Application Note

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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).

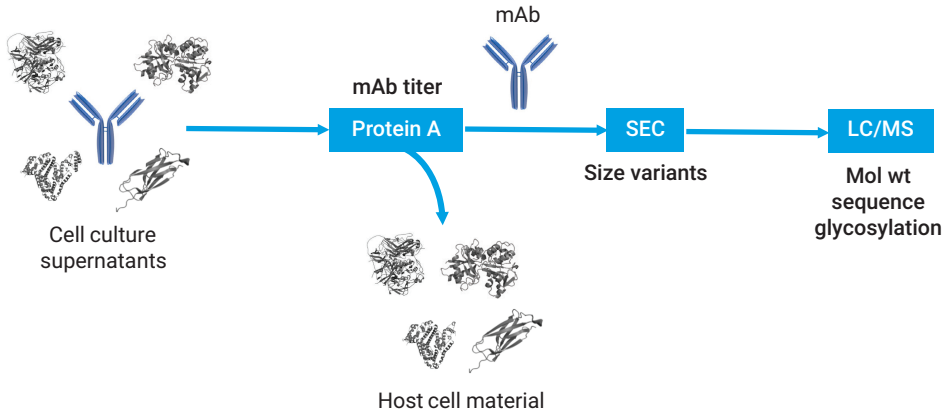

Figure 1. Multi-attribute analysis directly from cell culture supernatants by combining protein A affinity chromatography, size exclusion chromatography, and LC/MS in an online 3D setup.

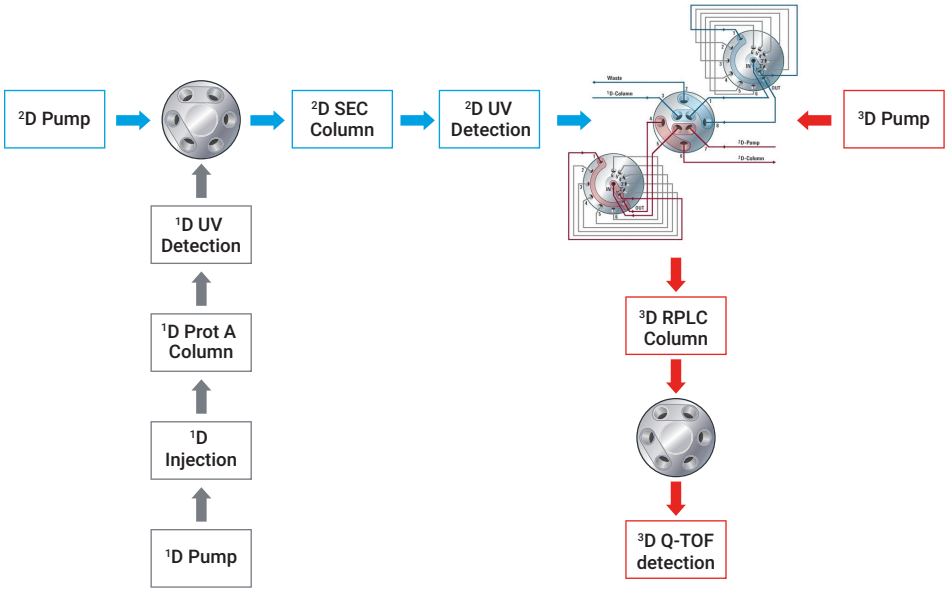

Figure 2. Configuration of the multi-attribute analyzer.

[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

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.

^{3}D: RPLC for Desalting	
Column	Polymer-based desalting cartridge, 2.1×10 mm
Temperature	30 °C
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

