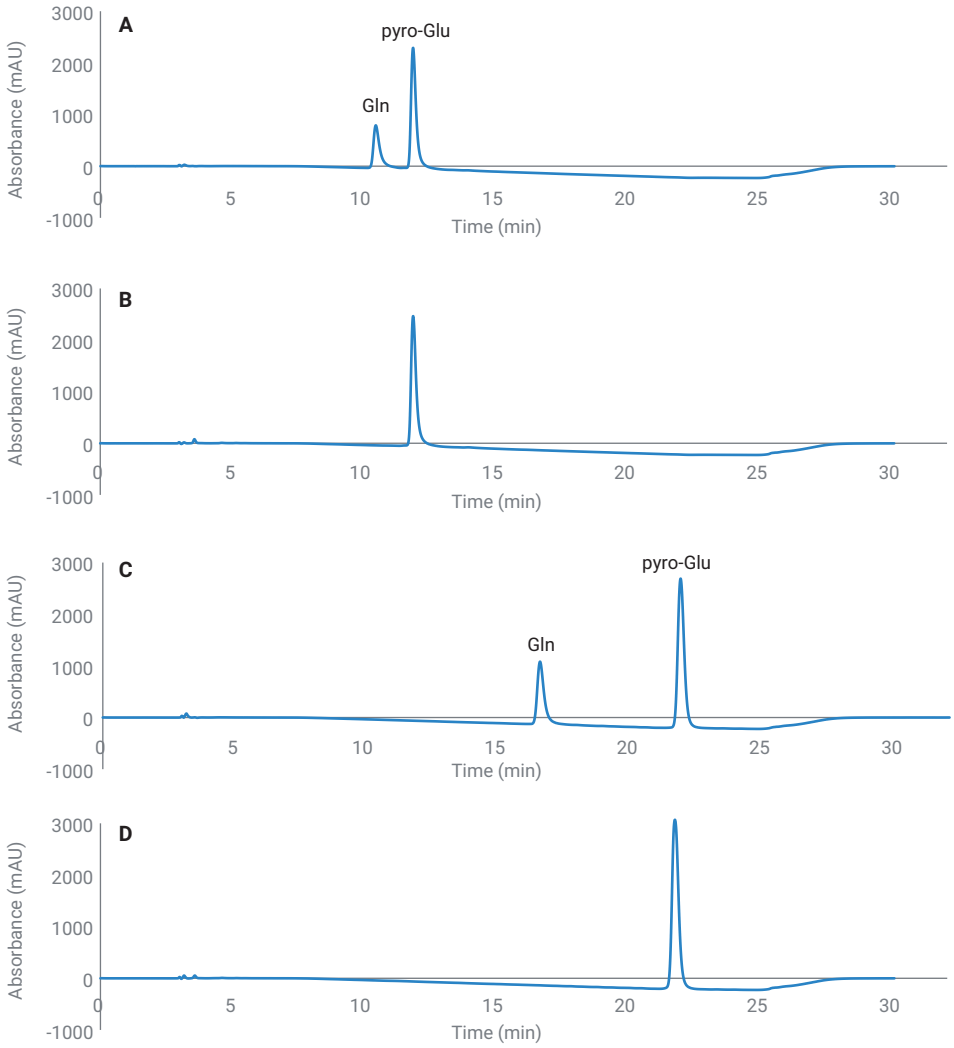

Figure 6. HIC analysis of (A) anti-PD1 sdAb, (B) anti-PD1 sdAb + QPCT, (C) anti-PDL1 sdAb, and (D) anti-PDL1 sdAb + QPCT. For enzyme treatment, 20 µL of sdAb (1 mg/mL) was incubated with 4 µL QPCT at 37 °C for 16 to 18 hours.

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Monitoring Product Quality Attributes of Biotherapeutics at the Peptide Level Using the Agilent InfinityLab LC/MSD XT System

Authors

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Agilent Technologies, Inc.

Abstract

Single quadrupole (SQ) LC/MS has been adopted in the biopharmaceutical QC labs for its low-cost, robustness, and simple operation. This Application Note describes a simple, generic method for routine biotherapeutic peptide map analysis using the Agilent InfinityLab liquid chromatography/mass selective detector XT (LC/MSD XT), an SQ system with an extended mass range up to *m/z* 3,000, in combination with an Agilent 1290 Infinity II LC System and Agilent OpenLab ChemStation software. Streamlined data processing and reporting were demonstrated for pre-identified peptides of a recombinant monoclonal antibody (mAb), including complementary-determining regions (CDR) peptides, deamidated peptides, oxidized peptides, and glycopeptides using OpenLab ChemStation. This study serves as a proof of concept for monitoring multiple product quality attributes (PQAs) using an SQ LC/MS system with software that is recommended for laboratories requiring regulatory compliance.

Introduction

In the biotherapeutic industry, optically based chromatographic methods have widely been used for quality control (QC). However, protein-based biotherapeutics are generally very complex, making an orthogonal detection method (for example, mass spectrometry) very attractive or necessary to assess product quality attributes at a molecular level. Therefore, SQ-based LC/MS has been adopted in the QC environment. Due to the product complexity, comprehensive analysis of protein-based therapeutics often requires running a panel of analytical methods. The concept of using a single LC/MS analytical method to monitor multiple PQAs has gained momentum in the biopharmaceutical industry. Therefore, it is valuable to develop an SQ-based LC/MS assay for monitoring multiple PQAs.

In the QC environment, an important need is to support regulatory compliance. OpenLab ChemStation in combination with central data storage (OpenLab ECM or OpenLAB Server) provides functionality that labs need to achieve compliance: controls for managing system access, audit trail, versioning of data, electronic signature, secured records and data archival.[1,2]

This Application Note develops a simple, untargeted, generic LC/MS method for routine biotherapeutic peptide map analysis using the InfinityLab LC/MSD XT system, coupled with a 1290 Infinity II LC and OpenLab ChemStation software. In a stress study using NIST monoclonal antibody (NISTmAb), we demonstrate that this compliance-ready system allows streamlined data processing and reporting for multiple PQAs in a single analysis, such as product identification confirmation, post translation modification (PTM) analysis, and glycopeptide analysis.

Experimental

Materials

All reagents and solvents were LC/MS grade. The NISTmAb reference material was purchased from National Institute of Standards and Technology.

Sample preparation

To induce asparagine deamidation, NISTmAb was exposed to elevated temperature (37 °C) in a Tris-HCl buffer system at pH 8.7 for six days. To induce methionine oxidation, NISTmAb was incubated in Tris-HCl buffers containing 0.002% (v/v) oxidizing agent H_2O_2 overnight at room temperature. Both reference and stress-induced NISTmAb were denatured, reduced, alkylated, and trypsin-digested followed by desalting using the Agilent AssayMAP Bravo platform.[3] Digested samples were injected at a concentration of approximately 0.5 µg/µL onto the LC/MS system.

LC/MS analysis

LC separation was carried out using an Agilent 1290 Infinity II LC, consisting of an Agilent 1290 Infinity II High-Speed Pump (G7120A), an Agilent 1290 Infinity II Multisampler (G7167B) with sample cooler (option 100), and an Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with an Agilent ZORBAX 300StableBond C18 column (2.1 × 150 mm, 300 Å, 1.8 µm, p/n 863750-902) (Table 1). The MS system used was the Agilent InfinityLab LC/MSD XT system (G6135BA) with the Agilent Jet Stream source (G1958-65138). Agilent OpenLab ChemStation (version C 01.09) was used for data acquisition, processing, and reporting. The data were acquired in positive scan mode ranging from *m/z* 360 to 1,400 (Table 2).

Table 1. LC conditions.

LC Parameters	
Analytical Column	Agilent ZORBAX RRHD 300Å StableBond C18, 2.1 × 150 mm, 1.8 µm (p/n 863750-902)
Mobile Phase A	H_2O with 0.1% (v/v) formic acid
Mobile Phase B	Acetonitrile with 0.1% (v/v) formic acid
Flow Rate	0.25 mL/min
Injection Volume	5 µL
Gradient	Time (min) %B 0 1 5 1 6 10 70 35 72 90 77 90 79 1 81 1
Column Temperature	50 °C

Results and discussion

Monitoring multiple PQAs in a single analysis

To evaluate the InfinityLab LC/MSD XT system for monitoring multiple attributes of biomolecules, NISTmAb was stressed under two conditions to induce deamidation and oxidation, respectively. The LC/MS method using MS positive scan mode described above was applied to collect the full peptide map for each sample. Figure 1 shows the total ion chromatogram of the peptide map data with 2.5 µg of NISTmAb digest loaded on-column, showing the sample complexity, as well as the high sensitivity and ultrafast scan speeds of the MSD within the InfinityLab LC/MSD XT system. The full scan of the NISTmAb peptide map allows monitoring of multiple attributes of interest using customized data processing methods. The scan also avoids re-acquiring data if additional attributes are of interest in the future.

Table 2. MS conditions.

Agilent MSD XT Parameters	
Drying Gas Flow	11 L/min
Drying Gas Temperature	325 °C
Sheath Gas Flow	10 L/min
Sheath Gas Temperature	325 °C
Nebulizer Pressure	35 psi
Capillary Voltage	4,000 V
Nozzle Voltage	0 V
Peak Width	0.07 minutes
Scan	360 to 1,400 *m/z* in positive mode from 5 to 80 minutes, step size 0.1
Fragmentor Ramp	Mass Value 300 125 V 2,000 200 V
Cycle Time	0.62 sec/cycle

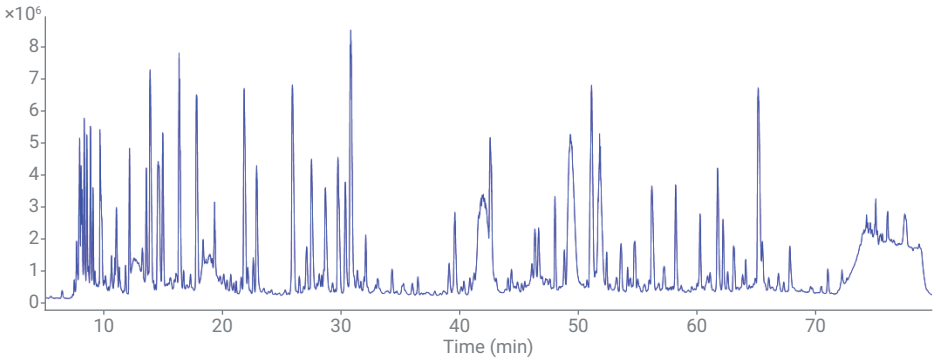

Figure 1. Total ion chromatogram of peptide map detection by Agilent LC/MSD XT with positive scan.

OpenLab ChemStation software supports automated data processing and reporting. To avoid manual extraction and integration of each peptide, a processing method can be created for extracted ion chromatograms (EICs) of multiple peptides of interest. Figure 2 shows screen captures of the EIC method setup for multiple peptides by the following steps:

1. MS chromatograms for the peptides of interest are defined with targeted *m/z*, then the targeted MS chromatograms are extracted accordingly (Figure 2A).
2. These targeted EICs are added to the processing method with adjustable retention time windows for automatic signal extraction and loading (Figure 2B).
3. The compound names, associated retention times, and EIC signals are linked through the Calibration Table setup (Figure 2C).

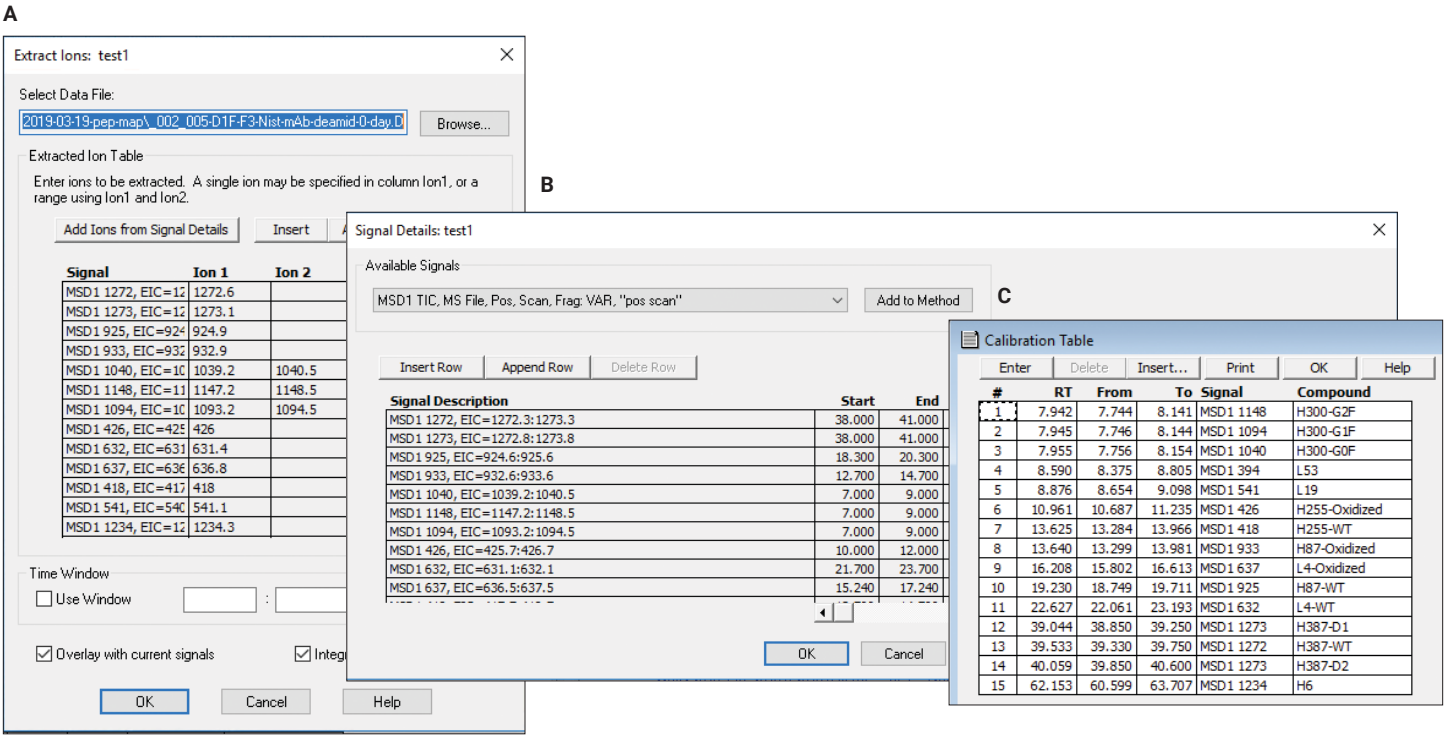

Figure 2. ChemStation screen captures of EIC method setup for multiple peptide attributes.

EICs for monitoring product attributes

To evaluate the performance using the InfinityLab LC/MSD XT, 15 precharacterized peptides were selected for identification and quantification analysis for the NISTmAb stress study (Table 3).[4,5] The identity and retention time of these peptides was predetermined using a high-resolution LC/Q-TOF system with the same LC gradient as in Table 1. A processing method, including all 15 peptides, was created using the steps described earlier, and a single dominant charge state was used to identify each of the peptides. If desired, the user could sum up additional charge states for each peptide.

The peptides listed in Table 3 can be separated into three categories according to the different monitoring purposes. The first category is the CDR peptides including peptides L4, L19, L53, H6, and H87. During product monitoring, an important need is to confirm the identity of a given biomolecule product. The sequences of CDR peptides are variable among different mAbs and can be used to confirm the product identity. Figure 3 shows the EIC of the CDR peptides that can be used to confirm protein identity.

Table 3. Peptide information for monitored attributes.

Peptide	Peptide sequence	Modification	Calculated *m/z*	Charge state (z)	Expected retention time (min)	mAb region
L4	DIQMTQSPSTLSASVGDR	Oxidation	637.0	3	16.24	CDR
L4	DIQMTQSPSTLSASVGDR	WT	631.6	3	22.68	CDR
L19	VTITCSASSR	WT	541.3	2	8.966	CDR
L53	LASGVPSR	WT	393.7	2	8.353	CDR
H6	ESGPALVKPTQTLTLTCTFSGFSLSTAGMSVGWIR	WT	1234.3	3	62.105	CDR
H87	VTNMDPADTATYYCAR	WT	924.9	2	13.64	CDR
H87	VTNMDPADTATYYCAR	Oxidation	932.9	2	19.23	CDR
H255	DTLMISR	Oxidation	426.2	2	11.01	CH2
H255	DTLMISR	WT	418.2	2	13.71	CH2
H300	TKPREEQYNSTYR	G0F	1039.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G1F	1093.5	3	7.97	CH2
H300	TKPREEQYNSTYR	G2F	1147.5	3	7.98	CH2
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	39.07	CH3
H387	GFYPSDIAVEWESNGQPENNYK	WT	1272.6	2	39.56	CH3
H387	GFYPSDIAVEWESNGQPENNYK	Deamidation	1273.1	2	40.06	CH3

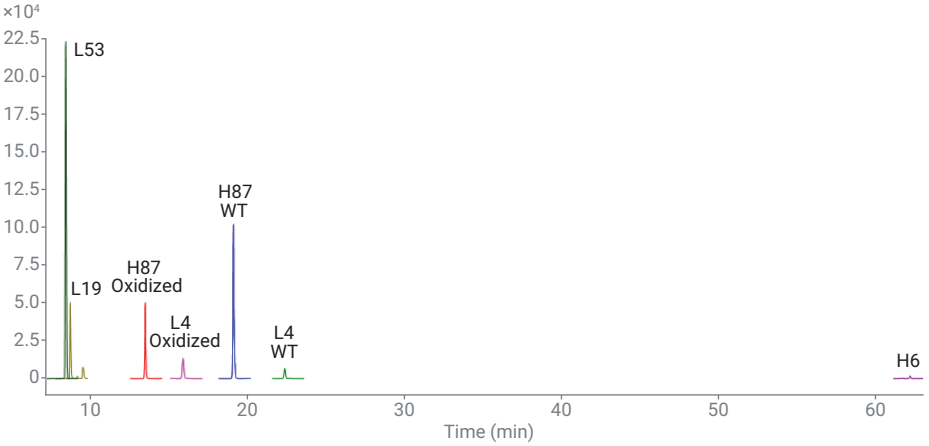

Figure 3. EICs of the CDR peptides.

The second category is peptides with variable modification sites, which are responsive for chemically induced deamidation and oxidation (L4, H87, H255, and H387).[6,7] PTMs such as asparagine deamidation, aspartate isomerization, and methionine oxidation lead to degradation products typical for recombinant antibodies. Process changes during manufacturing or storage conditions can affect the rate and extent of these modifications, which could potentially impact the stability and function of the protein drug. Therefore, these PTMs are closely monitored during process development and drug production. Figure 4A shows EICs of the wild type H387 peptide and its

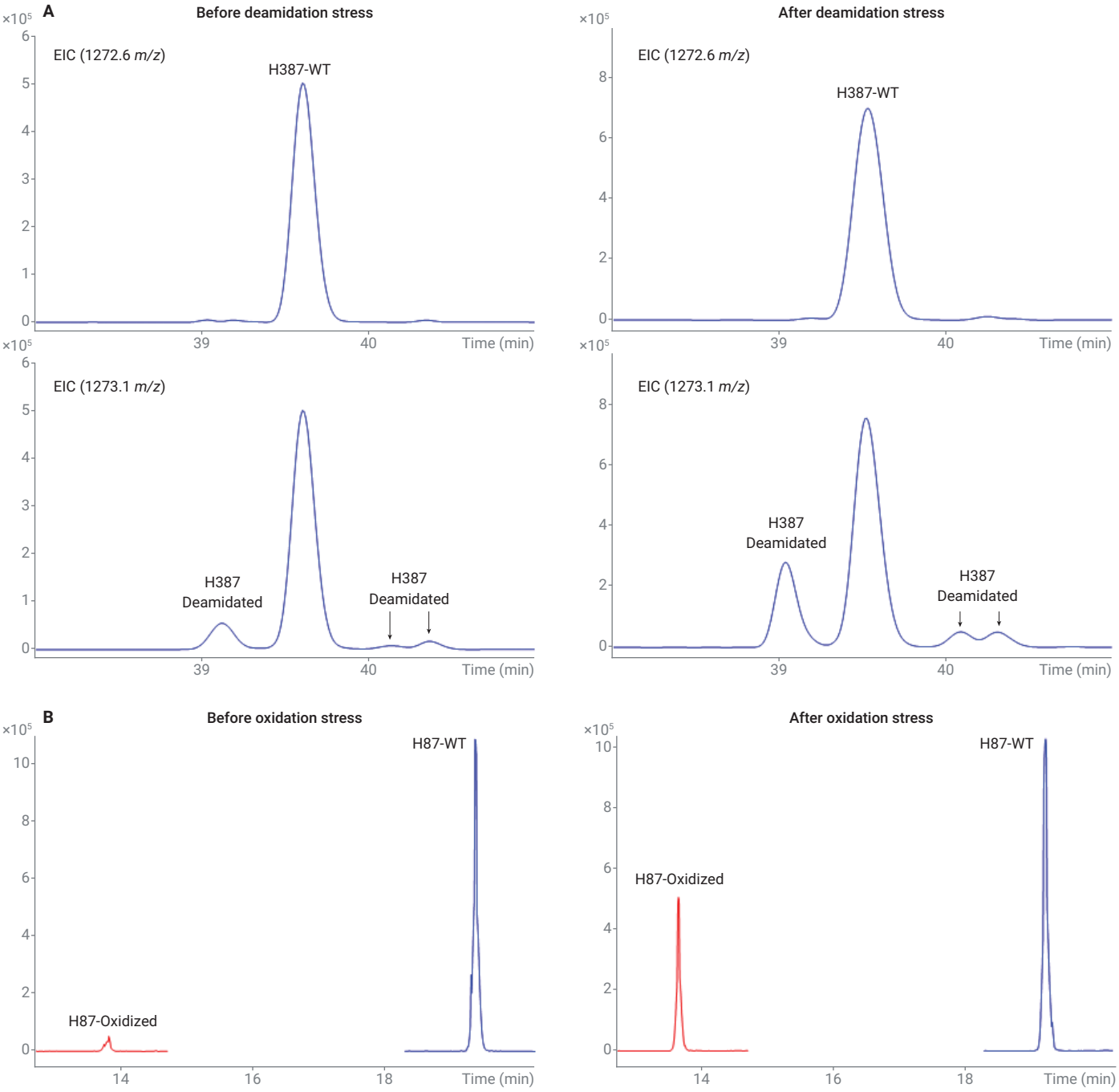

Figure 4. EICs of the peptides with variable PTMs. A) EIC comparison of the WT and deamidated H387 peptides before and after deamidation stress induction. B) EIC comparison of the WT and oxidized H87 peptides before and after oxidation stress induction.

deamidation forms, which is also called the PENNY peptide, in the reference and deamidated samples. The deamidated forms of H387 are elevated after deamidation induction. Figure 4B shows the overlaid EICs of the wild type peptide and its oxidized form from peptide H87 in both NISTmAb reference and oxidized samples. As expected, the extent of oxidation of H87 peptide was increased after oxidation induction.

The third category is glycopeptide (H300). Relative abundance of each glycopeptide can provide valuable information about the abundance of protein glycoforms. According to a previous publication on glycoanalysis in the NISTmAb tryptic digest using high-resolution LC/MS/MS, the glycopeptide located at heavy chain 292–304 (TKPREEQYNSTYR) was chosen as the dominant tryptic form[5]. Figure 5 shows the overlaid EICs of three glycopeptides (G0F, G1F, and G2F) used for determining their relative abundance. This result is consistent with a previous report on the relative abundance of these NISTmAb glycopeptides obtained using high-resolution LC/MS/MS.[5]

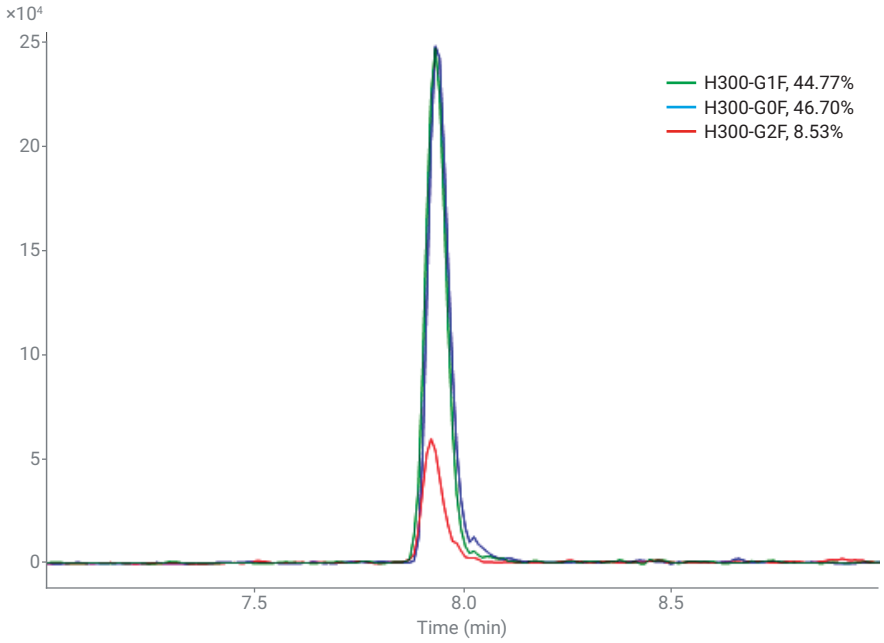

Figure 5. EICs of the three glycopeptides for determining relative abundance.

Intelligent reporting

OpenLab ChemStation software enables automated intelligent reporting. Intelligent reporting provides superior flexibility and allows the user to customize their report templates as desired. Figures 6A and 6B show examples of intelligent reports generated for monitoring multiple attributes.

Single Injection Report

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Data file:
Sample name:
Description:
Sample amount:

Instrument:
Injection date:
Acq. method:
Analysis method:

Location:
Injection:
Injection volume:
Acq. operator:

Analyst: _____

Date: _____

Pass/Fail: _____

Peak Summary Table: Glycopeptides

Name	RT [min]	Area	Area Percent
H300-G0F	7.955	1207180	46.70%
H300-G1F	7.945	1157171	44.77%
H300-G2F	7.942	220399	8.53%
	AreaSum	2584749	

Peak Summary Table: Deamidation

Name	RT [min]	Area	Area Percent	P/F
H387-D1	39.112	146688	2.86%	Pass
H387-WT	39.592	4914364	95.93%	Pass
H387-D2	40.123	61809	1.21%	Pass
	AreaSum	5122861		

Peak Summary Table: Oxidation

Name	RT [min]	Area	Area Percent	P/F
H87-Oxidized	13.811	113448	3.47%	Pass
H87-WT	19.367	3160505	96.53%	Pass
	AreaSum	3273952		

Name	RT [min]	Area	Area Percent	P/F
L4-Oxidized	16.208	105370	9.00%	Pass
L4-WT	22.627	1065867	91.00%	Pass
	AreaSum	1171237		

Name	RT [min]	Area	Area Percent	P/F
H255-Oxidized	10.961	1967116	16.80%	Fail
H255-WT	13.625	9738910	83.20%	Fail
	AreaSum	11706026		

Figure 6A. Intelligent reporting by Agilent OpenLab ChemStation. Example of a single injection report. Peak tables summarize the relative abundance of WT and PTM forms for each peptide sequence by custom calculation.

