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Biopharma Compendium

Accelerate Your CQA Efficiency and Approach it with Multi-Attribute Methodology

Breakthroughs to Increase the Efficiency of Critical Quality Attribute (CQA) Testing and Allow for a Multi- Attribute Method (MAM)

Production of therapeutic monoclonal antibodies (mAbs) relies on living cells or organisms for producing these biotherapeutics. Characterization of the structure of the mAb is extremely complicated due to the diversity of modifications that can occur during the production, storage and formulation process. It can be challenging to establish well-characterized antibody therapeutics and meet current regulatory expectations. This compendium demonstrates the possibility of using multidimensional technologies like 2D-LC, high-resolution Mass Spectrometry (MS), and novel chemistries and columns to enhance the efficiency in Critical Quality Attributes (CQAs) analysis and explore the possibility of measuring multiple attributes simultaneously to decrease overall analysis time and obtain faster turn-around results.

On line two-dimensional liquid chromatography is an emerging technology. The Agilent InfinityLab 2D-LC system is ideal to solve complex separations and makes it possible to separate co-eluting compounds and achieve the highest peak capacity. Increased resolution can be achieved with heart-cutting and high-resolution sampling or comprehensive 2D-LC for complex mixtures. 2D-LC can also be used for making buffer separations compatible with MS detection and thus eliminates tedious manual sampling handling steps.

The Agilent 6545XT AdvanceBio LC/Q-TOF system is designed to handle multiple workflows in biopharmaceutical characterization. The 6545XT Q-TOF offers higher resolution, increased sensitivity and a larger dynamic range. The hardware and software are geared towards the analysis of intact proteins, large protein fragments (e.g., mAb fragments), peptides and glycans. The added capabilities of the 6545XT Q-TOF, like iterative data acquisition allow greater access to the most information at the intact protein level, enabling you to automatically confirm a sequence through peptide mapping, and confidently understand PTMs. MassHunter BioConfirm software is part of the comprehensive solution for characterizing biopharmaceutical products. The software performs data analysis and reporting. In addition, tools like the drug-to-antibody ratio (DAR) calculator, flexible report templates, and integrated operation with MassHunter WalkUp bring expert LC/MS capabilities to non-experts.

Glycosylation plays an important role in many biological processes. It also affects the therapeutics efficacy, stability, pharma-kinetics and immunogenicity. Glycan characterization can be done with several techniques, including HPLC with fluorescence or MS detection. 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 dye is a novel instant glycan labeling reagent that provides markedly increased MS and fluorescent sensitivity. The traditional glycan analysis is starting with enzymatic glycan release by N-Glycanase (PNGase F) digestion. Followed by sample cleanup and labeling with InstantPC which forms a stable urea linkage with glycosylamines released by rapid N-Glycanase (PNGase F) digestion. This GlykoPrep N-glycan sample preparation kit uses a rapid digestion, instant labeling, and cleanup of excess InstantPC label. The Agilent GlykoPrep N-glycan sample preparation kit offers rapid digestion, instant labeling, and cleanup.

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Multi-Attribute Analysis of Monoclonal Antibodies Using the Agilent InfinityLab 2D-LC and 6530 Q-TOF MS Systems

This study demonstrates the use of the Agilent 1290 Infinity II 2D-LC and the Agilent 6530 Q-TOF LC/MS for multi-attribute analysis directly from cell-culture supernatants. The multi-attribute analyzer combines protein A affinity chromatography with size exclusion chromatography and liquid chromatography/mass spectrometry in a (multiple) heart-cutting three-dimensional setup. This workflow enables simultaneous assessment of mAb titer, size variants, molecular weight, amino acid sequence, and post-translational modifications.

Application Note

Pharma & Biopharma

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Multi-Attribute Analysis of Monoclonal Antibodies Using the Agilent InfinityLab 2D-LC Solution and Q-TOF MS

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Abstract

In recent years, two-dimensional liquid chromatography (2D-LC) has been shown to be highly promising for the detailed characterization of monoclonal antibodies (mAbs). This Application Note describes the use of the Agilent 1290 Infinity II 2D-LC System and the Agilent 6530 Q-TOF LC/MS for multi-attribute analysis directly from cell culture supernatants. The multi-attribute analyzer combines protein A affinity chromatography with size exclusion chromatography (SEC) and liquid chromatography/mass spectrometry (LC/MS) in a (multiple) heart-cutting three-dimensional (3D) setup. This workflow enables simultaneous assessment of mAb titer, size variants, molecular weight (mol wt), amino acid sequence, and post-translational modifications.

Introduction

mAbs have emerged as important therapeutics for the treatment of life-threatening diseases such as cancer and autoimmune diseases.[1,2,3] In contrast to small molecule drugs, mAbs are large (150 kDa) and heterogeneous as a result of the biosynthetic process and subsequent manufacturing and storage. Hundreds of different variants may co-exist, differing in aspects such as N-glycosylation, N- and C-terminal processing, deamidation, oxidation, amino acid sequence, and disulfide bridges. Fragmentation and aggregation further add to the complexity.

As a result, there are many different characteristics to monitor during mAb development, such as mAb titer (with affinity chromatography), size variants (with SEC), charge variants (with ion-exchange chromatography), amino acid sequence, and molecular weight (with MS).

Each of these quality-defining attributes generally requires a different setup in terms of equipment, analytical approach, and detection. The possibility of combining some of these techniques in one system and analytical run is attractive. 2D-LC has shown promise in this respect.[4,5] We pushed the limits of multidimensional chromatography by combining protein A affinity chromatography with SEC and LC/MS in a (multiple) heart-cutting three-dimensional (3D) setup. Such a multi-attribute analyzer allows for simultaneous assessment of mAb titer as well as important structural aspects such as aggregation, fragmentation, mol wt, amino acid sequence, and post-translational modifications such as glycosylation directly from cell culture supernatants (Figure 1).

Experimental

Instrumentation

An Agilent 1290 Infinity II 2D-LC System equipped with the multiple heart-cutting option and an additional Agilent 1260 Infinity II Quaternary Pump and two additional valves was used. Figure 2 represents the configuration schematically, and it is further summarized in the next section. DAD detection was used in the first and second dimension (protein A affinity chromatography and SEC). An Agilent 6530 Q-TOF LC/MS with a Jet Stream ESI source was used for detection after the third and final dimension (reversed-phase desalting).

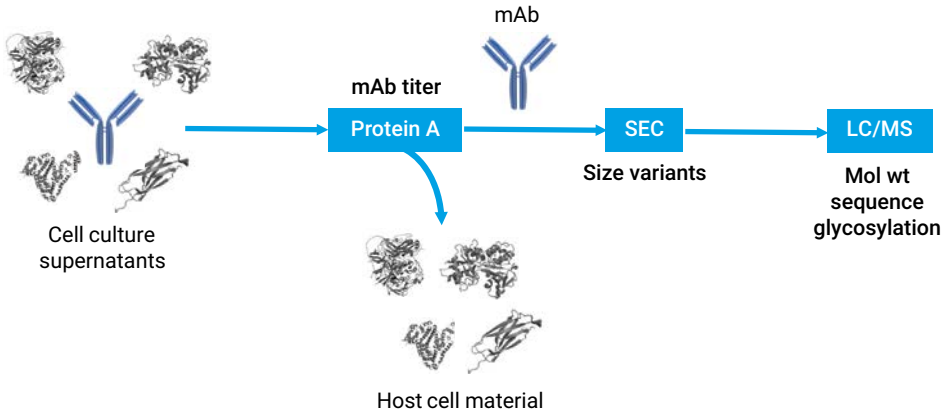

Figure 1. Multi-attribute analysis directly from cell culture supernatants by combining protein A affinity chromatography, size exclusion chromatography, and LC/MS in an online 3D setup.

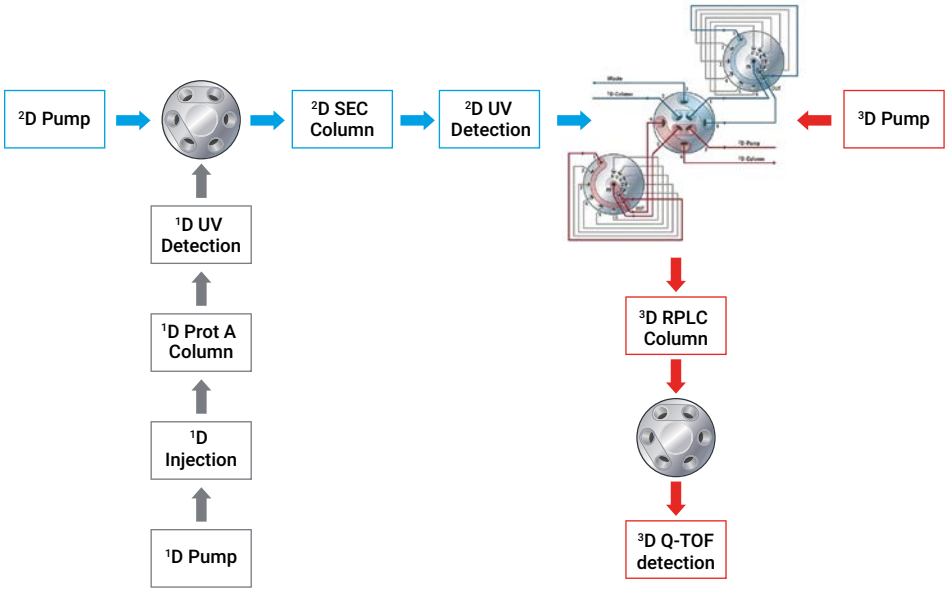

Figure 2. Configuration of the multi-attribute analyzer.

[1]D: Protein A affinity chromatography

Agilent Bio-Monolith protein A column (p/n 5069-3639)

- Agilent 1260 Infinity II Quaternary Pump with active inlet valve (AIV) (G7111B, option 032)
- Agilent 1290 Infinity II Multisampler with sample thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (MCT) with valve drive installed (G7116B, option 058) equipped with an Agilent InfinityLab Quick Change 2-Position/6-Port Valve, 1300 bar (G4231C) with one 80 µL loop (p/n 5067-5426) installed
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with a 3.7 mm HDR Max-Light Cartridge Cell (G4212-60032)

[2]D: SEC

Agilent AdvanceBio SEC column, 300 Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5301)

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multicolumn Thermostat (MCT) (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) with a 10 mm Max-Light Cartridge Cell (G4212-60008)

2D-LC with multiple heart-cutting

- Agilent 1290 Infinity Valve Drive (G1170A) with 2D-LC Valve (G4236A)
- Two Agilent 1290 Infinity Valve Drives (G1170A) with multiple heart-cutting valves (G4242-64000) equipped with 40 µL loops

[3]D: Reversed-phase chromatography (RPLC) for desalting

Polymer-based desalting cartridge, 2.1 × 10 mm

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity Valve Drive (G1170A) with an Agilent InfinityLab Quick Change 2-Position/6-Port Valve, 800 bar (G4231A) (used as a diverter valve)

Software

- Agilent OpenLab CDS ChemStation edition revision C.01.07 SR4 [505]
- 2D-LC software revision A.01.04 [017]
- Agilent MassHunter for instrument control (B.05.01)
- Agilent MassHunter with BioConfirm add-on for data analysis (B.07.00)

Method parameters

[1]D: Protein A Affinity Chromatography	
Column	Bio-Monolith protein A column
Temperature	23 °C
Mobile phase A	50 mM sodium phosphate pH 7.4
Mobile phase B	500 mM acetic acid
Flow rate	0.75 mL/min (flow rate was reduced to 0.2 mL/min during SEC and desalting steps)
Gradient	Time (min) %B 0.0 to 0.2 0 (binding) 0.2 to 0.3 0 to 100 (fast gradient to elution conditions) 0.3 to 1.4 100 (elution) 1.4 to 2.0 100 to 0
Injection	20 µL (needle wash in flush port, 5 seconds with water/acetonitrile 75/25 v/v)
Detection	DAD, 3.7 mm Max-Light Cartridge Cell Peak width >0.013 minutes (20 Hz) Signal wavelength/bandwidth: 220/4 and 280/4 nm, no reference
Heart-Cutting [1]D > [2]D	
Loop	80 µL (installed on a 2-Position/6-Port Valve in [1]D MCT)
Timetable	1.35 minutes: switch valve position (inject loop content on second dimension column)
[2]D: SEC	
Column	AdvanceBio SEC, 300 Å, 7.8 × 300 mm, 2.7 µm
Temperature	30 °C
Mobile Phase	150 mM sodium phosphate pH 7 (isocratic)
Flow Rate	0.70 mL/min
Detection	DAD, 10 mm Max-Light Cartridge Cell Peak width >0.05 minutes (5 Hz) Signal wavelength/bandwidth: 220/4 and 280/4 nm, no reference
Multiple Heart-Cutting [2]D > [3]D	
Valve and Loop Configuration	2-Position/4-Port Duo-Valve, 2 × 6 loops (concurrent)
Loop Size	40 µL
Sampling Timetable	HMW2: 9.37 minutes HMW1: 9.99 minutes Main: 10.73 minutes LMW1: 11.43 minutes
[3]D: RPLC for Desalting	
Column	Polymer-based desalting cartridge, 2.1×10 mm
Temperature	30 °C

Chemicals and solvents

Water (ULC/MS), acetonitrile (HPLC-S), formic acid (ULC/MS), and acetic acid (ULC/MS) were from Biosolve (Valkenswaard, The Netherlands). Sodium phosphate dibasic and monobasic were purchased at Merck (Darmstadt, Germany). mAbs and supernatants were obtained from a local biotechnology company.

Sample preparation

Samples were diluted to the desired concentration in the protein A binding buffer (50 mM sodium phosphate, pH 7.4). The buffer was also used as a blank.

Results and discussion

Figures 3 to 5 show the multi-attribute analysis of a therapeutic mAb in development using protein A affinity chromatography, SEC, and LC/MS in a 3D setup.

Mobile Phase A	0.1% (v/v) formic acid in water
Mobile Phase B	0.1% (v/v) formic acid in acetonitrile
Flow Rate	0.50 mL/min (idle flow rate 0.35 mL/min)
Gradient	Time (min) %B 0.0 to 10.0 5 10.0 to 15.0 5 to 80 15.0 to 17.0 80 17.0 to 18.0 80 to 5
Gradient Stop Time	19.5 minutes
Cycle Time	20 minutes
	Switch diverter valve to MS after 11.5 minutes
Detection	Agilent 6530 Q-TOF LC/MS
Ionization	Agilent Jet Stream Technology, positive ionization
Source Settings	
Drying Gas Temperature	320 °C
Drying Gas Flow	9 L/min
Nebulizer Pressure	40 psi
Sheath Gas Temperature	350 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	3,500 V
Nozzle Voltage	1,000 V
Acquisition Settings	
Fragmentor	350 V
Mode	High mass range (1 GHz)
Data Acquisition Range	*m/z* 800 to 10,000
	Profile Acquisition
	1 spectrum/s
Deconvolution	
	Maximum entropy and pMod algorithms incorporated in MassHunter BioConfirm

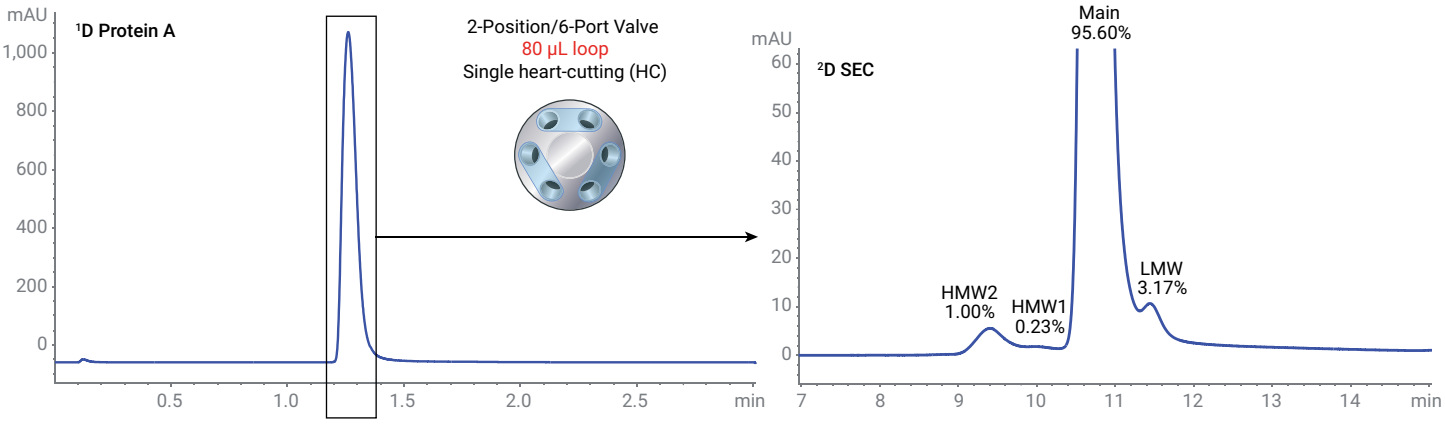

Figure 3. [1]D protein A affinity chromatogram (280 nm) of therapeutic mAb, and [2]D SEC chromatogram of transferred protein A peak (220 nm). The protein A peak was completely sampled in an 80 µL loop installed on an Agilent InfinityLab Quick Change 2-Position/6-Port Valve and transferred to the second dimension. The [2]D SEC chromatogram reveals various HMW and LMW variants and a peak purity of 95.6%.

The analysis starts with the determination of the mAb titer using affinity chromatography. The sample is injected on a protein A column (from *Staphylococcus aureus*), which retains the mAb. Protein A has strong affinity for the Fc domain of the therapeutic mAb, which allows its separation from matrix components (for example, cell culture supernatant). Retention conditions feature a phosphate buffer (pH 7.4). The mAb is quickly eluted as a sharp peak using a fast gradient towards an acidic mobile phase. This first dimension allows integration of the eluting peak and quantitation of the mAb concentration (titer) using UV or DAD detection. In this setup, a short 3.7 mm detector flow cell was installed to reduce the signal intensity, and prevent saturation of the UV signal.