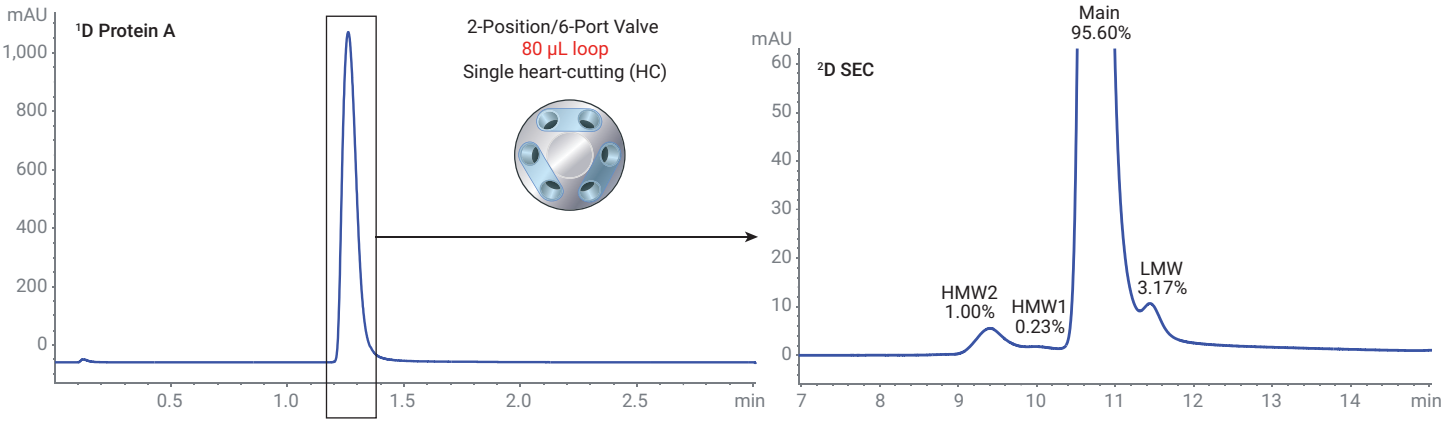

Figure 3. ^{1}D protein A affinity chromatogram (280 nm) of therapeutic mAb, and ^{2}D SEC chromatogram of transferred protein A peak (220 nm). The protein A peak was completely sampled in an 80 µL loop installed on an Agilent InfinityLab Quick Change 2-Position/6-Port Valve and transferred to the second 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.