Sequence Summary Report

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Sequence name:

Acquisition date:

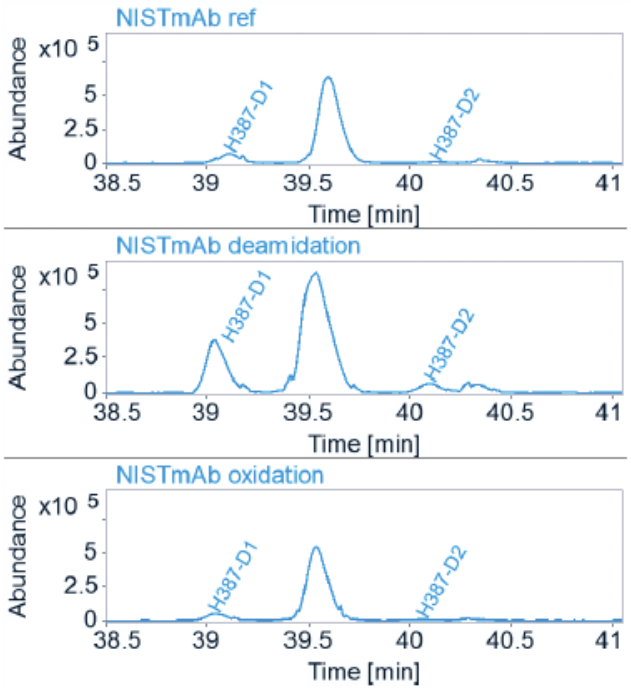
Acquired by:

Approved by: _____

Signature: _____

Date: _____

Extracted Ion Chromatogram **EIC=1272.8 :1273.8**



			H387-D1	H387-WT	H387-D2
			RT (min)	RT (min)	RT (min)
1	NISTmAb ref	_001_005-D1F-F3-Nist-mAb-deamid-0-day.D	39.11	39.59	40.12
2	NISTmAb deamidation	_002_006-D1F-F4-Nist-mAb-deamid-6-day.D	39.04	39.53	40.10
3	NISTmAb oxidation	_003_003-D1F-F2-Nist-mAb-H2O2-002pct.D	39.04	39.53	40.06

Figure 6B. Intelligent reporting by Agilent OpenLab ChemStation. Example of a sequence summary report comparing a NISTmAb reference sample, deamidated sample, and oxidized sample for the EIC and retention time of H387 peptide.

Conclusion

The Agilent InfinityLab LC/MSD XT system provides a simple and cost-effective solution for monitoring multiple PQAs in a development and quality control environment, assuming those attributes that have been precharacterized using a high-resolution MS instrument. This Application Note demonstrates that the InfinityLab LC/MSD XT system can deliver quantitative analysis for monitoring multiple attributes of biotherapeutics at the peptide level, including CDR peptides, oxidized and deamidated peptides, and glycopeptides in a single analysis. Automated data processing and reporting through Agilent OpenLab ChemStation software avoid manual interrogation and allow high-throughput analysis. OpenLab ChemStation in combination with central data storage provides a compliance solution for chromatography and mass spectrometry data collected in compliant environments.

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Critical Quality Attribute Monitoring of mAbs at the Intact and Subunit Levels Using a Cost-Effective, Simple and Robust LC/MS Solution

Authors

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Agilent Technologies, Inc.

Abstract

Recombinant monoclonal antibodies (mAbs) represent a fast-growing biotherapeutics drug category. Due to the structural complexity of mAbs, a variety of tests are required to monitor Critical Quality Attributes (CQAs) throughout the development and manufacturing process, for example, monitoring molecular mass and major glycoforms of protein product. Single quadrupole liquid chromatography/mass spectrometry (LC/MS) has been used as an effective mass detection technique in the quality control (QC) environment for its robustness, simple operation, and cost-effective characteristics.

This Application Note describes use of the Agilent LC/MSD XT mass selective detector, a single quadrupole LC/MS system with a mass range of 10 to 3,000 *m/z*, for mass determination of mAbs at intact and subunit levels. Two mAbs, NISTmAb and an immunoglobulin 1 (IgG1) mAb (mAb1), were prepared using four different approaches:

- Intact protein dilution
- Deglycosylation
- IdeS digestion
- Reduction

Samples were generated with various molecular mass and glycosylation patterns and were analyzed using the Agilent InfinityLab Liquid Chromatography/Mass Selective Detector XT (LC/MSD XT), and deconvoluted using Agilent OpenLab ChemStation software to determine the molecular mass and monitor the major glycoforms.

Introduction

In the biotherapeutic industry, production of protein-based biotherapeutics is a complex process that often requires running a panel of tests to ensure product efficacy and safety. Protein molecular mass and glycosylation are important product quality attributes, which are closely monitored during the development and production process.[1] For this purpose, single quadrupole-based LC/MS has been adopted in the QC environment for monitoring complex biomolecules. Using single quadrupole-based LC/MS, analysis of an intact protein drug and its subunits in a QC environment has become feasible for identifying variations in protein mass and glycosylation pattern, providing a rapid and cost-effective approach to monitoring the product CQAs.

This study demonstrates that the InfinityLab LC/MSD XT, a single quadrupole-based LC/MS system with a mass range up to 3,000 *m/z*, is suitable for mass determination of mAbs at intact and subunit levels. Two mAbs, NISTmAb and mAb1, were selected as test cases. The samples were prepared using four approaches, intact protein dilution, deglycosylation, IdeS digestion, and reduction experiments to produce intact protein and various subunit fragments (Figure 1). These experiments produced protein compounds with molecular masses ranging from 23 to 148 kDa, and various glycoform patterns at three mass ranges including ~25, ~50, and ~148 kDa. All the samples were analyzed using the InfinityLab LC/MSD XT, coupled with an Agilent 1290 Infinity II LC and Agilent OpenLab ChemStation Software. The molecular mass of each compound peak was determined using the LC/MS deconvolution tool within OpenLab ChemStation software, and then compared to their theoretical average mass for mass accuracy evaluation.

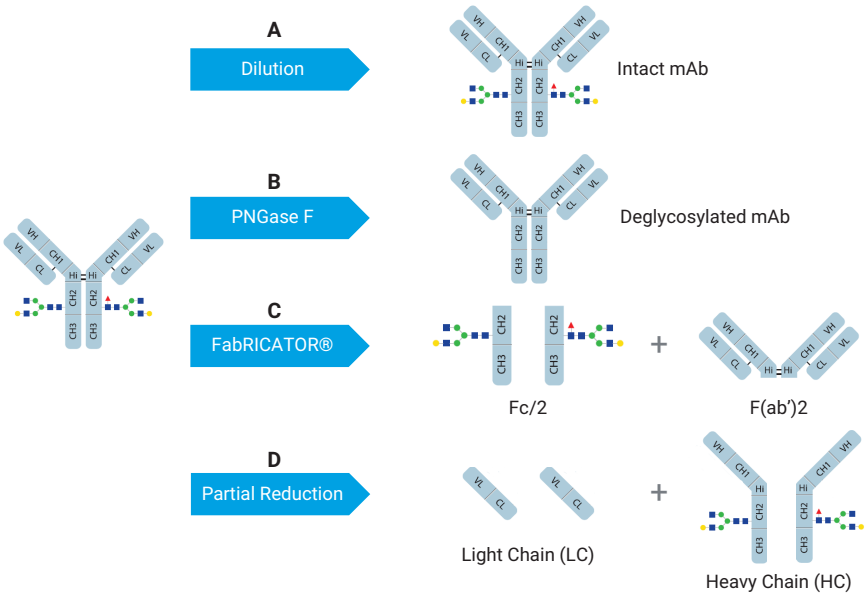

Figure 1. Scheme of sample preparation workflow. A: Dilution of Intact mAb. B: Deglycosylateion of mAb by PNGase F enzyme. C: Digestion of mAb by IdeS enzyme (FabRICATOR). D: Partial reduction of mAb. Image as kindly provided by Genovis.

Experimental

Materials

The NISTmAb reference material was purchased from the National Institute of Standards and Technology. The recombinant IgG1 mAb (mAb1) was obtained from a partner lab. PNGase F enzyme (P0705L) was purchased from New England BioLabs. IdeS enzyme (FabRICATOR) was purchased from Genovis.

Sample preparation

- **Intact mAbs:** Intact mAb stocks (10 µg/µL NISTmAb or 21 µg/µL mAb1) were diluted in H_2O to a final concentration of 0.5 µg/µL.
- **Deglycosylation of mAbs:** 20 µg mAb (NISTmAb or mAb1) was added into 2 µL of 10x G7 reaction buffer that was supplied with the PNGase enzyme by the vendor, diluted with H_2O to a total volume of 19 µL, then increased with 1 µL of PNGase F enzyme to give a final mAb concentration of 1 µg/µL. The resulting sample was incubated at 37 °C for one hour.
- **IdeS digestion:** 20 µg mAb (NISTmAb or mAb1) was incubated in 40 µL of reaction buffer containing 2 mM Na_2HPO_4, 6 mM NaCl, pH 6.6, and ~25 units of FabRICATOR at 37 °C for 30 minutes.
- **Partial reduction:** 40 µL of 0.5 µg/µL mAb (NISTmAb or mAb1) was incubated with 20 mM DTT at 60 °C for 30 minutes.

All the samples were injected immediately after sample preparation for LC/MS analysis.

LC/MS analysis

Liquid chromatography separation was carried out on a PLRP-S column (1,000 Å, 2.1 × 50 mm, 5 µm, p/n PL1912-1502) using an Agilent 1290 Infinity II LC, consisting of an Agilent 1290 Flexible Pump (G7104A), a Multisampler (G7167B) with sample cooler (Option 100), and a Thermostatted Column Compartment (G7116B). The MS system used was the Agilent LC/MSD XT (G6135BA) with the Agilent Jet Stream source (G1958-65138). Tables 1 and 2 list the LC/MS conditions.

Software

Agilent OpenLab ChemStation (version C 01.09) was used for data acquisition, data processing, and reporting.[2,3] The LC/MS deconvolution tool in ChemStation was used to determine the molecular mass of each protein compound. The theoretical average mass of each protein compound was calculated using NIST Mass and Fragment Calculator (v2.0) with NIST defined elemental average mass.[4]

Results and discussion

Agilent LC/MSD XT mass selective detector has an extended mass range up to 3,000 *m/z*, which covers the whole charge envelope of most mAb subunits and a portion of the charge envelope of intact mAb. To evaluate the reliability of mass determination of mAbs at both intact and subunit levels, NISTmAb and mAb1 were prepared by four difference methods as previously described, then injected onto the LC/MSD XT using the methods in Tables 1 and 2. To determine the mass of each protein compound peak, the deconvolution tool of the OpenLab ChemStation software was used to deconvolute the mass spectral data. Average spectra were selected at half peak height. Ion Peak Width at Half Height (PWHH) was set to 0.6 Da and Gaussian Curve fitting was used for molecular weight (MW) assignment.

Table 1. LC conditions

LC parameters					
Analytical Column	PLRP-S 1000Å, 2.1 x 50 mm, 5 µm (p/n: PL1912-1502)				
Mobile Phase A	H_2O with 0.1% (v/v) formic acid				
Mobile Phase B	Acetonitrile with 0.1% (v/v) formic acid				
Column Temperature	80 °C				
Flow Rate	0.5 mL/min				
Gradient	Intact mAb				
	Time (minutes)	%A	%B	%C	%D
	0.0	90	10	0	0
	5.0	40	60	0	0
	6.0	90	10	0	0
	8.0	90	10	0	0
	mAb Subunits				
	Time (minutes)	%A	%B	%C	%D
	0	95	5	0	0
	1.0	80	20	0	0
	8.0	60	40	0	0
	8.1	50	50	0	0
	9.1	95	5	0	0
	11.0	95	5	0	0

Table 2. MS conditions.

MSD XT parameters	
Drying Gas Flow	12 L/min
Drying Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Sheath Gas Temperature	360 °C
Nebulizer Pressure	50 psi
Capillary Voltage	4,500 V
Nozzle Voltage	2,000 V
Peak Width	0.1 minutes
Scan	positive mode, step size 0.1, 1,000 to 3,000 *m/z* for intact mAb, 500 to 3,000 *m/z* for mAb subunits

Figure 2 shows intact NISTmAb and mAb1 analysis using the InfinityLab LC/MSD XT, including Total Ion Chromatogram (TIC), average mass spectra at half peak height, and deconvoluted mass. Both mAbs have a MW of ~148 kDa. Since the mass range of LC/MSD XT is up to 3,000 *m/z*, only a portion of the charge envelope of intact mAbs was detected, which falls within the range of 2,000 to 3,000 *m/z*. The spectral zoom-in views of one of the charge states (Figures 2C and 2D) show multiple peaks from NISTmAb and mAb1, which correspond to their major glycoforms observed on a high-resolution LC/Q-TOF.[5] Using this partial charge envelope, the deconvoluted mass yields five glycoforms of NISTmAb (Figure 2E) and three glycoforms of mAb1 (Figure 2F). Comparing the theoretical average molecular mass and experimental mass shows most intact protein glycoforms have a mass deviation of approximately 10 Da, ranging from −3.1 to −20.2 Da, that is, a mass accuracy of −21 to −136 ppm (Table 3).

To further test the application of the InfinityLab LC/MSD XT for large protein analysis, the PNGase F enzyme was used to remove N-linked oligosaccharides from mAbs. The deglycosylated mAbs have a MW of ~145 kDa. Figure 3 shows the analysis of deglycosylated NISTmAb and mAb1 using this system. It shows the multiple glycoforms were collapsed into one major peak (Figures 3C and 3D). The deconvoluted mass shows a mass error of −7.5 Da/−52 ppm for deglycosylated NISTmAb and 0.2 Da/1 ppm for deglycosylated mAb1 (Figures 3E and 3F; Table 3).

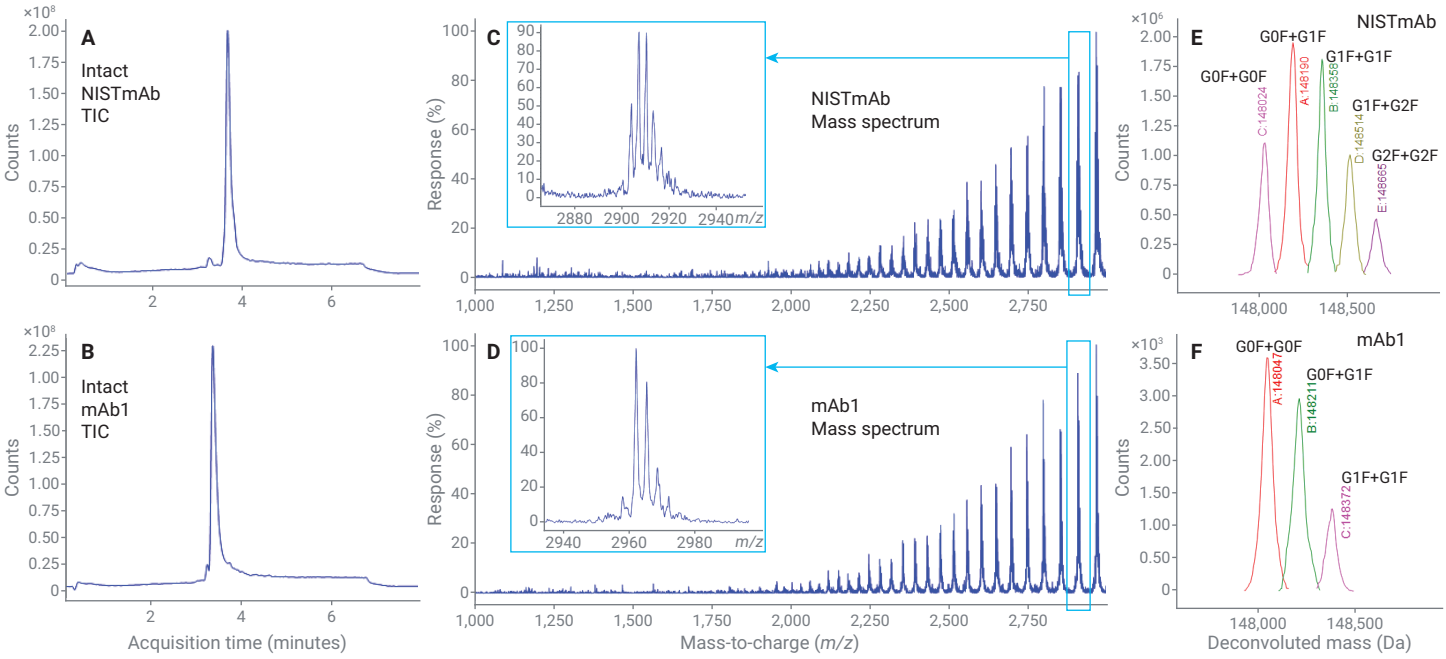

Figure 2. Intact NISTmAb and mAb1 analysis. Panels A,B: TIC. Panels C,D: Average Mass spectra at half peak height with the zoom-in view of one of the charge states in the insets. Panels E,F: Deconvoluted mass.

Table 3. Comparison summary of the theoretical average molecular mass and experimental mass as determined by deconvolution using Agilent ChemStation software.

Experiment	Subunits	Modification	Average Mass (Da)	Experimental Mass (Da)	Δ Mass (Da)	Mass Error (ppm)
Intact Protein Dilution	Intact NISTmAb	G0F + G0F	148,036.5	148,023.7	-12.8	-86
		G0F + G1F	148,198.6	148,189.5	-9.1	-62
		G1F + G1F	148,360.8	148,357.7	-3.1	-21
		G1F + G2F	148,522.9	148,514.2	-8.7	-59
		G2F+G2F	148,685.1	148,664.9	-20.2	-136
	Intact mAb1	G0F + G0F	148,055.9	148,047.3	-8.6	-58
		G0F + G1F	148,218.0	148,211	-7.0	-47
		G1F + G1F	148,380.2	148,372.2	-8.0	-54
PNGaseF Digest	Deglycosylated NISTmAb	None	145,145.8	145,138.3	-7.5	-52
	Deglycosylated mAb1	None	145,165.2	145,165.4	0.2	1
IdeS Digest	NISTmAb F(ab')2	None	97,608.6	97,606.3	-2.3	-24
	NISTmAb Fc/2	G0F	25,232.0	25,230.8	-1.2	-46
		G1F	25,394.1	25,393.1	-1.0	-39
		G2F	25,556.2	25,555.3	-0.9	-37
	mAb1 F(ab')2	None	97,628.0	97,622.1	-5.9	-61
	mAb1 Fc/2	G0F	25,232.0	25,230.8	-1.2	-46
		G1F	25,394.1	25,392.8	-1.3	-51
Partial Reduction	NISTmAb Light Chain	None	23,123.5	23,122.3	-1.2	-51
	NISTmAb Heavy Chain	G0F	50,898.8	50,900.7	1.9	38
		G1F	51,060.9	51,060.7	-0.2	-4
		G2F	51,223.1	51,222.3	-0.8	-15
	mAb1 Light Chain	None	23,438.8	23,437.8	-1.0	-42
	mAb1 Heavy Chain	G0F	50,593.2	50,593.2	0.0	0
		G1F	50,755.3	50,752.1	-3.2	-64

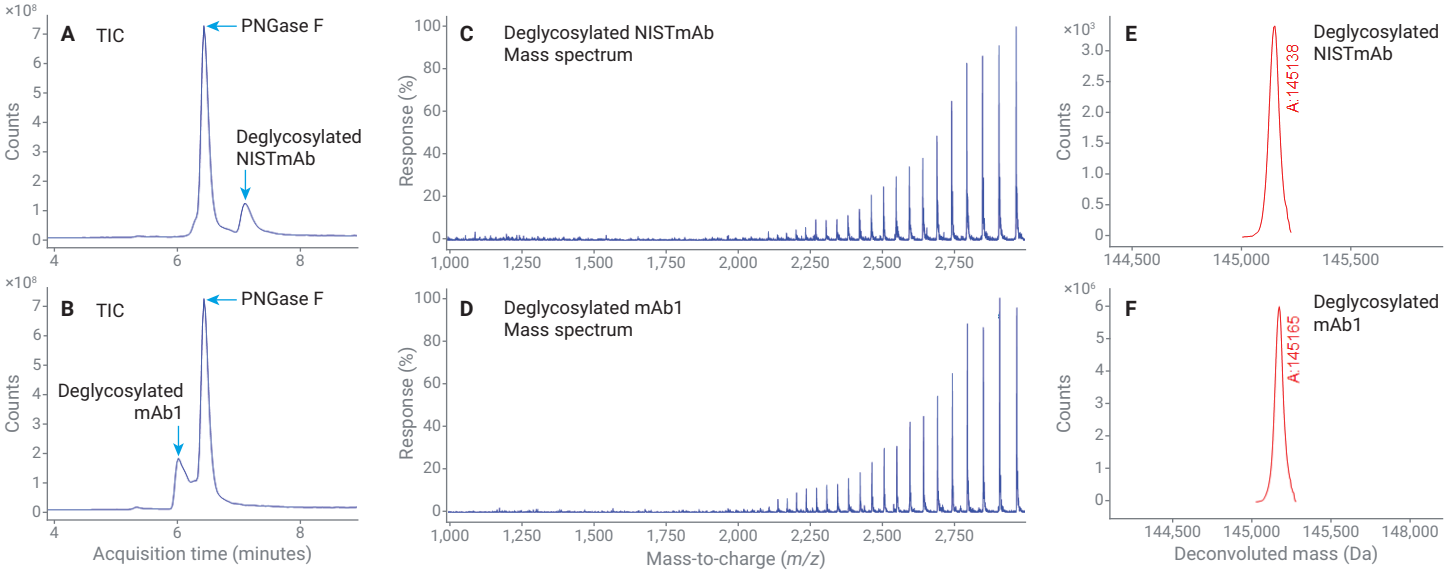

Figure 3. Deglycosylated NISTmAb and mAb1. Panels A,B: TIC. Panels C,D: Average mass spectra at half peak height. Panels E,F: Deconvoluted mass.

To assess the application of LC/MSD XT for subunit analysis, the commonly used IdeS enzyme was used to generate subunit fragments from NISTmAb and mAb1. By performing IdeS digestion of each mAb without reduction, two fragments–Fc/2 and F(ab')2–were produced, which are ~25 and ~98 kDa in size, respectively (Figures 4 and 5). Most of the charge envelope from the two fragments was observed by LC/MSD XT, even for the larger F(ab')2 fragment (Figures 4B, 4C, 5B, and 5C). After digestion, the N-glycosylation site was retained on the small fragment Fc/2. The Fc/2 fragment of NISTmAb shows three major peaks corresponding to G0F, G1F, and G2F glycoforms with the mass error ranging from −0.9 to −1.2 Da. The Fc/2 fragment of mAb1 shows two major peaks corresponding to G0F and G1F with the mass error of −1.2 and −1.3 Da, respectively. The F(ab')2 fragments from NISTmAb and mAb1 show a single major peak, and their deconvoluted mass have a mass error of −2.3 and −5.9 Da, that is, −24 and −61 ppm, respectively (Figures 4E and 5E; Table 3).

In addition to the preceding application,

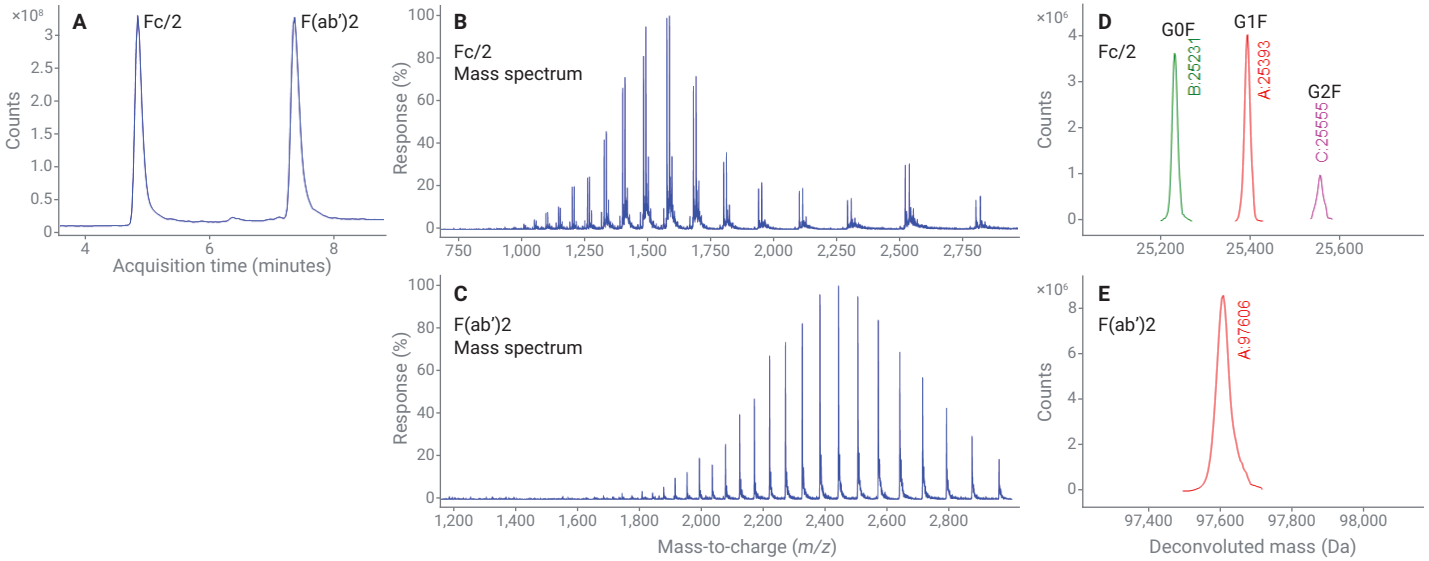

Figure 4. NISTmAb fragments after IdeS digestion. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

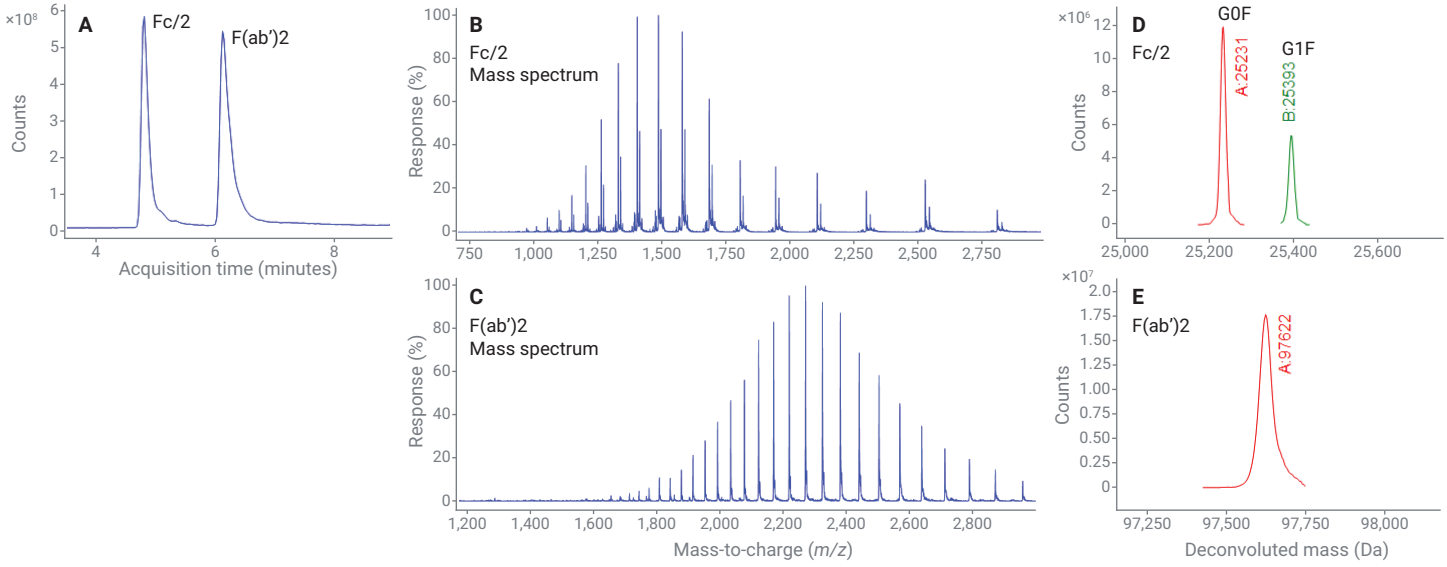

Figure 5. mAb1 fragments after IdeS digestion. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

the mAbs were also partially reduced as described in the experimental method to obtain data for the light and heavy chains. As expected, the deconvolution of the light chain collapses the charge envelope to a single peak while the deconvolution of the heavy chain identifies three major glycoforms from NISTmAb and two major glycoforms from mAb1 (Figures 6 and 7). The mass accuracy of the light and heavy chains from these two mAbs ranges from −64 to 38 ppm (Table 3).