The protein A peak was subsequently transferred to the second dimension. An 80 µL loop was installed on a 2-Position/6-Port Valve located in the column compartment of the protein A column. The retention time of the mAb in the protein A separation is very stable, allowing reproducible heart-cutting of the peak. The transfer to the second dimension was done by switching the valve at a time predefined in the general method, not controlled by the 2D-LC software. This loads the loop content onto the SEC column for separation of the mAb size variants. The SEC column was operated with a phosphate buffer pH 7, and separated the high mol wt variants (HMWs) from the main peak (the mAb) and from low mol wt variants (LMWs). These variants are detected by a second DAD, and the amount of HMWs and LMWs can be calculated.

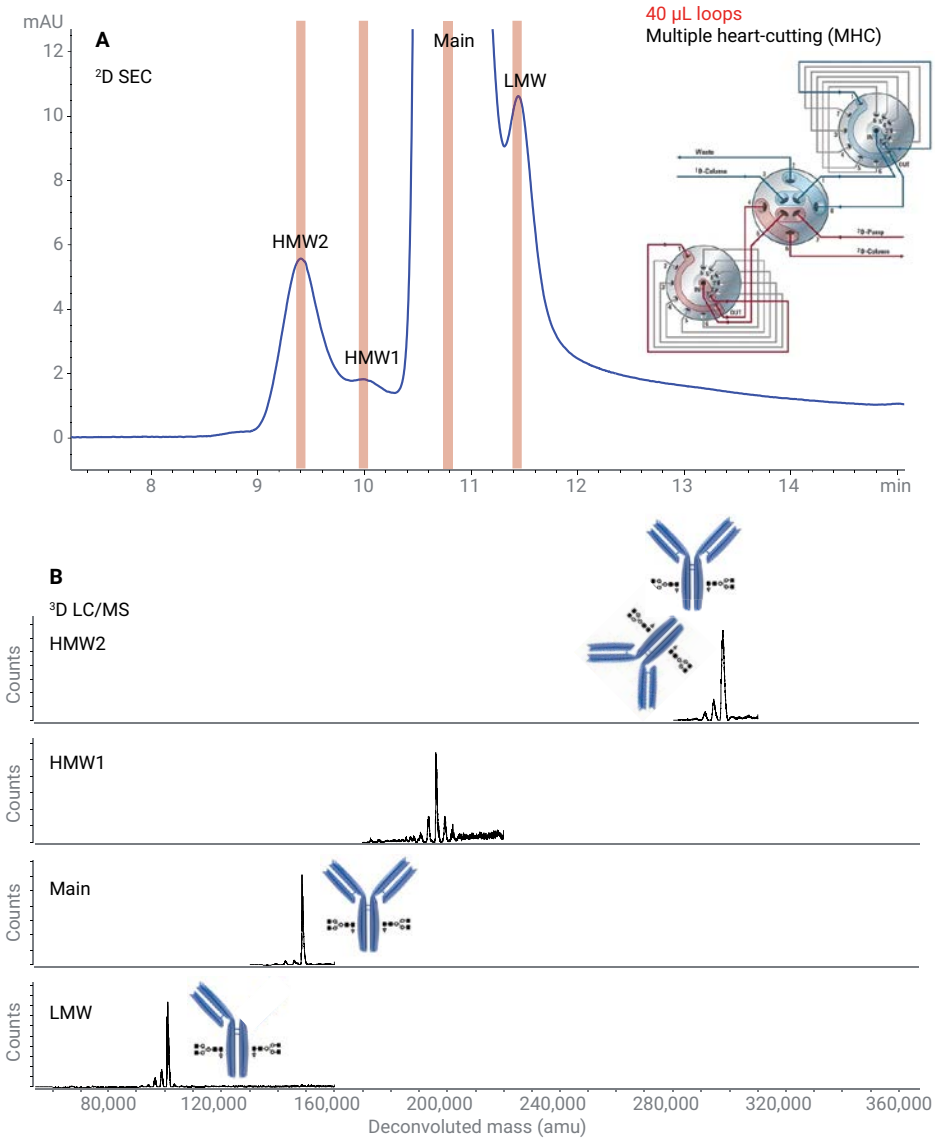

Figure 4. 2D SEC chromatogram of transferred protein A peak (220 nm) and 3D LC/MS analysis of SEC fractions. The different HMW and LMW variants as well as the main peak were collected in 40 µL loops installed on a MHC valve and subsequently transferred individually to the LC/MS. The third chromatographic dimension (RPLC) was primarily used to desalt the SEC peaks prior to MS analysis.

The actual mol wt of the various size variants is determined online using a third chromatographic dimension using 2D-LC software and the MHC valves. This setup allows sampling of multiple fractions from the SEC second dimension and analysis of each of them on RPLC. Peaks are parked in one of the 12 loops available on the MHC valves and then desalted on the online desalting cartridge. The parking location and time programming of the next dimension separation are controlled by the 2D-LC software in the OpenLab CDS ChemStation.

The RPLC cartridge serves as a desalting tool to separate the protein from the sodium phosphate present in the SEC mobile phase. This is necessary to prepare the sample for the final step in the analysis: high-resolution mass spectrometry (HRMS). The polymeric-based stationary phase retains the mAb-related analytes while the salts are flushed through the column under highly aqueous mobile phase conditions. A gradient towards stronger mobile phase conditions elutes the desalted mAb or size variants from the column into the MS, where its mol wt is determined. An additional 2-Position/6-Port Valve is installed between the column and the MS source to divert the salts to the waste. The RPLC gradient and diverter valve switching are repeated several times (depending on the number of peaks sampled from SEC) during one multi-attribute run. Both are fully controlled by the 2D-LC software.

The 6530 Q-TOF LC/MS equipped with the Jet Stream technology ESI source generates mass spectra populated with multiple-charged mAb ions (charge-state envelope). These mass spectra are then deconvoluted to reveal the actual mol wt of the compounds.

These steps are fully automated on a single system. The determination of mAb titer, size variants, and mol wt is completed in 90 minutes.

For the therapeutic mAb in development, an SEC peak purity of 95.6% is revealed (Figure 3). The MS data confirm the cloned amino acid sequence, and provides information in the glycosylation profile (Figure 5). The HMW2 variant present at 1.0% with a measured mol wt of ±300 kDa is identified as an mAb dimer (Figures 3 and 4). Based on the MS data, the LMW variant present at 3.2% corresponds to the truncated mAb resulting from a clip in the hinge region (Figures 3 and 4).

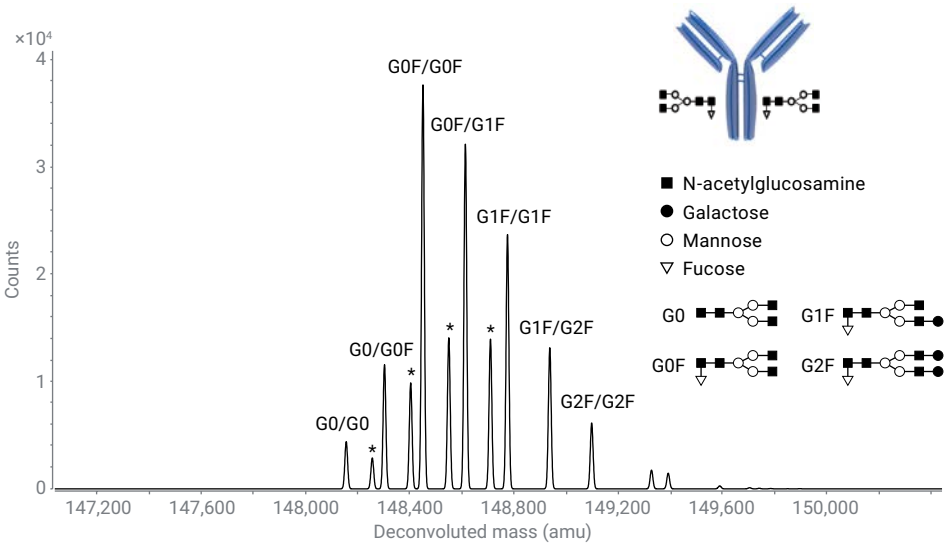

Figure 5. [3]D LC/MS analysis of main SEC peak. This deconvoluted spectrum confirms the cloned amino acid sequence and reveals the glycosylation pattern. * Corresponds to phosphate adducts.

The method performance was evaluated for both [1]D protein A chromatography (one peak) and [2]D SEC (four peaks). The precision of retention time, peak area, ratio of the size variants, and linearity were determined. The stability of retention time in both dimensions is crucial for accurate and reproducible heart-cutting of the targeted compounds. Figure 6 shows the results of four consecutive analyses. These data show that the method is fit for purpose, and that heart-cutting can be done accurately in first and second dimensions.

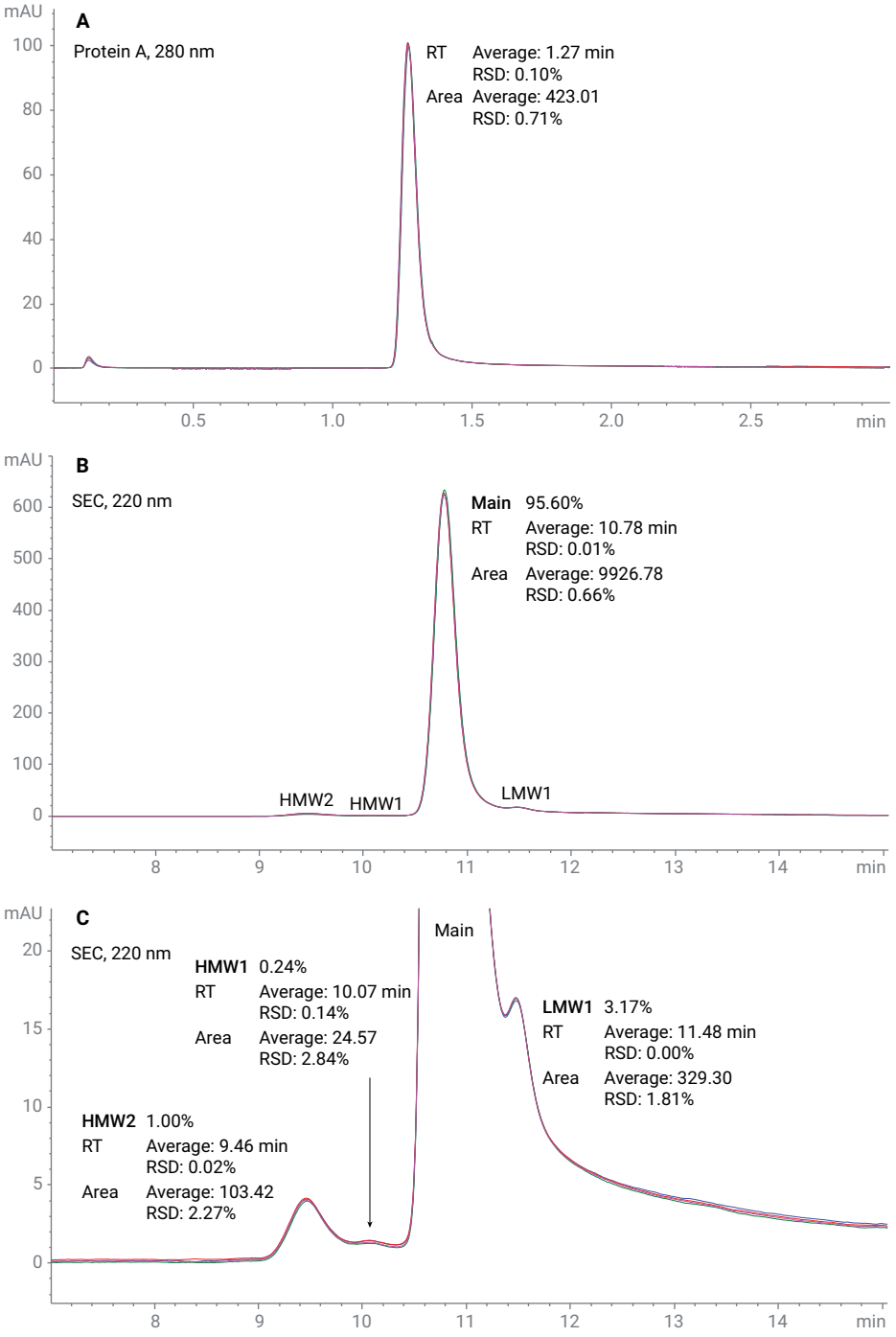

Figure 6. [1]D protein A (280 nm) and [2]D SEC (220 nm) chromatograms of four consecutive injections of the sample (10 µg load). Precision data are included in the chromatograms.

A calibration curve was composed by injection of the therapeutic mAb at a concentration of 0.5 µg/µL (two replicates), 2, and 5 µg/µL (single injections). With an injection volume of 20 µL; this corresponds to 10, 40, and 100 µg loaded on the protein A column (Figures 7 and 8).

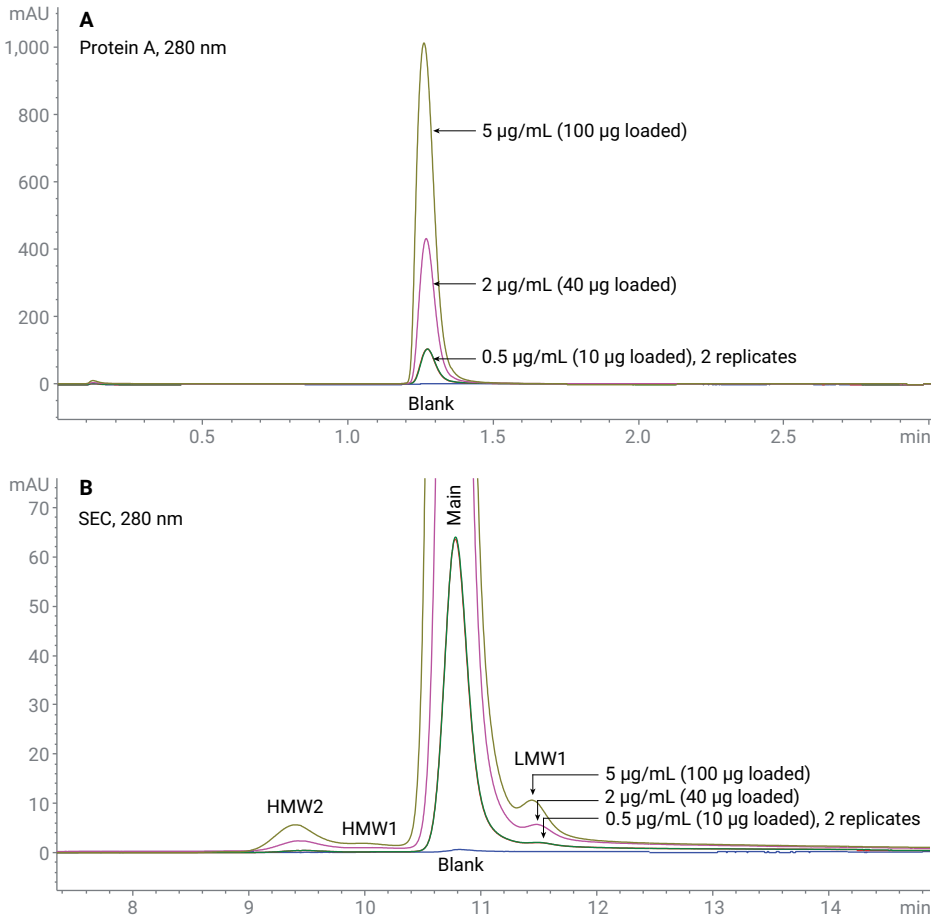

Figure 7. [1]D Protein A (280 nm) and [2]D SEC (280 nm) chromatograms for a blank injection and three calibration levels. 10/40/100 µg sample was loaded.

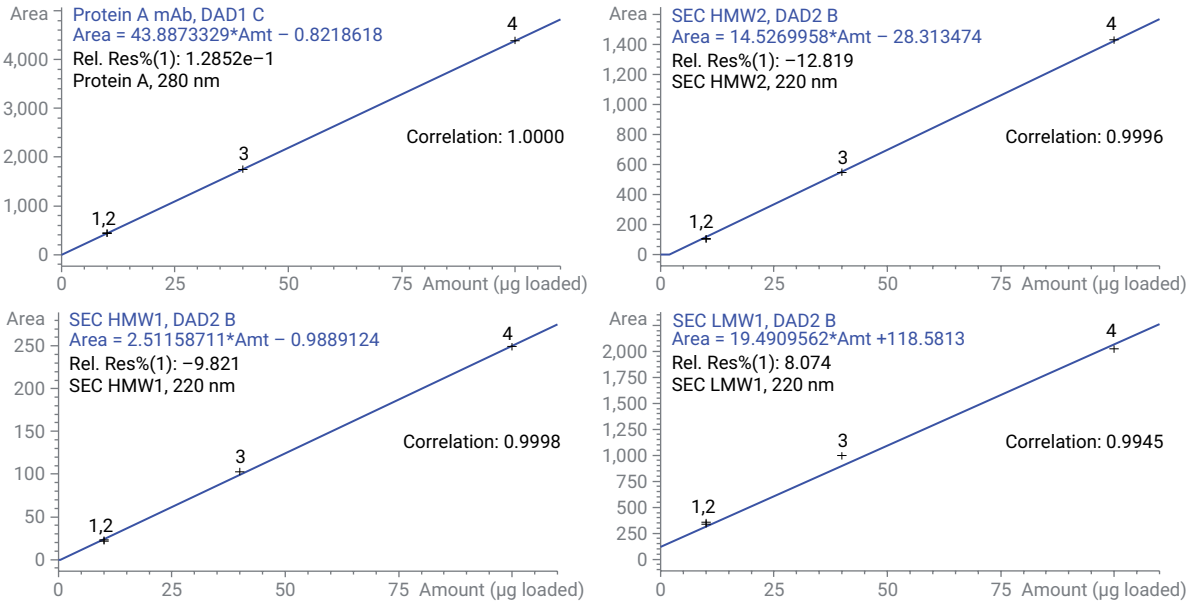

Figure 8. [1]D Protein A (280 nm) and [2]D SEC (220 nm) calibration curves for 10/40/100 µg sample loaded.