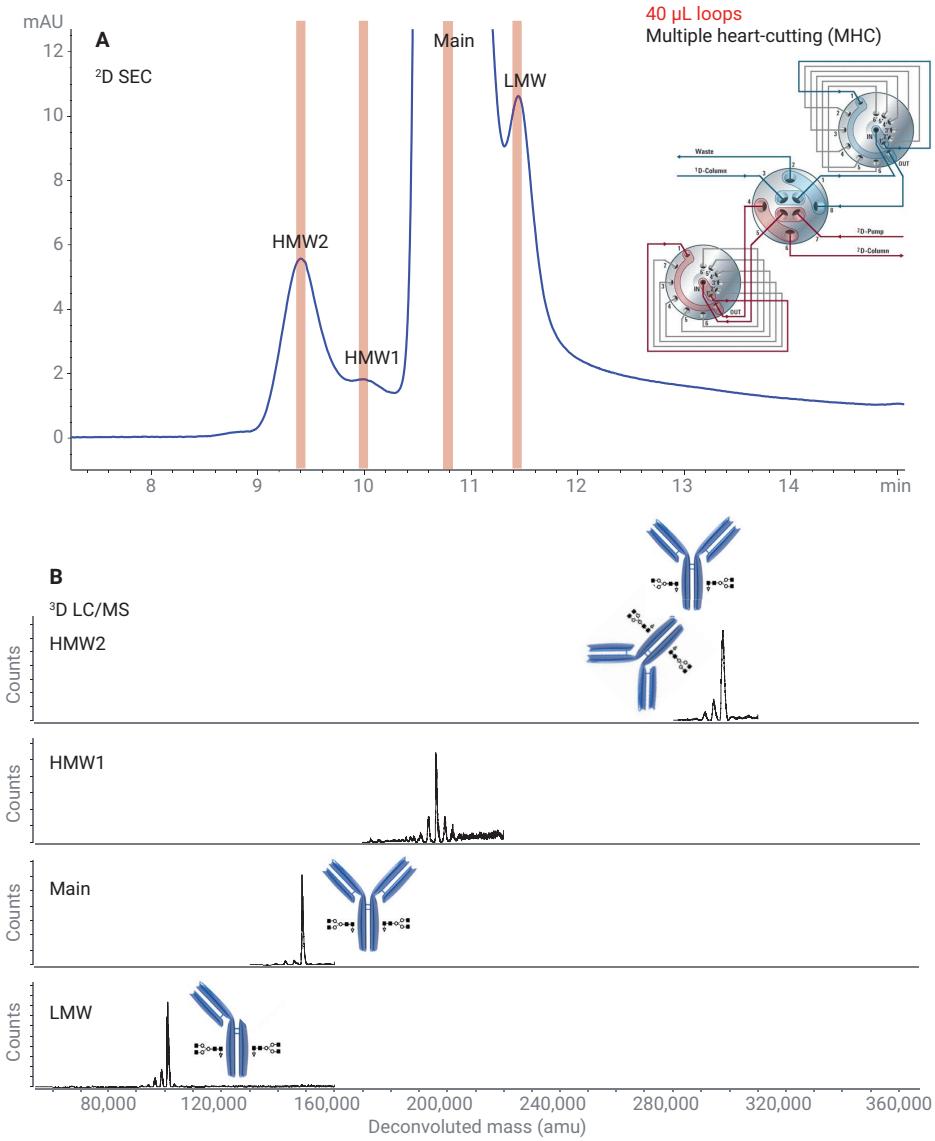

Figure 4. ²D SEC chromatogram of transferred protein A peak (220 nm) and ³D LC/MS analysis of SEC fractions. The different HMW and LMW variants as well as the main peak were collected in 40 µL loops installed on a MHC valve and subsequently transferred individually to the LC/MS. The third chromatographic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

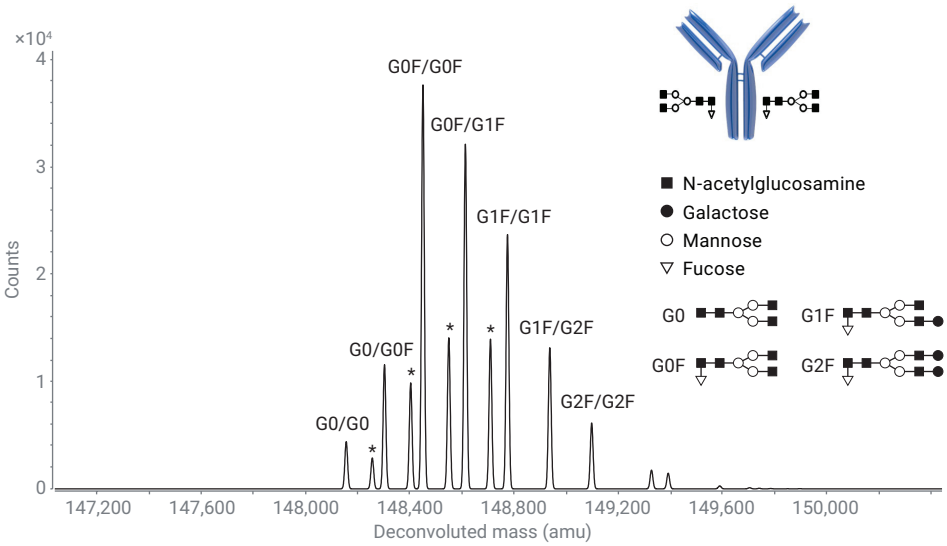

Figure 5. [3]D LC/MS analysis of main SEC peak. This deconvoluted spectrum confirms the cloned amino acid sequence and reveals the glycosylation pattern. * Corresponds to phosphate adducts.

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.