In summary, these results show that the InfinityLab LC/MSD XT yields a mass error less than ±70 ppm for most protein and subunits tested. Only two glycoforms from intact mAbs showed a mass error greater than ±70 ppm.

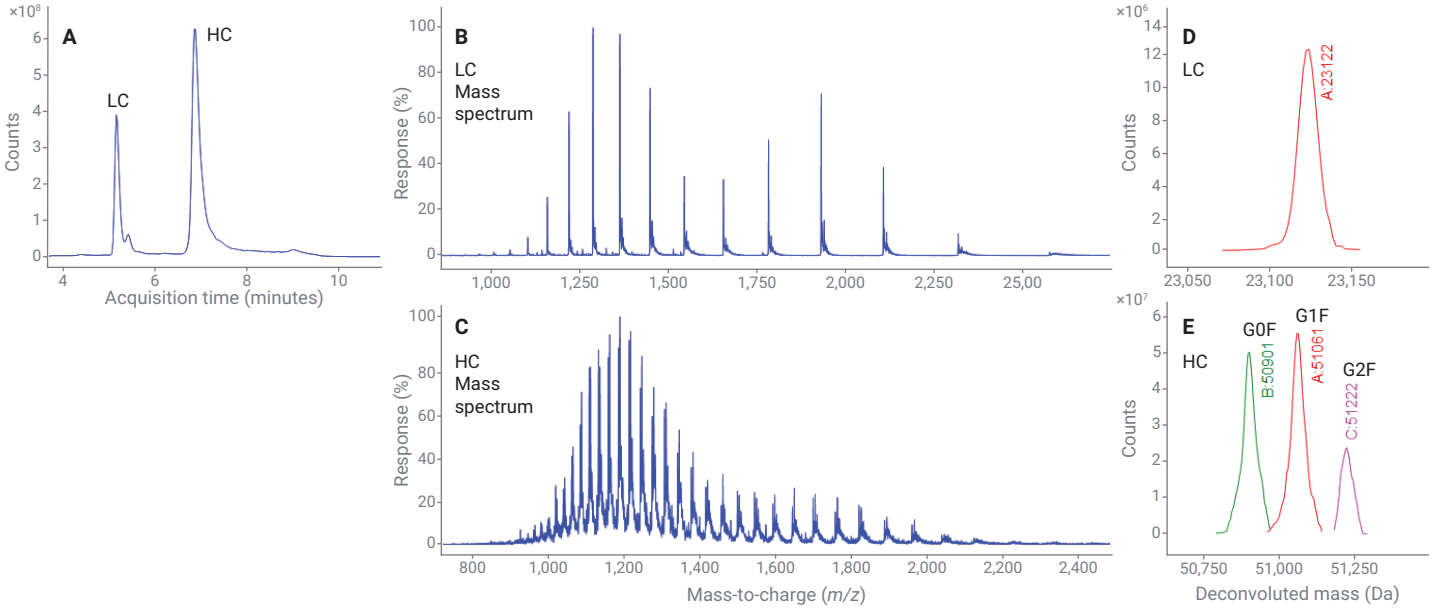

Figure 6. NISTmAb Light Chain (LC) and Heavy Chain (HC) subunits after partial reduction. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

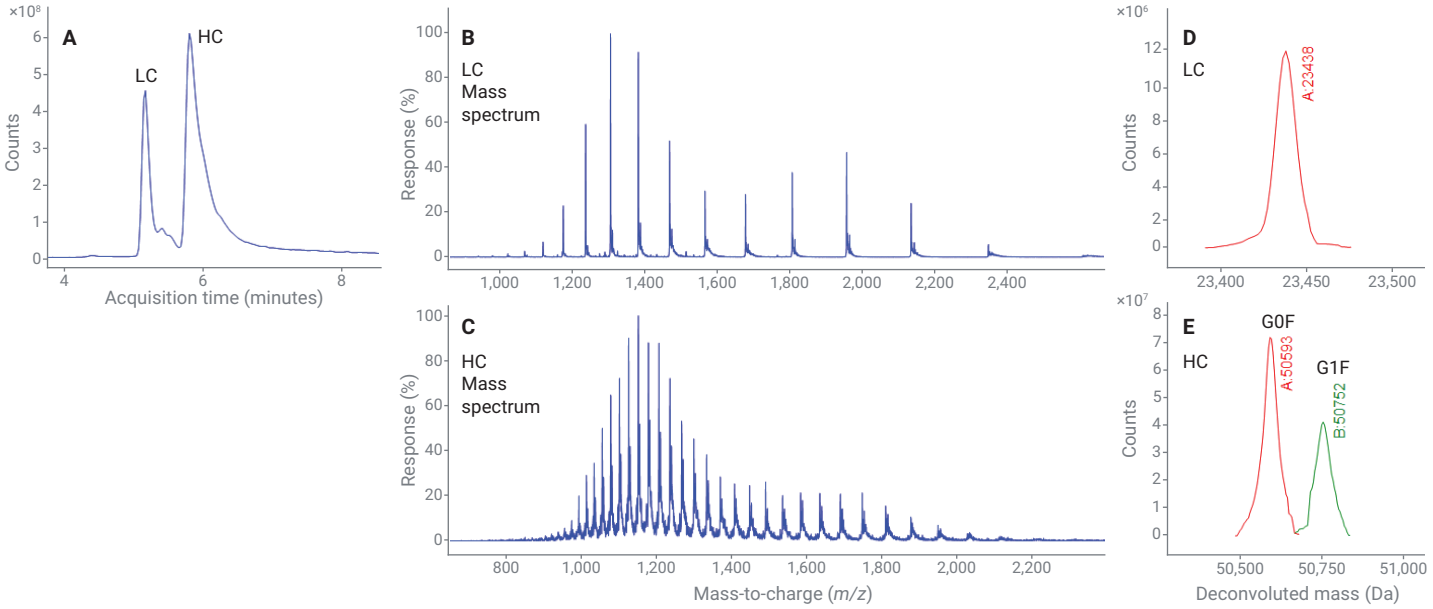

Figure 7. mAb1 subunits after partial reduction. Panel A: TIC. Panels B,C: Average mass spectra at half peak height. Panels D,E: Deconvoluted mass.

Conclusion

This Application Note demonstrates that the Agilent InfinityLab LC/MSD XT can be used for quick and reliable mass determination of mAbs at both intact and subunit levels using four different sample preparations. These four procedures produced various mAb samples, with the molecular weight ranging from ~23 to ~148 kDa. This mass range covers most protein samples at intact and subunit levels. This study shows that the InfinityLab LC/MSD XT can provide a simple, rapid, and cost-effective way to monitor variations in the molecular weight and in the glycoform pattern in an analytical development and quality control environment.

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Charge Variant and Aggregation Analysis of Innovator and Biosimilars of Rituximab

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Abstract

Monoclonal antibodies are an important class of biomolecules used for treatment of various diseases. Biosimilars, the copy versions of an innovator molecule, need to be characterized in detail for their critical quality attributes (CQAs) such as aggregates and charge variants. The attributes must fall within a desired range compared to the innovator for approval by regulatory agencies. This study compares two rituximab biosimilars from different manufacturers to the innovator for their aggregation and charge variant profiles by following two analytical workflows using Agilent 1260 Infinity II bio-inert LC and Agilent AdvancedBio columns. The results show the similarities or differences between innovator and biosimilars in their aggregates and charge variant profiles. Biosimilar 1 has more similarities with the innovator than biosimilar 2 in terms of aggregates and charge variants. Excellent intraday and interday reproducibility of the methods was demonstrated. Agilent OpenLab CDS software featuring peak explorer facilitates easy data review at a glance. This work is part of a series of biosimilarity studies of rituximab.

Introduction

Monoclonal antibody (mAb) drugs are one of the fastest growing biotherapeutics in the pharma market. The majority of mAbs are for treatment of cancers.[1] The investment during the discovery, development, manufacturing, and clinical trials is huge for innovator mAb drugs. As a result, the cost of innovator drug treatment is usually high for patients. Therefore, more affordable generic versions of innovator drugs, called biosimilars, are in high demand. The first biosimilar was approved for the European market in 2006, and the U.S. market opened nine years later after the introduction of the Affordable Care Act in March 2010. The development of biosimilars is gaining traction due to patent expiry of innovator molecules.

For biosimilars to be approved by regulatory agencies, manufacturers need to demonstrate that there are no clinically meaningful differences between the biological product and the reference product in terms of the safety, purity, and potency.[2] A critical part in this process is an extensive comparative analytical study to understand the physicochemical similarities between the innovator and biosimilars.

Aggregates, truncation, and other modified forms (deamidation, isomerization, and so forth) are product-related impurities that arise during the manufacturing process or storage. Their presence in the drug negatively impact drug stability, activity, and efficacy. Therefore, they are usually considered CQAs, and are closely monitored and tested throughout the manufacturing process.[3]

This Application Note uses two analytical workflows to demonstrate a comparison between two biosimilars of rituximab and their reference innovator in terms of aggregate and charge variant profiles. Rituximab is a well known biotherapeutic drug for the treatment of rheumatoid arthritis, lupus, vasculitis, and dermatomyositis. The two biosimilars were obtained from two manufacturers in different geographical locations. Both workflows are based on the 1260 Infinity II bio-inert LC system together with advancedBio columns and OpenLab CDS. Charge variants were separated on a weak cation exchange (WCX) column, while aggregates were separated on a size exclusion (SEC) column. Figure 1 shows the two workflow details. Good reproducibility on intraday and interday results ensured reliability of the workflows and demonstrated clear similarities or differences between the innovator and biosimilars.

Experimental

Instrumentation

The systems were composed of the following modules:

- 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 II Diode Array Detector WR (G7115A) with bio-inert flow cell
- Agilent 1260 Infinity II Bio-inert MultiDetector Suite (MDS) (G7805A) featuring dual-angle static and DLS detection (G7809A)

Columns

- Agilent Bio mAb, nonporous, 2.1 × 250 mm, 5 µm HPLC, PEEK (p/n 5190-2411) for charge variants analysis
- Agilent AdvanceBio SEC 300Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5301) for aggregation analysis.

Software

- Agilent OpenLab CDS Version 2.3
- Agilent Buffer Advisor A.01.01 [009]
- Agilent Bio-SEC Software version A.02.01 Build 9.34851[21]

LC instrument control as well as LC data analysis was carried out using Agilent OpenLab CDS Version 2.3. It provides a smooth user interface with customized and interactive reporting with drag-and-drop template creation. The peak explorer feature of the software was used to compare the results between the innovator and biosimilars.

Chemicals and samples

All solvents used were LC grade. Fresh ultrapure water was obtained from a Milli-Q Integral system equipped with a 0.22 µm membrane point-of-use cartridge (Millipak). Sodium phosphate monobasic, sodium phosphate dibasic, and sodium chloride were purchased from Sigma-Aldrich, St. Louis, USA. The mAb drugs, including the innovator and two biosimilars, were purchased from a local distributor. Before analysis in the DLS system, the mobile phase was triple filtered through a 0.1 µm hydrophilic PTFE membrane filter (Merck Millipore).

Samples were taken from the original container and centrifuged at 13,000 g for two minutes. Supernatant was aliquoted to an LC sample vial for analysis.

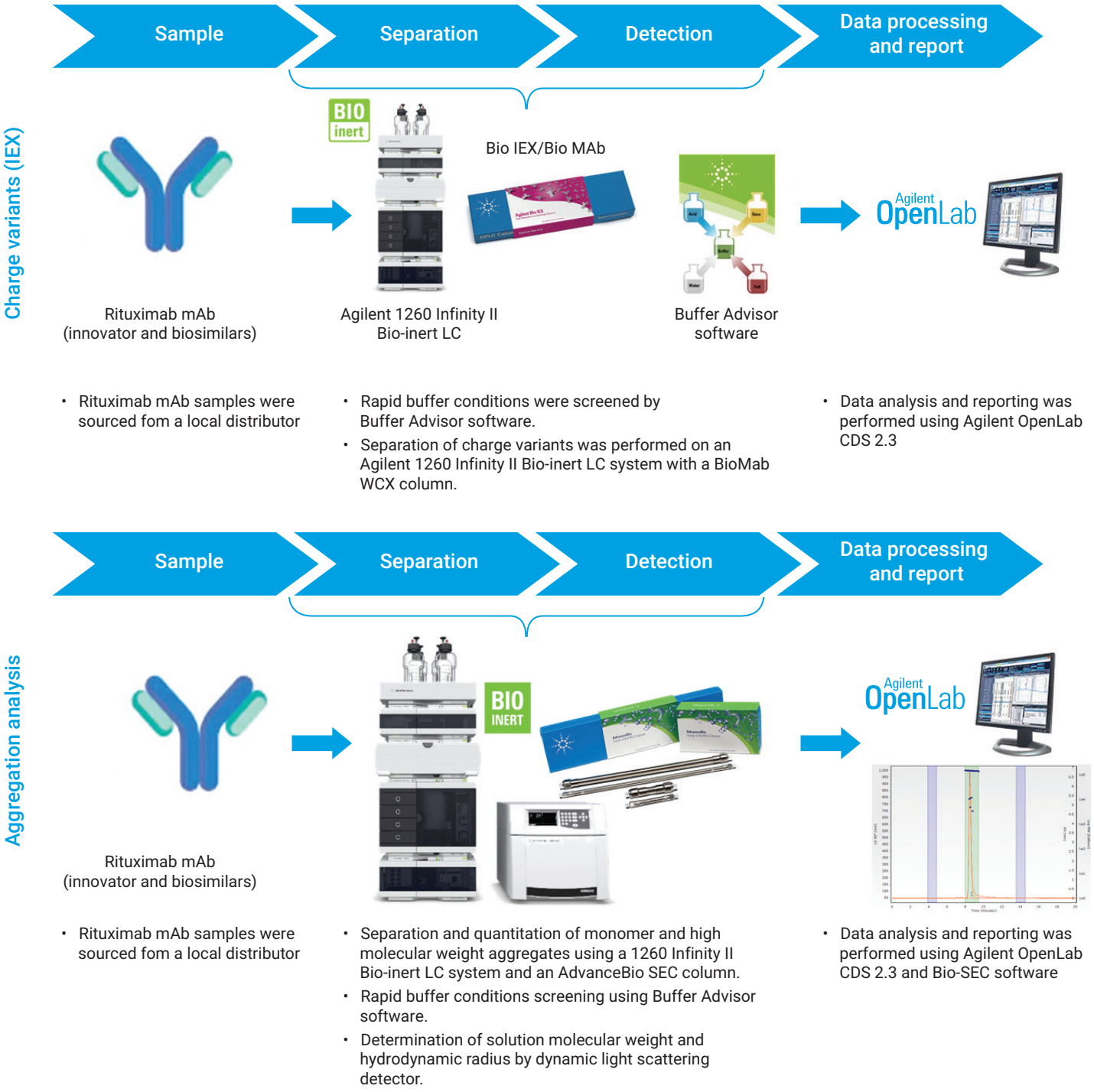


Figure 1. Charge variant and aggregation analysis workflow for the analysis of rituximab innovator and biosimilars.

Experimental methods

Charge variant: Table 1 shows the chromatographic parameters used for ion exchange chromatography of rituximab innovator and biosimilars. The gradient used in the study was calculated from the buffer advisor software. Samples were directly injected without dilution (10 mg/mL). Retention time (RT), area, and area percent were used to calculate relative standard deviation (RSD %) values. Relative percent area was used to quantify the charge variants of the mAbs.

Aggregates analysis: Table 2 shows the chromatographic parameters used for aggregation analysis of rituximab innovator and biosimilars. Samples were directly injected without dilution (10 mg/mL). RT, area, and percent area were used to calculate RSD% values. Relative percent area was used to quantify the high molecular weight species (HMWS) and low molecular weight species (LMWS) in the samples. Average molecular weight and hydrodynamic radius of rituximab were obtained from DLS analysis.

Results and discussion

Charge variant (IEX)

Figure 2 shows the charge variant profiles of innovator and biosimilars on a BioMAb PEEK column, demonstrating high resolution separation of charge variants in 16 minutes. The overlay of six replicates of rituximab innovator and biosimilars shows excellent reproducibility. The RSD of RT and area for main peak and variants are all within 0.3 and 1%, respectively.

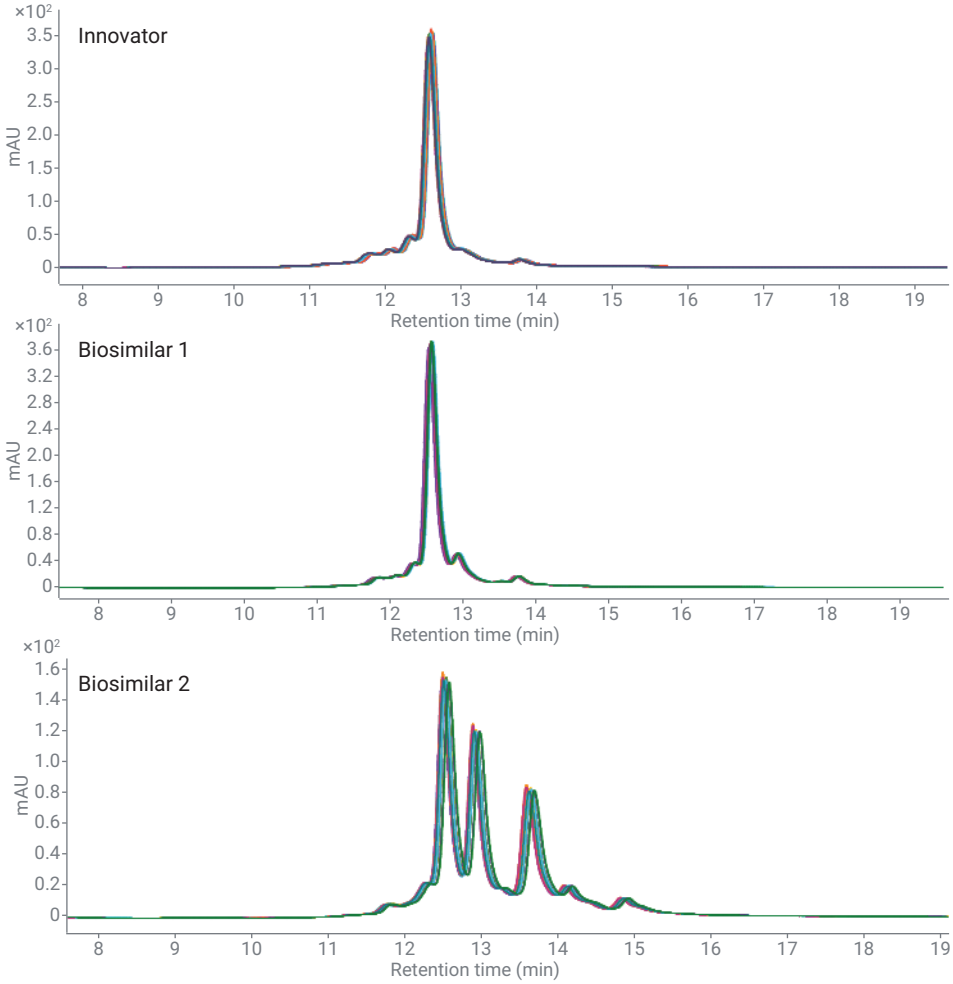
Table 1. IEX chromatographic conditions.

Parameter	Value
Salt Gradient 0 to 200 mM NaCl, 30 mM Sodium Phosphate Buffer, pH 6.8	Time (min) A) Water B) NaCl (1,000 mM) C) NaH_2PO_4 (55 mM) D) Na_2HPO_4 (50 mM) 0.0 43.1 0.0 31.0 25.9 30.0 22.3 20.0 22.7 35.0 35.0 22.3 20.0 22.7 35.0
Stop Time	35 minutes
Post Time	30 minutes
Flow Rate	0.25 mL/min
Injection Volume	2 µL
Sampler Temperature	10 °C
Column Temperature	25 °C
DAD	280 nm/4 nm, Ref:OFF
Peak Width	>0.025 minutes (10 Hz)

Table 2. Aggregation analysis chromatographic conditions.

Parameter	Value
Mobile Phase	100 mM sodium phosphate buffer+150 mM NaCl, pH 7.0
Flow Rate	0.8 mL/min
Stop Time	20 minutes
Injection Volume	10 µL (for UV) / 25 µL (for DLS)
Sampler Temperature	10 °C
Column Temperature	25 °C
DAD	280 nm/4 nm, Ref:off
Peak Width	>0.05 minutes (1.0 second response time) (5 Hz)
LS Detector	25 °C
DLS Operational Parameters	
Correlator Run Time	5 seconds
Correlator Function Clip Time	10 µs
R^2	0.80
Eluent Viscosity	0.0079 (viscosity of water at 30 °C)
Eluent Refractive Index	1.333 (refractive index of water)

Figure 3 shows overlaid chromatograms for comparison between innovator and biosimilars. The peak at ~12.5 minutes is attributed to the main peak, and the peaks to the left and right of the main peak are assigned to acidic and basic charge variants, respectively. The profiles of acidic variants were similar between the innovator and biosimilar 1, while biosimilar 2 shows a slight difference. The profiles of basic variants mainly attributed to lysine truncation showed huge differences between biosimilar 2 and innovator due to the incomplete lysine truncation.[4]



Innovator	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
Acidic Variants	0.12	0.42	0.31	0.68
Main Peak	0.10	0.15	0.29	0.50
Basic Variants	0.10	0.90	0.28	1.91

Biosimilar 1	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
Acidic Variants	0.08	0.60	0.16	1.07
Main Peak	0.07	0.32	0.15	0.85
Basic Variants	0.07	0.75	0.14	1.05

Biosimilar 2	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
Acidic Variants	0.20	0.19	0.26	0.80
Main Peak	0.21	0.52	0.25	0.89
Basic Variants	0.22	0.32	0.26	0.70

Figure 2. Overlay of six replicates of innovator and biosimilars of rituximab on an Agilent 1260 Infinity Bio-inert quaternary LC using an Agilent Bio Mab, 2.1 × 250 mm, 5 µm, PEEK column. The tables in the figure show the precision of retention time and area for main peak and charge variants, n = 6.

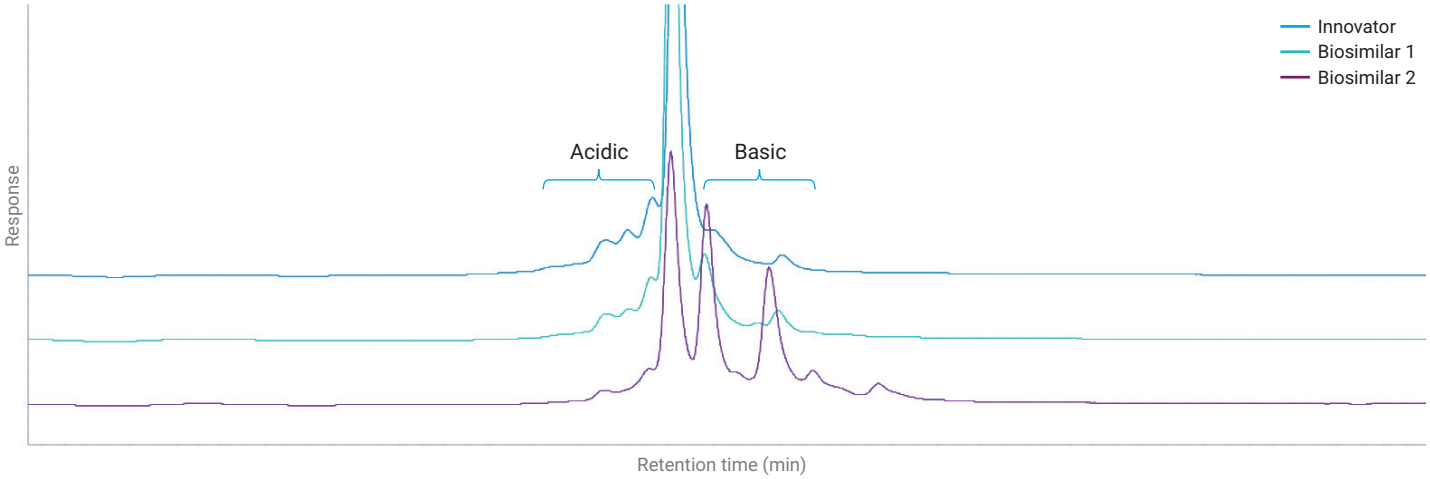


Figure 3. Expanded view of the charge variant profile comparison of innovator and biosimilars of rituximab.

Figure 4 shows the charge variants and main form distribution across innovator and biosimilars. The main form in innovator rituximab was found to be 65.35 ±0.27 %, with 65.14 ±0.10% in biosimilar 1, but only 28.8 ±0.07 % in biosimilar 2. The major charge variant in biosimilar 2 rituximab was 62.99 ±0.06% basic variants compared to the innovator product (12.97 ±0.23%). The innovator and biosimilar 1 are similar in their charge variant profile, except biosimilar 1 showed slightly more basic variants (20.11 ±0.12% versus 12.97 ±0.23%) and fewer acidic variants (14.53 ±0.09% versus 21.68 ±0.13%).

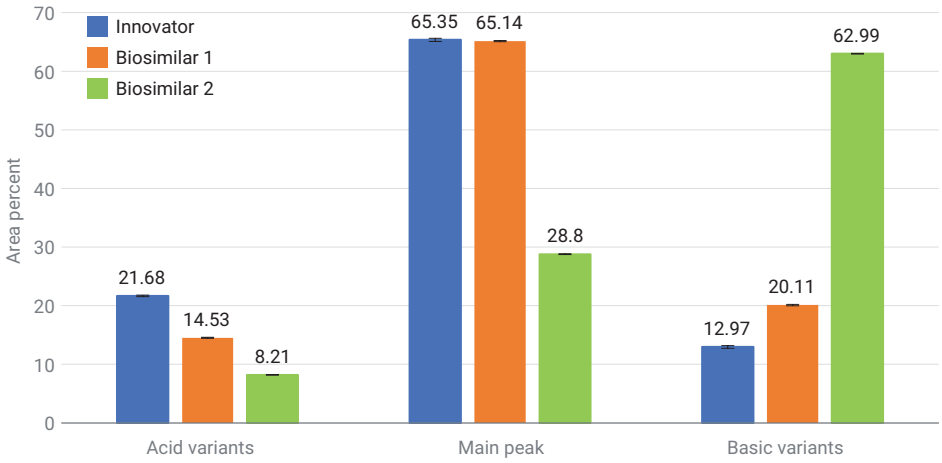


Figure 4. Comparison of the charge variants (acidic, main, and basic) area percentage between innovator and biosimilars.

Another useful data analysis capability feature found in the OpenLab CDS is the Peak Explorer. This feature promotes quick data review for complex samples by visualizing large data sets to discover trends, retention time shifts, outliers, artifacts, and so forth. Peak Explorer was used to examine the charge variant data and compare the innovator and biosimilars. Figure 5 shows the visualization of comparison from Peak Explorer for innovator and biosimilars. Each bubble corresponds to the acid and basic variants and the main peak. The size of the bubble represents the area percent of the variant. The comparison shown with Peak Explorer is in accordance with the Figure 4 conclusion.

Aggregate analysis

Figure 6 shows the aggregates profiles of innovator and biosimilars demonstrating high-resolution separation of aggregates in 20 minutes. The overlay of six replicates of innovator and biosimilars shows excellent reproducibility. The RSD of RT and area for the main peak and variants are all within 0.1 and 2%, respectively.