The multi-attribute analyzer was subsequently applied to guide mAb clone selection in the development of an Actemra biosimilar (scientific name: tocilizumab). Therefore, the Actemra originator and supernatants of two tocilizumab-producing CHO clones were subjected to the 3D setup. Obtaining complimentary information is vital for well considered clone selection during the development of biosimilar mAbs. Biosimilar developers try to select the clone that produces the mAb at high concentration, and with similar characteristics to the originator product. Figure 9 shows the ^{1}D Protein A and ^{2}D SEC chromatograms as well as the ^{3}D LC/MS spectra associated with the main peak of an Actemra originator and two tocilizumab-producing CHO clones. The spectrum of clone A is similar to the spectrum of the originator, and corresponds to the mAb carrying the complex type mammalian N-glycans G0F, G1F, and G2F with the N- and C-termini of the heavy chains being, respectively, cyclic (pyroglutamate) and truncated Lys. The spectrum associated with tocilizumab-producing CHO clone C shows a deviating profile with a shift of the entire glycosylated envelope to mol wt values that are 68 Da lower as a result of a point mutation in the variable part of the heavy chain (Phe to Ile/Leu). According to US and European regulatory authorities, an identical primary sequence is required for similarity, ruling out CHO clone C from further development.

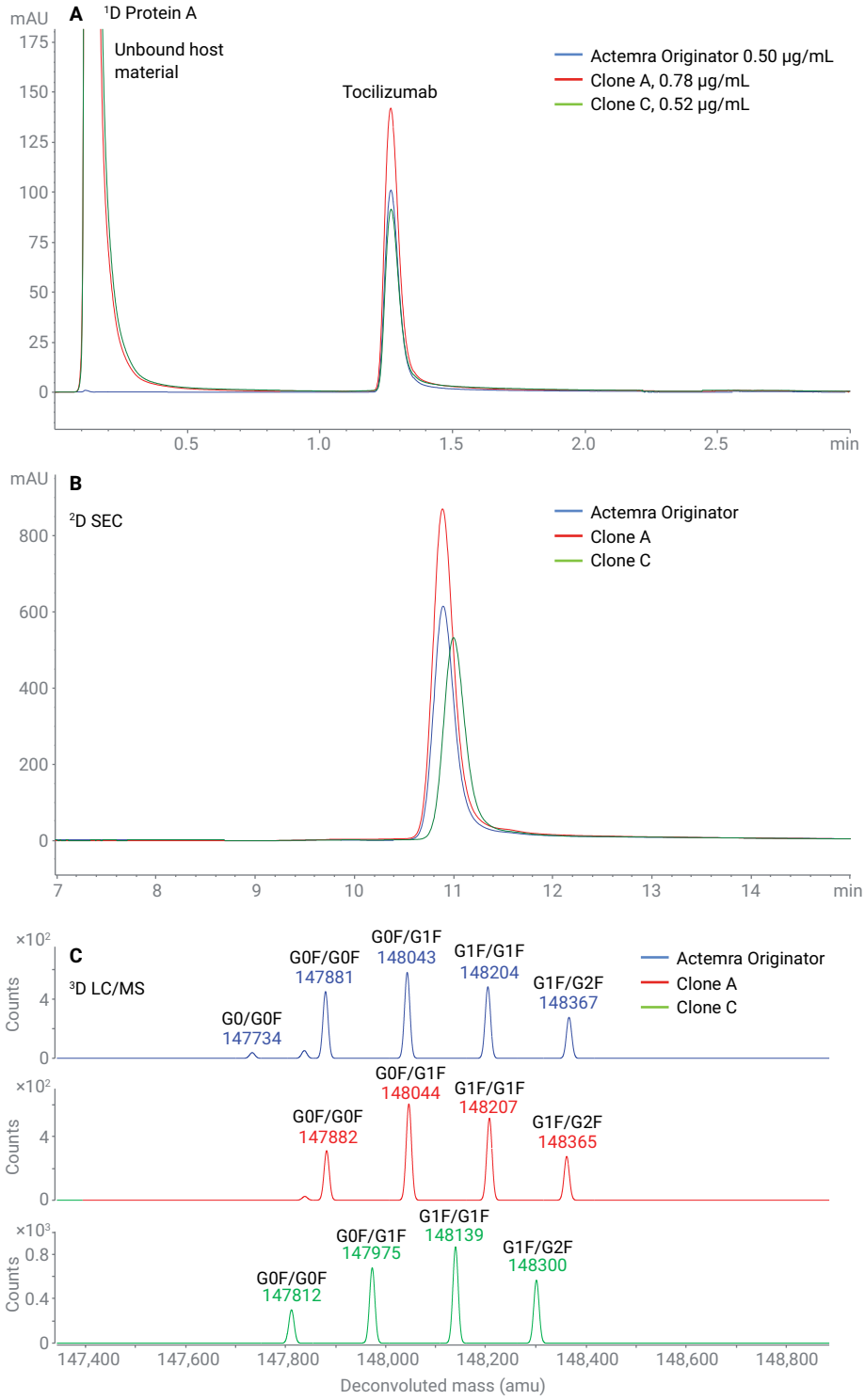

Figure 9. Multi-attribute analysis in the context of biosimilar development. An Actemra originator and supernatants of two tocilizumab-producing CHO clones (clones A and C) were subjected to protein A affinity chromatography, SEC, and LC/MS in a 3D setup. (A) ^{1}D protein A affinity chromatogram (280 nm). The peak at retention time 1.2 minutes was sampled in an 80 µL loop installed on an Agilent InfinityLab Quick Change 2-Position/6-Port Valve, and transferred to the second dimension. (B) ^{2}D SEC chromatogram (220 nm). The main peak was collected in a 40 µL loop installed on a multiple heart-cutting valve, and subsequently transferred to the LC/MS. (C) ^{3}D LC/MS spectra of the main peak.

Conclusion

Protein A affinity chromatography, SEC, and LC/MS were combined in an MHC 3D setup making use of the Agilent 1290 Infinity II 2D-LC System and the Agilent 6530 Q-TOF LC/MS. This setup allowed the fully automated determination of mAb titer and structural aspects such as aggregation, fragmentation, mol wt, amino acid sequence, and post-translational modifications directly from cell culture supernatants. The multi-attribute analyzer was successfully used for the characterization of a therapeutic mAb in development, and to guide mAb clone selection. The performance of the method was further assessed by replicate injections of an mAb, and by running a dilution series of an mAb.

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Online 2D-LC Characterization of Monoclonal Antibodies with Size Exclusion and Weak Cation Exchange Chromatography

In this study, heart-cutting 2D-LC enables online transfer of peaks from the first dimension to the second dimension, allowing highly precise aggregate analysis and charge variant profiling within 13 minutes, and offering a combined online analysis of two of the most important quality attributes. In addition, high-resolution sampling 2D-LC enables the detection of coeluting compounds and delivers accurate 2D-LC quantification by the transfer of the entire 1D peak area to the second dimension.

Application Note

Biotherapeutics
and Biologics

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Online 2D-LC Characterization of Monoclonal Antibodies with Size Exclusion and Weak Cation Exchange Chromatography

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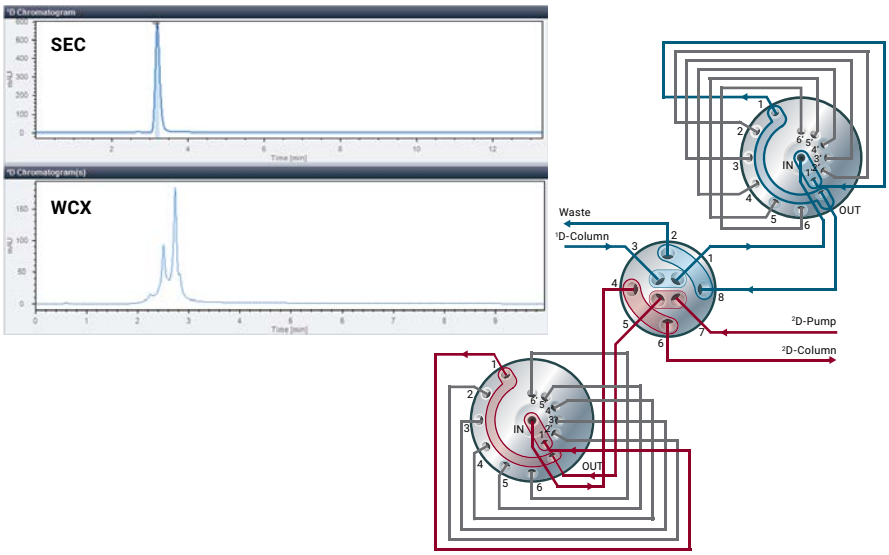
Abstract

The characterization of biopharmaceuticals can be highly complex and extensive to evaluate all important quality attributes. Online 2D-LC analysis offers a combination of different analysis methods to monitor different parameters in one run. This Application Note shows a combination of aggregate analysis with size exclusion chromatography in the first dimension, followed by ion exchange chromatography for charge variant profiling in the second dimension. Heart-cutting 2D-LC enables online transfer of peaks from the first dimension to the second dimension, allowing highly precise aggregate analysis and charge variant profiling within 13 minutes, and offering a combined online analysis of two of the most important quality attributes. In addition, high-resolution sampling 2D-LC enables the detection of coeluting compounds and delivers accurate 2D-LC quantification by the transfer of the entire 1D peak area to the second dimension.



Introduction

Detailed and in-depth characterization of biopharmaceuticals is of extreme importance to ensure their safety and efficacy from triggering unpredictable immunogenic responses. Different parameters have to be monitored during various stages in the development and production of the product. In early phases of process development, an enormous number of samples need to be screened. Those extensive studies include important quality attributes such as titer analysis, aggregation studies, charge variant and glycan profiling, peptide mapping, and many others. Due to the extent and complexity of the used methods, full characterization of biopharmaceuticals is an elaborative, time-consuming, and cost-intense business. Two of the most important quality attributes in monoclonal antibodies (mAbs) are aggregation and charge variant analysis[1].

With the Agilent 1290 Infinity II 2D-LC solution, two analysis types can be combined in an online setup to increase efficiency and reduce hands-on time (no fraction collection and reinjection necessary). In addition to the two classical 2D-LC modes: (multiple) heart-cutting and comprehensive 2D-LC, acting complementary to each other, high-resolution sampling 2D-LC combines advantages of both heart-cutting and comprehensive 2D-LC. Up to 10 consecutive cuts can be defined for the first dimension with the same valve setup that is used for multiple heart-cutting 2D-LC. Especially in biochromatography such as affinity chromatography, size exclusion (SEC), or ion exchange chromatography (IEX), high-resolution sampling can be helpful by enabling the analysis of larger areas of interest or broad unresolved peaks in the first dimension.

This Application Note shows the combination of aggregation analysis using SEC with weak cation exchange chromatography (WCX) for a subsequent charge variant profiling of an mAb using heart-cutting 2D-LC for a short online analysis in 13 minutes. In addition, high-resolution sampling offers the option to transfer the complete 1D peak to the second dimension to identify potential coeluting impurities as well as to enable accurate 2D-LC quantification.

Experimental

Instrumentation

The Agilent 1290 Infinity II 2D-LC Solution was comprised of the following modules:

- **First Dimension Pump**: Agilent 1260 Infinity Bio-Inert Quaternary Pump (G5611A)
- **Second Dimension Pump**: Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B) with cooler
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity Valve Drive (G1170A) with 2-position/4-port duo-valve (2D-LC) valve head (G4236A)
- 2× Agilent 1290 Infinity Valve Drives (G1170A) with 2x multiple heart-cutting valves (G4242-64000) equipped with 40-µL loops
- 2× Agilent 1290 Infinity II Diode Array Detectora (G7117B) with 10-mm Max-Light cartridge cell (G4212-60008)

Columns

Agilent AdvanceBio SEC 300Å, 4.6 × 150 mm, 2.7 µm (p/n PL1580-3301)

Agilent Bio MAb, non-porous, 4.6 × 50 mm, 1.7 µm, stainless steel (p/n 5190-2401)

Software

Agilent OpenLab CDS ChemStation Edition software, version C.01.07 [27] with Agilent 1290 Infinity 2D-LC software, version A.01.03

Solvents 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 chloride and monobasic and dibasic sodium phosphate were purchased from Sigma-Aldrich, St. Louis, MO, USA.

An mAb solution was injected in a 50 mM phosphate buffer, pH 6.2 (10 mg/mL). It was filtered using an Agilent Captiva Premium Syringe Filter, regenerated cellulose membrane, 15-mm diameter, 0.45 µm pore size (p/n 5190-5109).

Results and Discussion

An mAb was analyzed for aggregates using the Agilent AdvanceBio SEC 300Å in the first dimension. Almost baseline separated, a small aggregate peak was detected in front of the main peak (Figure 1 inset). After integration, an aggregate amount of 1 % was calculated.

Chromatographic conditions

Parameter	Value
First-dimension parameters	
1D column	Agilent AdvanceBio SEC 300 Å
1D mobile phase	50 mM Phosphate buffer, pH 6.2
1D flow rate	0.5 mL/min
Second-dimension parameters	
Mode	Heart cutting and High-resolution sampling
2D column	Agilent Bio mAb
2D mobile phase	A) 25 mM Phosphate buffer, pH 6.2 B) 500 mM Sodium chloride in 25 mM phosphate buffer, pH 6.2
2D gradient stop time	6 minutes
2D cycle time	10 minutes
2D flow rate	0.5 mL/min
2D gradient	0.00 minutes – 5 % B 5 minutes – 30 % B 6 minutes – 40 % B
2D time segments	**Heart cutting** Time 1D 3.13 Mode Time based Sampling time 0.11 **High-resolution sampling** Time 1D 3.05 Mode Time based Sampling time 5 seconds Cuts 8
Injection volume	5 µL
Thermostat autosampler	6 °C
Column temperature	RT
DADs	280 nm, 4 nm, Ref. 360 nm, 100 nm
Peak widths	0.025 minutes (0.5 seconds response time) (10 Hz)

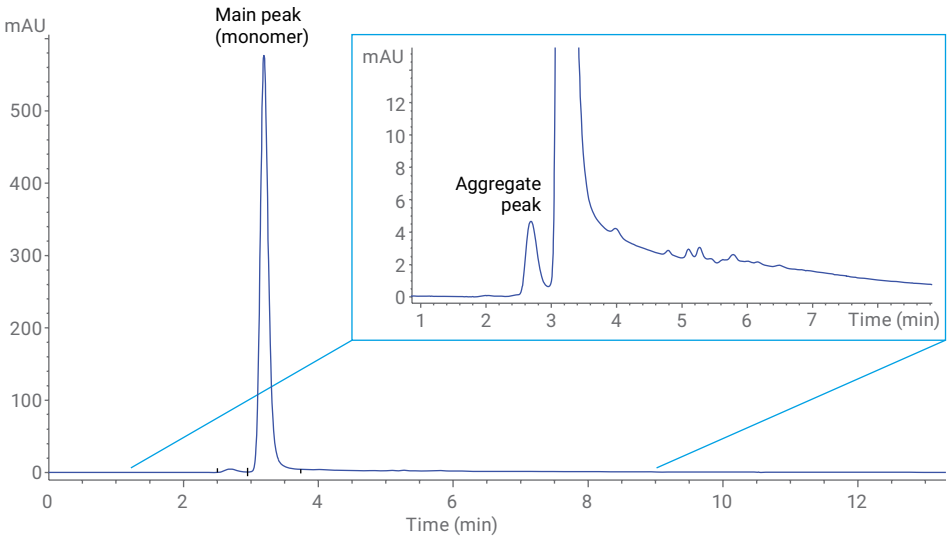


Figure 1. Aggregate analysis after size exclusion chromatography using an Agilent AdvanceBio SEC 300Å, 4.6 × 150 mm, 2.7 µm column.

A heart-cut from the main peak was transferred to the second dimension for charge variant analysis using a sub-2 µm column, the Agilent Bio MAb, nonporous, 4.6 × 50 mm, 1.7 µm for optimal resolution in a short run time. Figure 2 shows the 2D-LC Viewer with the first dimension (aggregate analysis) in the upper chromatogram, where the heart-cut that is transferred into the second dimension for charge variant analysis (lower chromatogram) is marked. In addition to the main peak, the charge variant analysis reveals two acidic and one basic variant. Acidic and basic species are defined based on their retention times (RTs) relative to the main peak. The variants eluting before the main peak in cation exchange chromatography are defined as acidic, whereas the variants eluting later than the main peak are defined as basic[2].

The short (50-mm) sub-2 µm Agilent Bio MAb column enables highly resolving charge variant analysis in the second dimension, even for shorter run times. Compared to traditional charge variant analyses with 30 to 40 minutes

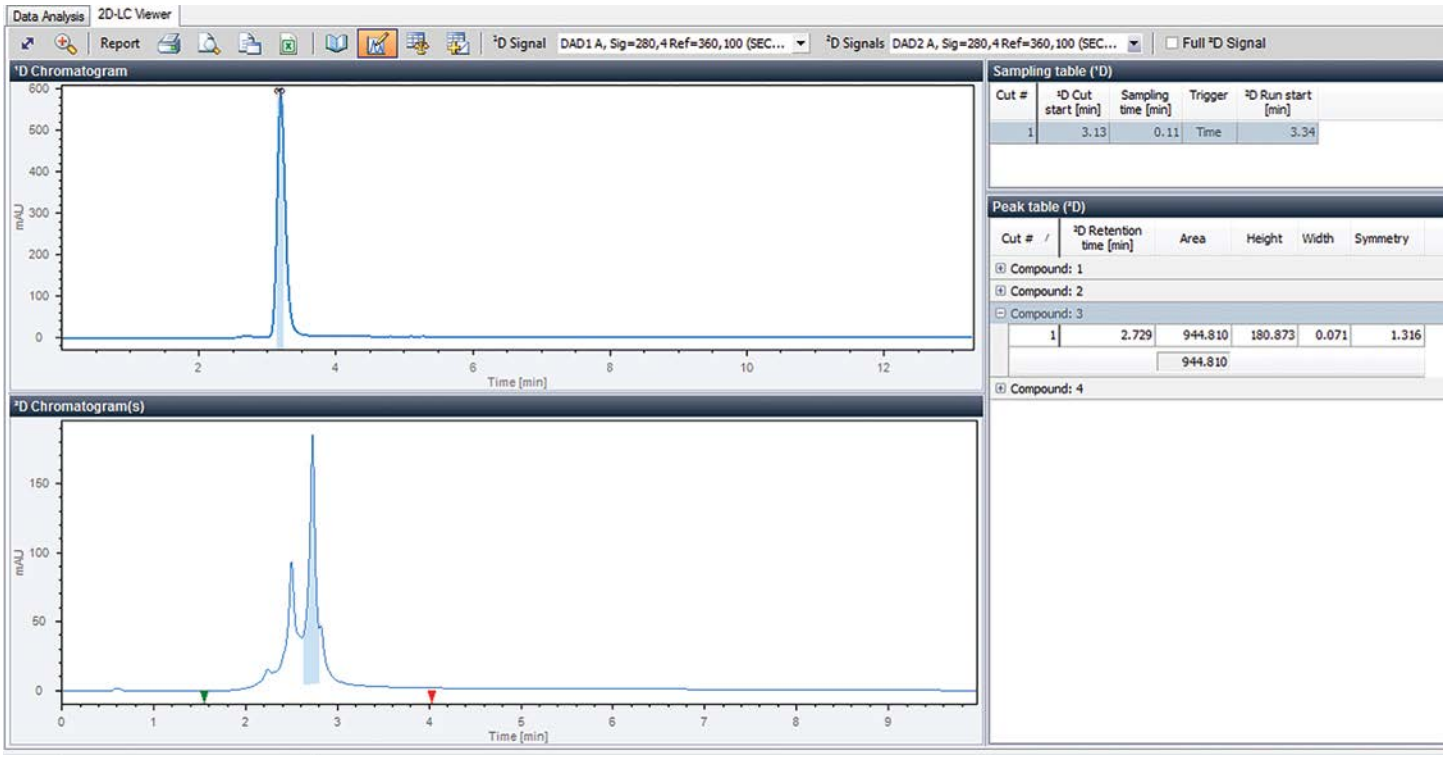


Figure 2. 2D-LC viewer with SEC aggregate analysis in the first dimension (A), and WCX charge variant analysis in the second dimension (²D signal, B). The ²D peak table (C) allows a closer look into the second dimension chromatogram. The marked compound in the ²D peak table is shown as the blue region in the ²D chromatogram. The green and the red arrows mark the integrated area in the second dimension.

run times on 250-mm columns (5-µm particle size), here, a total run time of 10 minutes is enabled[3]. The cycle time for the complete 2D-LC run was approximately 13 minutes, including column regeneration.

