Application Note

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Automated Monoclonal Antibody Subunit Analysis by Online Reduction Using 2D-LC/MS

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Abstract

Recombinant monoclonal antibodies (mAbs) have become a popular class of biotherapeutics over recent years. To assess critical quality attributes in mAb products, a common sample preparation technique is the reduction of mAb disulfide bonds for subunit analysis. Reduction is then followed by analysis with liquid chromatography/mass spectrometry (LC/MS). This application note describes an automated two-dimensional liquid chromatography (2D-LC) workflow for the reduction of mAbs using Protein A affinity chromatography in the first dimension and MS-compatible size exclusion chromatography (SEC) in the second dimension. Two different mAbs were successfully reduced while trapped on a reversed-phase column, which was used instead of the sampling loop. Additionally, 2D-LC acted as a desalting tool and was used for subsequent MS analysis of mAbs resulting from the MS-incompatible Protein A purification step. Application of the Agilent 6545XT AdvanceBio LC/Q-TOF enabled identification of mAb subunits by matching their deconvoluted mass spectrum with their amino acid sequences.

Introduction

Over recent years, the attention to mAbs has increased dramatically. A rising number of blockbuster mAb products were approved and are now widely accepted because of their therapeutic efficacy and little side effects. Due to their heterogeneity, the characterization of critical quality attributes of mAbs requires sophisticated analytical techniques. LC/MS has become the most popular technique for bioanalysis of mAbs because of its superior sensitivity and accuracy. The bioanalysis workflow of mAbs can be broadly divided into top-down, middle-down, and bottom-up analysis. Each approach requires specific sample preparation steps before LC/MS analysis, involving several complex and usually manual steps.[1]

In biochromatography, separation of analytes is usually achieved using salt-containing or nonvolatile buffer additives that can result in severe contamination of the electrospray ion source and MS signal suppression. 2D-LC can be used as an effective desalting tool to allow online coupling of chromatographic methods using MS-incompatible mobile phases before MS detection.[2] Agilent 1290 Infinity II Bio LC modules are specially designed for conditions used in biochromatography. These modules are built of MP35N, a nickel-cobalt alloy, which can reduce potential corrosion from high salt-containing buffers and avoid protein modifications caused by the presence of iron ions (e.g., oxidation, protein complex formation).[3] Due to their excellent resolution at high mass range, quadrupole time-of-flight (Q-TOF) MS systems are usually applied for analysis of intact proteins or mAbs. The 6545XT AdvanceBio LC/Q-TOF offers the ability to measure biomolecules up to *m/z* 30,000 and includes software features to automatically process data resulting from mAb product analysis.[4]

Reduction of disulfide bonds divides mAbs into its subunits—heavy and light chain—and is a common sample preparation step prior to LC/MS analysis. 2D-LC can be operated with a trap column instead of the sampling loop. Therefore, an mAb can be retained and treated with a reduction agent after the first chromatographic dimension and before the second chromatographic dimension. This technical versatility enables purification of mAbs in the first chromatographic dimension, online reduction of disulfide bonds on the trap column, and subsequent separation of light and heavy chain in the second chromatographic dimension. This application note describes a fully automated online reduction workflow for subunit analysis of mAbs using 2D-LC with a trap column instead of a sampling loop.

Experimental

Instrument

- 2× Agilent 1290 Infinity II Bio High-Speed Pump (G7132A) with Agilent Bio Jet Weaver mixer kit, 35 µL volume (part number G7132-68135)
- Agilent 1290 Infinity II Bio Multisampler (G7137A) with Agilent InfinityLab Sample Thermostat (G4761A, option #101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) equipped with Agilent Thermal Equilibration Device (part number G7116-60013)
- Agilent 1260 Infinity II Multicolumn Thermostat (G7116A) equipped with Agilent Quick-Connect Bio Heat-Exchanger, Standard Flow (part number G7116-60071, option #065)
- Agilent 1290 Infinity Valve Drive (G1170A) equipped with Agilent InfinityLab Bio 2D-LC ASM Valve (G5643A). Two 0.12 × 500 mm MP35N capillaries (part number 5004-0045) were used to connect the trap column (Pos 1 to column inlet, Pos 8 to column outlet). A 0.12 × 150 mm MP35N capillary (part number 5004-0047) was used to connect Pos 4 and 5. A graphical representation of the valve setup is depicted in Figure 1.
- Agilent 1290 Infinity II Variable Wavelength Detector (G7114B) equipped with an Agilent Bio Standard Flow Cell for VWD (option #028, part number G1314-60188)
- Agilent 6545XT AdvanceBio LC/Q-TOF with Agilent Dual Jet Stream ESI Source