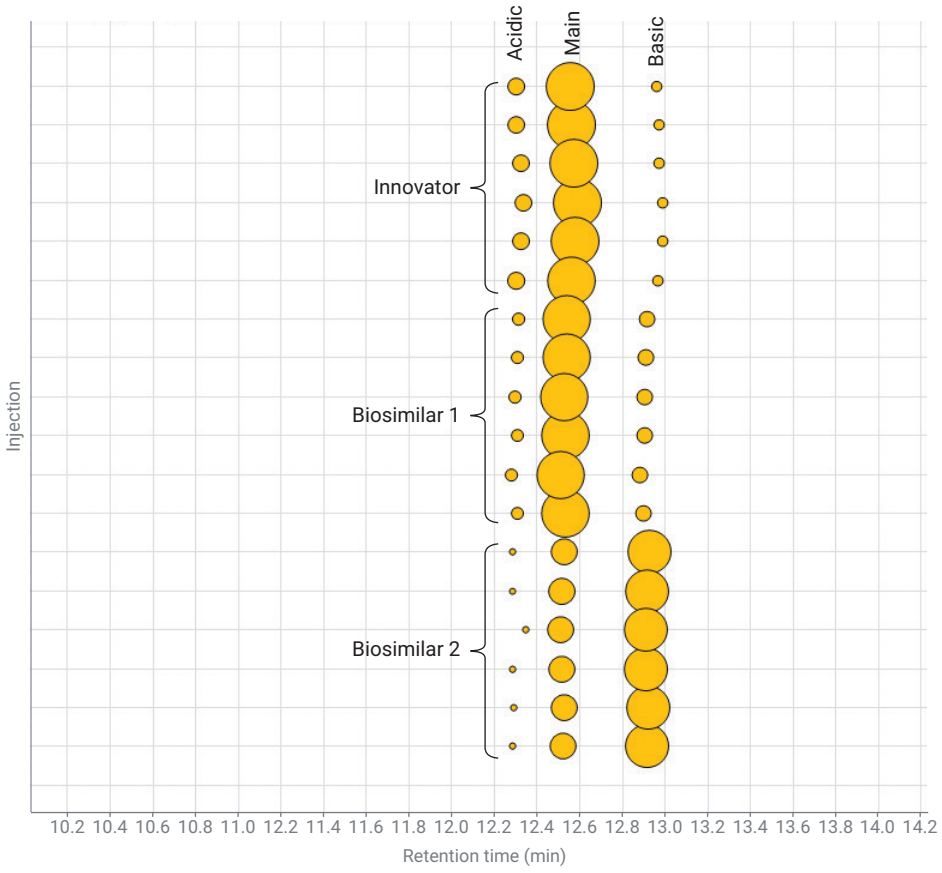
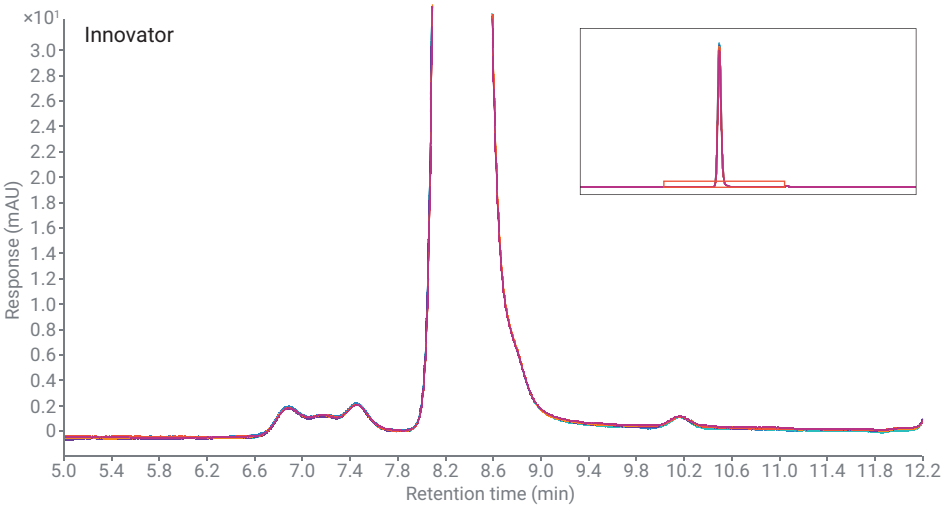
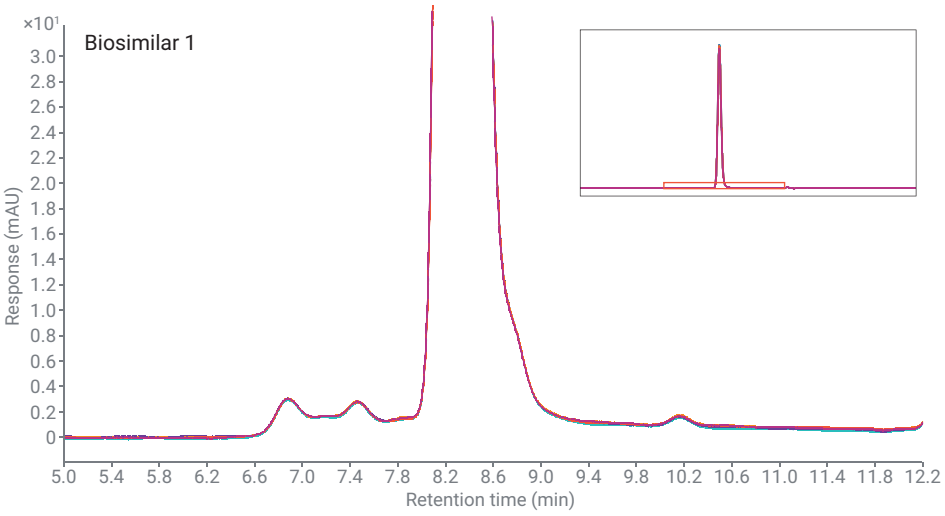


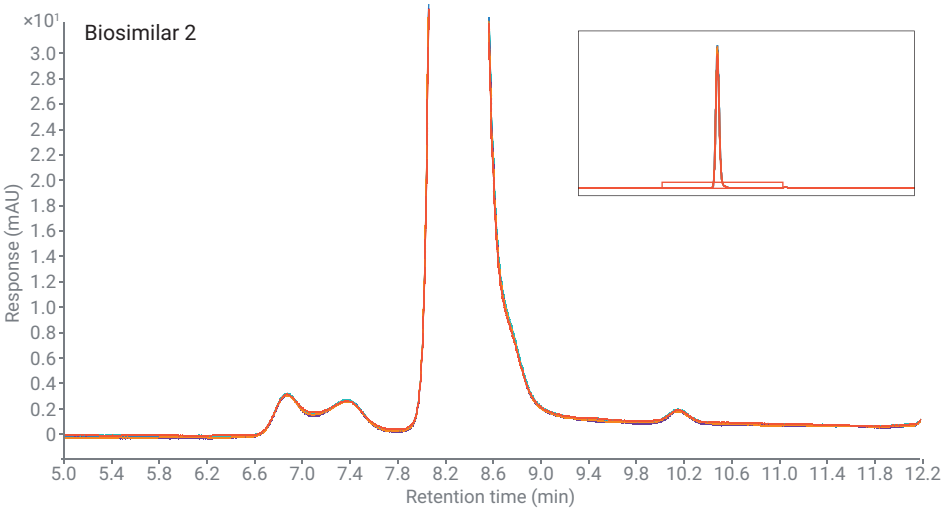
Figure 5. A Peak Explorer data presentation snapshot. The X-axis is retention time, and the Y-axis is the injection number of the data set loaded. Each bubble represents each variant and main peak. The size of the bubble represents peak area percent.



Innovator	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
HMWS	0.05	0.35	0.05	0.56
Monomer Peak	0.02	0.91	0.04	0.86
LMWS	0.03	1.12	0.06	1.77



Biosimilar 1	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
HMWS	0.04	0.72	0.08	1.29
Monomer Peak	0.00	0.68	0.02	0.62
LMWS	0.04	1.75	0.04	2.25



Biosimilar 2	Intraday (n = 6)		Interday (n = 6)	
	%RSD RT	RSD% Area	%RSD RT	RSD% Area
HMWS	0.04	0.66	0.04	0.63
Monomer Peak	0.01	0.51	0.01	0.61
LMWS	0.02	2.11	0.05	2.29

Figure 6. Overlay of six replicates of innovator and biosimilars of rituximab on an Agilent 1260 Infinity Bio-inert quaternary LC using an Agilent AdvancedBio SEC, 7.8 × 300 mm, 2.7 µm column. The tables show the precision of retention time and area for HMWS, monomer, and LMWS, n = 6.

Figure 7 shows overlaid chromatograms for comparison between innovator and biosimilars. The peak at 8.4 minutes is attributed to the monomer, and the peaks to the left and right of the main peak are assigned to HMWS and LMWS, respectively. As shown in the figure, the LMWS are similar across samples (0.08, 0.08, and 0.11%) whereas the HMWS shows different profiles; the difference between innovator and biosimilar 2 is more prominent than with biosimilar 1.

Figure 8 shows the HMWS, LMWS, and monomer distribution within the samples. The monomer peak in all three samples was found to be 98 to 99%. Slightly higher levels of HMWS were observed in both biosimilars (0.93 ±0.01%, 0.94 ±0.01%).

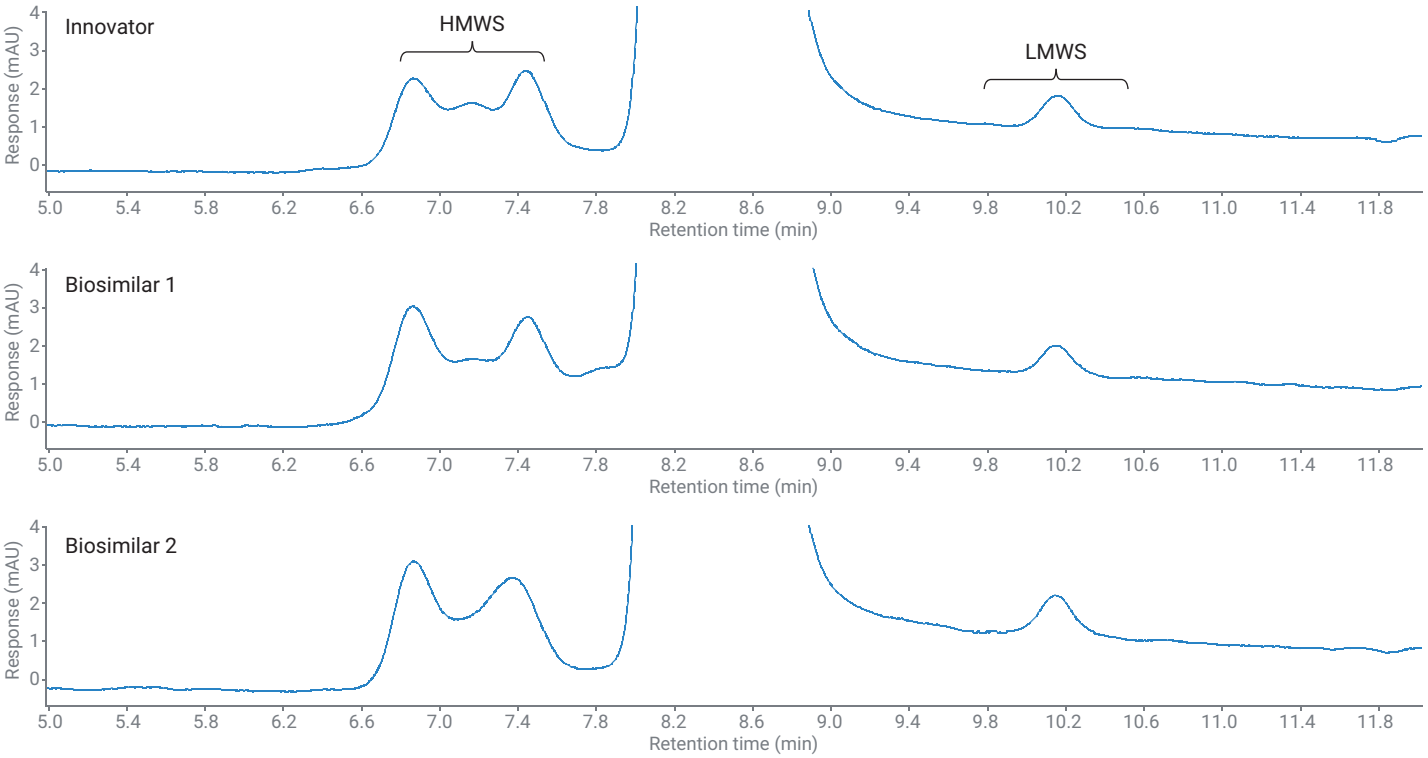


Figure 7. Zoom-in comparison of the aggregate's profiles of innovator and biosimilars.

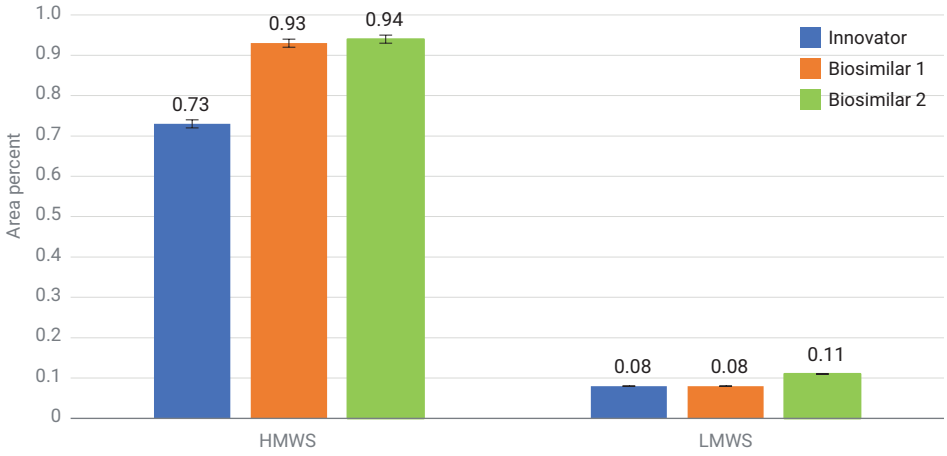
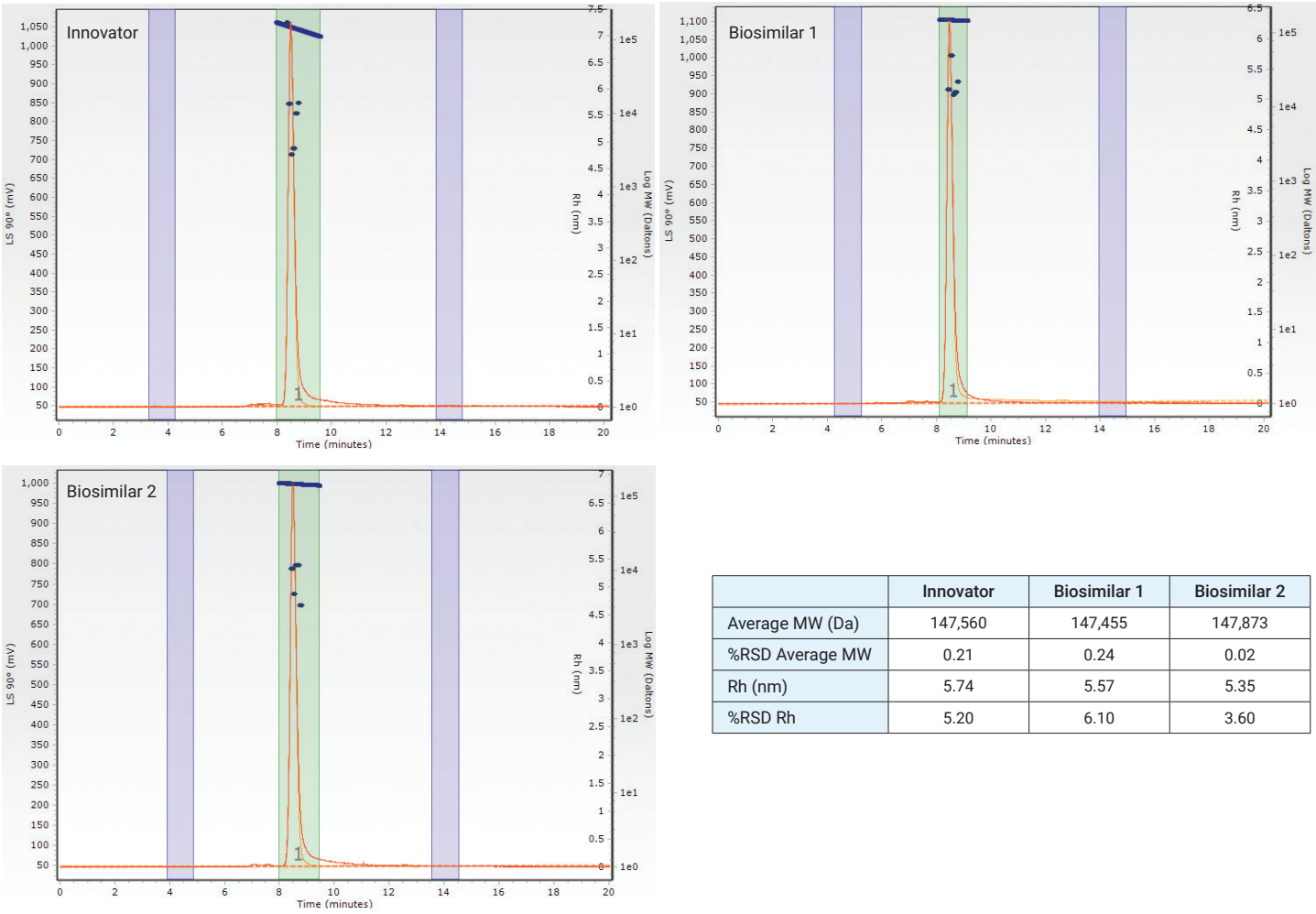


Figure 8. Comparison of the HMWS and LMWS area percentage between innovator and biosimilars.

Biosimilar 2 also presented a higher level of LMWS (0.11%) compared to the innovator (0.08%) and biosimilar 1 (0.08%). However, all the differences are subtle.

Figure 9 demonstrates the results acquired with the bio-MDS system with DLS detection. Absolute average molecular weight can be read directly from the results together with the hydrodynamic radius (Rh) of the mAb monomer. Results showed good reproducibility of DLS analysis and accurate measurement of molecular weight and Rh values.



	Innovator	Biosimilar 1	Biosimilar 2
Average MW (Da)	147,560	147,455	147,873
%RSD Average MW	0.21	0.24	0.02
Rh (nm)	5.74	5.57	5.35
%RSD Rh	5.20	6.10	3.60

Figure 9. Comparison of the average molecular weight and Rh from DLS analysis between innovator and biosimilars.

Conclusion

This Application Note demonstrates two analytical workflows, charge variant analysis and aggregation analysis, to analyze rituximab innovator and its two biosimilars. Good reproducibility in RT and area were achieved for both workflows. In the charge variant analysis, the biosimilar 2 sample presented distinct differences with a high percentage of basic variants that are believed to be lysine truncation variants. In the aggregation analysis, biosimilar 2 presented a slightly different HMWS profile compared to the innovator. In terms of charge and aggregate variants properties of the drug samples, biosimilar 1 demonstrated more similarities to the innovator. The results are also in line with the published data of rituximab characterization.[5] This shows that Agilent charge variant and aggregation analysis workflows are reliable for biosimilar comparability studies. To facilitate easy data review in batch mode, increasing analytical efficiency, Agilent Openlab CDS software offers features such as Peak Explore.

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Streamlined Workflows for N-Glycan Analysis of Biotherapeutics Using Agilent AdvanceBio Gly-X InstantPC and 2-AB Express Sample Preparation with LC/FLD/MS

Authors

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Abstract

This Application Note describes the preparation and analysis of released N-glycans from biotherapeutic glycoproteins using two labels, InstantPC and 2-aminobenzamide (2-AB). N-Glycan analysis is vital to the development and production of biotherapeutics, as glycosylation can influence the therapeutic function of the final drug product. The workflows described here use the Agilent AdvanceBio Gly-X with InstantPC and Gly-X 2-AB Express kits (formerly ProZyme) for the release of N-glycans using PNGase F followed by instant glycosylamine labeling with InstantPC or reductive amination labeling with 2-AB Express, respectively. Labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC), with detection using both fluorescence and mass spectrometry (MS). Gly-X sample preparation offers a high level of reproducibility and throughput, with a one hour preparation time for InstantPC and two hours for 2-AB Express. In addition, the InstantPC label offers improved fluorescence response and MS ionization efficiency.

Introduction

The characterization of N-glycans is an essential part of the biotherapeutic development process, as the structure of N-linked glycans can influence the function of glycosylated biotherapeutics, frequently making glycosylation a critical quality attribute (CQA).[1] N-Glycan analysis often involves the labeling of released glycans with a tag to allow for detection by fluorescence (FLD), and to enhance ionization for mass spectrometry (MS), followed by N-glycan separation, detection, and relative quantitation. Many of the frequently used fluorescent tags such as 2-AB[2] are limited concerning MS sensitivity compared with recently introduced dyes such as InstantPC, and pre-existing N-glycan sample preparation workflows can be time-consuming.[3] However, 2-AB has been used for over 20 years and so is well-established in the literature and in many laboratories.

This Application Note presents streamlined workflows for preparation of InstantPC and 2-AB labeled N-glycans coupled with analysis using Agilent LC/FLD/MS instrumentation. Gly-X N-glycan sample preparation kits for InstantPC or 2-AB Express labeling (formerly ProZyme) include all reagents for N-glycan sample preparation: denaturation, deglycosylation, labeling, and sample cleanup, as illustrated in Figure 1.

Experimental

N-Glycan sample preparation

Agilent AdvanceBio Gly-X N-glycan prep with InstantPC (p/n GX96-IPC) and Gly-X 2-AB Express (p/n GX96-2AB) Kits were used to prepare labeled N-glycans from monoclonal antibody rituximab (Rituxan, lot number M190170) and Fc fusion protein etanercept (Enbrel, lot number 1092537), 40 µg protein per preparation. Four replicates of each sample were analyzed with fluorescence/MS detection and relative percent glycan peak areas calculated.

InstantPC and 2-AB labeled samples were prepared by standard manual protocols. The Gly-X in-solution deglycosylation protocol uses a three-minute denaturation at 90 °C, opening up the glycoprotein target to enable a five-minute deglycosylation reaction at 50 °C with PNGase F. Following in-solution deglycosylation, InstantPC labeled samples are prepared by one-minute glycosylamine labeling of released N-glycans (Figure 2), followed

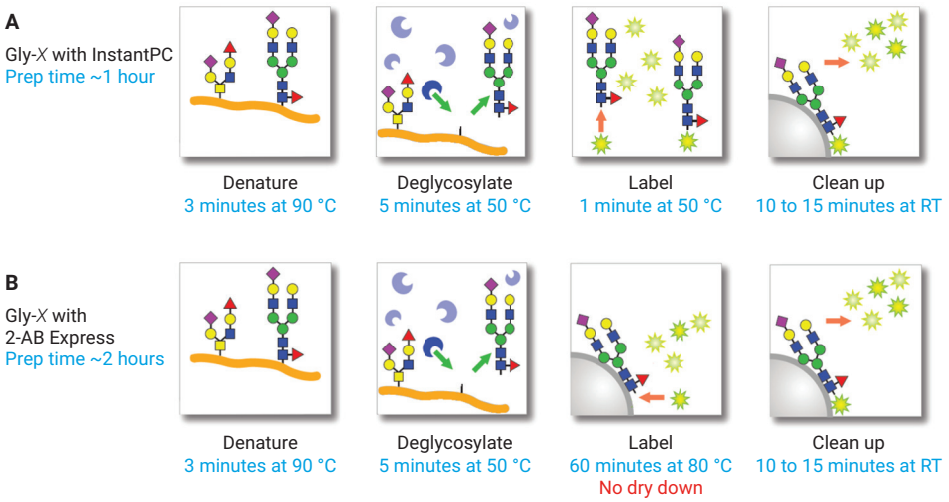
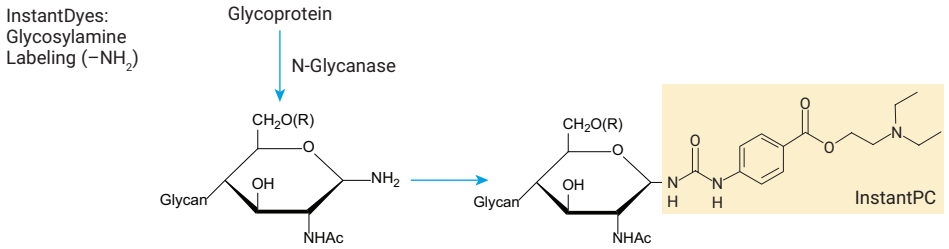


Figure 1. Gly-X N-glycan sample prep. A) InstantPC workflow with in-solution deglycosylation and labeling followed by on-matrix cleanup; B) 2-AB workflow with deglycosylation in-solution, followed by on-matrix labeling and cleanup.



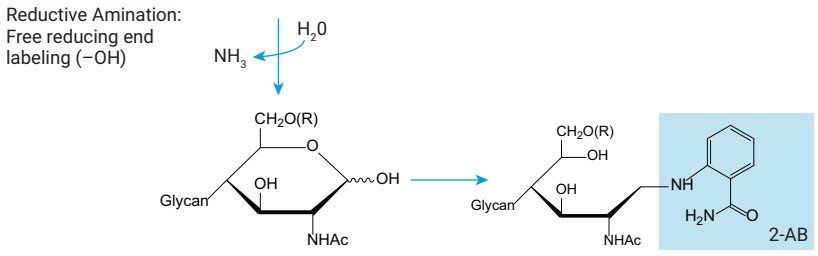


Figure 2. Comparison of InstantPC glycosylamine labeling and traditional reductive amination with 2-AB.

by vacuum-driven cleanup of free dye using HILIC solid-phase extraction (SPE).

N-Glycan samples labeled with 2-AB were prepared using the standard Gly-X 2-AB Express protocol with reductive amination chemistry. Following the Gly-X five-minute deglycosylation with PNGase F, released N-glycans are converted from the glycosylamine form ($-NH_2$) to free reducing end form (–OH) to allow for 2-AB labeling with reductive amination. N-Glycans are then desolvated by vacuum filtration onto a solid-state matrix followed by an on-matrix 2-AB labeling step. This process eliminates the need for glycan drying prior to the 2-AB labeling step, thereby reducing total sample preparation time.

N-Glycan analysis

InstantPC and 2-AB labeled N-glycans were separated by hydrophilic interaction liquid chromatography (HILIC) using an Agilent AdvanceBio Glycan Mapping column, 2.1 × 150 mm, 1.8 µm (p/n 859700-913) with an Agilent 1290 Infinity II LC system with in-line fluorescence detection (Table 1) coupled to an Agilent AdvanceBio 6545XT LC/Q-TOF (Table 2).

All HILIC separations were conducted under the conditions described in Table 1. A fixed flow splitter (IDEX Health & Science p/n UH-427) was used post-FLD, diverting approximately 50% of the flow to waste and 50% to the MS. Agilent MassHunter BioConfirm software was used for data processing, with a personal compound database (PCD).

Materials

LC/MS grade acetonitrile and water were

Table 1. Agilent 1290 Infinity II UHPLC HILIC/FLD conditions.