Precision of retention time and area was determined in the first and second dimension for seven consecutive injections. In the first dimension, the aggregate and main peak were evaluated. Four peaks were evaluated in the second dimension: two acidic variants, one main peak, and one basic variant. Figure 3 shows an overlay of seven consecutive runs in the 2D-LC Viewer for the first and second dimension, together with the precision values for RT and peak area. For the first dimension, the RT and area precision was excellent, with < 0.08 and < 0.5 %, respectively. The second dimension precision for RT and area was also excellent, with < 0.09 and < 2.1 %, respectively.

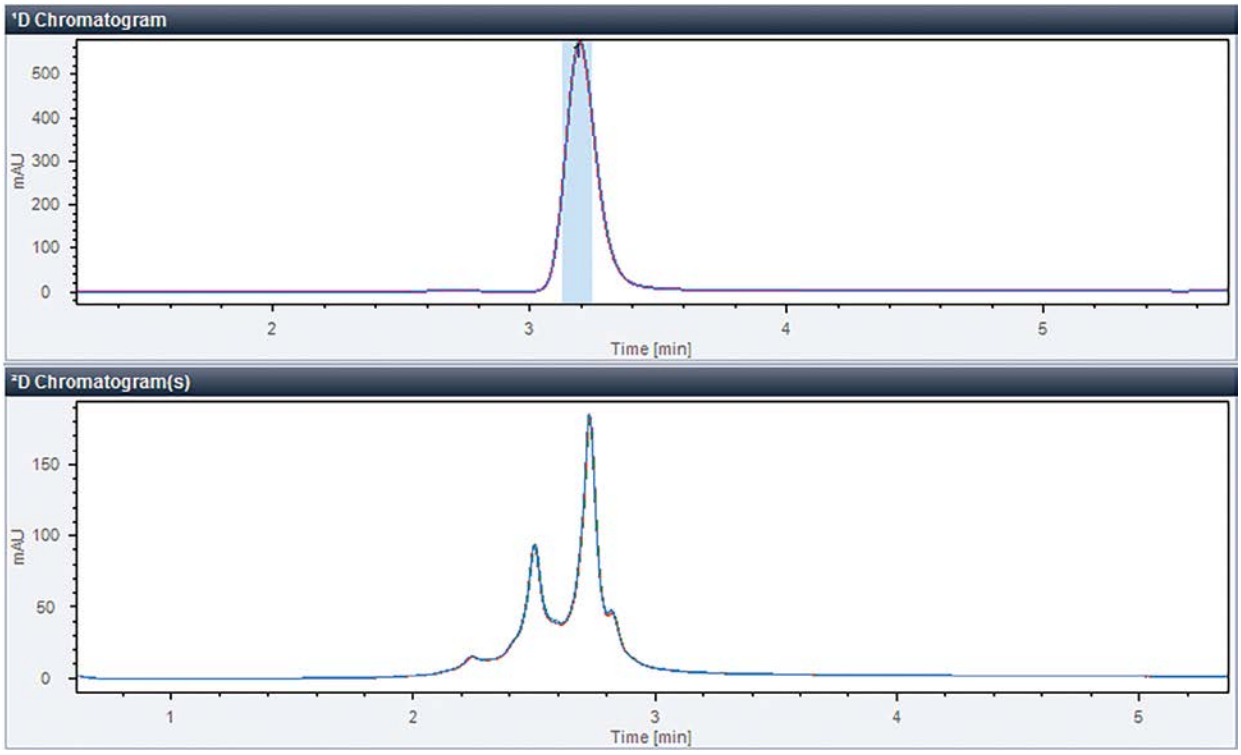


Figure 3. Overlay of seven consecutive 2D-LC runs: 1D aggregate analysis and 2D charge variant analysis.

To check for coeluting impurities after SEC analysis, the main peak was sampled into eight cuts using high-resolution sampling to include the complete peak from the first dimension (Figure 4). No coeluting impurities were detected after WCX in the second dimension, as all resulting eight chromatograms (eight cuts) were similar, only differing in signal intensity.

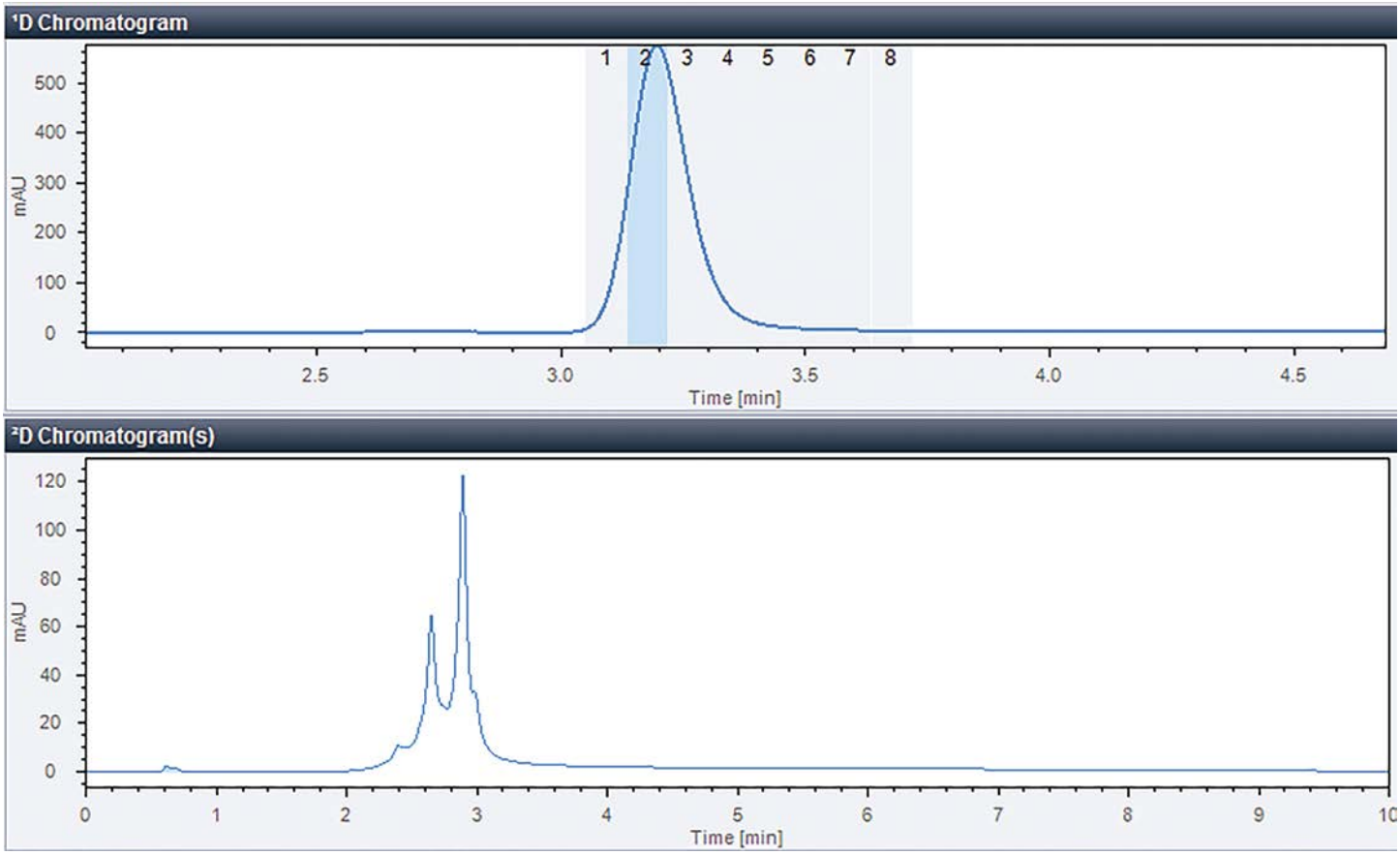


Figure 4. The 2D-LC analysis with SEC aggregate analysis in the first dimension, showing the chosen HiRes sampling set of cuts for subsequent charge variant analysis in the second dimension. No coeluting impurities were detected.

Conclusion

Aggregation and charge variant analysis were combined using the Agilent 1290 Infinity II 2D-LC solution for the analysis of mAbs. High resolution and excellent precision was found for both dimensions in a short combined SEC-WCX run within 13 minutes total cycle time using heart-cutting 2D-LC chromatography. The number of aggregates was determined to be approximately 1 % of the main peak. Charge variant analysis in the second dimension revealed two acidic and one basic variant, in addition to the main peak. Potential coeluting impurities could ideally be detected using high-resolution sampling where the relatively broad peak from the first SEC dimension was sampled in eight cuts, and transferred to the second dimension. In this case, no coeluting peaks were detected after WCX analysis in the second dimension.

References

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2. Du, Y.; *et al.* Chromatographic analysis of the acidic and basic species of recombinant monoclonal antibodies, *MAbs* Sep 1 **2012**, *4(5)*, 578–585.
3. Schneider, S. Simple Method Optimization in mAb Charge Variant Analysis using pH Gradients Generated from Buffer Advisor with Online pH and Conductivity Monitoring, Agilent Technologies Application Note, publication number 5991-3365EN, **2014**.

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Identifying Monoclonal Antibody Mutation Sites Using the Agilent 1290 Infinity II 2D-LC Solution with Q-TOF LC/MS

We describe the use of the Agilent 1290 Infinity II 2D-LC solution in the comparison of an infliximab originator and candidate biosimilar. RPLC×RPLC and SCX×RPLC peptide mapping revealed important differences that could be attributed to a double mutation in the heavy chain of the candidate biosimilar using quadrupole time-of-flight mass spectrometry.

Application Note

Biopharmaceuticals and Biosimilars

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Identifying Monoclonal Antibody Mutation Sites Using the Agilent 1290 Infinity II 2D-LC Solution with Q-TOF LC/MS

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Abstract

In recent years, 2D-LC has been highly promising for the detailed characterization and comparability assessment of protein biopharmaceuticals such as monoclonal antibodies. This Application Note describes the use of the Agilent 1290 Infinity II 2D-LC solution in the comparison of an infliximab originator and candidate biosimilar. RPLC×RPLC and SCX×RPLC peptide mapping revealed important differences that could be attributed to a double mutation in the heavy chain of the candidate biosimilar using quadrupole time-of-flight mass spectrometry.

Introduction

Monoclonal antibodies (mAbs) have emerged as important biopharmaceuticals. Today, more than 40 mAbs are marketed in the United States and Europe, of which 18 have attained blockbuster status, with sales more than doubled since 2008[1,2,3]. Over 50 are in late-stage clinical development.

The knowledge that the top-selling mAbs are, or will become, open to the market in the coming years has resulted in an explosion of biosimilar activities. In 2013, we witnessed the European approval of the first two mAb biosimilars (Remsima and Inflectra), which both contain the same active substance, infliximab[4]. In April 2016, Inflectra also reached marketing authorization in the US, and a third infliximab biosimilar (Flixabi) was recently approved in Europe. Remicade, infliximab's originator, reached global sales of $8.9 billion in 2013[3].

It is clear that the biosimilar market holds great potential, but it is simultaneously confronted with major hurdles. In contrast to generic versions of small molecules, exact copies of recombinant mAbs cannot be produced due to differences in the cell clone and manufacturing processes used. Even originator companies experience lot-to-lot variability. As a consequence, regulatory agencies evaluate biosimilars based on their level of similarity to, rather than the exact replication of, the originator. In demonstrating similarity, an enormous weight is placed on analytics, and both biosimilars and originators need to be characterized and compared in great detail. In contrast to small-molecule drugs, mAbs are large (approximately 150 kDa) and heterogeneous (due to the biosynthetic process and subsequent manufacturing and storage), making their analysis highly challenging[1,2].

Online two-dimensional liquid chromatography (2D-LC) is an emerging tool used to tackle this analytical complexity[5]. In online 2D-LC, peaks, parts, or the whole chromatogram are subjected to two different separation mechanisms. Online 2D-LC can be divided into two main types. In comprehensive two-dimensional LC (LC×LC), the entire effluent stream of the first (^{1}D) column is transferred to the second (^{2}D) column. In heart-cutting two-dimensional LC (LC-LC), one peak or one part of the chromatogram is transferred to the 2D column. Multiple peaks or multiple parts of the chromatogram also can be selected for transfer to the ^{2}D column (mLC-LC). Multiple heart-cutting two-dimensional LC (mCEX-RPLC) combined with high-resolution MS has recently been used at the protein level to identify the main isoforms of the mAb rituximab, and to characterize the antibody drug conjugate (ADC) ado-trastuzumab emtansine (marketed as Kadcyla)[6,7]. Comprehensive LC×LC has shown promise at the peptide level for the detailed characterization and comparability assessment of a Herceptin innovator and biosimilar, and assessment of drug conjugation sites in Kadcyla[7,8,9,10].

To further illustrate the attractiveness of LC×LC for the detailed characterization and comparability assessment of biopharmaceuticals, we describe the comparison of the tryptic digests of the originator infliximab (Remicade) and a biosimilar candidate for which the recombinant expression in Chinese hamster ovary (CHO) cells was going wrong. An Agilent 1290 Infinity II 2D-LC solution, operated in LC×LC mode, was combined with an Agilent 6530 Quadrupole Time-of-Flight (Q-TOF) LC/MS.

Experimental

Materials

Acetonitrile, methanol, formic acid (FA), and water were acquired from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid, dithiothreitol (DTT), iodoacetamide (IAM), NaCl, NH_4HCO_3, NaH_2PO_4, and phosphoric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA), and porcine sequencing-grade modified trypsin was from Promega (Madison, MA, USA). Tris-HCl was from Thermo Fisher Scientific (Waltham, MA, USA), and Rapigest was from Waters (Milford, MA, USA). Monoclonal antibodies were obtained from a local biotechnology company.

Sample preparation

Preparation of light and heavy chains

A 100 mM amount of DTT was added to the sample to a final concentration of 10 mM DTT following sample dilution to 0.2 mg/mL using 100 mM Tris-HCl, pH 8.0. Reduction was performed at 60 °C for 1 hour.

Trypsin digestion

To a volume corresponding to 100 µg of protein, 105 µL of 0.1 % Rapigest in 100 mM Tris-HCl, pH 8, was added followed by the addition of 100 mM Tris-HCl, pH 8, to a final volume of 192.5 µL. The sample was subsequently reduced at 60 °C for 30 minutes by the addition of 5 mM DTT (2.5 µL of 400 mM DTT in 100 mM Tris-HCl), and alkylated at 37 °C for 1 hour by adding 10 mM IAA (5 µL of 400 mM IAA in 100 mM Tris-HCl). Digestion proceeded for 16 hours at 37 °C using trypsin as protease, added at an enzyme-to-substrate ratio of1:25 (w:w). Lyophilized trypsin (20 µg) dissolved in 100 mM Tris-HCl (50 µL) was added in a volume of 10 µL, for a final sample volume of 210 µL.