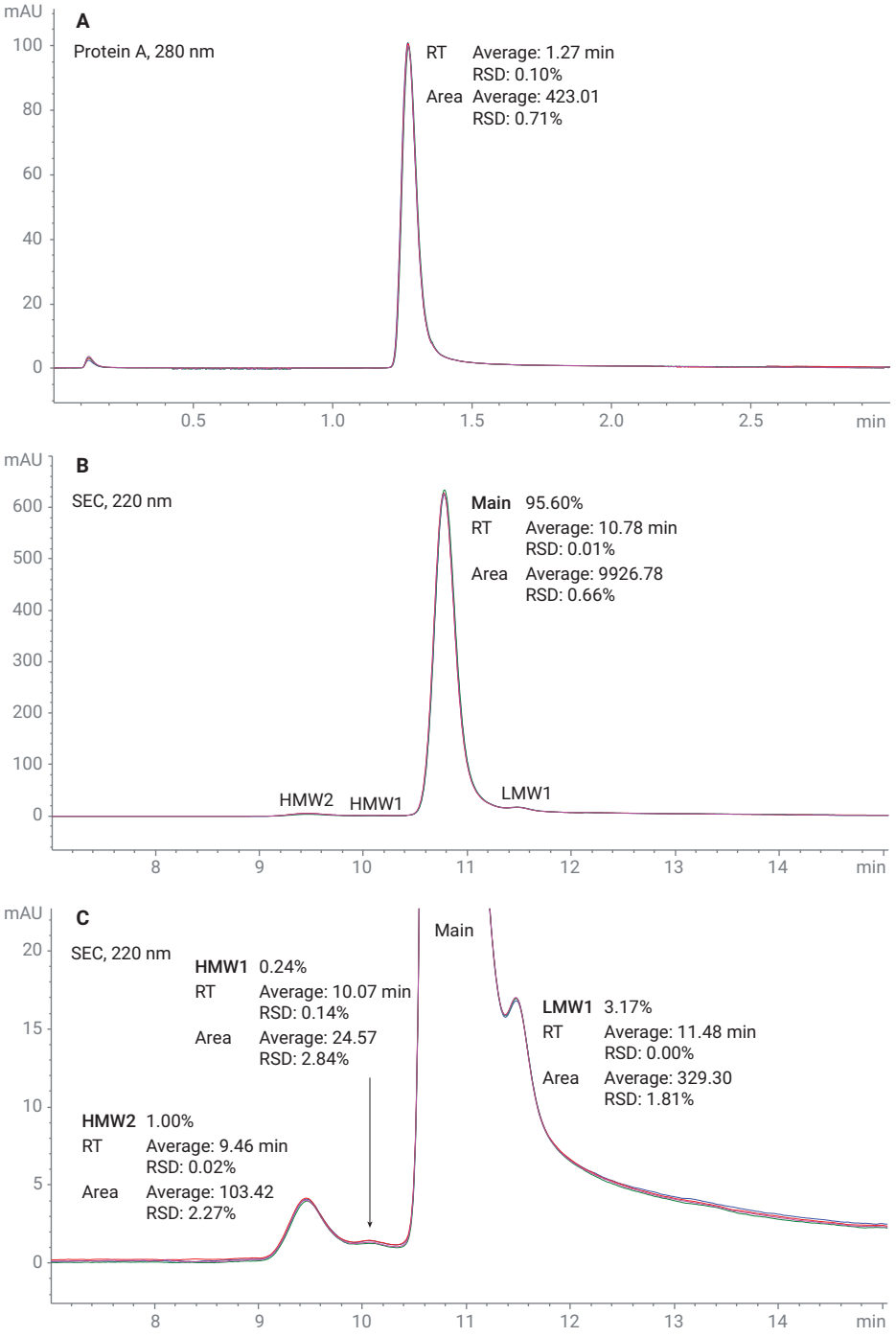

Figure 6. [1]D protein A (280 nm) and [2]D SEC (220 nm) chromatograms of four consecutive injections of the sample (10 µg load). Precision data are included in the chromatograms.

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).