Parameter	Value		
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 150 mm, 1.8 µm (p/n 859700-913)		
Column Temp	40 °C		
Mobile Phase	A) 50 mM ammonium formate, pH 4.5 B) Acetonitrile		
Gradient Program	InstantPC and 2-AB labeled glycans		
	Time (minutes)	%B	Flow rate (mL/min)
	0	80	0.5
	2	75	0.5
	48	62	0.5
	49	40	0.5
	51.5	80	0.5
	52	80	0.5
	60	80	0.5
Injection Volume	1 µL (equivalent to glycans from 0.4 µg protein)		
Detection	Agilent 1260 Infinity II FLD InstantPC: λ_{Ex} 285 nm, λ_{Em} 345 nm 2-AB: λ_{Ex} 260 nm, λ_{Em} 430 nm		

Table 2. Agilent 6545XT Q-TOF parameters.

Agilent 6545XT Q-TOF	
Source	Dual AJS ESI
Gas Temperature	150 °C
Drying Gas Flow	9 L/min
Nebulizer	35 psi
Sheath Gas Temperature	300 °C
Sheath Gas Flow	10 L/min
Vcap	3,000 V
Nozzle Voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Mass Range	*m/z* 600 to 3,000
Scan Rate	1 spectra/sec
Acquisition Mode	High resolution (4 GHz)

purchased from Honeywell Research Chemicals.

Instrumentation

Labeled N-glycan samples were separated using an Agilent AdvanceBio Glycan Mapping column (Table 1 shows the method details) on an Agilent LC/MS setup composed of:

- Agilent 1290 Infinity II high speed pump (G7120A)
- Agilent 1290 Infinity II multisampler (G7167B)
- Agilent 1290 Infinity II multicolumn thermostat (G7116B)
- Agilent 1260 Infinity fluorescence detector (G1321B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (parameters in Table 2)

Software

- Agilent MassHunter Acquisition
- Agilent MassHunter Qualitative Analysis software

Results and discussion

HILIC Separation of InstantPC and 2-AB N-Glycans

HILIC separation of labeled N-glycans from Rituxan and Enbrel labeled with InstantPC or 2-AB results in well resolved peaks for major glycan species with the 60-minute method used (Figures 3 and 4). Rituxan (Figure 3A, InstantPC; Figure 4A, 2-AB), an IgG, has an N-glycan profile typical of monoclonal antibodies with one N-glycosylation site in the Fc region produced in Chinese hamster ovary (CHO) cells: predominantly neutral biantennary complex N-glycans with core fucose, some Man5, and a relatively low proportion of sialylated glycans. The N-glycan profile of Enbrel (Figure 3B, InstantPC; Figure 4B, 2-AB), an Fc fusion protein, contains a higher level of sialylated glycans owing to two additional N-glycosylation sites in the fusion partner, TNF-α receptor (TNFR) extracellular domain, in addition to the single N-glycan site in the Fc portion.[5]

The HILIC retention time of 2-AB N-glycans is shorter than for InstantPC N-glycans, although the elution order of N-glycan species is comparable. Critical pairs such as G0F/Man5 and Man5/G1, which are often monitored during the development process of biotherapeutics, are well separated with both InstantPC and 2-AB labels, leading to confident determination of relative percentage composition. G1F isomers G1F[6] and G1F[3] are also separated. Relative percent areas, standard deviation, and relative standard deviation are reported in Tables 3 through 6, and show a low degree of variability between the four sample preparation replicates. This variability rises for lower abundance glycans.

An added benefit of InstantPC is the separation of isoforms G2S1[6]/[3] and G2FS1[6] from Enbrel (Figure 3B) compared to 2-AB (Figure 4B) using the previously described chromatography conditions. Analysis with fluorescence detection of InstantPC and 2-AB labeled N-glycans from biotherapeutics Rituxan and Enbrel results in comparable relative

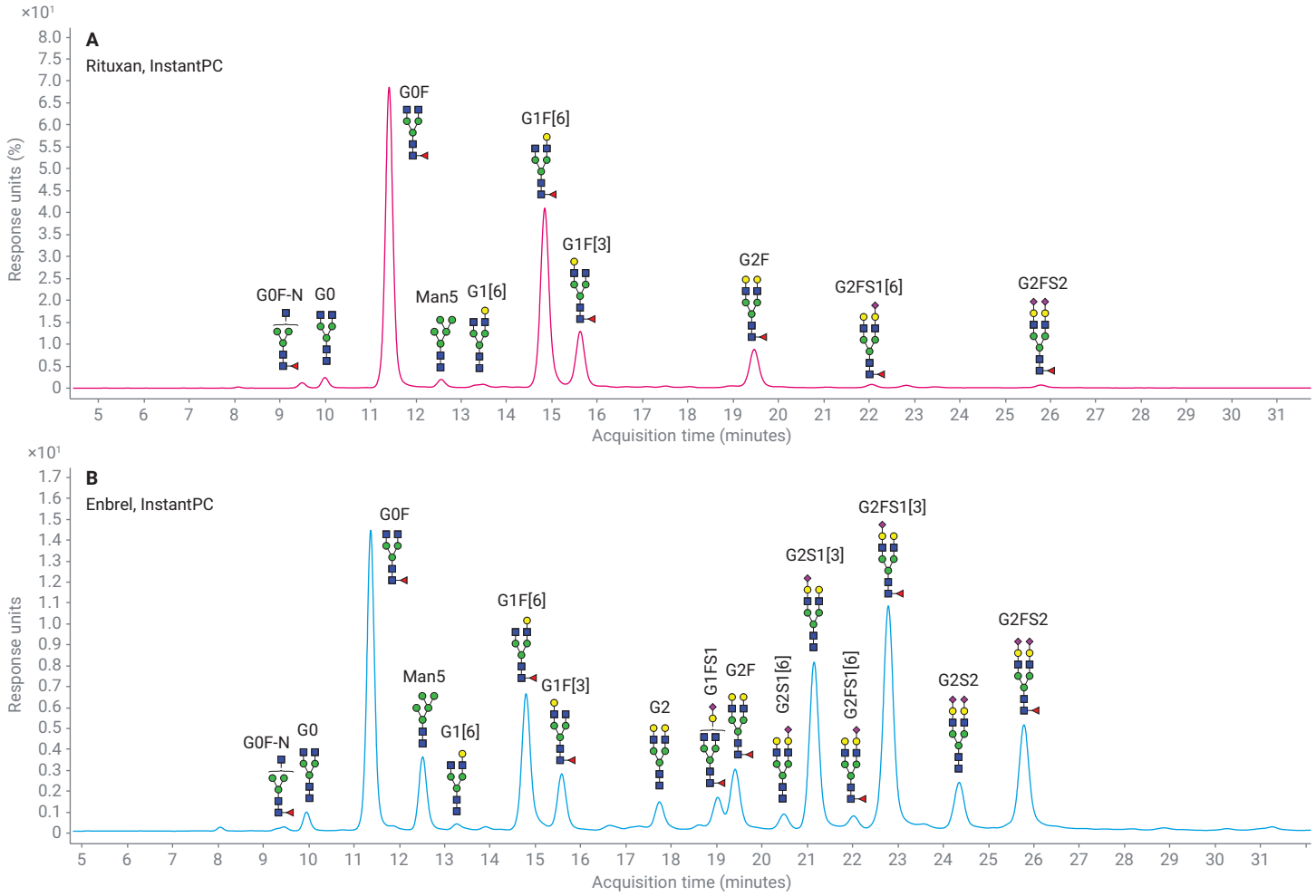


Figure 3. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with InstantPC. N-Glycan relative percent areas are shown in Table 3 and Table 4, n = 4.

Table 3. Figure 3A relative % area, SD, and %CV values for Rituxan N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.75	0.01	1.55
G0	1.47	0.02	1.18
G0F	46.82	0.07	0.15
Man5	1.21	0.01	0.83
G1[6]	0.75	0.02	2.67
G1F[6]	31.21	0.11	0.35
G1F[3]	9.27	0.05	0.54
G2F	7.04	0.04	0.51
G2FS1[6]	0.67	0.02	2.29
G2FS1[3]	0.37	0.06	15.98
G2FS2	0.45	0.03	6.67

Table 4. Figure 3B relative % area, SD, and %CV values for Enbrel N-glycans labeled with InstantPC, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0	1.10	0.02	2.09
G0F	19.36	0.16	0.84
Man5	5.08	0.03	0.52
G1[6]	0.48	0.00	0.00
G1F[6]	10.48	0.04	0.39
G1F[3]	3.97	0.01	0.25
G2	2.08	0.01	0.55
G1FS1	1.84	0.05	2.49
G2F	4.26	0.09	1.99
G2S1[6]	1.18	0.01	0.49
G2S1[3]	13.91	0.04	0.31
G2FS1[6]	0.89	0.00	0.00
G2FS1[3]	20.54	0.08	0.37
G2S2	4.26	0.01	0.14
G2FS2	10.54	0.08	0.78

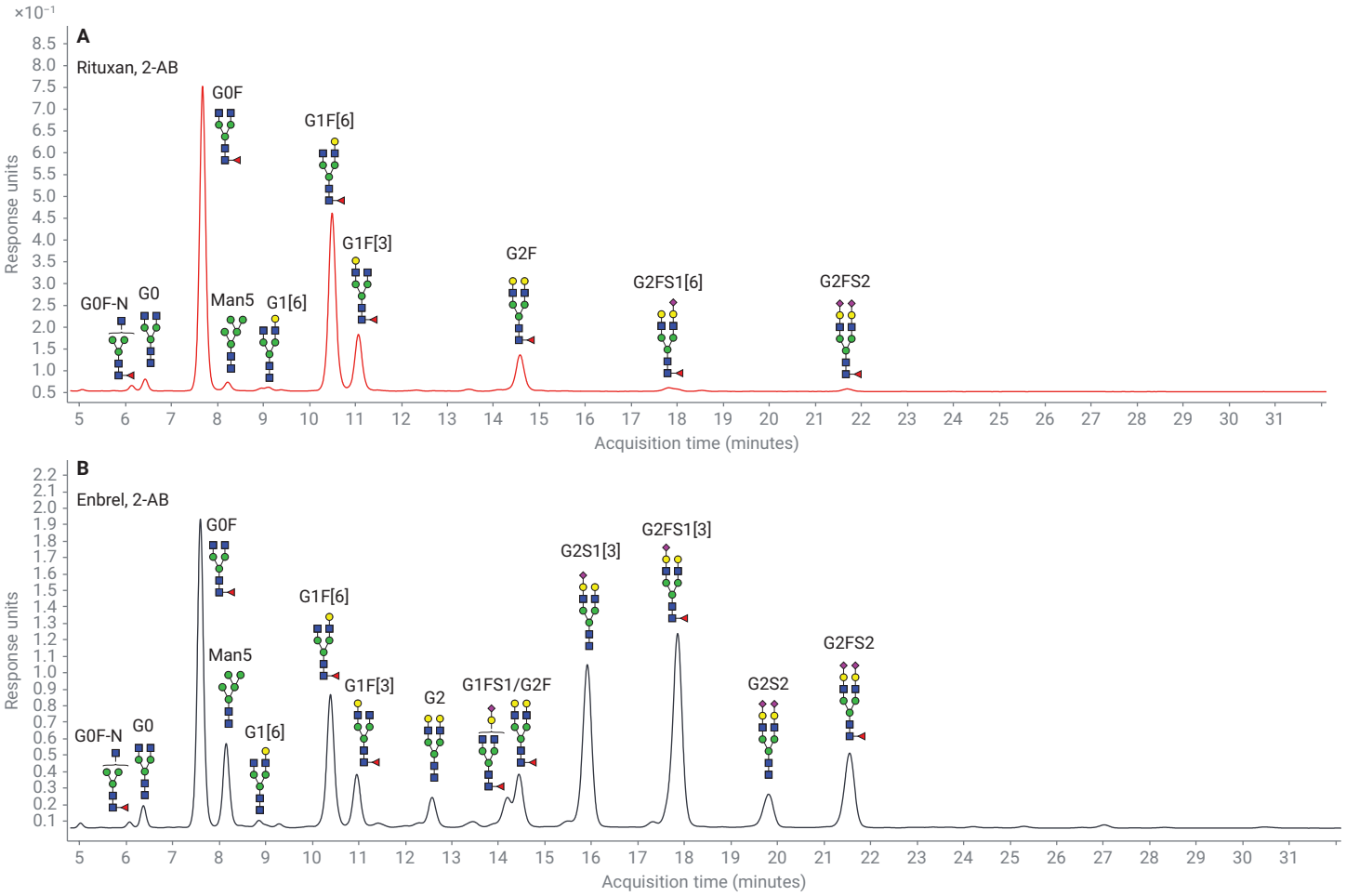


Figure 4. HILIC-UHPLC fluorescence profile of A) Rituxan and B) Enbrel N-glycans labeled with 2-AB. N-Glycan relative percent areas are shown in Table 5 and Table 6, n = 4.

Table 5. Figure 4A relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.78	0.09	11.94
G0	1.64	0.05	3.12
G0F	44.89	0.39	0.87
Man5	1.54	0.14	8.83
G1F[6]	31.39	0.09	0.27
G1F[3]	10.40	0.14	1.34
G2F	7.52	0.16	2.10
G2FS1	1.17	0.03	2.13
G2FS2	0.67	0.02	3.58

Table 6. Figure 4B relative % area, SD, and %CV values for Rituxan N-glycans labeled with 2-AB, n = 4.

	Average Rel % Area	Standard Deviation	%CV
G0F-N	0.32	0.02	7.44
G0	1.27	0.07	5.34
G0F	20.18	0.45	2.22
Man5	5.50	0.34	6.17
G1[6]	0.45	0.02	3.89
G1F[6]	10.35	0.33	3.18
G1F[3]	3.92	0.17	4.39
G2	2.21	0.15	6.78
G2F/G1FS1	7.00	0.25	3.63
G2S1	15.19	0.17	1.09
G2FS1	20.10	0.32	1.59
G2S2	4.19	0.25	5.95
G2SF2	9.35	0.74	7.93

percent areas for major glycoforms G0F, G1F[6]/[3], G2F, G2S2, and G2FS2.

FLD and MS detection of InstantPC and 2-AB N-Glycans

InstantPC displays higher fluorescence and MS signal compared to 2-AB (Figure 5), when using the same amount of glycoprotein starting material (40 µg), and injecting the same relative volume for HILIC separations (1 µL of 100 µL kit eluent). Individual spectra for InstantPC

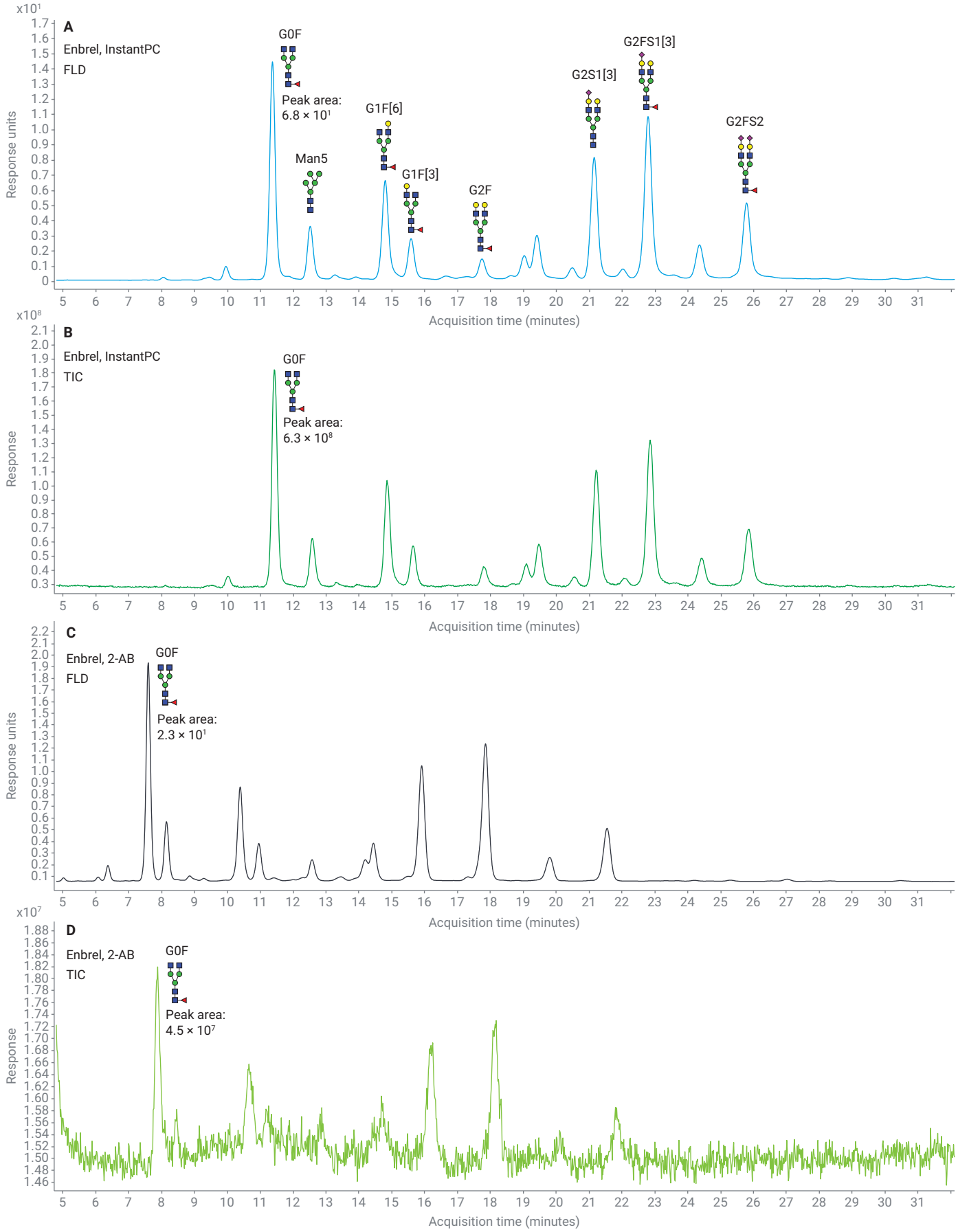


Figure 5. FLD and MS of InstantPC and 2-AB labeled N-glycans from Enbrel. A) InstantPC FLD; B) InstantPC TIC (total ion chromatogram); C) 2-AB FLD; D) 2-AB TIC.

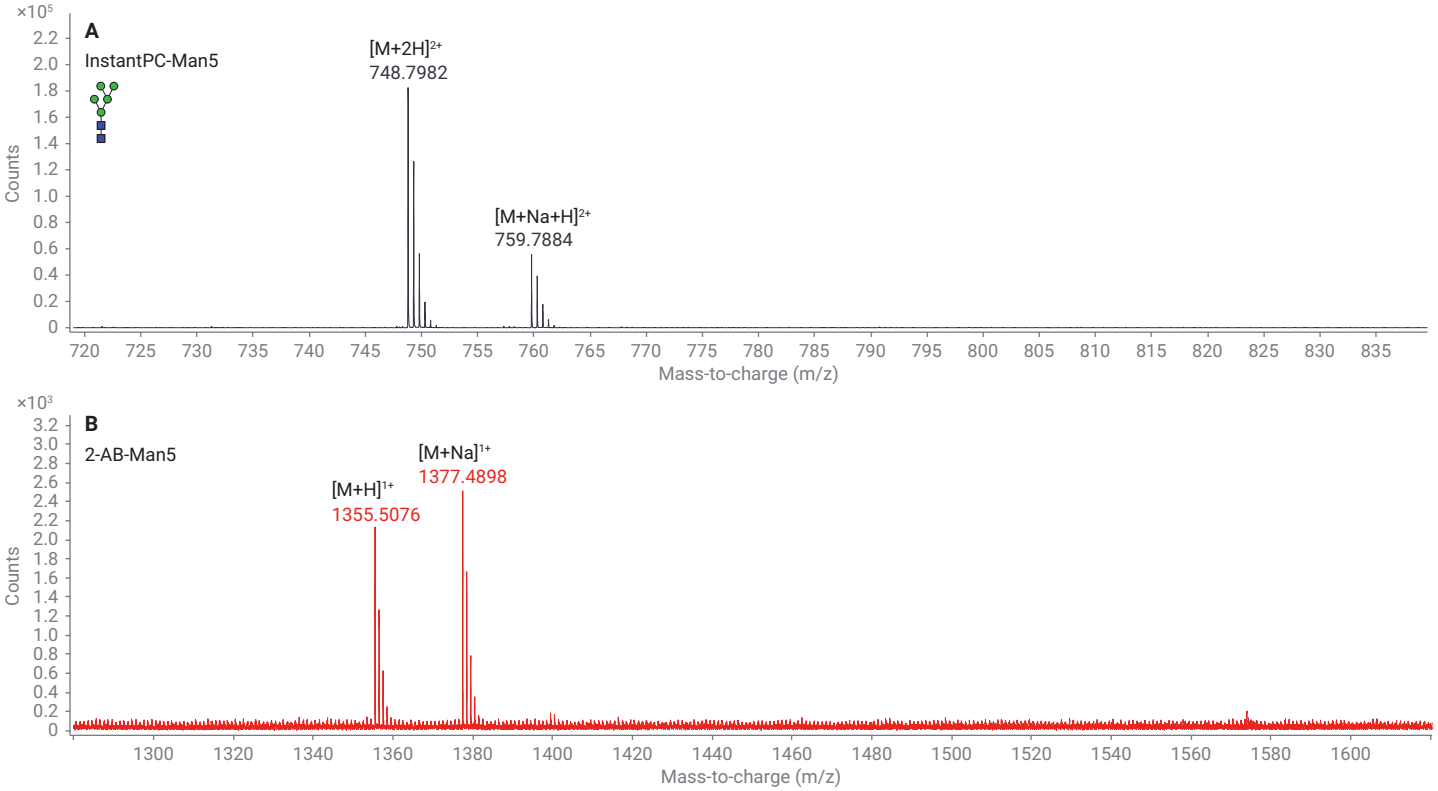


Figure 6. Mass spectrum comparison of Man5 from Enbrel, labeled with A) InstantPC and B) 2-AB.

and 2-AB labeled Man5 illustrates the higher MS signal of InstantPC (Figure 6).

Conclusion

Glycosylation is a feature of many biotherapeutic proteins and is often a CQA that must be monitored. N-Glycan analysis is important in the development and production of therapeutic proteins. Gly-X N-glycan sample preparation workflows enable a five minute release of N-linked glycans suitable for labeling both by glycosylamine labeling with InstantPC and reductive amination chemistry with 2-AB. These workflows allow for instant glycosylamine labeling with InstantPC or no dry down on-matrix reductive amination labeling with 2-AB. Glycan species were profiled by relative fluorescence peak area % and peak assignments confirmed by high resolution mass spectrometry. Compared to 2-AB, InstantPC labeled glycans display higher FLD signal and greater MS ionization efficiency in positive mode, allowing for confident detection of low abundance glycan species. Although the performance benefits of InstantPC are clear, 2-AB is an N-glycan label that has been used for many years. Therefore, a rapid 2-AB workflow enables continuity with historical 2-AB N-glycan data sets.

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A Comprehensive Approach for Monoclonal Antibody N-linked Glycan Analysis from Sample Preparation to Data Analysis

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Introduction

Monoclonal antibodies (mAbs) and their derivatives comprise a very important class of biopharmaceutical molecules with a wide range of applications. Due to the heterogeneous nature of these mAbs, comprehensive analytical characterization is required. These analyses include determining the complete amino acid sequences of the mAbs and their variants, as well as characterization of post-translational modifications (PTMs) including glycosylation, oxidation, and deamidation.

Glycosylation plays an important role in many biological processes. It also affects the therapeutics' efficacy, stability, pharmacokinetics, and immunogenicity[1]. Glycan characterization usually involves techniques such as NMR, HPLC, or mass spectrometry (MS). Since glycans are very diverse in composition/structures and are poorly ionized by electrospray, the MS-based approach for glycan characterization has been challenging. InstantPC is a novel fluorescence tag from ProZyme Inc. (Figure 1) that has been developed to improve MS ionization efficiency, and sensitivity for N-glycan molecules.

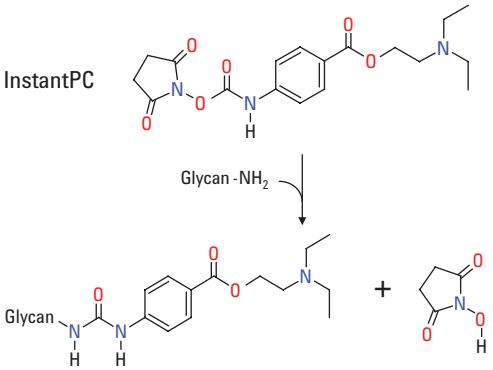


Figure 1. Diagram of InstantPC-labeled N-glycans released from an mAb.

The traditional method of glycan analysis is laborious, and involves many steps, starting with enzymatic glycan release by PNGaseF (overnight), followed by sample cleanup, labeling with a fluorescence tag by reductive amination (2-AB or InstantPC), and finally cleanup of the released labeled N-glycans prior to LC-FLD or LC/MS analysis[2,3]. Despite the significant improvement of MS sensitivity using fluorescent tags, the labor intensiveness of manual sample preparation, low reproducibility, and limitation to scale-up on sample processing have been major issues for the biopharmaceutical industry.

This study demonstrates how to increase sample throughput for glycan characterization workflows using the Agilent AssayMAP Bravo liquid handling platform. The solution incorporates the Agilent 1290 Infinity II LC system, Agilent AdvanceBio Glycan Mapping column, Agilent highly sensitive fluorescence detection (FLD), and the Agilent 6545XT AdvanceBio LC/Q-TOF. The Q-TOF data are analyzed automatically with Agilent MassHunter BioConfirm B.09.00 software (Figure 2). This solution dramatically improves productivity by allowing convenient sample preparation, streamlined data acquisition, and data analysis. This solution provides the flexibility to perform quantitation based on FLD or MS signals with accurate mass peak assignment from an N-glycan mass database.

Experimental

Sample preparation

Four monoclonal antibody (mAb) samples were used in this study:

- The monoclonal antibody standard, RM 8671, was from National Institute of Standards & Technology (NIST) A.K.A. NISTmAb.
- Formulated Herceptin (Trastuzumab) was from Genentech (So. San Francisco, California, USA).
- Sigma SiLu mAb was purchased from Sigma-Aldrich (SiLu Lite, P/N: MSQC4).
- CHO mAb1 was expressed and purified from the Agilent R&D lab.

All mAb samples were diluted with DI water to 1.0 µg/µL prior to sample preparation using the AssayMAP Bravo liquid handling system (G5542A) with the GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) from ProZyme Inc. A detailed procedure for the sample preparation is described in ProZyme's application note (product code: GPPNG-PC). After the final cleanup step, the eluted, released, labeled N-glycans had a final volume of 50 µL, so that each 1 µL of the prepared sample contained N-glycans from 1 µg of mAb.

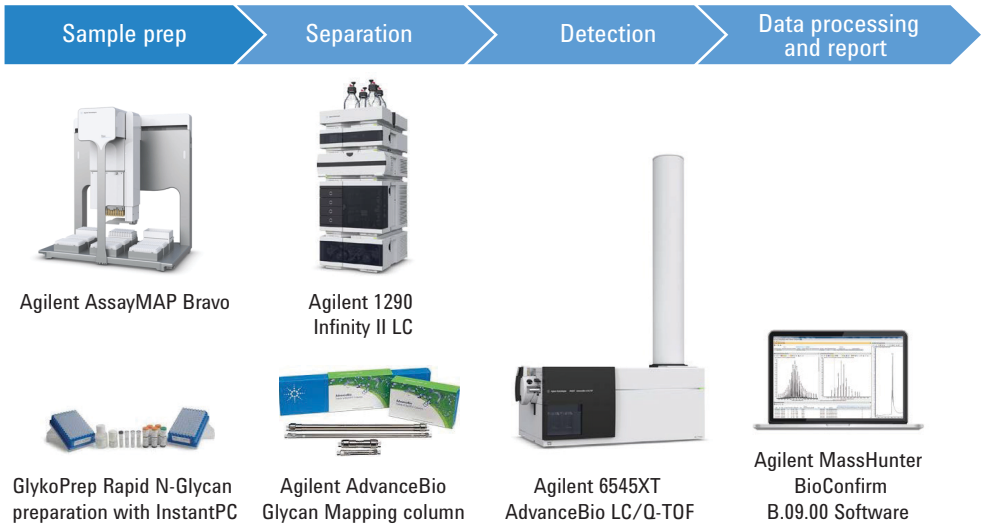


Figure 2. mAb Glycan characterization workflow.