LC instrumentation (middle-up)

- Agilent 1290 Infinity II High Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with a 10-mm flow cell
- Agilent 6540 Accurate-Mass Quadrupole Time-of-Flight LC/MS (G6540A)

2D-LC instrumentation (peptide mapping)

- Agilent 1290 Infinity II High Speed Pump (G7120A) with seal wash option (first dimension)
- Agilent 1290 Infinity II High Speed Pump (G7120A) (second dimension)
- Agilent 1290 Infinity II Multisampler (G7167B)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B)
- Agilent 1290 Infinity Valve Drive (G1170A)
- Agilent InfinityLab Quick Change 2-position/4-port duo valve for 2D-LC equipped with two 40-µL loops (G4236A)
- Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS (G6530A)

Software

- Agilent OpenLab CDS ChemStation, revision C.01.07 with 2D-LC add-on software, revision A.01.02
- Agilent Technologies MassHunter for instrument control (B05.01)
- Agilent Technologies MassHunter for data analysis (B07.00)
- Agilent Technologies BioConfirm software for MassHunter (B07.00)
- GC Image LC×LC Edition Software for 2D-LC data analysis (GC Image, LLC., Lincoln, NE, USA)

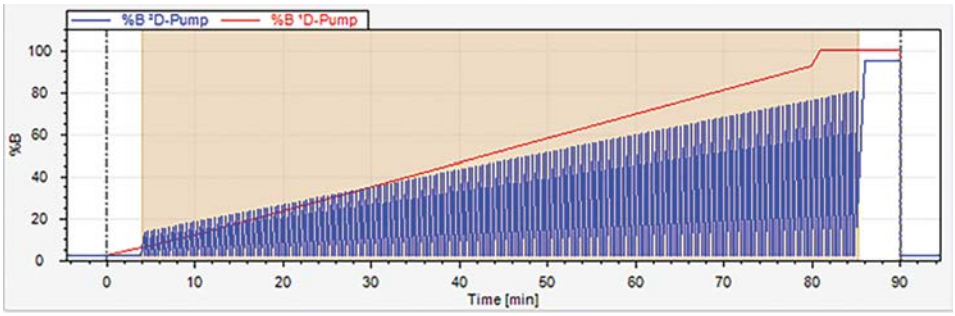
LC/MS method parameters (middle-up analysis)

RPLC-UV-MS	
Column	Agilent AdvanceBio RP-mAb C4, 50 × 4.6 mm, 3.5 µm
Solvent A	0.1 % TFA
Solvent B	0.1 % TFA in ACN
Flow rate	1 mL/min
Gradient	0 to 6 minutes – 30 to 42.5 %B 6 to 6.1 minutes – 42.5 to 95 %B 6.1 to 7.1 minutes – 95 %B 7.1 to 7.2 minutes – 95 to 30 %B 7.2 to 9.2 minutes – 30 %B
Temperature	80 °C
Injection	
Volume	10 µL
Temperature	4 °C
Needle wash	6 seconds flush port (solvent B)
Detection DAD	
Wavelength	Signal 214/8 nm
Detection MS	
Ionization	Agilent Jet Stream technology source, positive ionization
Drying gas	350 °C, 10 L/min
Nebulizer	50 psig
Sheath gas	350 °C, 11 L/min
Capillary voltage	3,500 V
Nozzle voltage	1,000 V
Fragmentor	200 V
Acquisition	High resolution (4 GHz), resolution 40,000 for m/z 1,000, 2 spectra/s

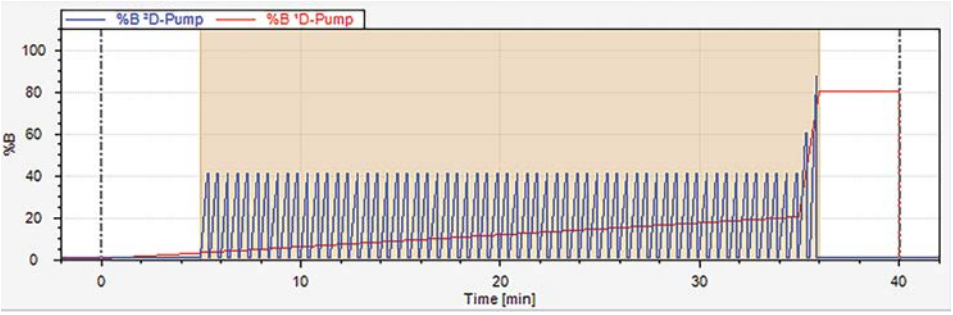
LC×LC/MS method parameters (peptide mapping)

	SCX×RPLC	RPLC×RPLC
First dimension		
Column	Agilent Bio SCX, NP 10 (PEEK), 2.1 × 250 mm, 10 µm	Agilent ZORBAX Bonus-RP, 2.1 × 150 mm, 3.5 µm
Solvent A	10 mM Phosphate pH 3	10 mM NH_4-Bicarbonate pH 8
Solvent B	10 mM Phosphate pH 3 + 1 M NaCl	MeOH:ACN 50:50
Flow rate	65 µL/min	80 µL/min
Gradient	0 to 35 minutes – 0 to 20 %B 35 to 36 minutes – 20 to 80 %B 36 to 40 minutes – 80 %B Post time – 10 minutes at 0 %B	0 to 80 minutes – 2 to 92 %B 80 to 81 minutes – 92 to 100 %B 81 to 90 minutes – 100 %B Post time – 9 minutes at 2 %B
Temperature	23 °C	23 °C
Second dimension		
Column	Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 3.5 µm	Agilent ZORBAX Eclipse Plus C18, 4.6 × 50 mm, 3.5 µm
Solvent A	0.1 % Formic acid in water	0.1 % Formic acid in water
Solvent B	ACN	ACN
Flow rate	3.5 mL/min	3.5 mL/min
Idle Flow rate	0.4 mL/min	0.5 mL/min
Initial gradient	0 to 0.35 minutes – 1 to 41 %B 0.35 to 0.40 minutes – 41 %B 0.40 minutes – 1 %B	0 to 0.35 minutes – 2 to 10 %B 0.35 minutes – 2 %B
Gradient modulation	Full-in-fraction with increased %B at end for cleaning 1 %B at 0 minutes 41 %B at 0.35 minutes to 41 %B at 35 minutes to 95 %B at 36 minutes	Constantly shifted %B and Δ%B 2 %B at 0 minutes to 2 %B at 85 minutes to 95 %B at 86 minutes (10 %B at 0.35 minutes to 80 %B at 85 minutes)
Temperature	50 °C	55 °C
Modulation		
Modulation on	5 to 36 minutes	4 to 85 minutes
Loops	Two 40-µL loops, cocurrent configuration	Two 40-µL loops, cocurrent configuration
Modulation time	0.50 minutes	0.40 minutes
Injection		
Volume	8 µL	8 µL
Temperature	4 °C	4 °C
Needle wash	6 seconds flush port (50 % ACN)	6 seconds flush port (50 % ACN)
Detection MS		
Ionization	Agilent Jet Stream technology source, positive ionization	Agilent Jet Stream technology source, positive ionization
Drying gas	350 °C, 11 L/min	350 °C, 11 L/min
Nebulizer	55 psig	55 psig
Sheath gas	400 °C, 9 L/min	400 °C, 9 L/min
Capillary voltage	3,500 V	3,500 V
Nozzle voltage	1,000 V	1,000 V
Fragmentor	175 V	175 V
Acquisition		
	Extended Dynamic Range (2 GHz)	Extended Dynamic Range (2 GHz)
Resolution	10,000 for *m/z* 1,000	10,000 for *m/z* 1,000
MS	8 spectra/s	8 spectra/s
MS/MS	Data Dependent MS/MS, 8 spectra/s	Data Dependent MS/MS, 8 spectra/s
MS/MS Threshold (Abs)	1,000	1,000
Isolation width	~4 *m/z*	~4 *m/z*
Collision energy	(3.6 × *m/z*)/100−4	(3.6 × *m/z*)/100−4

RPLC×RPLC gradient



SCX×RPLC gradient



Results and Discussion

Figure 1A shows the LC/UV-MS middle-up analysis of an infliximab originator and a candidate biosimilar after chemical reduction to cleave the disulfide bridges. Separation was obtained on a wide-pore superficially porous C4 column (AdvanceBio RP-mAb). The light-chain (Lc) peaks nicely overlay, but a retention difference is noted for the heavy-chain (Hc) peaks. When combining the separation with Q-TOF MS, a 99-Da mass increase is demonstrated on the biosimilar Hc (*m/z* 50,833.0 in the originator, and 50,931.9 in the biosimilar) explaining this retention time shift (Figure 1B).

LC×LC peptide mapping was performed subsequently to elucidate the origin of this retention time and mass difference. Compared to 1D-LC, LC×LC is known to increase substantially the chromatographic resolution as long as the two dimensions are orthogonal, and the separation obtained in the first dimension is maintained upon transfer to the second dimension. Orthogonal combinations for 2D-LC-based peptide mapping are: strong cation exchange × reversed-phase LC (SCX×RPLC), hydrophilic interaction chromatography × reversed-phase LC (HILIC×RPLC) and reversed-phase LC × reversed-phase LC (RPLC×RPLC) at different pH values in the two dimensions[8].

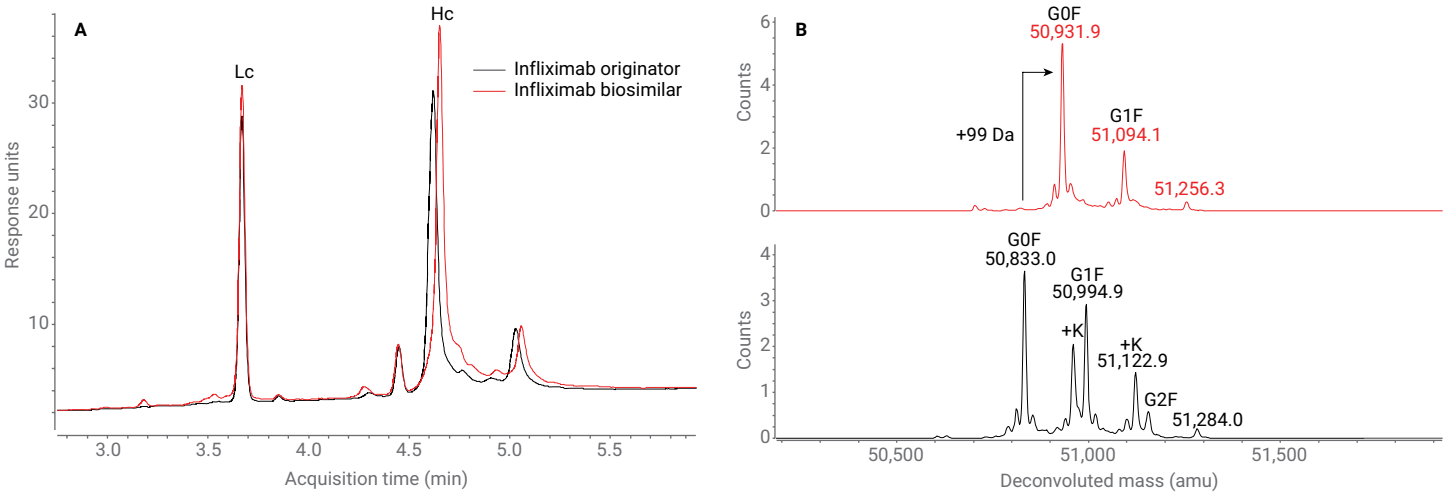


Figure 1. RPLC/Q-TOF MS middle-up analyses of infliximab originator (black) and candidate biosimilar (red) on an Advance Bio RP-mAb column. A) Chromatogram showing the Lc and Hc peaks, and (B) the deconvoluted mass spectra recorded for the Hc showing the glycoforms (G0F, G1F, and G2F) and the +99-Da shift.

Figure 2 displays the LC×LC tryptic peptide maps of the infliximab originator and candidate biosimilar using the combination RPLC×RPLC. Both plots are similar, but some striking differences are noted, taking the MS data into consideration. The spots SLSLSPG and SLSLSPGK clearly present in the originator are replaced by one spot SLSLSPGI in the biosimilar, which, according to the mass spectral data, corresponds to the addition of an isoleucine (I) to SLSLSPG, or the replacement of lysine (K) by isoleucine in SLSLSPGK at the C-terminus of the heavy chain. From a biochemical standpoint, this makes sense and results in a positive move of 113 Da. The origin of the two spots SLSLSPG and SLSLSPGK in the originator mAb can be explained by the knowledge that heavy chains are historically cloned with a C-terminal lysine, but during cell culture production, host cell carboxypeptidases act on the antibody, resulting in the partial removal of these lysine residues. The presence of the lysine residues is also apparent in the deconvoluted Q-TOF MS spectra shown in Figure 1B. A small retention shift was also noted in another spot that could be identified by MS/MS as NYYGSTYDYWGQGTTLTVSSASTK in the originator, and as NYYGSSYDYWGQGTTLTVSSASTK in the biosimilar (Figure 3). From threonine (T) to serine (S) is −14 Da, and combining both modifications (+113 Da, −14 Da) results in a difference of 99 Da. Two point mutations are at the origin of this wrong recombinant expression (Figure 2). According to US and European regulatory authorities, identical primary sequence is primordial to similarity, thereby ruling out this candidate biosimilar from further development.

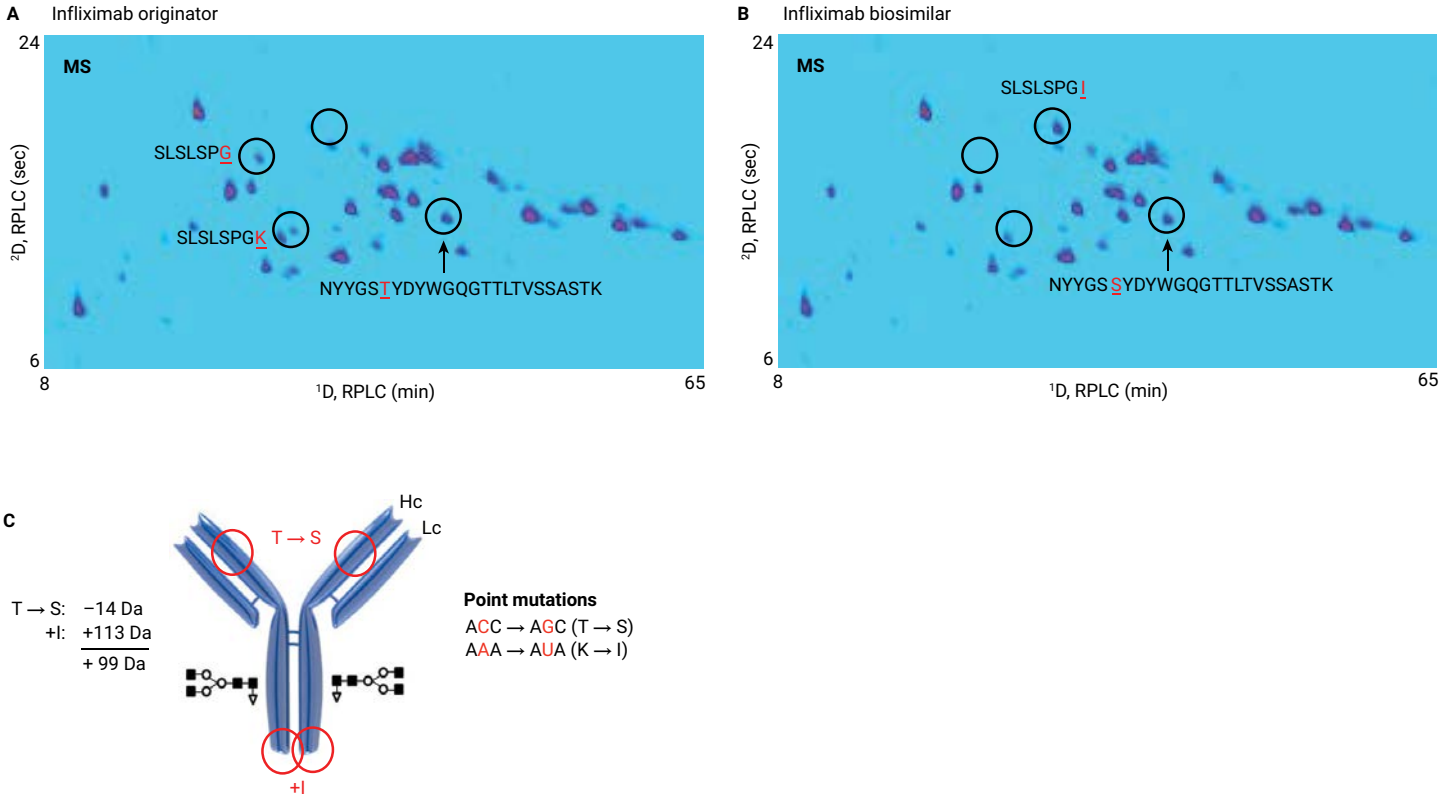


Figure 2. RPLC×RPLC/Q-TOF MS analysis of infliximab originator and candidate biosimilar, and schematic of the mAb with annotation of the modifications.

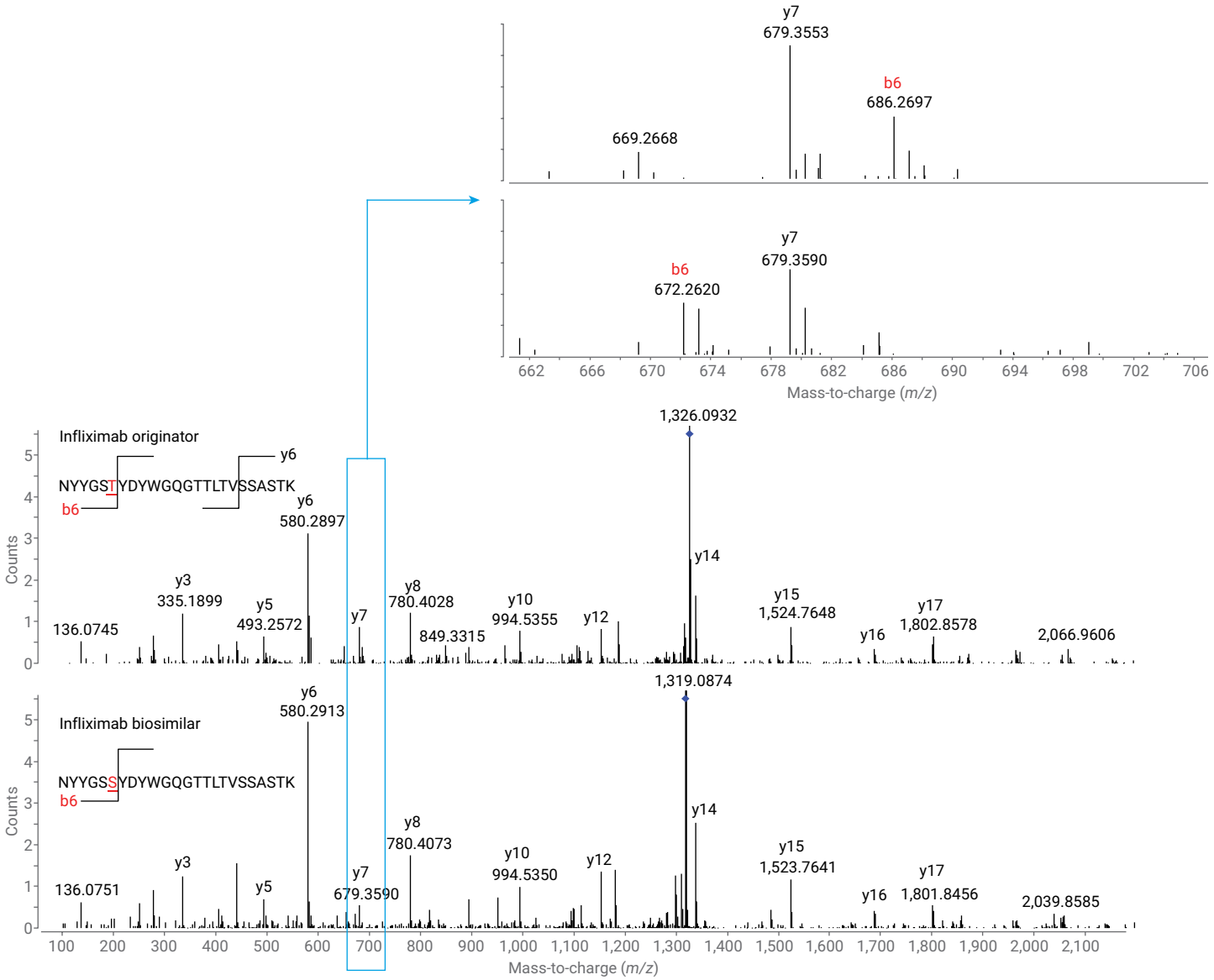


Figure 3. LC×LC/MS/MS data acquired on-the-fly in data-dependent mode, confirming the threonine to serine substitution.