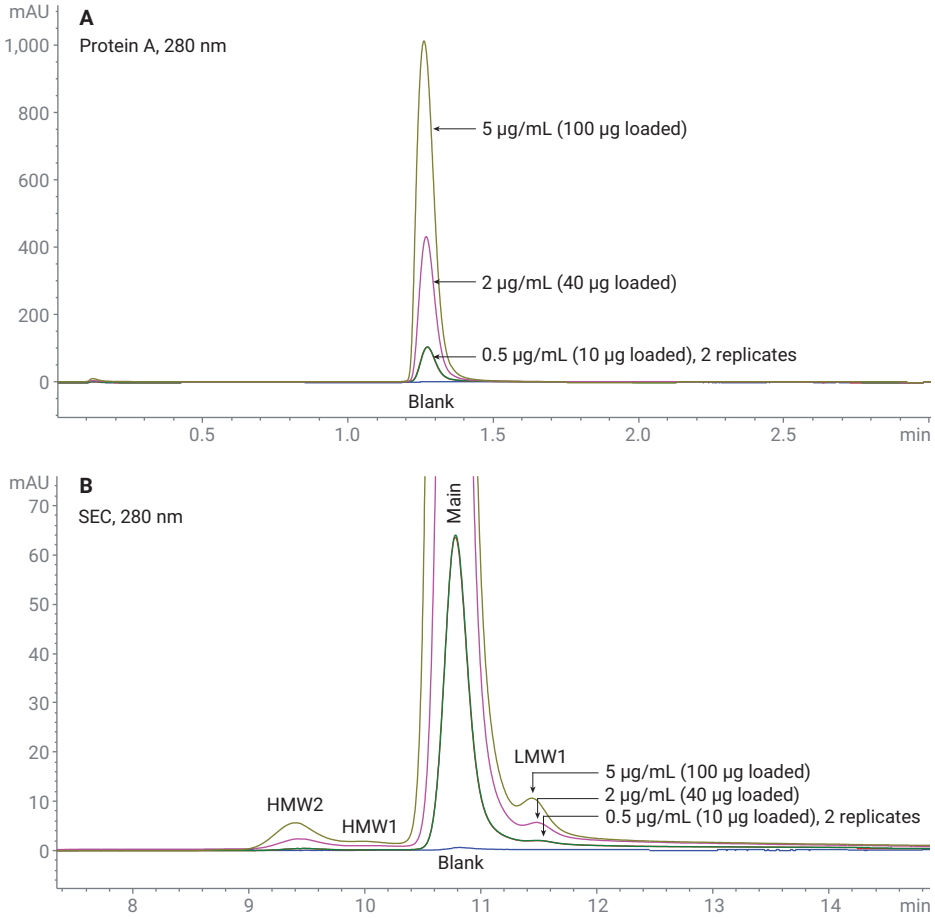

Figure 7. [1]D Protein A (280 nm) and [2]D SEC (280 nm) chromatograms for a blank injection and three calibration levels. 10/40/100 µg sample was loaded.

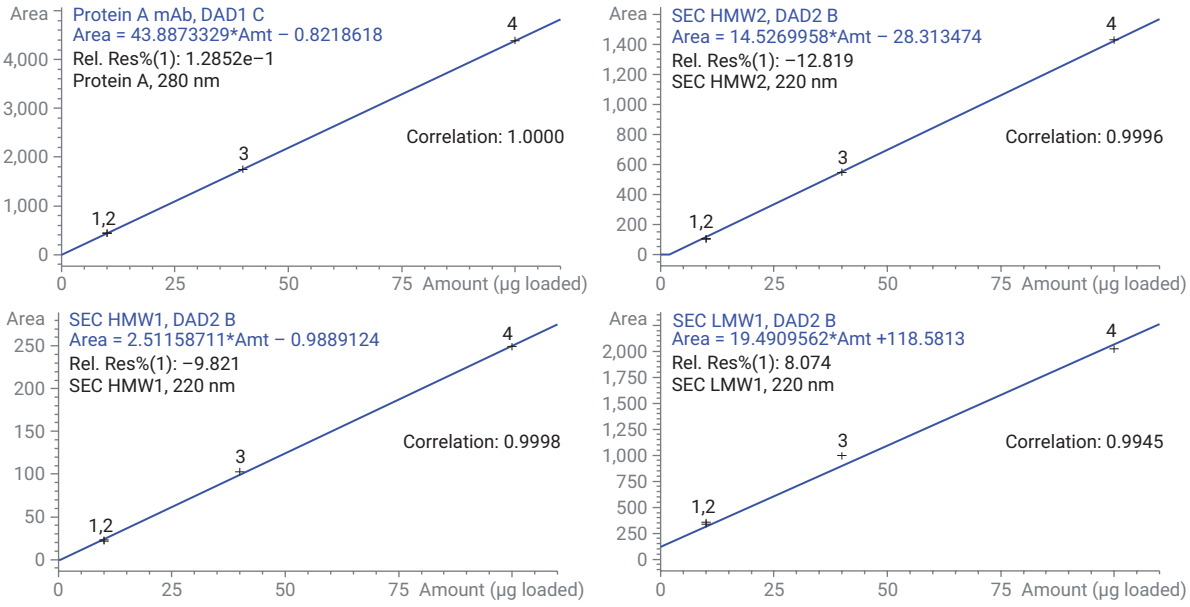

Figure 8. [1]D Protein A (280 nm) and [2]D SEC (220 nm) calibration curves for 10/40/100 µg sample loaded.