LC/MS analysis

LC/MS analyses were conducted on a 1290 Infinity II LC system equipped with an Agilent 1260 Infinity Fluorescence Detector (G1321B), coupled with a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. The detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10. Glycans were chromatographically separated with an AdvanceBio Glycan Mapping column (2.1 × 100 mm, 1.8 µm). Tables 1 and 2 list the LC/MS parameters used. Approximately 1–2 µL of each N-glycan sample were injected for LC/MS analysis.

Data processing

The InstantPC-labeled released N-glycans were analyzed using the Released Glycans Workflow of MassHunter BioConfirm B.09.00 software. This analytical workflow uses the Agilent Personal Compound Database (PCD) glycan database. The PCD glycan database provides accurate glycan identification and confirmation. Finally, a summarized report of the analyses was generated in PDF format using the Report Builder program in BioConfirm B.09.00.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System	
Column	Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm
Thermostat	4 °C
Solvent A	50 mM formic acid adjusted to pH 4.5 with ammonium hydroxide
Solvent B	Acetonitrile
Gradient	0–0.5 minutes, 75–71 %B 0.5–16 minutes, 71–67.5 %B 1–22 minutes, 67.5–60 %B 22–22.5 minutes, 60–40 %B 22.5–23.5 minutes, 40 %B (0.7 mL/min) 23.5–24 minutes, 40–75 %B (0.7 mL/min) 24–30 minutes, 75 %B (0.9 mL/min)
Column temperature	40 °C
Flow rate	0.4 mL/min
Injection volume	2.0 µL

Agilent 1260 Infinity Fluorescence Detector (G1321B) was used. The detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10.

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF system	
Gas temperature	150 °C
Drying gas	9 L/min
Nebulizer (psig)	35
Sheath gas temperature	300 °C
Sheath gas flow	10 L/min
VCap	3,000 V
Nozzle voltage	500 V
Fragmentor	120 V
Skimmer	65 V
Quad AMU	95
Acquisition mode	Low mass range, HiRes (4 GHz)
Mass range	*m/z* 300–1,700
Acquisition rate	2 spectra/sec

Results and Discussion

LC-FLD analysis of released labeled glycans is one of the most widely used approaches to determining therapeutic protein glycosylation. We have previously published application notes showing optimized separation of several mAb glycan profiles using various column dimensions and run conditions[4,5]. The separation method in this report represents the best overall performance with maximum peak resolution and excellent robustness for the different mAb N-glycan samples in this study.

Figure 3 shows the representative chromatograms of N-glycans (FLD and MS EIC) from the NISTmAb. The FLD chromatogram (Figure 3 top, zoom in) reveals that more than 15 glycan peaks were detected. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F was comparable between the fluorescent and MS data (Figure 7).

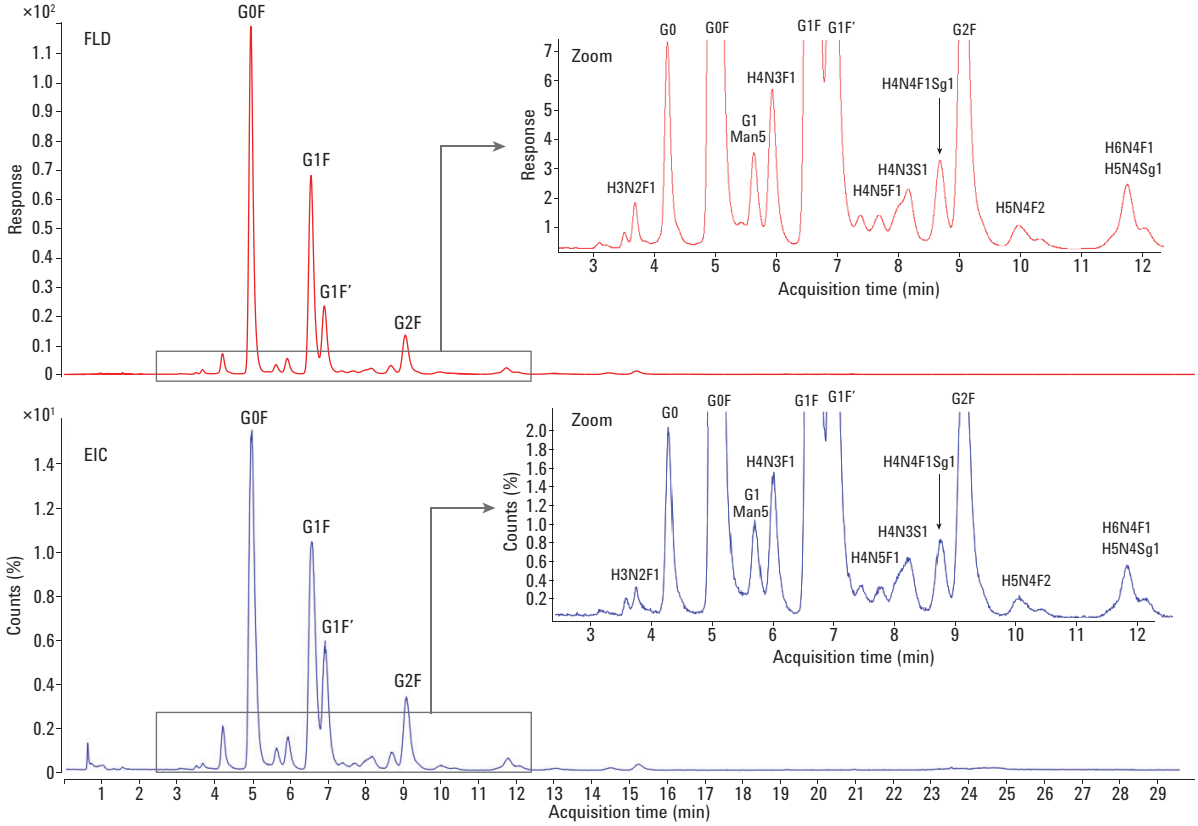


Figure 3. FLD chromatogram and mass spectra (EIC) of InstantPC-labeled N-glycans from NISTmAb.

While fluorescence detection does not allow for direct structure elucidation, MS analysis of mAb glycans can be used to determine glycan monosaccharide composition. In the case of many mAb N-glycans, this composition is sufficient to achieve a high-confidence structural assignment. The combination of the positively charged InstantPC tag and sensitive Agilent Jet Stream (AJS) electrospray ionization (ESI) source technology dramatically increases MS detection sensitivity for N-glycans. In addition, we have optimized the MS parameters to maximize the sensitivity of the InstantPC-labeled N-glycans while minimizing in-source fragmentation of these fragile molecules. The optimized conditions have significantly improved the MS spectrum quality, leading to accurate N-glycans identification and relative quantification results. Figure 4 shows the MS spectrum of an InstantPC-labeled N-glycan (G2F) where only the doubly charged ions of its protonated form, $[M+2H]^{2+}$, as well as its adducts $[M+H+Na]^{2+}$ and $[M+H+K]^{2+}$ were observed (Note: InstantPC tag causes a mass increment of 261.1477 Da compared to the free reducing end form of the glycan).

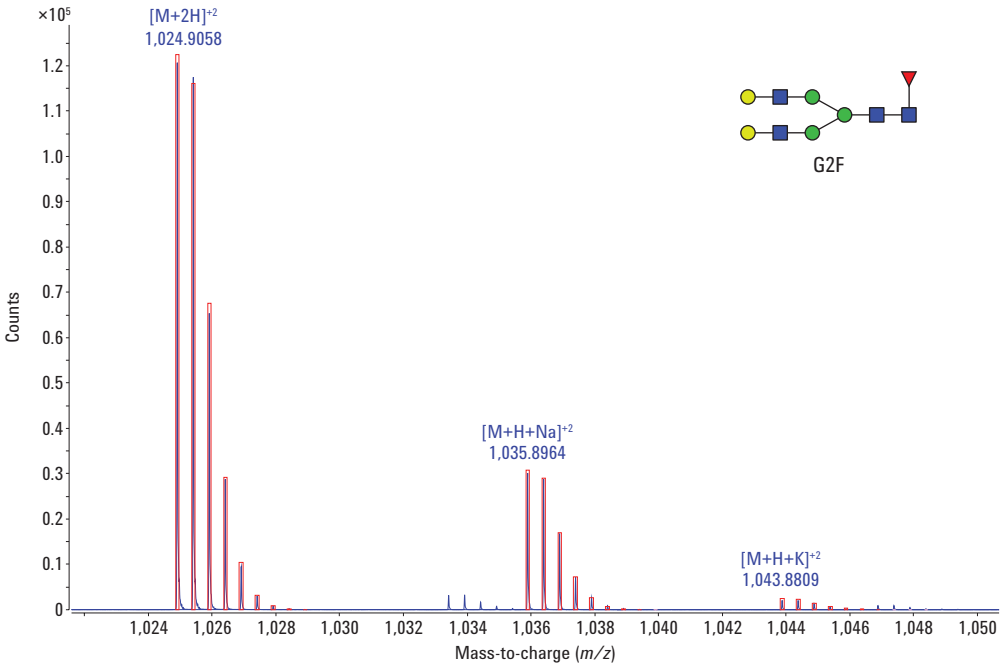


Figure 4. Representative spectrum of an InstantPC-labeled N-glycan (G2F). Excellent isotopic fidelity of the charge states of the InstantPC-labeled G2F glycan and its adducts. The red boxes represent the theoretical isotopic pattern, and the blue lines represent the actual raw MS spectrum.

We have introduced a workflow in MassHunter BioConfirm software for released glycan profiling. This workflow enables the easy setup of sample batch analysis. The software can accommodate many commercial or customized fluorescent tags. A Personal Compound Database (PCD) containing accurate mass and structural information of glycans is used for identification using the Agilent proprietary Find by Formula algorithm. Subsequently, a summary analytical report can be created in a customer-defined report format. Figure 5 shows the extracted ion chromatograms (EICs) of the identified glycans.

The Biomolecules results table (Figure 6) in BioConfirm allows quick review of detailed glycans information including names, mass, retention time, peak area, composition, and database matching score. Multiple IDs are displayed for glycans with possible isoform structures. It also allows users to review the TIC of the sample as well as the individual glycan MS spectra. In addition, multiple data files can be processed and analyzed in batch mode. The user may use peak areas of the selected glycans in the results table for relative quantitative analysis.

InstantPC-labeled glycans were previously shown to give similar relative quantitation results for MS and FLD analysis[4]. The FLD chromatogram for the CHO mAb1 sample was integrated using the Agilent MassHunter Qualitative Analysis software. The relative sum abundance of the top seven most abundant N-glycans was calculated and compared against the same data from the MS analysis (Figure 7). To get equivalent results, do not saturate the MS detector. An ideal quantity for this workflow would be to inject N-glycans released from approximately 0.5 µg of mAb.

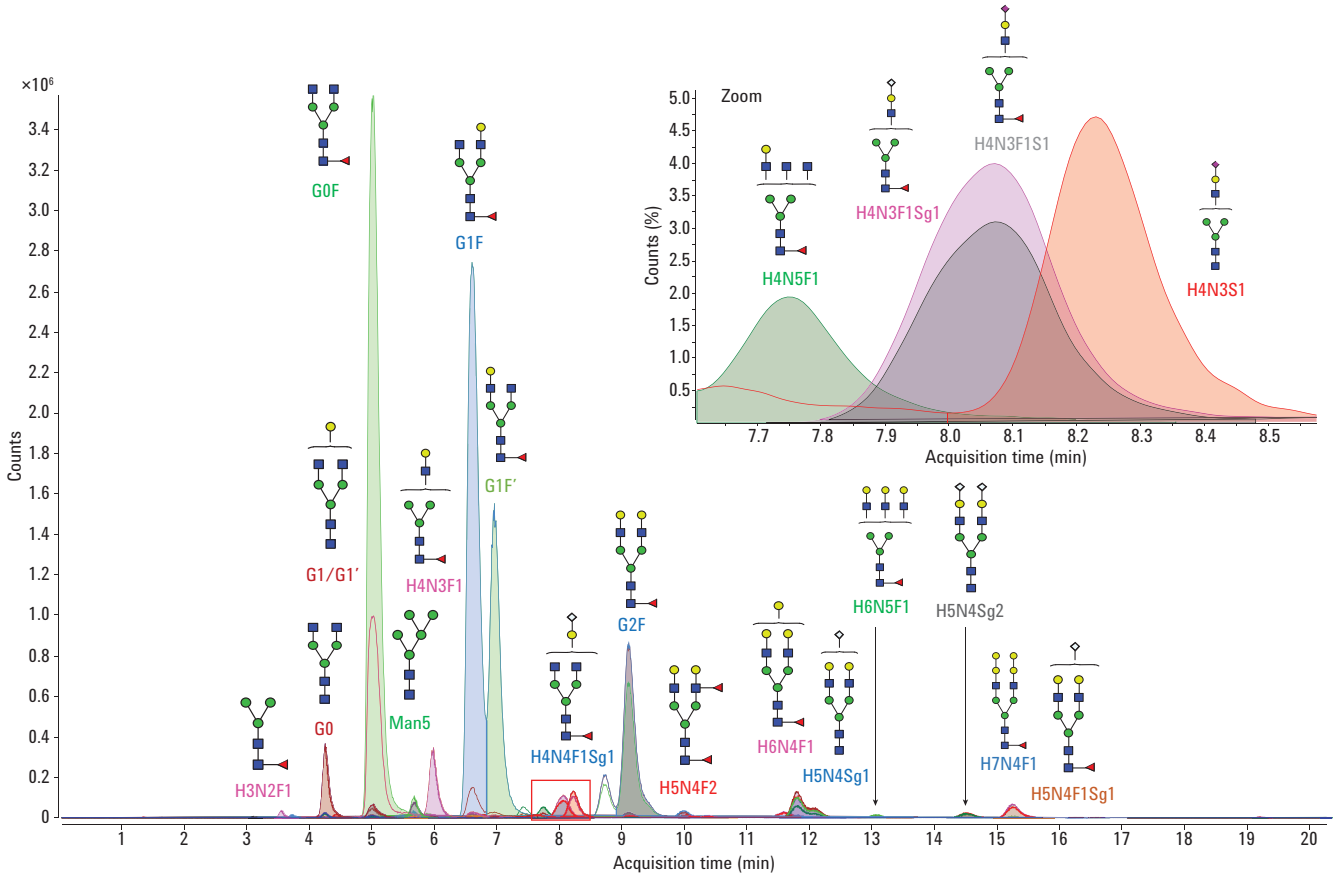
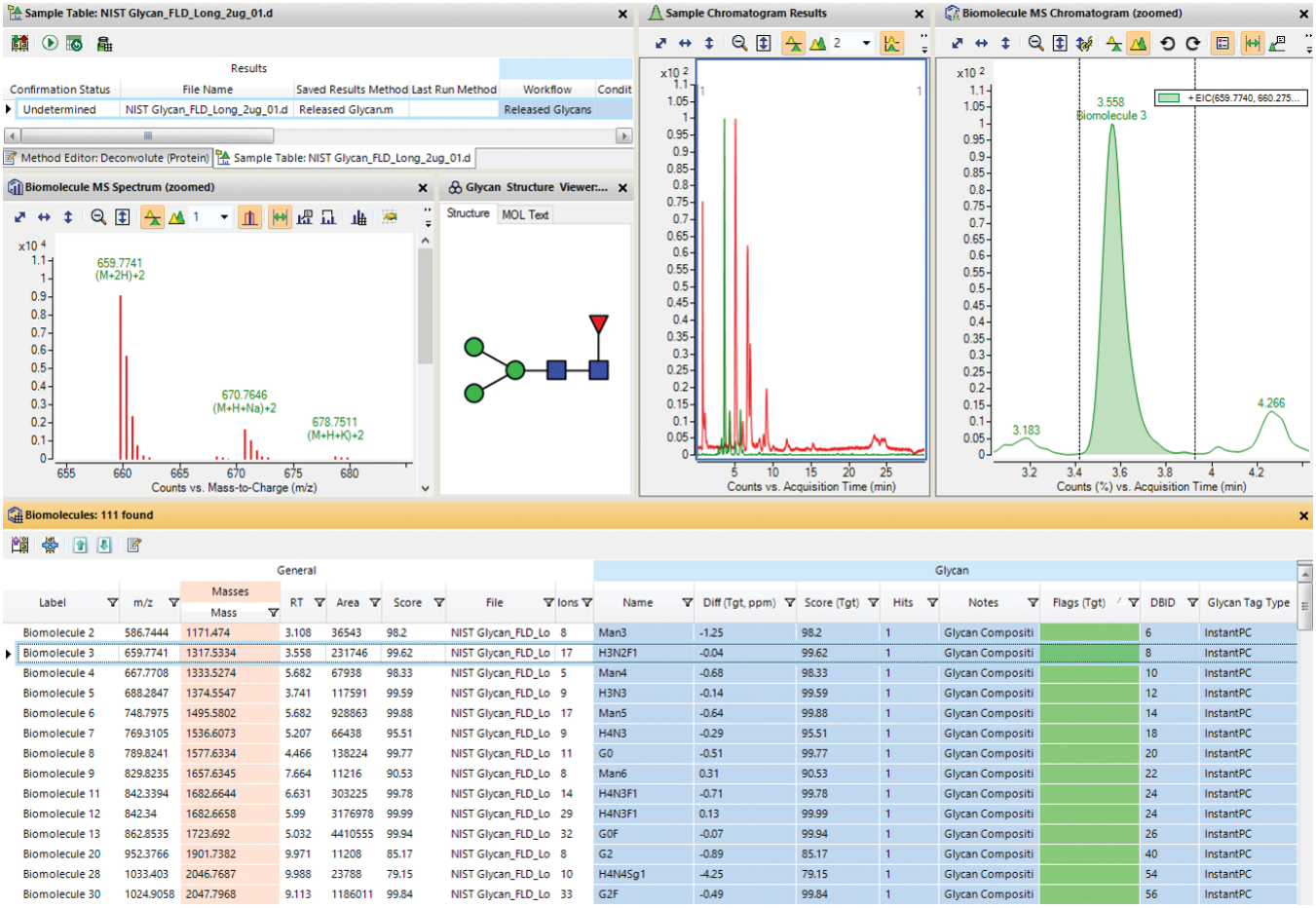


Figure 5. Extracted ion chromatograms of the identified glycans from NISTmAb. Inset: zoom of EICs of identified glycans eluted in the retention time range of 7.6–8.6 minutes.



Label	m/z	Masses Mass	RT	Area	Score	File	Ions	Name	Diff (Tgt, ppm)	Score (Tgt)	Hits	Notes	Flags (Tgt)	DBID	Glycan Tag Type
Biomolecule 2	586.7444	1171.474	3.108	36543	98.2	NIST Glycan_FLD_Lo	8	Man3	-1.25	98.2	1	Glycan Compositi		6	InstantPC
Biomolecule 3	659.7741	1317.5334	3.558	231746	99.62	NIST Glycan_FLD_Lo	17	H3N2F1	-0.04	99.62	1	Glycan Compositi		8	InstantPC
Biomolecule 4	667.7708	1333.5274	5.682	67938	98.33	NIST Glycan_FLD_Lo	5	Man4	-0.68	98.33	1	Glycan Compositi		10	InstantPC
Biomolecule 5	688.2847	1374.5547	3.741	117591	99.59	NIST Glycan_FLD_Lo	9	H3N3	-0.14	99.59	1	Glycan Compositi		12	InstantPC
Biomolecule 6	748.7975	1495.5802	5.682	928863	99.88	NIST Glycan_FLD_Lo	17	Man5	-0.64	99.88	1	Glycan Compositi		14	InstantPC
Biomolecule 7	769.3105	1536.6073	5.207	66438	95.51	NIST Glycan_FLD_Lo	9	H4N3	-0.29	95.51	1	Glycan Compositi		18	InstantPC
Biomolecule 8	789.8241	1577.6334	4.466	138224	99.77	NIST Glycan_FLD_Lo	11	G0	-0.51	99.77	1	Glycan Compositi		20	InstantPC
Biomolecule 9	829.8235	1657.6345	7.664	11216	90.53	NIST Glycan_FLD_Lo	8	Man6	0.31	90.53	1	Glycan Compositi		22	InstantPC
Biomolecule 11	842.3394	1682.6644	6.631	303225	99.78	NIST Glycan_FLD_Lo	14	H4N3F1	-0.71	99.78	1	Glycan Compositi		24	InstantPC
Biomolecule 12	842.34	1682.6658	5.99	3176978	99.99	NIST Glycan_FLD_Lo	29	H4N3F1	0.13	99.99	1	Glycan Compositi		24	InstantPC
Biomolecule 13	862.8535	1723.692	5.032	4410555	99.94	NIST Glycan_FLD_Lo	32	G0F	-0.07	99.94	1	Glycan Compositi		26	InstantPC
Biomolecule 20	952.3766	1901.7382	9.971	11208	85.17	NIST Glycan_FLD_Lo	8	G2	-0.89	85.17	1	Glycan Compositi		40	InstantPC
Biomolecule 28	1033.403	2046.7687	9.988	23788	79.15	NIST Glycan_FLD_Lo	10	H4N4Sg1	-4.25	79.15	1	Glycan Compositi		54	InstantPC
Biomolecule 30	1024.9058	2047.7968	9.113	1186011	99.84	NIST Glycan_FLD_Lo	33	G2F	-0.49	99.84	1	Glycan Compositi		56	InstantPC

Figure 6. Screenshot of Agilent MassHunter BioConfirm B.09.00 software with representative glycan profiling results.

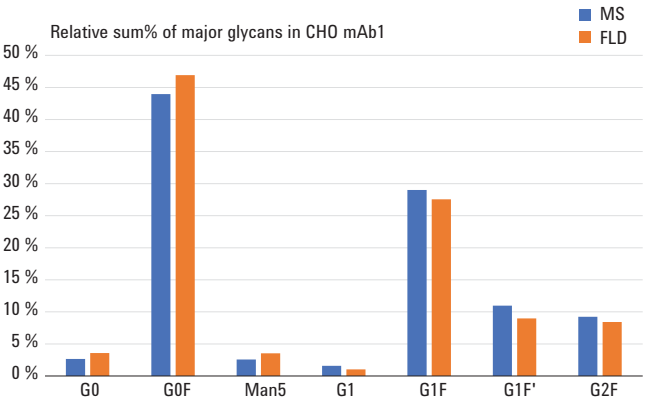


Figure 7. Relative sum % of the major N-glycans in the CHO mAb1 (0.5 µg), comparing results from MS-based quantitation (blue) with FLD-based quantitation (orange).

To summarize and compare the MS results, the top five most abundant N-glycans for each mAb sample were used to calculate relative sum %. Figure 8 presents the data.

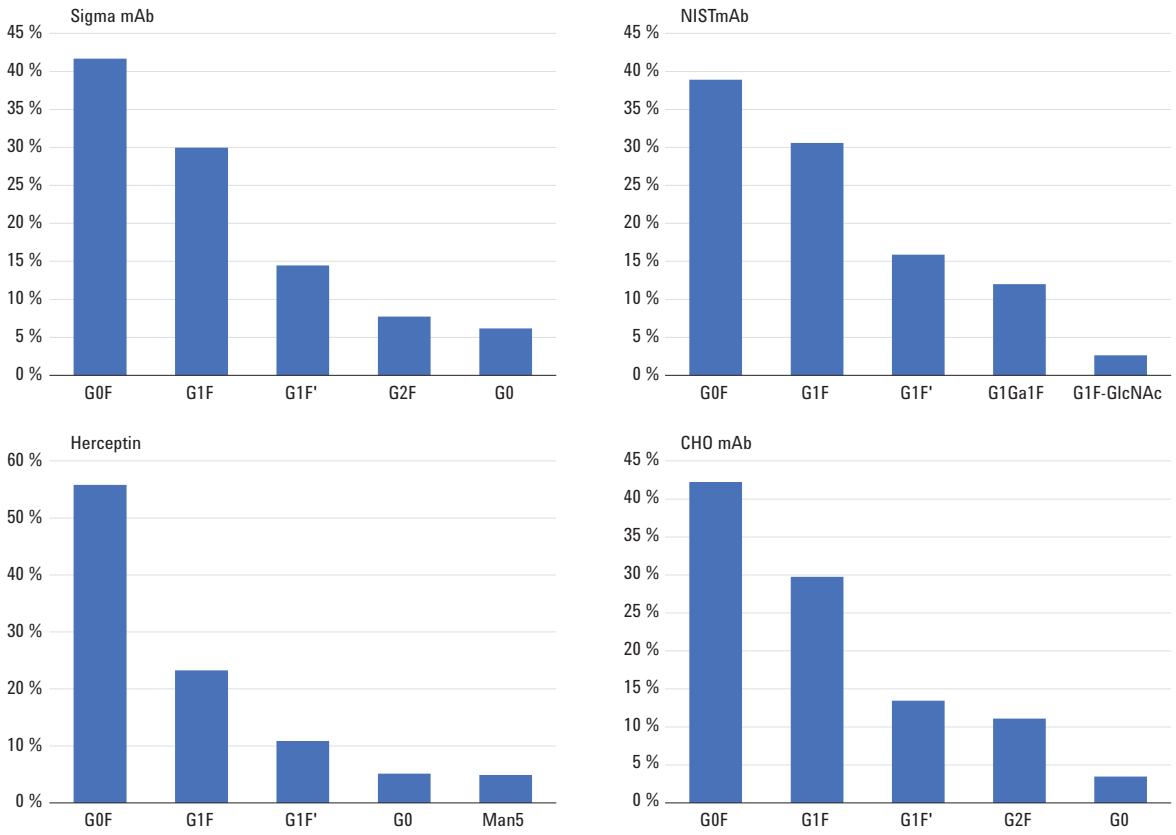


Figure 8. Relative sum % of the top five N-glycans in each of the four mAb samples. Note: The NISTmAb contained a structure suspected to be G1F with an additional alpha-1,3-galactose, and this was labeled as G1Ga1F.

The BioConfirm B.09.00 software allows users to generate their own glycan profile reports using the Report Builder program. Figure 9 shows an example of a released glycan report. In the Report Builder, users can customize the report sections with information such as Sample Information, Sample Chromatogram, Biomolecule Summary, and Biomolecule Details. The corresponding glycan structures are displayed along with the identified glycans.