Figure 4 displays the LC×LC tryptic peptide maps of infliximab originator and candidate biosimilar generated on the combination SCX×RPLC. From these measurements, the same conclusions can be drawn, yet the chromatographic behavior of the differential spots is altered. The peptide SLSLSPGK separates out from the peptides SLSLSPG and SLSLSPGI on the first SCX dimension as a result of the net +1 charge (at pH 3) of the former peptide, as opposed to the net 0 charge (at pH 3) of the latter two peptides. Again, a minor retention difference is noticed for peptides NYYGSTYDYWGQGTTLTVSSASTK and NYYGSSYDYWGQGTTLTVSSASTK. Note that a shorter method was used in this case.

Observed mutations were confirmed with reverse transcription polymerase chain reaction (RT-PCR) and DNA sequencing, as illustrated in Figure 5, for a CHO clone producing a candidate biosimilar with the correct sequence, and with the incorrect sequence.

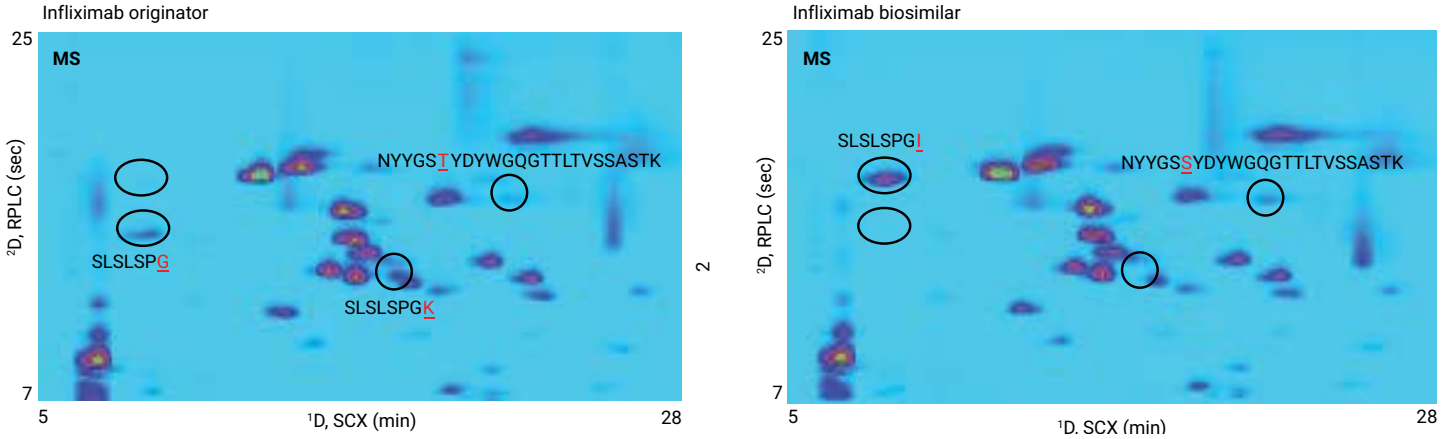


Figure 4. SCX×RPLC/Q-TOF MS analysis of infliximab originator and candidate biosimilar.

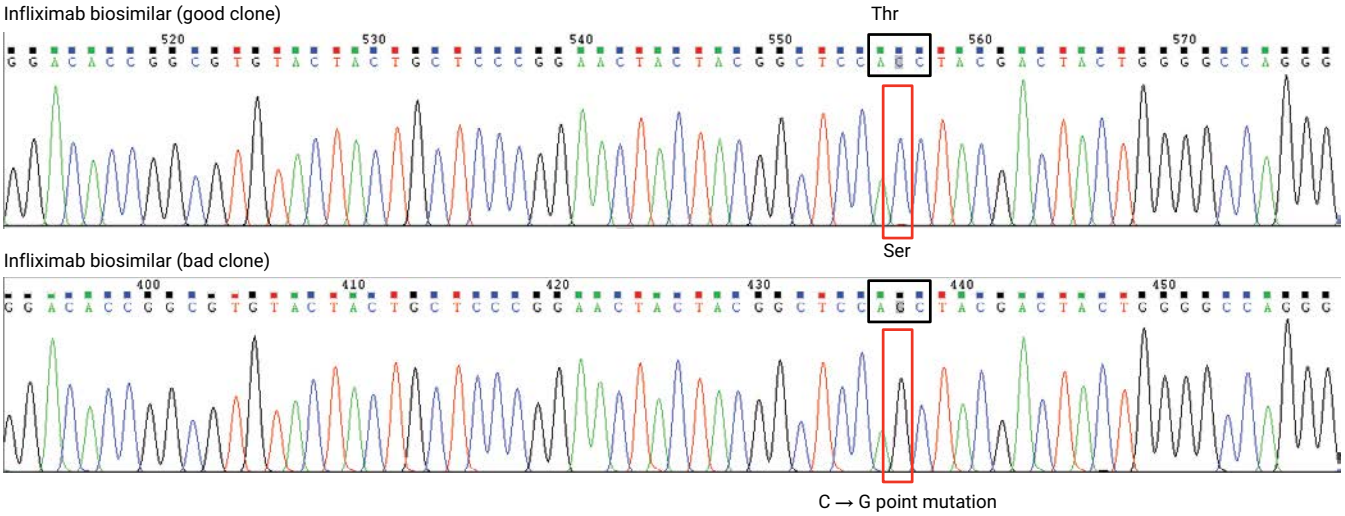


Figure 5. RT-PCR and DNA sequencing of CHO clones expressing the correct (top) and incorrect (bottom) infliximab sequence, illustrating the C-to-G point mutation and, thus, the threonine-serine substitution in the latter case.

Conclusion

The Agilent 1290 Infinity II 2D-LC solution, combined with an Agilent 6530 Accurate-Mass Quadrupole Time-of-Flight LC/MS, successfully enabled pinpointing critical differences between an infliximab originator and candidate biosimilar. A difference in the amino acid sequence of the heavy chain is revealed that *a priori* excludes this candidate biosimilar for further development since authorities require the amino acid sequence to be identical.

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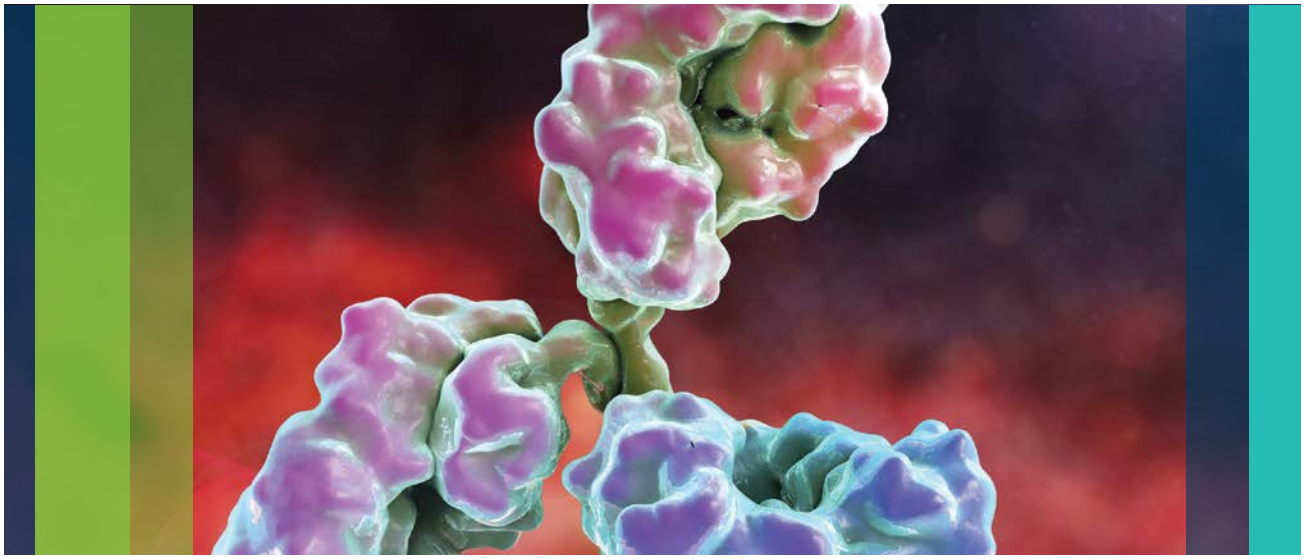

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Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies

We demonstrate simultaneous identification and quantification of chemical-induced deamidation and oxidation on recombinant mAbs with a peptide-mapping method using an integrated workflow that includes an Agilent AssayMAP Bravo liquid-handling platform, an Agilent 1290 Infinity II LC, an Agilent 6545XT AdvanceBio LC/Q-TOF, and Agilent MassHunter BioConfirm software.

Application Note

Biotherapeutics

Quantitation of Chemical-Induced Deamidation and Oxidation on Monoclonal Antibodies

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

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Introduction

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

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

Figure 1. Integrated workflow for peptide mapping.

Experimental

Materials

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

Instrumentation

- Agilent AssayMAP Bravo system (G5571AA)
- Agilent 1290 Infinity II LC system including:
  - Agilent 1290 Infinity II high-speed pump (G7120A)
  - Agilent 1290 Infinity II multisampler (G7167B) with sample cooler (option 100)
  - Agilent 1290 Infinity II thermostatted column compartment (G7116B)
- Agilent 6545XT AdvanceBio LC/Q-TOF (G6549A)
- Dual Agilent Jet Stream ESI source (G1958-65268)

Chemical induction and sample preparation

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

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

LC/MS analysis

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

Table 1. Liquid chromatography parameters.

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

Table 2. MS parameters.

Parameter	Value
Instrument	6545XT AdvanceBio LC/Q-TOF
Gas temperature	325 °C
Drying gas flow	13 L/min
Nebulizer	35 psig
Sheath gas temperature	275 °C
Sheath gas flow	12 L/min
VCap	4,000 V
Nozzle voltage	0 V
Acquisition mode	Extended Dynamic Range (2 GHz)
Mass range	*m/z* 300 to 1,700
Acquisition rate	8 spectra/sec
Auto MS/MS range	*m/z* 50 to 1,700
Min MS/MS acquisition rate	3 spectra/sec
Isolation width	Narrow (~ *m/z* 1.3)
Precursors/cycle	Top 10
Collision energy	3.1*(*m/z*)/100+1 for charge 2; 3.6*(*m/z*)/100−4.8 for charge 3 or greater than charge 3
Threshold for MS/MS	1,000 counts and 0.001 %
Dynamic exclusion on	1 repeat, then exclude for 0.2 minutes
Precursor abundance based scan speed	Yes
Target	25,000 counts/spectrum
Use MS/MS accumulation time limit	Yes
Purity	100 % stringency, 30 % cutoff
Isotope model	Peptides
Sort precursors	By charge state then abundance; +2, +3, >+3

Data processing

Data acquired from LC/MS/MS analysis were processed using MassHunter BioConfirm 10.0 software. For the chemical-induced deamidation study, searching parameters were set up as trypsin digest allowing semitryptic peptides and maximum two missed cleavages, fixed modifications containing cysteine (C) alkylation and N-terminal pyroGlu (E/Q), variable modifications containing asparagine (N) or glutamine (Q) deamidation, and methionine (M) oxidation. Mass tolerance allows 10 ppm for MS1 and 20 ppm for MS2. Peptide length was limited to 5 to 60 amino acids (AAs). Peptide-spectrum matches required MS/MS features, and were filtered by a 0.1 % false discovery rate (FDR). For the chemical-induced oxidation study, the searching parameters were the same as the induced deamidation study. An exception was that tryptophan (W) oxidation (+4 Da, +16 Da, or +32 Da) was added[6].

Results and discussion

Separation and identification of deamidated peptides

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

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

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

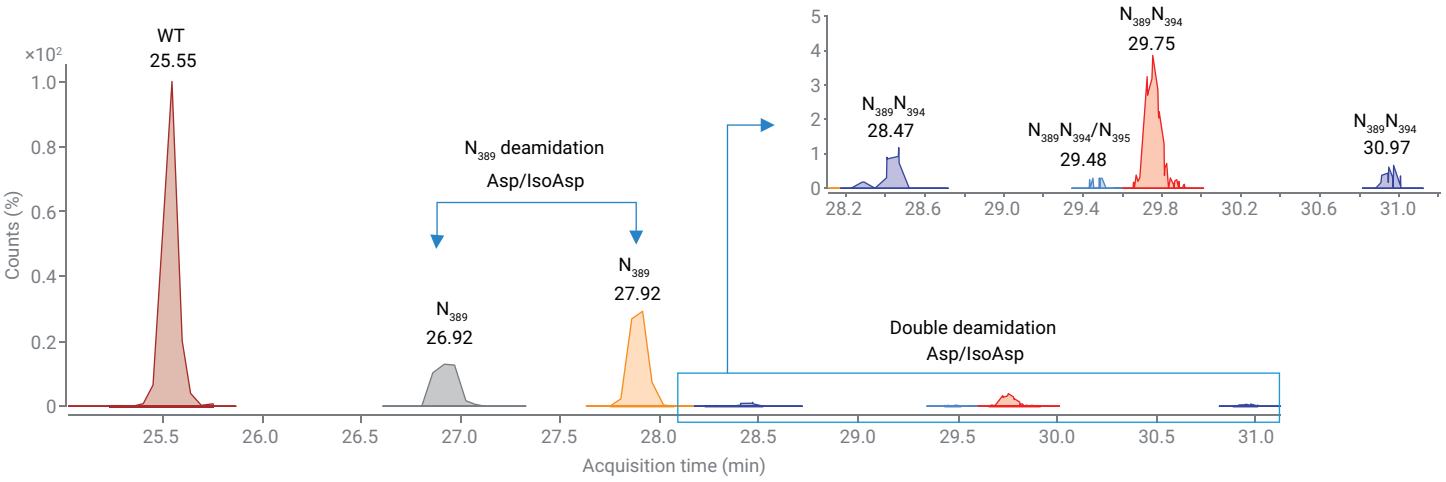

Figure 2. ECC of Asn deamidation, Asp isomerization, and wild type forms of the GFYPSDIAVEWESN$_{389}$GQPEN$_{394}$N$_{395}$YK peptide on a reversed-phase C18 column with charged surface using a 30-minute LC gradient .

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

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

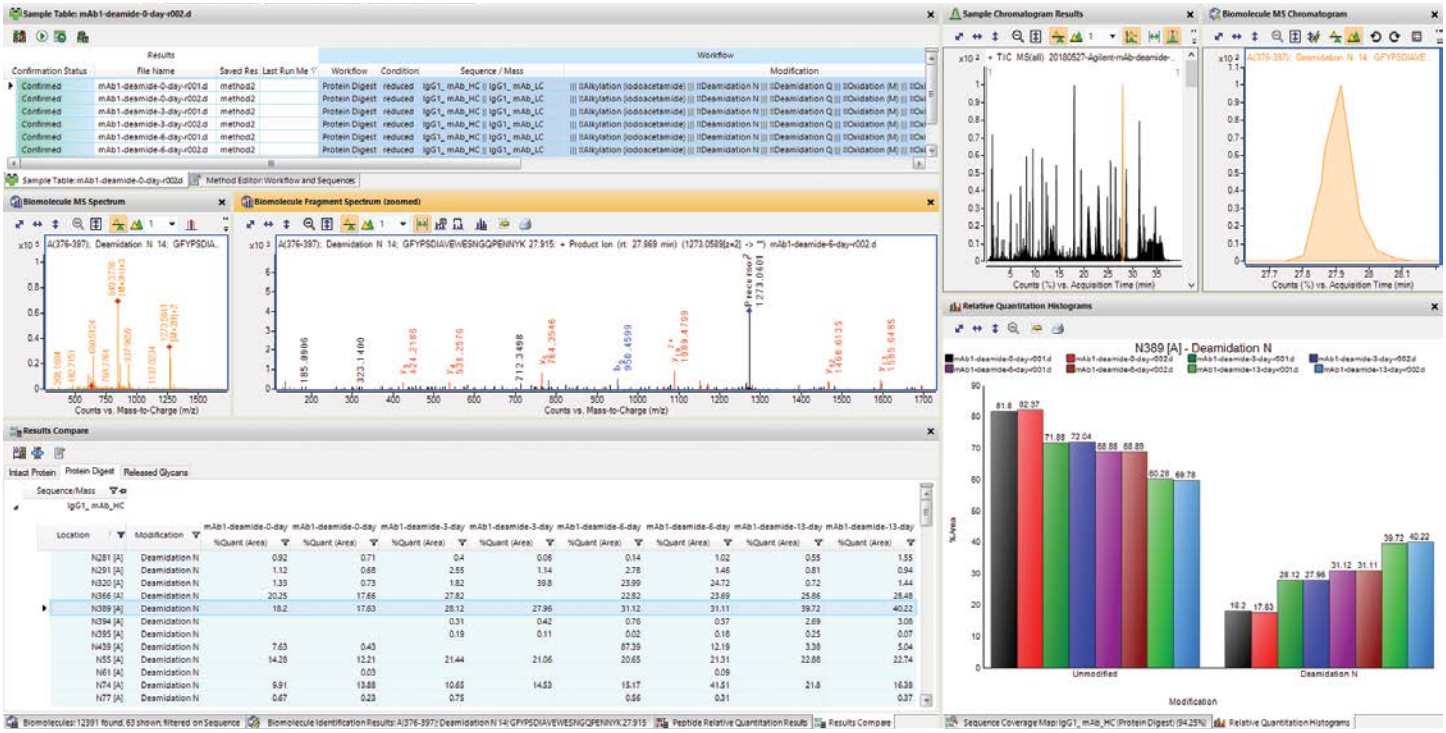

Figure 3. Screenshot of MassHunter BioConfirm 10.0 software for PTM identification and quantitation.