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.

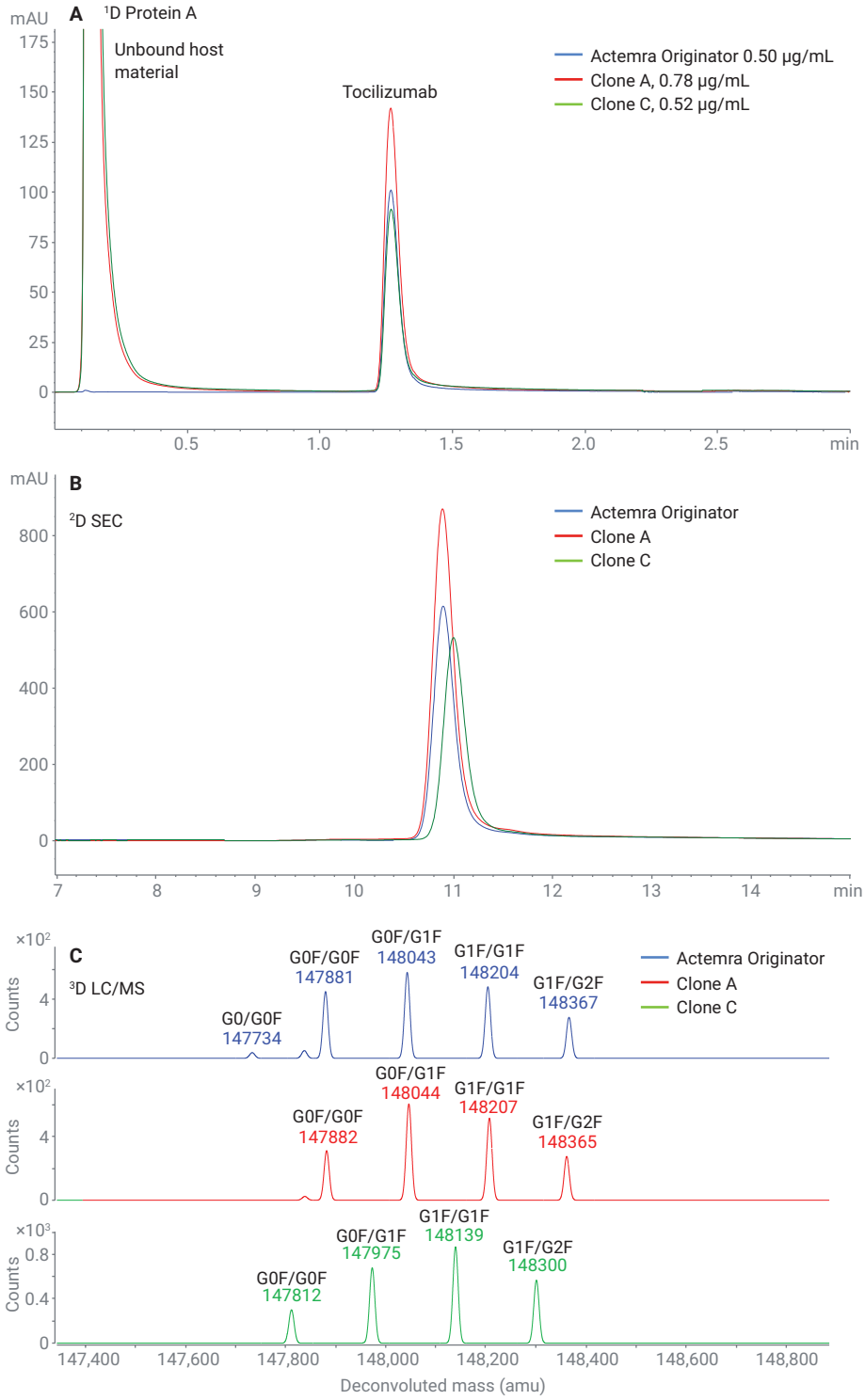

Figure 9. Multi-attribute analysis in the context of biosimilar development. An Actemra originator and supernatants of two tocilizumab-producing CHO clones (clones A and C) were subjected to protein A affinity chromatography, SEC, and LC/MS in a 3D setup. (A) ^{1}D protein A affinity chromatogram (280 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.

References

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