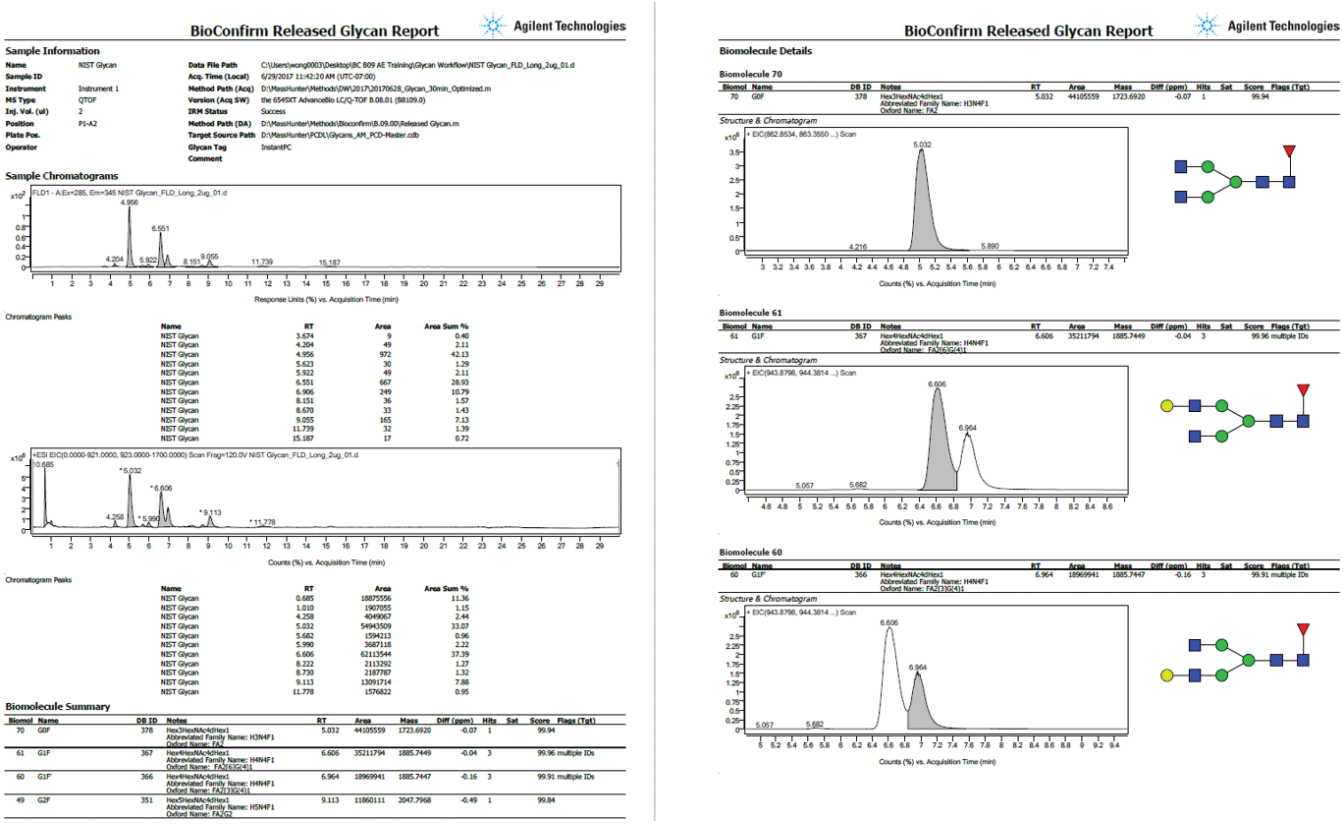


Figure 9. Agilent MassHunter BioConfirm B.09.00 Software – Released Glycan Report.

Conclusion

This study demonstrated the performance of the Agilent AssayMap Bravo, 6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software, when used as an integrated solution for released glycan analysis.

- This workflow combines high-throughput sample preparation with excellent chromatographic separation using the Agilent AdvanceBio Glycan Mapping column.
- The easy setup and use of the glycan database included with BioConfirm B.09.00 provided the ability to accurately profile, identify, and perform relative quantification.
- The 6545XT-based glycan analysis generated similar quantitative results to that of fluorescence analysis, making it possible to compare different N-glycans across different mAb samples.
- The Report Builder function in BioConfirm B.09.00 provides the ability to create custom reports.

In conclusion, the Agilent solution automated the entire process of N-linked glycan analysis from sample preparation to data analysis with high precision. This approach provided high sensitivity and best quantitation for glycan analysis using fluorescence and additional identification by mass spectrometric detection.

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2. Anumula, K. R. "Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates" *Anal. Biochem.* **2006**, *350*, 1-23.
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Profiling Glycosylation of Monoclonal Antibodies at Three Levels Using the Agilent 6545XT AdvanceBio LC/Q-TOF

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Introduction

Monoclonal antibodies (mAbs) and their derivatives represent a very important class of biopharmaceutical molecules with a wide range of applications. With the dramatic increase in approved mAb products and mAb product sales over recent years, there is an increased need for comprehensive analytical characterization capabilities. mAbs are heterogeneous molecules by nature, which are composed of various types of sequences, modifications, and structural variants. Protein glycosylation is one of the major post-translational modifications (PTMs) of mAbs that plays an important role in many biological processes. The distribution and composition of the glycans bound to the mAb molecules can have an effect on therapeutic efficacy and immunogenicity; consistent glycosylation-associated quality control of therapeutic mAbs has become a high priority in pharmaceutical bioprocessing[1].

Quadrupole Time-of-Flight (Q-TOF) Liquid Chromatography/Mass Spectrometer (LC/MS) systems are widely used to analyze intact mAbs and mAb subunits, perform mAb peptide sequence mapping, and characterize PTMs due to the excellent mass accuracy and resolution in the high mass range of these instruments[2-4].

Typically, there are four levels of LC/MS workflows for glycan/glycoforms in characterization (Figure 1):

- Level 1 and level 2 focus on the analysis of glycoforms on the intact and reduced mAb molecules.
- Level 3 is the analysis of glycopeptides generated from the proteolytical digestion of mAbs, commonly part of a peptide sequence mapping workflow.
- Level 4 is the characterization of glycans that have been released by enzymatic cleavage or other mechanism.

Since we reported on glycopeptide analysis (level 3) of an IgG protein in a previous Application Note[5], three other major LC/MS-based workflows (levels 1, 2, and 4) were evaluated in this study using the NISTmAb. In this study, all three approaches aim to quantitatively understand the glycosylation present for a given protein. These workflows incorporated the:

- Agilent AssayMAP Bravo liquid handling platform
- Agilent 1290 Infinity II LC system
- Agilent PLRP-S column or AdvanceBio Glycan Mapping column
- Highly sensitive Agilent fluorescence detection (FLD)
- Agilent 6545XT AdvanceBio LC/Q-TOF system

As data were acquired on the 6545XT AdvanceBio LC/Q-TOF, they were automatically analyzed using Agilent MassHunter BioConfirm B.09.00 software (Figure 2). This solution dramatically improves not only productivity by allowing convenient sample preparation and streamlined data acquisition, but also accuracy in data analysis.

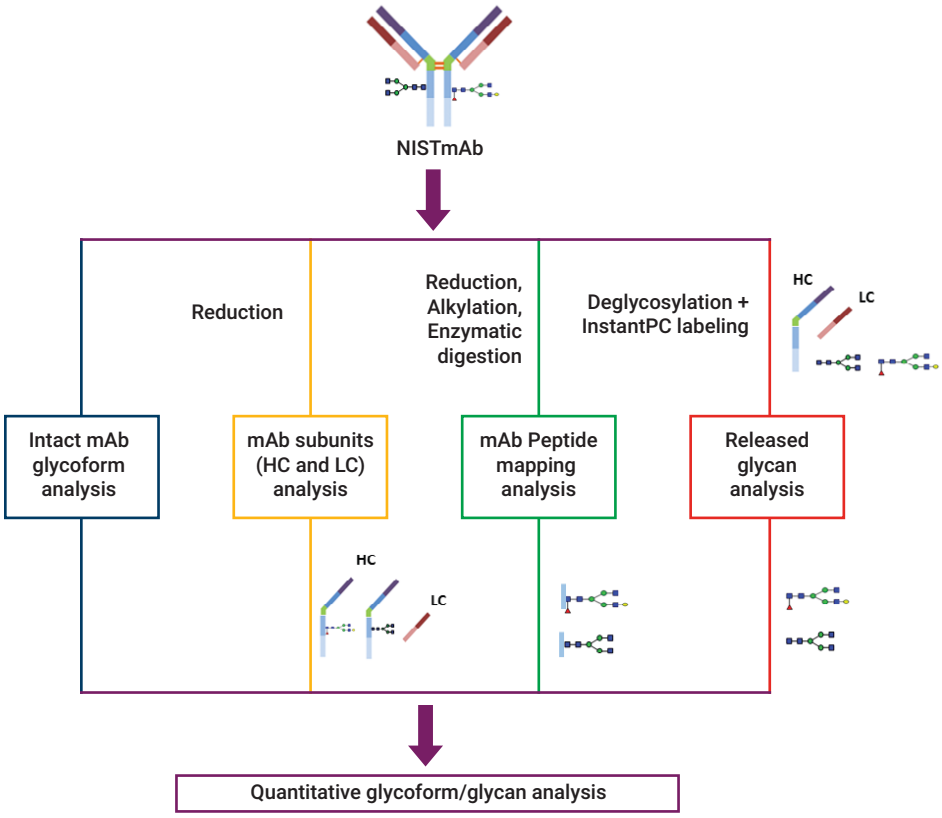


Figure 1. Various glycoforms/glycans quantitative analysis workflows.

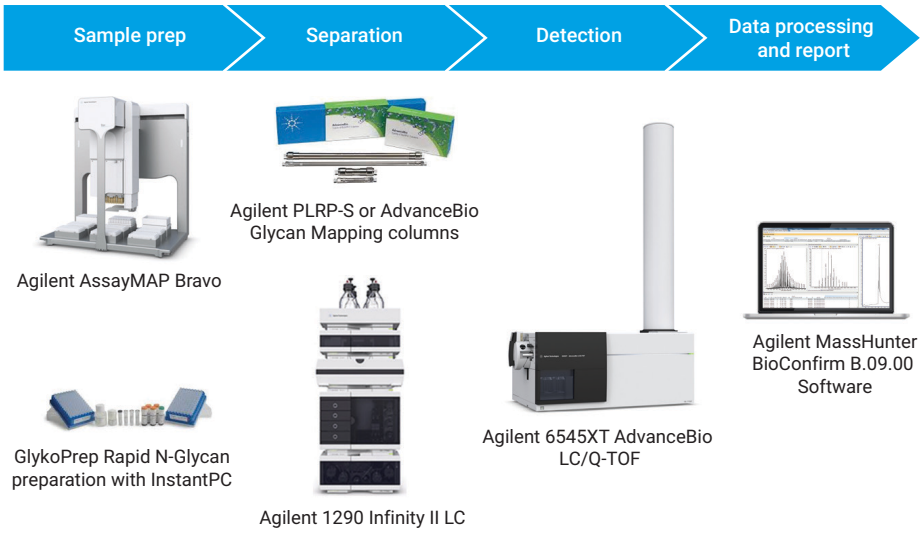


Figure 2. Analytical components of the mAb glycoform/glycan characterization workflow.

Experimental

Materials and Methods

Monoclonal antibody standard, RM 8671, was purchased from the National Institute of Standards and Technology (NIST), and is often referred to as the NISTmAb. 2,2,2-Trifluoroethanol (TFE), DL-Dithiothreitol (DTT), and *tris*(2-carboxyethyl) phosphine hydrochloride (TCEP) were purchased from Sigma-Aldrich. Rapid PNGase F was sourced from New England BioLabs. The GlykoPrep-plus Rapid N-Glycan Sample Preparation with InstantPC (96-ct) was purchased from ProZyme, Inc. The NISTmAb samples used in all workflows were diluted with DI water to 1.0 µg/µL.

Sample Preparation

No sample preparation was needed for the intact mAb glycoforms analysis workflow. For accurate quantitative analysis on the glycoforms of NISTmAb subunit (heavy chain), complete protein reduction was required. Therefore, a first reduction reaction with 40 mM DTT at 60 °C for 30 minutes, followed by an additional 25 mM TCEP reaction (30 minutes at room temperature) were performed. Finally, we used the Agilent AssayMAP Bravo liquid handling system (G5542A) in the released glycan quantitation workflow. The detailed procedure for the sample preparation is described in ProZyme's Application Note (product code: GPPNG-PC). After the final cleanup step, the released labeled N-glycan elution had a final concentration of 1.0 µg/µL.

LC/MS Analysis

LC/MS analyses were conducted on an Agilent 1290 Infinity II LC system equipped with an Agilent 1260 Infinity Fluorescence Detector (G1321B) and coupled to a 6545XT AdvanceBio LC/Q-TOF system with a Dual Agilent Jet Stream source. LC separation for the intact NISTmAb and the reduced NISTmAb was obtained with an Agilent PLRP-S column (2.1 × 50 mm, 1,000 Å, 5 µm). Glycans were chromatographically separated with an Agilent AdvanceBio Glycan Mapping column (2.1 × 100 mm, 1.8 µm). The fluorescence detector was set to λ_{Ex} = 285 nm, λ_{Em} = 345 nm, with PMT gain = 10. Tables 1 and 2 list the LC/MS parameters used. Approximately 0.5 µg of protein was injected for the intact and subunit analyses. The N-glycan experiments injected the free glycans released from 1–2 µg of intact protein.

Data Processing

MassHunter BioConfirm B.09.00 software featuring three major biopharma workflows (intact mAb, peptide mapping, and released glycan profiling) was used for all data processing in this study. This powerful software program simplifies downstream data analysis, enabling automatic identification and relative quantitation of targeted biomolecules. For the released glycan workflow, the Agilent Personal Compound Database and Library (PCDL) glycan database, which provides accurate glycan identification and confirmation, was used.

Table 1. Liquid chromatography parameters.

Agilent 1290 Infinity II LC System			
Sample type	Intact mAb	mAb Subunits (HC and LC)	mAb Released glycans
Column	Agilent PLRP-S, 2.1 × 50 mm, 1,000 Å, 5 µm (p/n PL1912-1502)	Agilent PLRP-S, 2.1 × 50 mm, 1,000 Å, 5 µm (p/n PL1912-1502)	Agilent AdvanceBio Glycan Mapping, 2.1 × 100 mm, 1.8 µm (p/n 858700-913)
Thermostat	4 °C	4 °C	4 °C
Solvent A	0.1 % Formic acid in DI water	0.1 % Formic acid in DI water	50 mM Formic acid adjusted to pH 4.5 with ammonium hydroxide
Solvent B	0.1 % Formic acid in 100 % acetonitrile	0.1 % Formic acid in 100 % acetonitrile	Acetonitrile
Gradient	0–1 minute, 0–20 %B 1–3 minutes, 20–50 %B 3–4 minutes, 50–70 %B	0 minutes, 25 %B 5 minutes, 45 %B 6 minutes, 60 %B 6–7 minutes, 60 %B	0–0.5 minutes, 75–71 %B 0.5–16 minutes, 71–67.5 %B 16–22 minutes, 67.5–60 %B 22–22.5 minutes, 60–40 %B 22.5–23.5 minutes, 40 %B (0.7 mL/min) 23.5–24 minutes, 40–75 %B (0.7 mL/min) 24–30 minutes, 75 %B (0.9 mL/min)
Column temperature	60 °C	60 °C	40 °C
Flow rate	0.5 mL/min	0.8 mL/min	0.4 mL/min
Injection volume	0.5 µL	1.0 µL	2.0 µL

Results and Discussion

Carbohydrate compositions, structures, and their relative quantitative levels are important for the safety and efficacy of therapeutic proteins. Detailed studies of these glycan structures will also potentially help to improve the discovery and development of novel drugs.

Characterization of mAb glycoforms at the intact protein level is the most widely used method for quick assessment and monitoring of mAb glycosylation during pharmaceutical bioprocessing.

Intact NISTmAb samples were analyzed with an Agilent PLRP-S column using the 1290 Infinity II LC system coupled to a 6545XT AdvanceBio LC/Q-TOF mass spectrometer. Raw mass spectra were deconvoluted by the Maximum Entropy algorithm in MassHunter BioConfirm B.09.00 software, as shown in Figure 3.

Table 2. MS Acquisition parameters.

Agilent 6545XT AdvanceBio LC/Q-TOF System			
Sample type	Intact mAb	mAb Subunits (HC and LC)	mAb Released glycans
Source	Dual Agilent Jet Stream	Dual Agilent Jet Stream	Dual Agilent Jet Stream
Gas temperature	350 °C	350 °C	150 °C
Gas flow	12 L/min	12 L/min	9 L/min
Nebulizer	60 psig	35 psig	35 psig
Sheath gas temperature	400 °C	350 °C	300 °C
Sheath gas flow	11 L/min	11 L/min	10 L/min
VCap	5,500 V	4,000 V	3,000 V
Nozzle voltage	2,000 V	500 V	500 V
Fragmentor	380 V	180 V	120 V
Skimmer	140 V	65 V	65 V
Quad AMU	500 *m/z*	300 *m/z*	95 *m/z*
Mass range	100–10,000 *m/z*	100–3,200 *m/z*	300–1,700 *m/z*
Acquisition rate	1.0 spectra/s	1.0 spectra/s	2.0 spectra/s
Reference mass	922.0098	922.0098	922.0098
Acquisition mode	Positive, extended (10,000 *m/z*) mass range	Positive, standard (3,200 *m/z*) mass range, HiRes (4 Gz)	Positive, low mass range, HiRes (4 Gz)

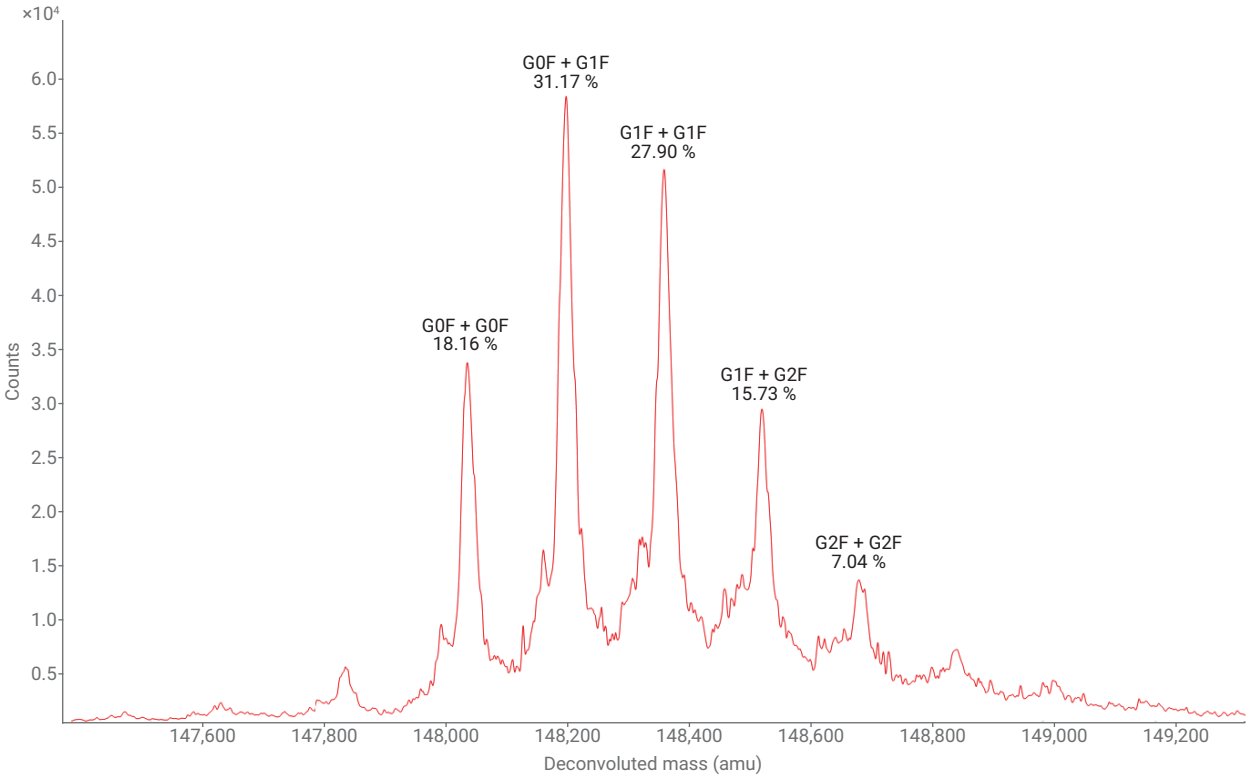


Figure 3. MS Deconvoluted spectrum (maximum entropy) of intact NISTmAb standard with relative quantitation labeled on five major glycoforms.

Typically, once the MS raw data are acquired, the BioConfirm Intact Protein Workflow can be used in an automatic mode to sum up the spectra across any chromatographic peaks, then deconvolute into the intact mass of the mAb. The biomolecule peaks were then confirmed by matching the measured masses with the theoretical masses based on the known mAb sequences in the protein database. The relative quantitation on all identified glycoforms was also automatically calculated using either the peak heights or peak areas of the deconvoluted mass spectra. BioConfirm can recalculate the relative quantitation percentages for any glycoform that is removed or added to the list.

Figure 4 summarizes the relative quantitation and the reproducibility results of five major glycoforms of the NISTmAb from 10 replicate sample injections of 0.5 µg on-column. The quantitative results from the peak height analysis were similar to those from the peak area calculation. However, the peak height analysis results show accuracy with the average standard deviations (SDs) of all glycoforms less than 1 %, while the average SDs of the peak area results were approximately 1.62 %.

One feature of the BioConfirm B.09.00 software allows the user to perform a relative quantitation comparison on the selected glycoforms among different samples. Figure 5 shows the mirror plot image of the deconvoluted spectra of two NISTmAb samples (1 and 2). The G1F + G1F glycoforms (shaded) were chosen for detailed analysis. The table in Figure 5 shows that both samples have very similar quantitation results using either peak height or the peak area data.

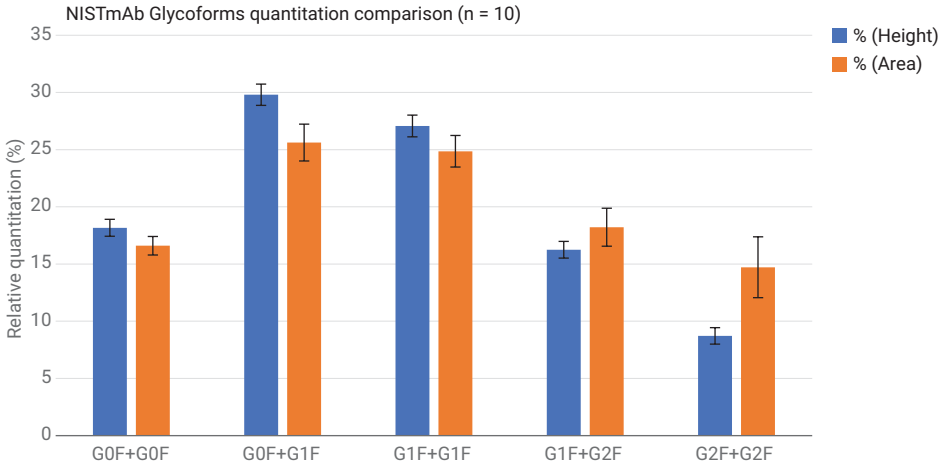
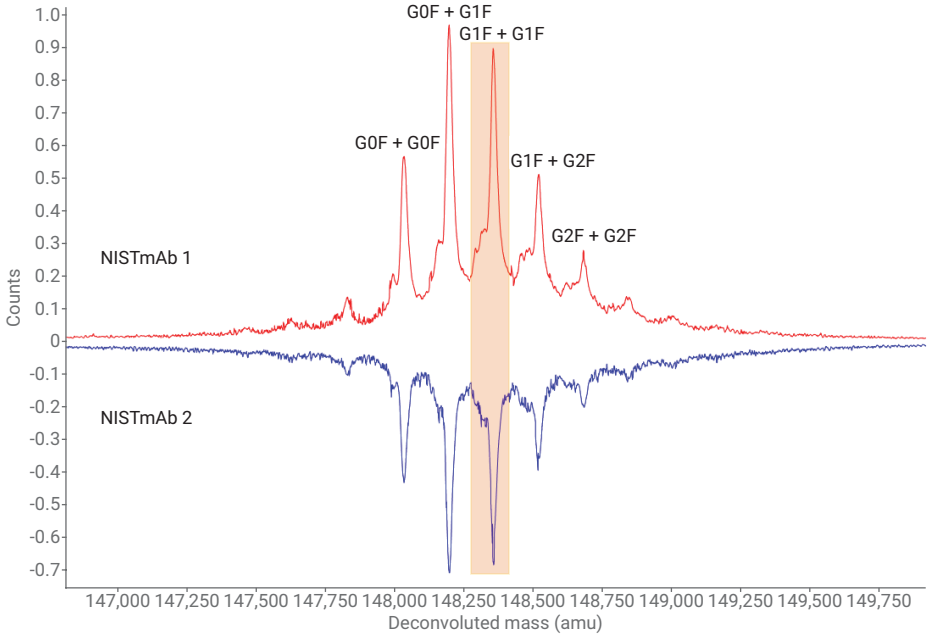


Figure 4. Quantitation results from intact NISTmAb glycoforms analysis (10 replicates).



General			% Quantitation					Sequence match
Mass	RT	File	Use for % quant	Height (MS)	% Quant (height)	Area (MS)	% Quant (area)	Pred mods
148,363.0381	2.229	NIST mab_1.d	☑	8,387	28.43	6,527,163	27.71	2*G1F(1607.5013)
148,362.5884	2.261	NIST mab_2.d	☑	6,268	29.5	4,936,695	28	2*G1F(1607.5013)

Figure 5. Quantitation results comparison of glycoforms (G1F+G1F) between two NISTmAb samples.

The NISTmAb sample was also used to perform mAb subunits analysis (level 2). To obtain accurate quantitation results on the glycoforms attached to the heavy chain of the NISTmAb, it is critical to generate the homogeneous forms of heavy (HC) and light chains (LC) of the NISTmAb. Therefore, full protein reduction with the combination of DTT and TECP reactions was performed to completely reduce all inter- and intra-disulfide bond linkages. Figure 6A shows the total ion chromatogram of the reduced NISTmAb separating the two major subunits. Excellent liquid chromatographic separation of LC and HC was achieved using a very short HPLC gradient. Figure 6B represents the deconvoluted spectrum of the NISTmAb heavy chain (shaded in light green in Figure 6A). Three major glycoforms (G0F, G1F, and G2F) were observed, and their relative abundances were calculated. Moreover, the average percent quantitation values of these three glycoforms from 10 technical replicates were also calculated to be 39.14 %, 47.68 %, and 13.18 %, respectively. The average SDs of these results were less than 0.24 % (Figure 7).

For released glycan analysis (level 4), we have developed a new workflow solution integrating UHPLC technologies, the Agilent AssayMAP Bravo liquid handling platform, the

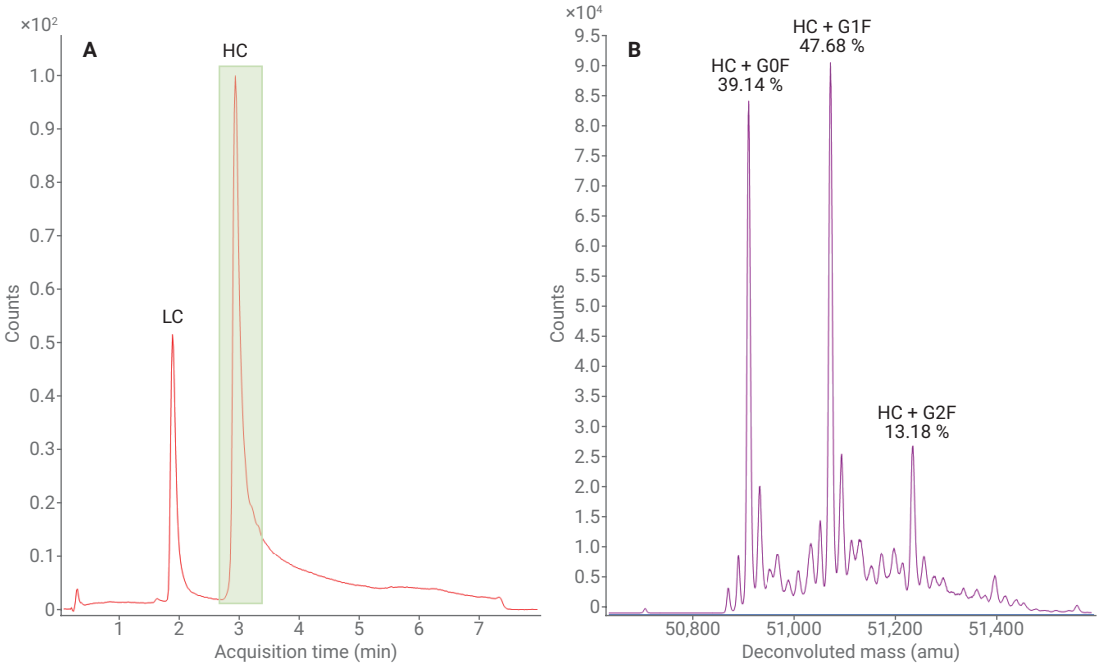


Figure 6. Total ion chromatogram (A), and MS deconvolution (B) of NISTmAb sub-units.