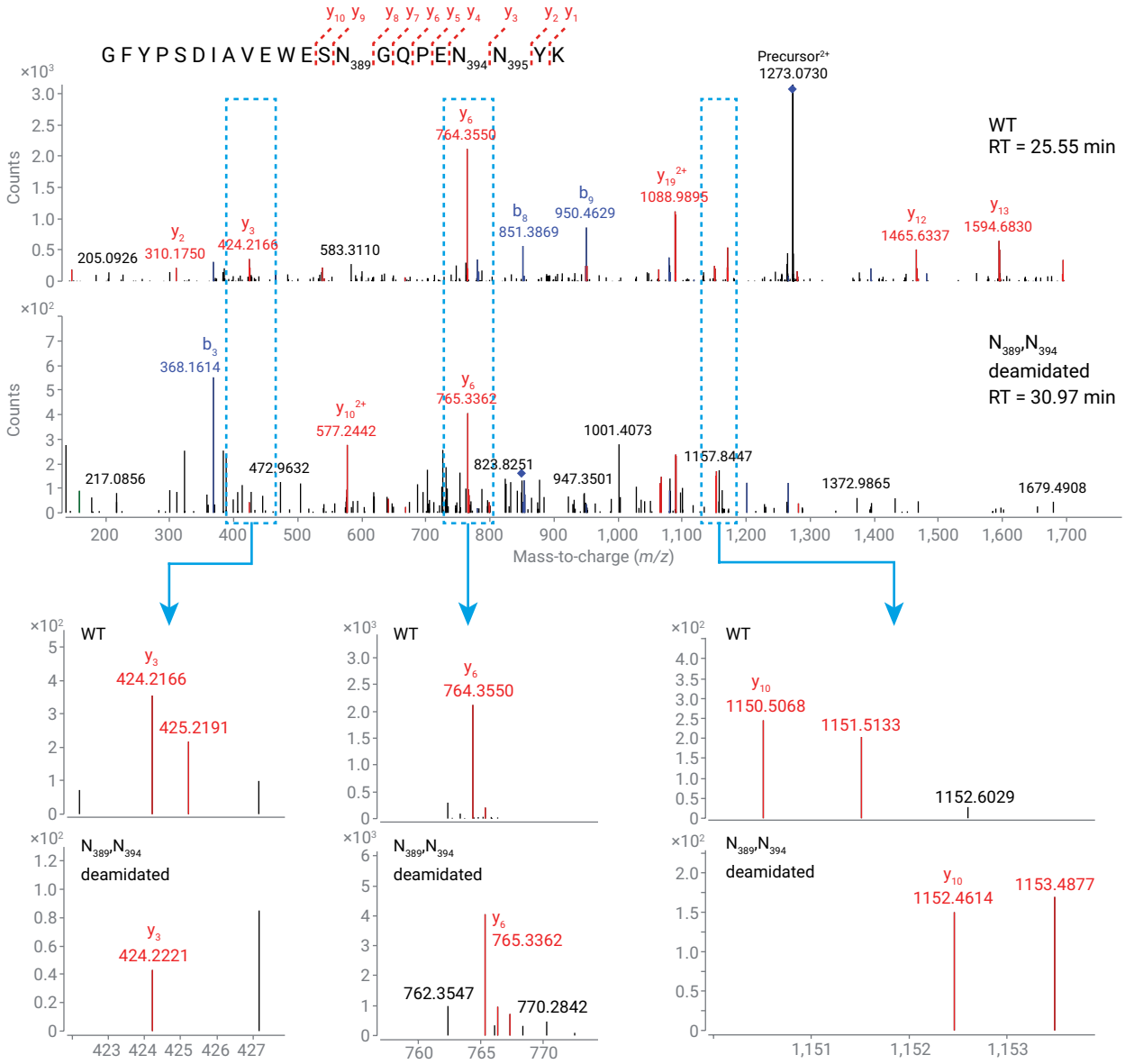

Figure 4. Comparison of MS/MS spectra between wild type and the double-deamidated form (N_{389}, N_{394}, RT = 30.97 minutes) of the peptide GFYPSDIAVEWESNGQPENNYK using MassHunter BioConfirm software. The fragment ions with a differential feature for PTM localization assignment were zoomed in for detailed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

Quantitation of deamidation

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

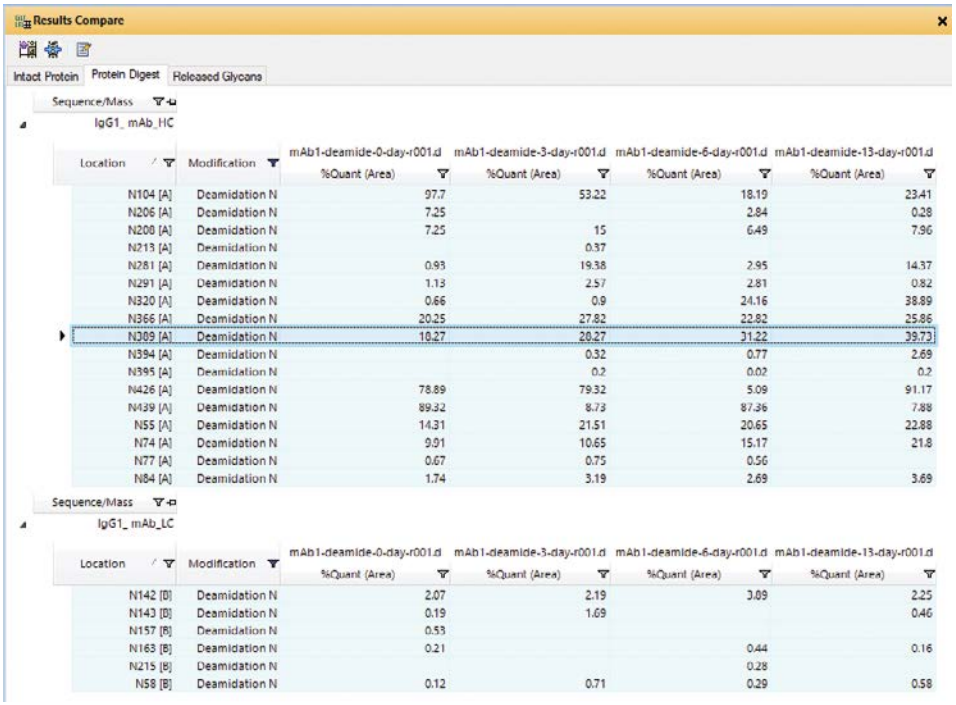

Figure 5. Results Compare table for PTM quantitation, showing a quantitative comparison of Asn deamidation in mAb1 heavy chain and light chain in high-pH conditions over a time course.

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

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

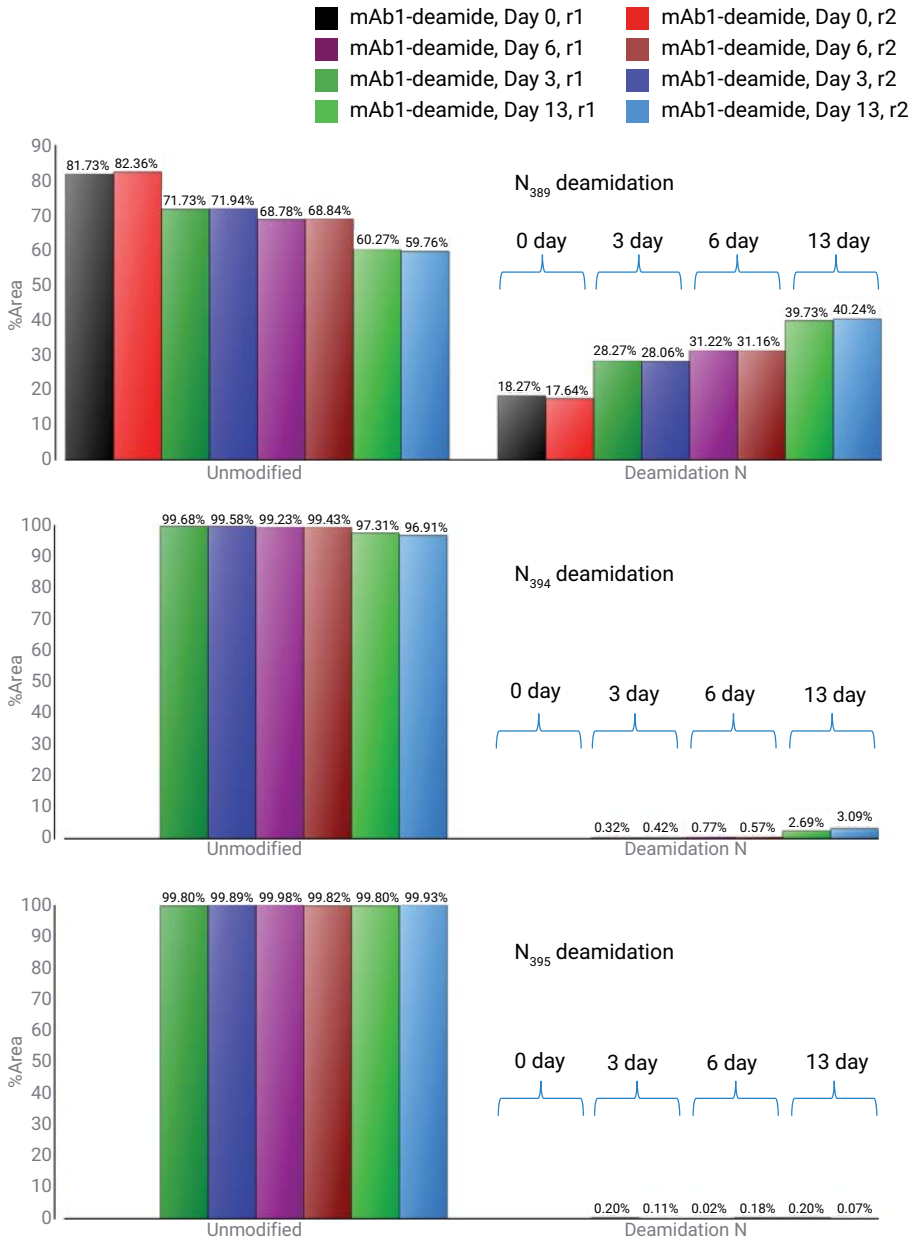

Figure 6. Histograms of quantitation for the three Asn deamidations (N_{389}, N_{394}, and N_{395}) on mAb1 heavy chain in high-pH conditions during a time course using MassHunter BioConfirm software. Two technical replicates were performed for each time point.

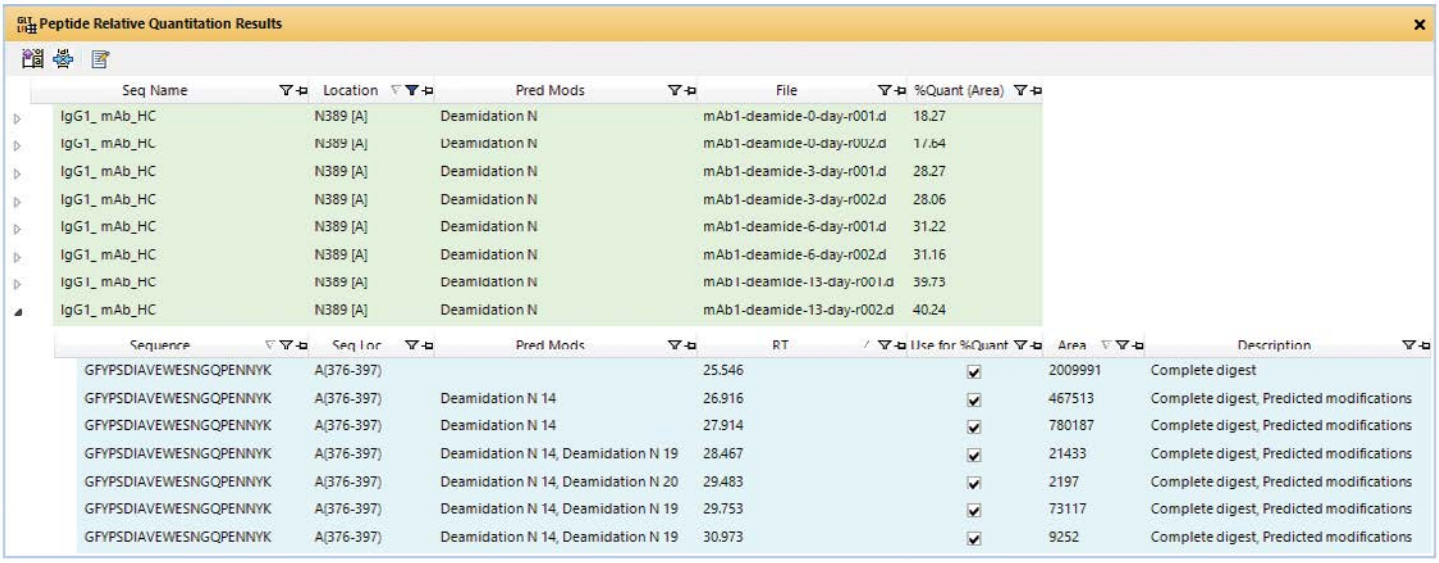

Figure 7. Screenshot of a portion of the Peptide Relative Quantitation Results table from MassHunter BioConfirm.

Quantitation of oxidation

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

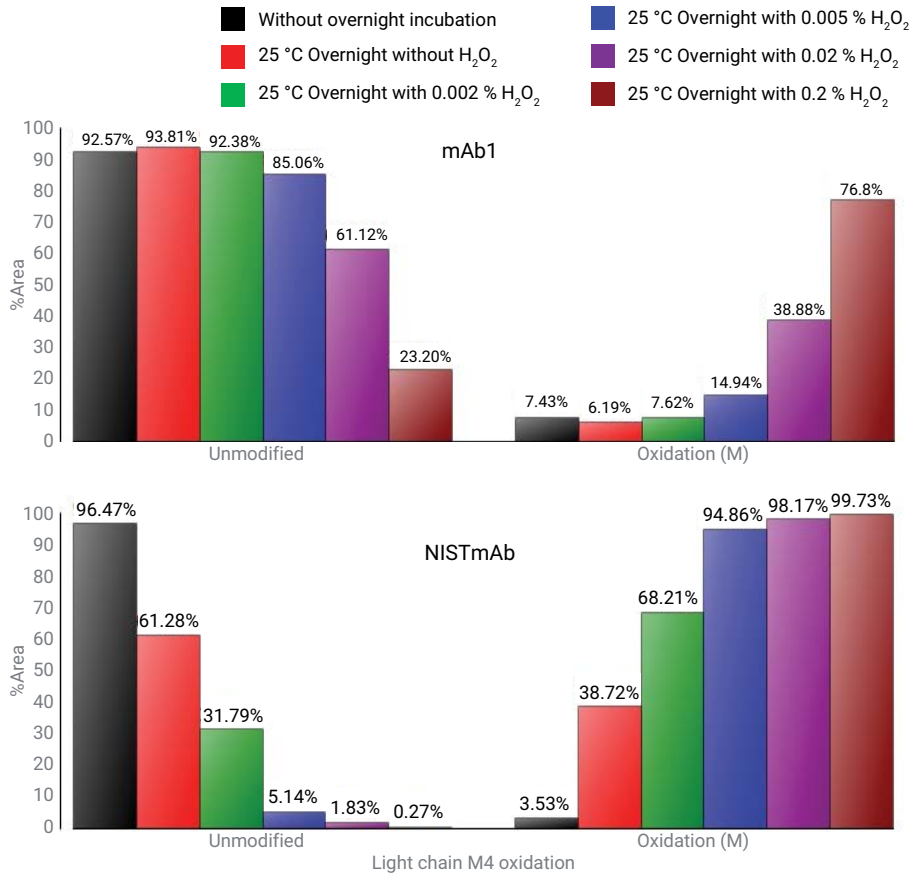

Figure 8. Quantitative histograms of light chain M4 oxidation for mAb1 and NISTmAb under accelerated oxidation conditions using MassHunter BioConfirm software.

Conclusion

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

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

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

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5994-0406EN

Profiling Glycosylation of Monoclonal Antibodies at Three Levels Using the Agilent 6545XT AdvanceBio LC/Q-TOF System

We present a complete workflow solution for antibody glycoforms characterization.

The intact workflow provided rapid assessment of the major glycoforms of the intact mAb. The mAb subunits workflow offered detailed quantitative information about individual glycans such as G0F, G1F, and G2F. The glycopeptide analysis through peptide-mapping workflow resulted not only in glycan-relative quantitation but also N-glycosylation sites information. The released glycan workflow provided high analytical sensitivity and the best quantitation for glycan analysis.

Application Note

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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 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.

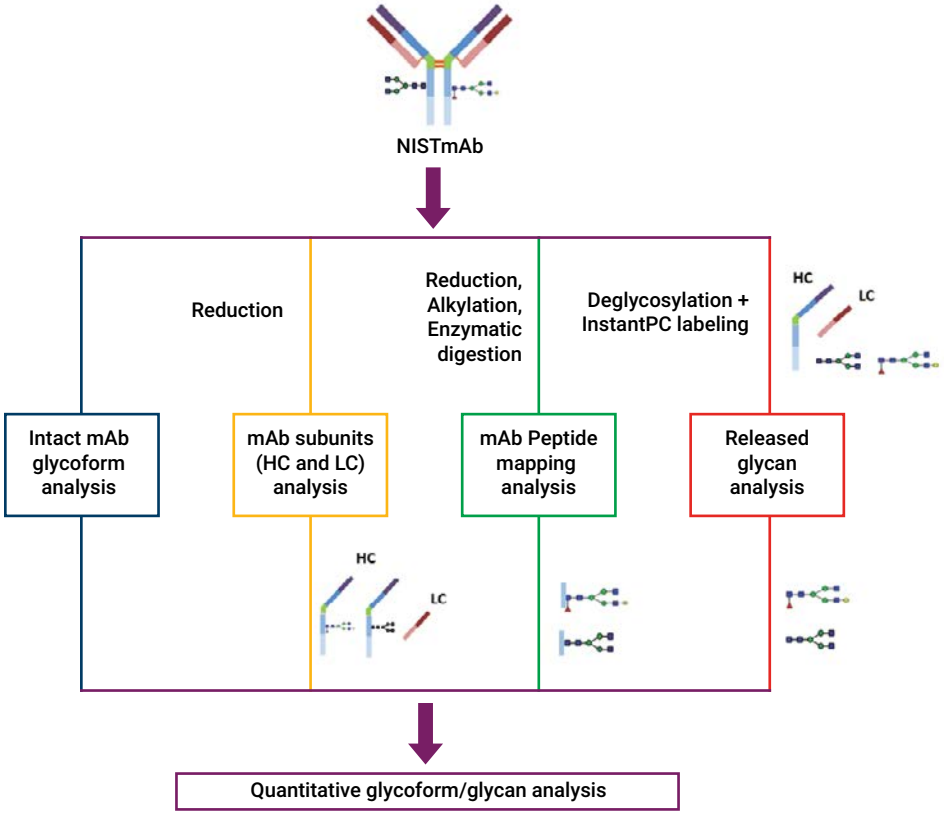


Figure 1. Various glycoforms/glycans quantitative analysis workflows.

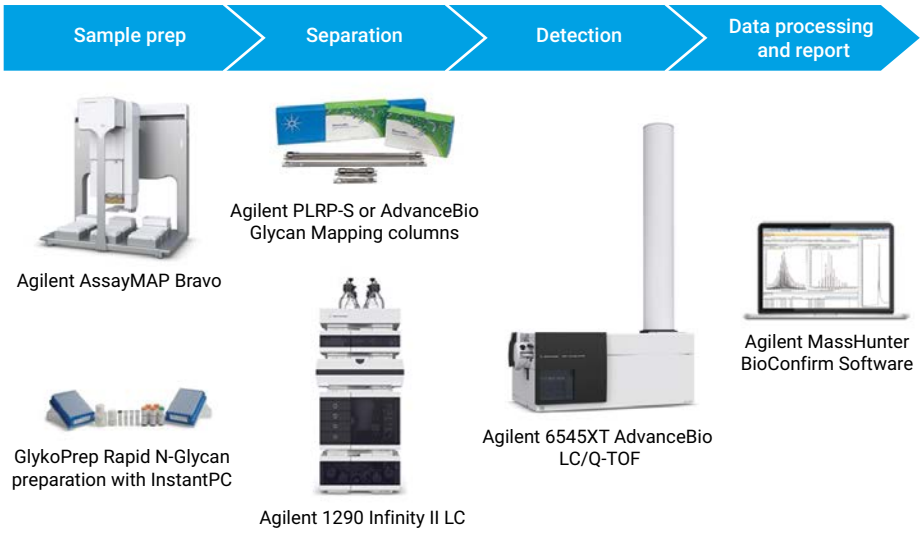


Figure 2. Analytical components of the mAb glycoform/glycan characterization workflow.

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)

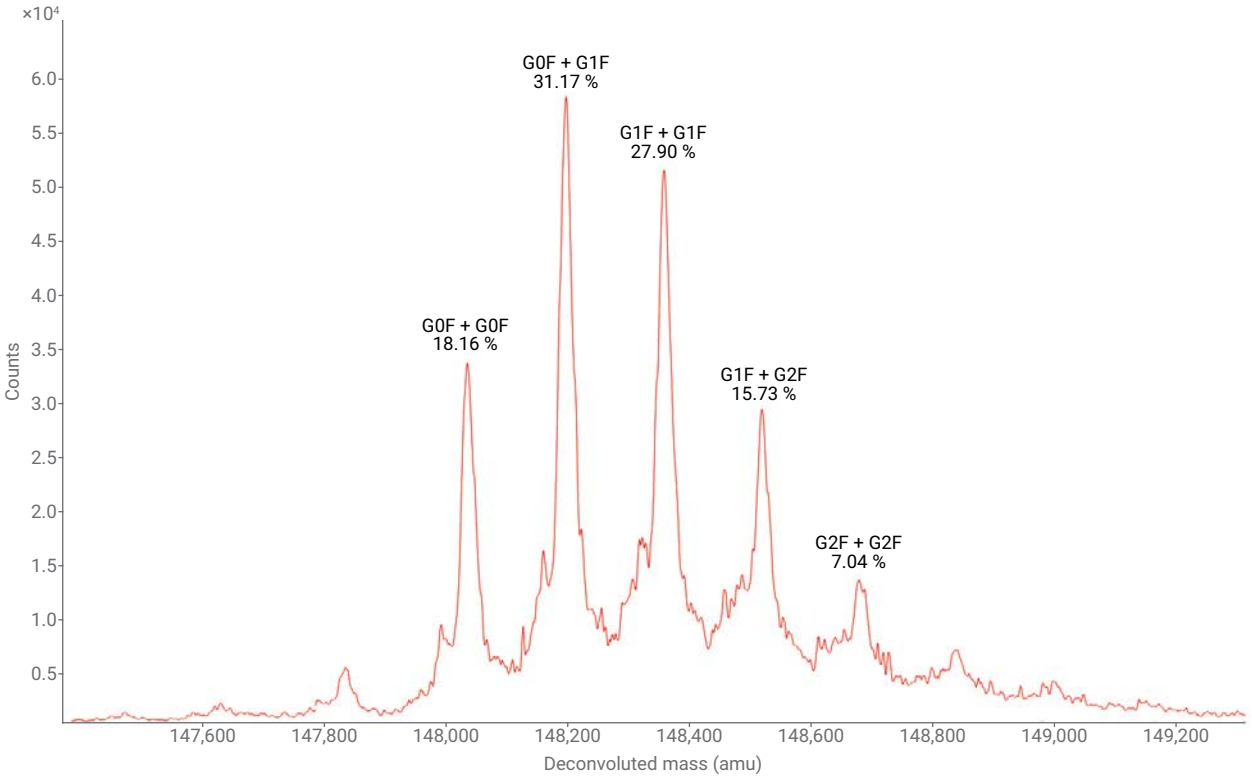


Figure 3. MS Deconvoluted spectrum (maximum entropy) of intact NISTmAb standard with relative quantitation labeled on five major glycoforms.

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.

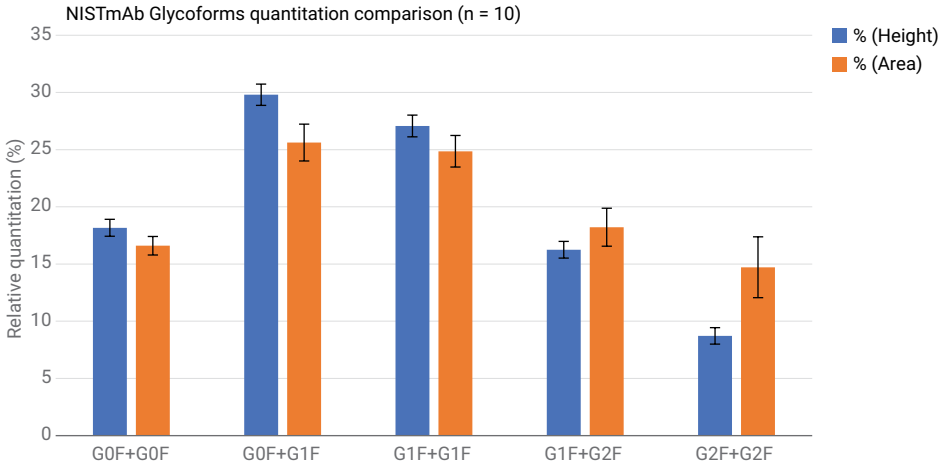
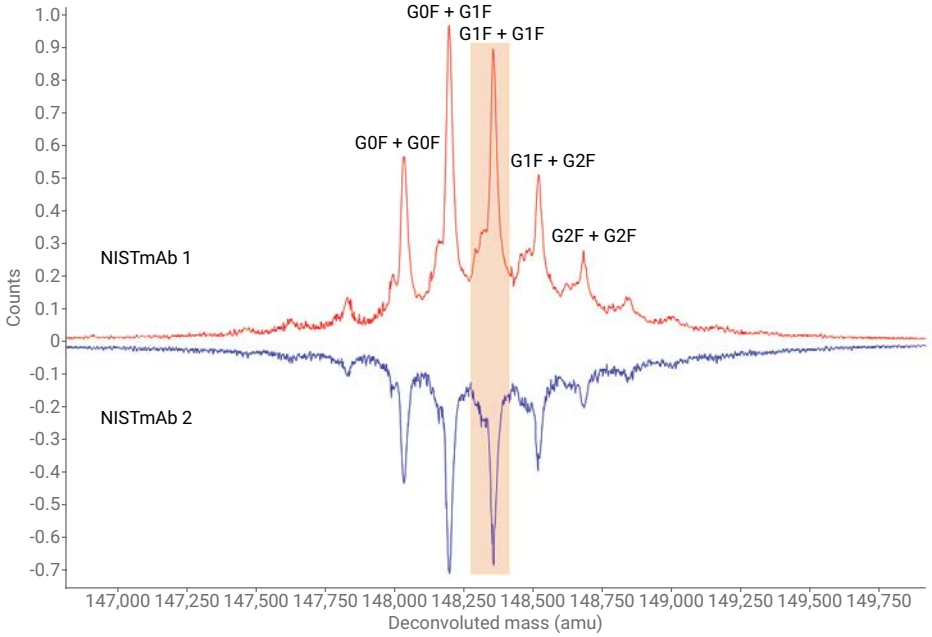


Figure 4. Quantitation results from intact NISTmAb glycoforms analysis (10 replicates).



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

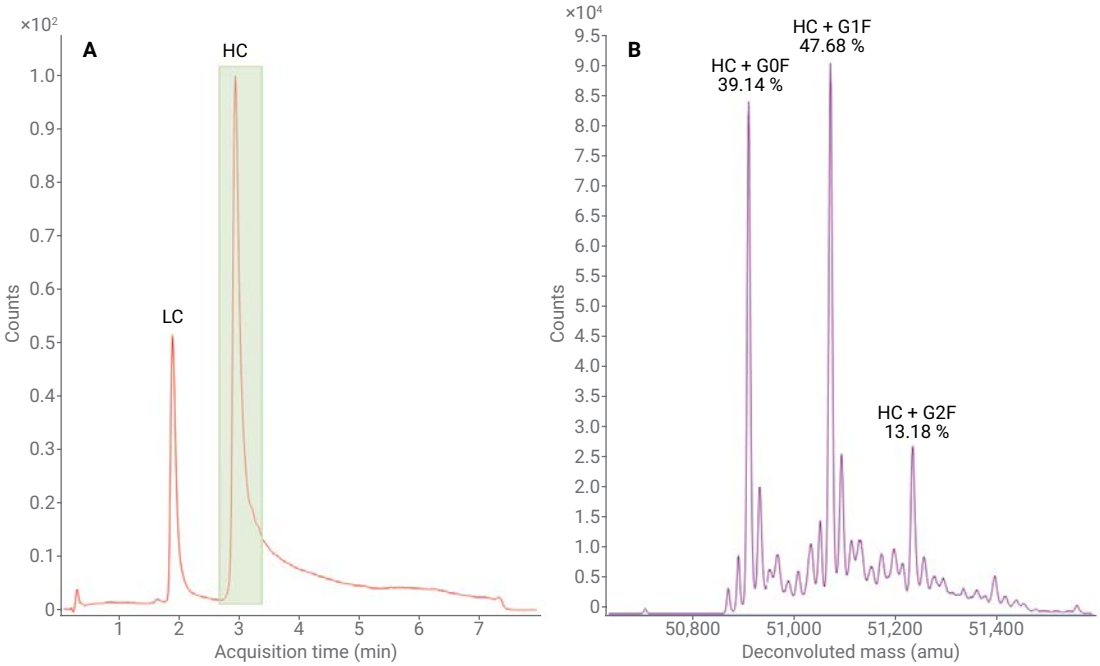


Figure 6. Total ion chromatogram (A), and MS deconvolution (B) of NISTmAb sub-units.

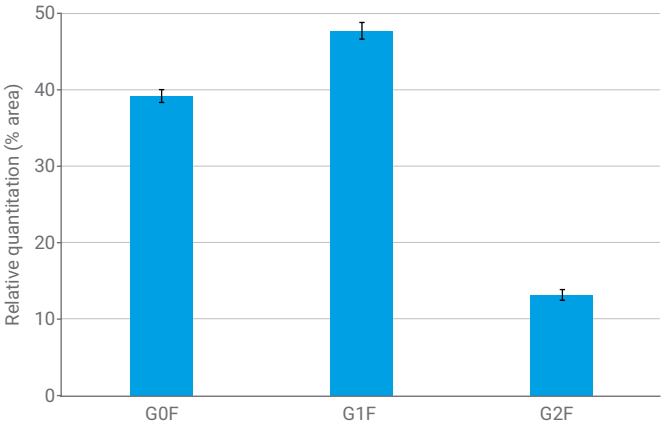


Figure 7. Quantitation results from NISTmAb sub-unit workflow.

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.

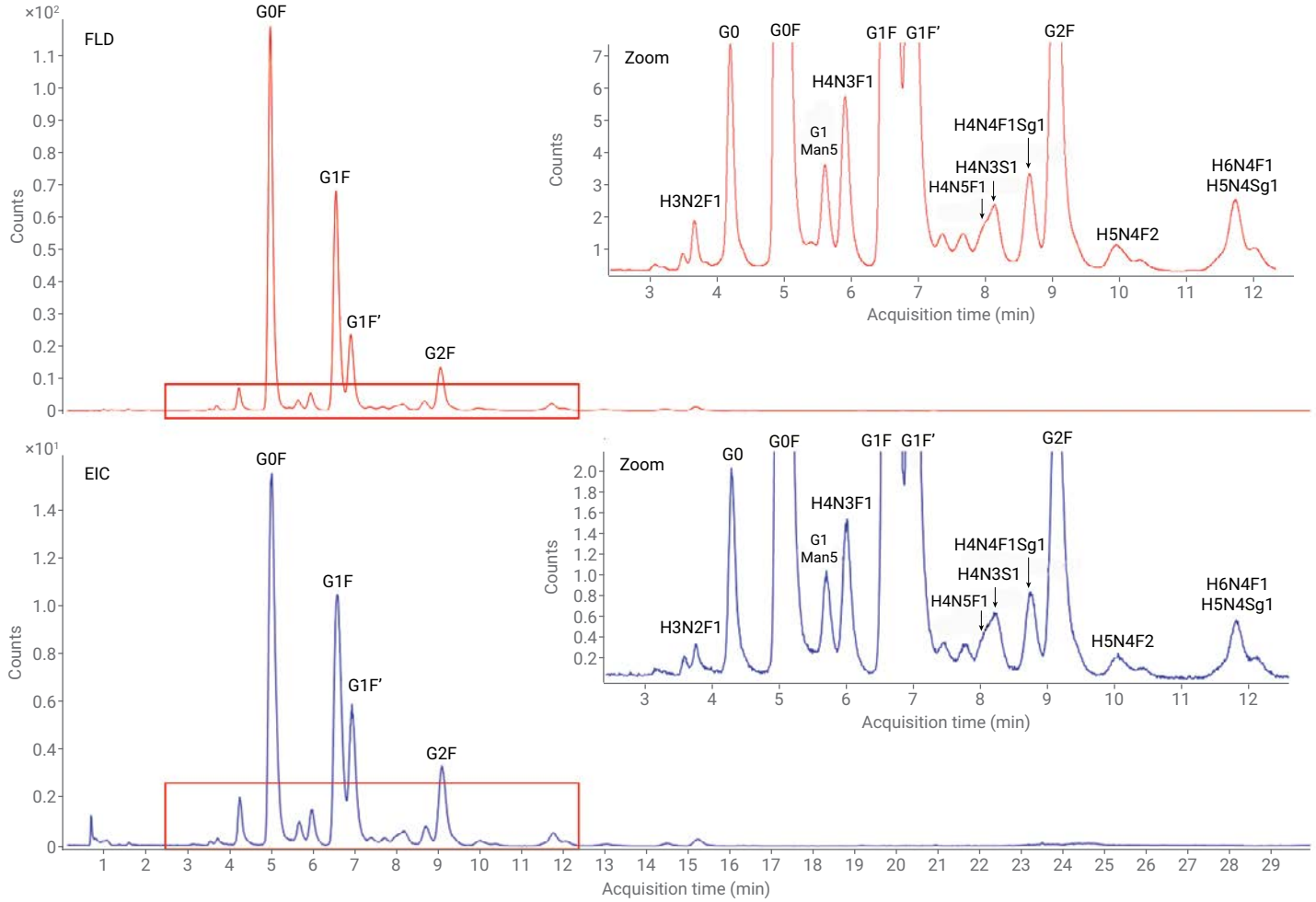


Figure 8. FLD chromatogram and mass spectra (EIC) of InstantPC labeled N-glycans from NISTmAb.

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.

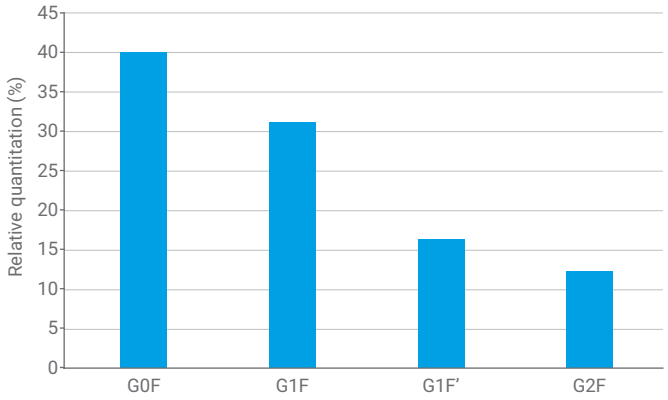


Figure 9. Quantitation results from NISTmAb released glycan workflow.

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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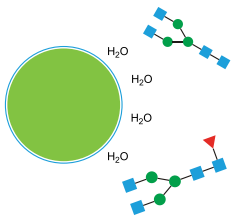

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