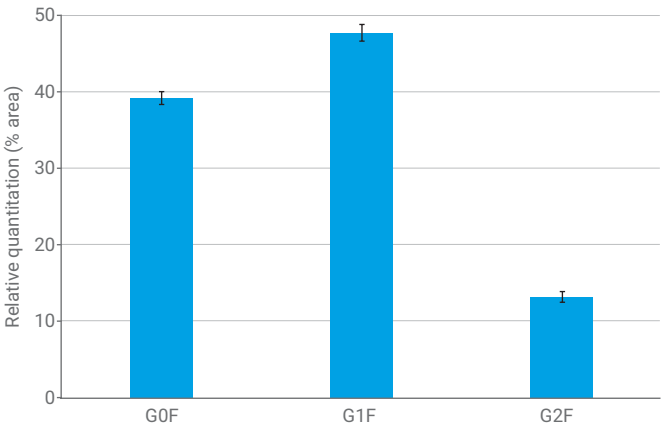


Figure 7. Quantitation results from NISTmAb sub-unit workflow.

6545XT AdvanceBio LC/Q-TOF, and MassHunter BioConfirm software for automatic data processing[4]. Briefly, N-glycans of NISTmAb were enzymatically released by PNGaseF, followed by labeling with a fluorescent tag (InstantPC), and LC-FLD or LC/MS analysis. All sample preparations were done using the AssayMAP Bravo liquid handling system (G5542A) in a high-throughput manner. A Personal Compound Database (PCD) containing accurate mass and structural information of glycans was used for identification using the Agilent proprietary Find-by-Formula algorithm.

Figure 8 shows the representative chromatograms of N-glycans (FLD and MS EIC) from the NISTmAb. The FLD chromatogram (Figure 8 top, zoom in) revealed that more than 15 glycan peaks were detected. The glycosylation pattern of the major abundant glycans, such as the G0F, G1F isoforms, and G2F, was comparable between the fluorescent and MS data.

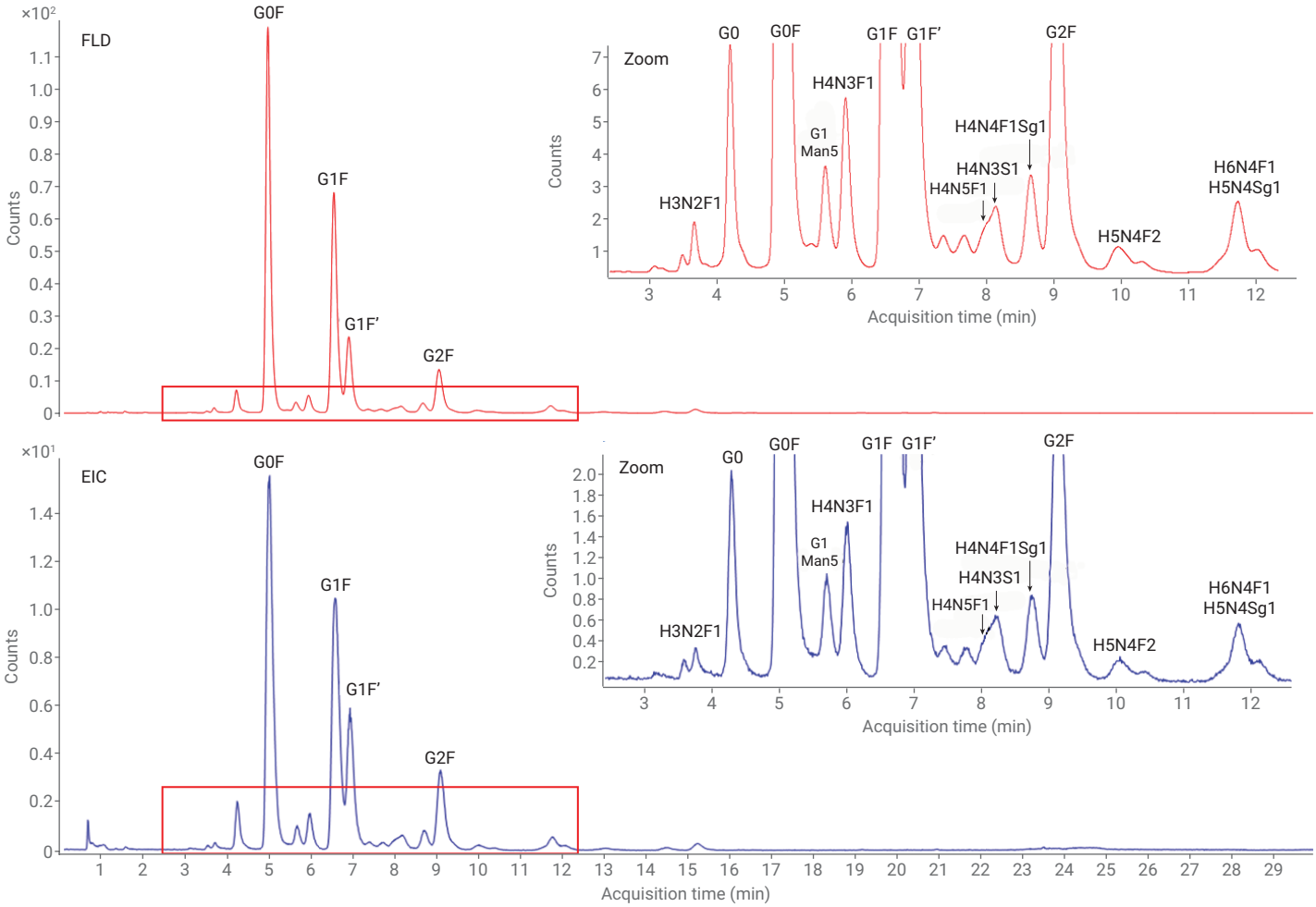


Figure 8. FLD chromatogram and mass spectra (EIC) of InstantPC labeled N-glycans from NISTmAb.

Figure 9 shows the relative sum % of the top four most abundant N-glycans of the NISTmAb sample. The relative quantitation (%) results of these glycoforms were also comparable to the results from the NISTmAb subunit workflow (level 2). The minor result discrepancy between levels 2 and 4 was likely due to the exclusion of minor glycoform peaks in the level 2 sample used for quantitative analysis. However, excellent chromatographic separation and accurate quantitation of the G1F isoforms were obtained using the AdvanceBio Glycan Mapping column. Overall, this approach can also eliminate ambiguity about glycan peak assignments and peak quantitation due to the sample heterogeneity caused by incomplete mAb reduction.

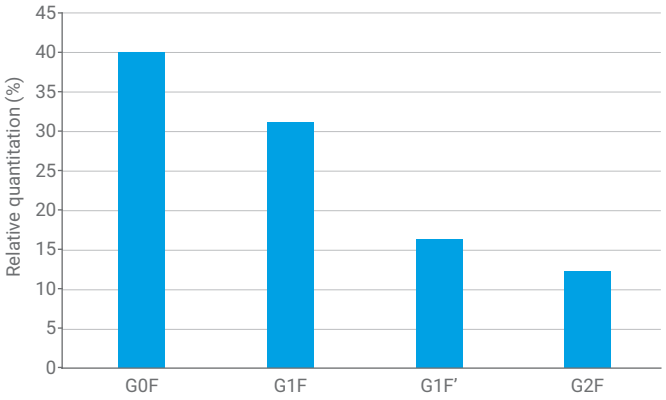


Figure 9. Quantitation results from NISTmAb released glycan workflow.

Conclusion

We have developed a complete workflow solution for antibody glycoforms characterization by integrating the Agilent AssayMAP Bravo liquid handling platform, UHPLC technologies, the Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software. This approach offers users flexible workflows for glycan relative quantitation at four different analytical levels:

- The intact mAb workflow provided rapid assessment of the major glycoforms of the intact mAb. The same glycoforms from various time points of the same sample or from different batch samples can easily be monitored and compared.
- The mAb subunits workflow offered detailed quantitative information about individual glycans such as G0F, G1F, and G2F. The overall high throughput of this workflow makes it an ideal method for accurate mass measurements of the majority of mAbs and their variants, including bispecific mAbs.
- The glycopeptide analysis through peptide mapping workflow resulted not only in glycan-relative quantitation but also N-glycosylation site(s) information. The Agilent AdvanceBio Glycan Mapping (HILIC) column demonstrated strong retention and increased resolution for the hydrophilic glycopeptides.
- The released glycan workflow provided high analytical sensitivity and the best quantitation for glycan analysis using both fluorescence and mass spectrometric detection. Excellence in glycans (G1F isoforms) separation, and the use of a glycan database provided in BioConfirm B.09.00 resulted in accurate glycan profiling: identification and relative quantitation.

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

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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.

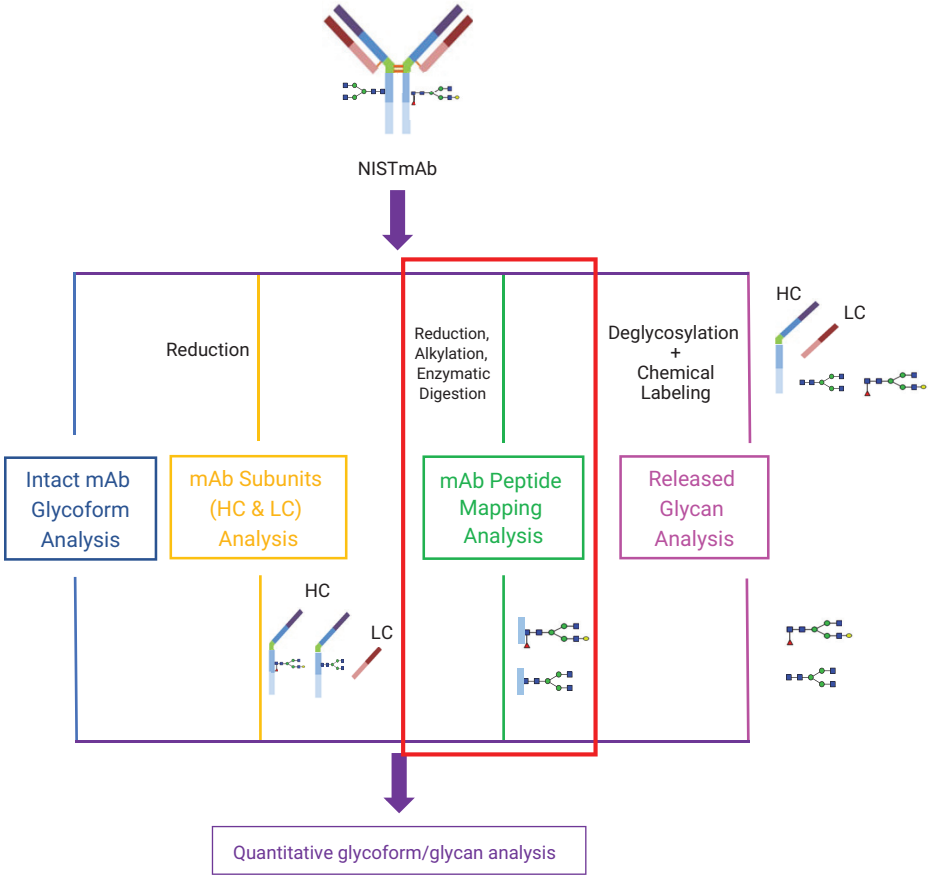


Figure 1. Various glycoform/glycan quantitative analysis workflows. The glycopeptide workflow is highlighted in the red box.

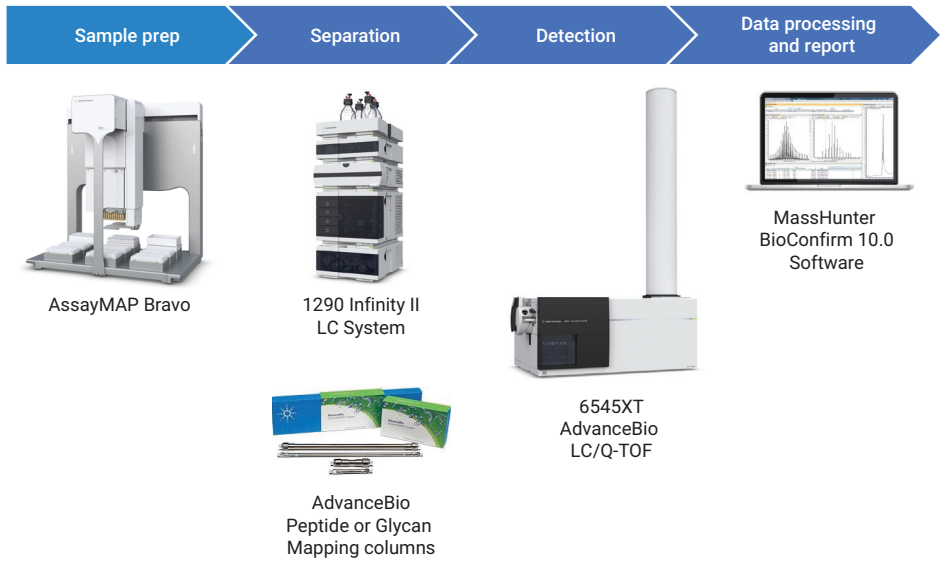


Figure 2. Analytical components of the mAb glycopeptide characterization workflow.

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.

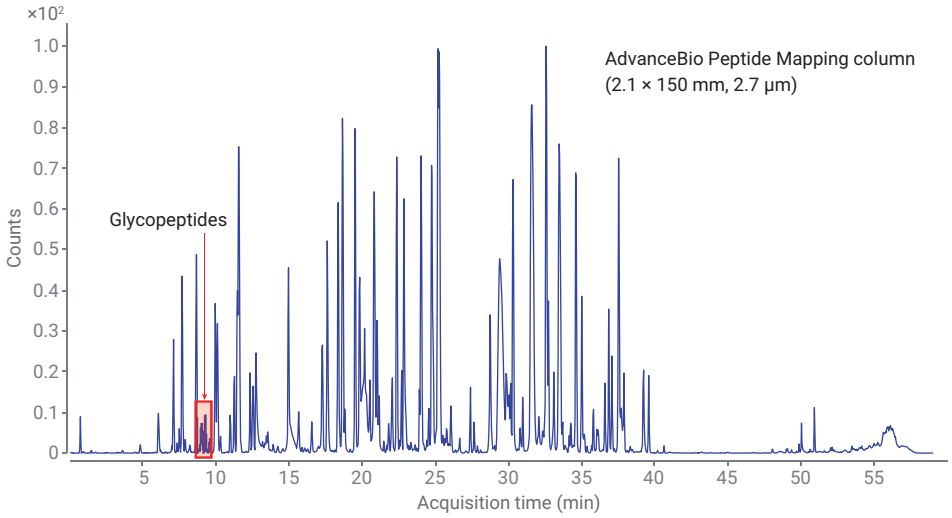


Figure 3. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Peptide Mapping (RP-C18, 2.1 × 150 mm, 2.7 µm) column.

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

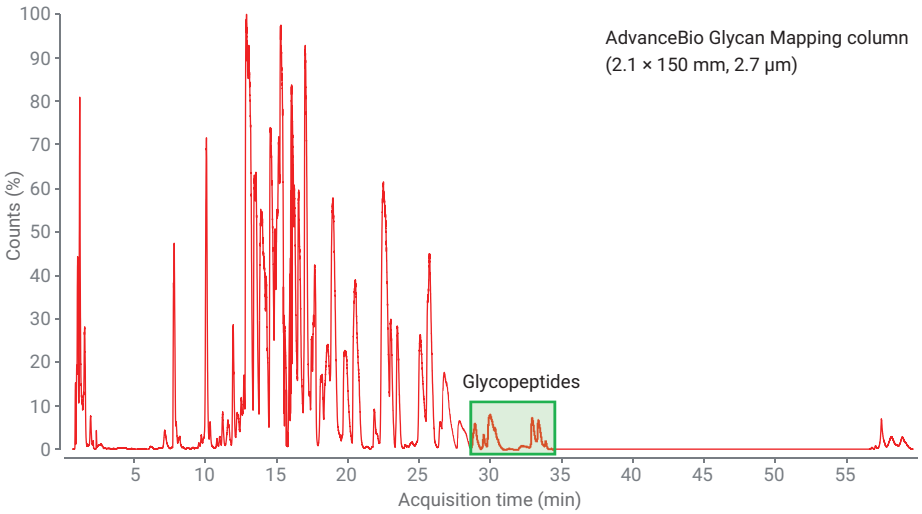


Figure 4. MS TIC of peptides from Trypsin/Lys-C digested NISTmAb on the AdvanceBio Glycan Mapping (HILIC, 2.1 × 150 mm, 2.7 µm) column.

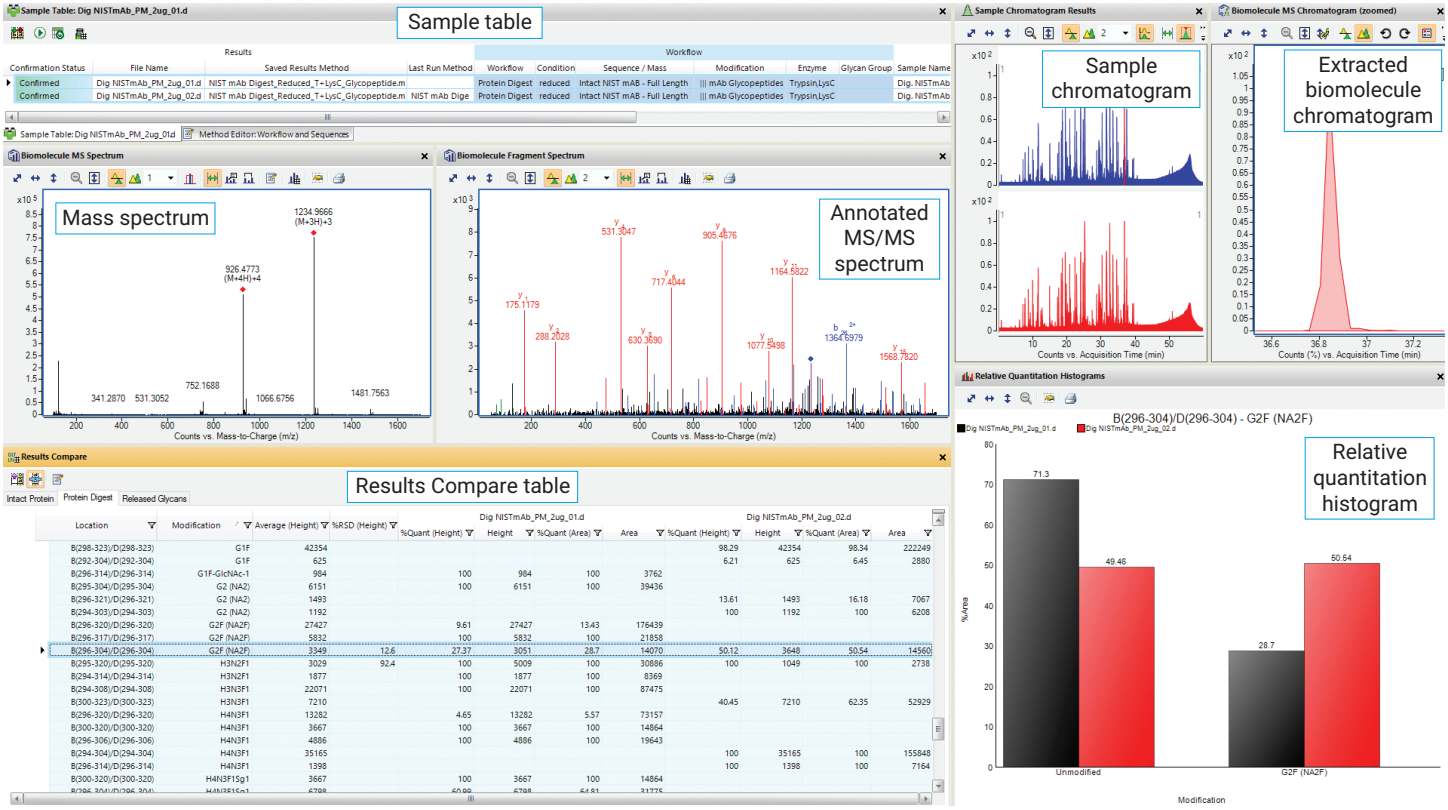
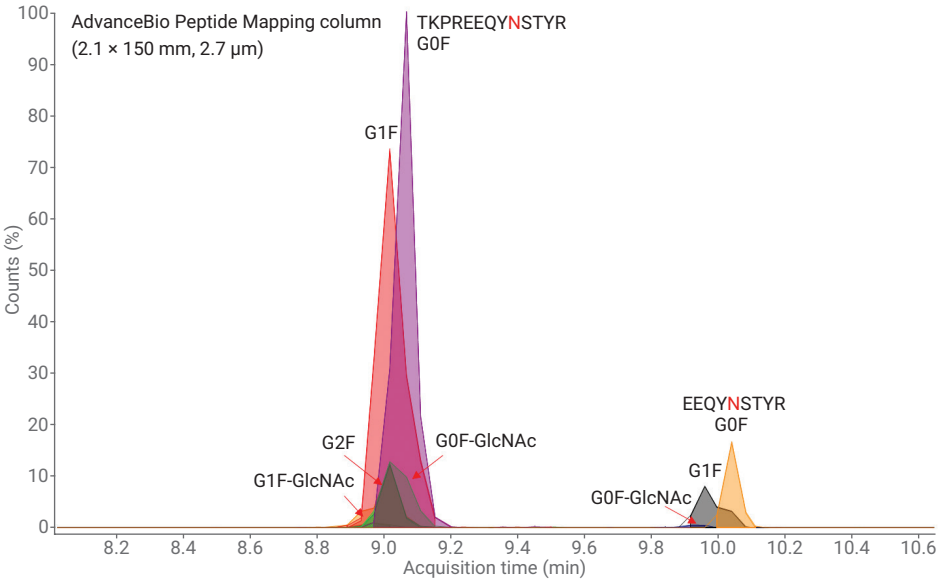


Figure 5. Screen capture of MassHunter BioConfirm 10.0 software with representative glycopeptide profiling results and histogram of relative quantitation on glycopeptides.

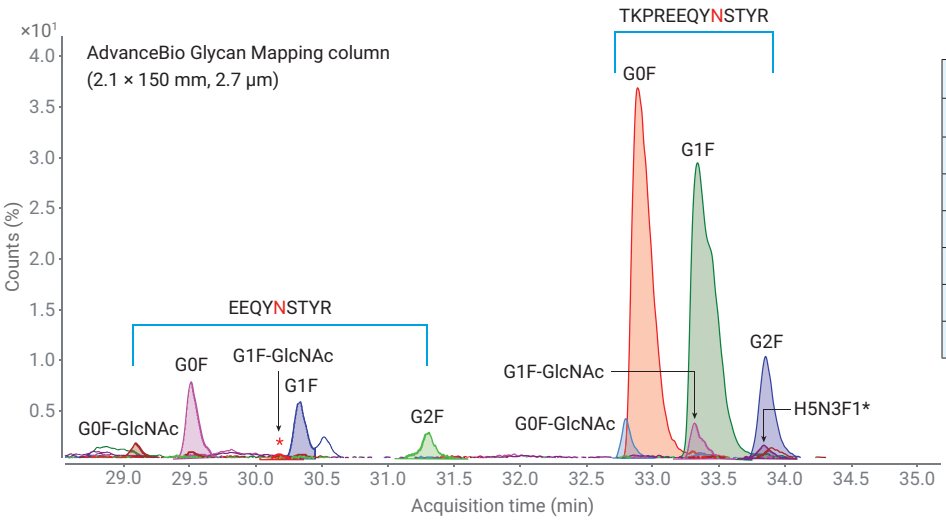
times, sequences, modifications, scores, and quantitative results by either peak heights or peak areas. One feature of the BioConfirm 10.0 software is that users have the ability to select or deselect certain peptides for grouping in relative quantitation analysis, with the results shown in histogram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

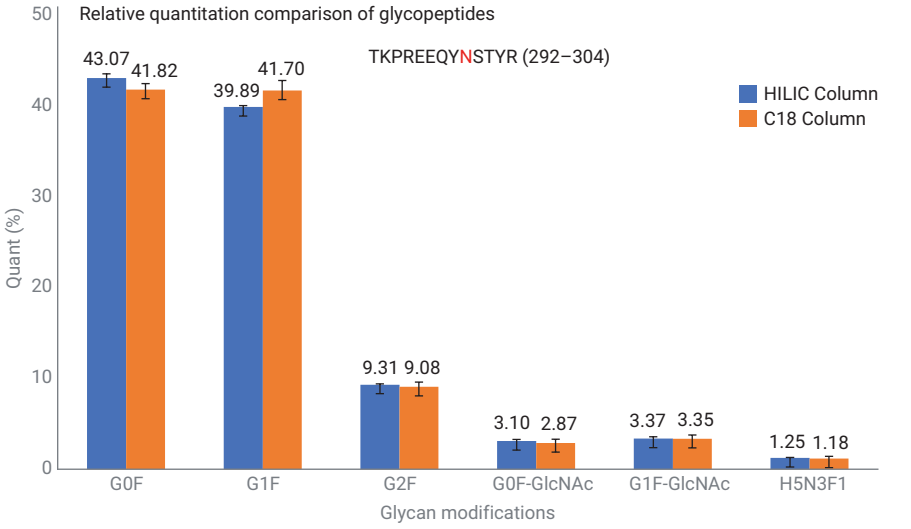


Figure 8. Relative quantitative comparison of NISTmAb glycopeptides analysis (RP-C18 versus HILIC, three replicates).

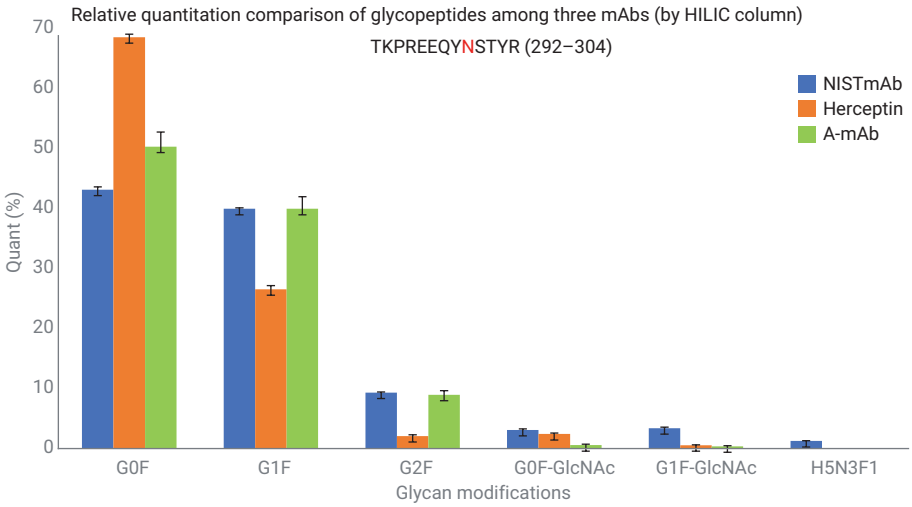


Figure 9. Relative % quantitation of the top six glycopeptides in each of the three mAb samples. All digested mAb samples were separated by the HILIC column (three replicates).

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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