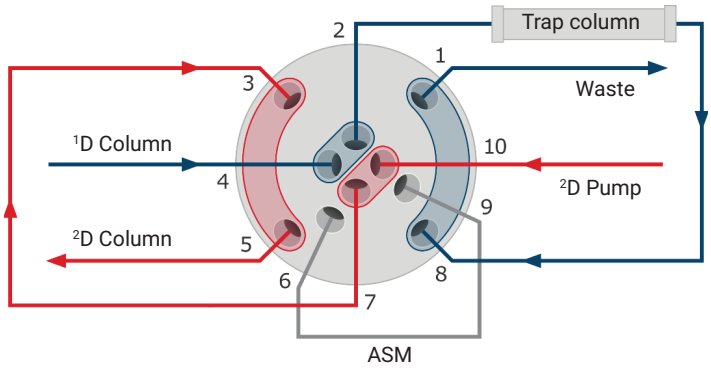

Figure 1. Schematic representation of the online reduction setup.

Software

- Agilent MassHunter Acquisition software 11.0
- Agilent MassHunter BioConfirm software 10.0 SP1
- Agilent MassHunter Qualitative Analysis software 10.0

Samples

- Agilent-NISTmAb, 4 × 25 µL, 10 mg/mL (part number 5191-5745)
- Trastuzumab (Herceptin, Roche, Switzerland), 10 mg/mL in water

Columns

- **^{1}D column:** Bio-Monolith Recombinant Protein A, 4.95 × 5.2 mm (part number 5190-6903)
- **Trap column:** Agilent AdvanceBio RP-mAb Diphenyl, 2.1 × 50 mm, 3.5 µm (part number 799775-944)
- **^{2}D column:** Agilent AdvanceBio SEC 300Å, 4.6 × 300 mm, 2.7 µm (part number PL1580-5301)

Chemicals

- **^{1}D mobile phase A:** 50 mM phosphate buffer pH 7.4
- **^{1}D mobile phase B:** 500 mM acetic acid
- **^{2}D mobile phase A:** 25 mM *tris*(2 carboxyethyl)phosphine hydrochloride (TCEP)
- **^{2}D mobile phase B:** 0.1% formic acid in acetonitrile:water (1:1)
- For 50 mM phosphate buffer pH 7.4, 10.107 g of sodium phosphate dibasic heptahydrate and 1.697 g of sodium phosphate monobasic monohydrate were added to an amber-colored 1 L bottle and filled up to 1 L using ultrapure water. The buffer was filtered using a 0.2 µm membrane filter and the pH value was checked and adjusted, if necessary, to pH 7.4.
- Fresh ultrapure water was obtained from a Milli-Q integral system equipped with LC-Pak polisher and a 0.22 µm membrane point-of-use cartridge (Millipak).
- For ^{2}D solvents, Agilent InfinityLab ultrapure LC/MS acetonitrile (part number 5191-4496) and Agilent InfinityLab ultrapure LC/MS water (part number 5191-4498) were used.
- TCEP was obtained from Alfa Aesar, Germany.
- Other mobile phase ingredients were obtained from Merck, Germany.

Method parameters

^{1}D pump	
Flow	0.5 mL/min, after 9 min the flow was adjusted to 0.1 mL/min to save solvent
Timetable	0 to 0.5 min: 0% B 0.6 to 3.6 min: 100% B 3.7 to 36.52 min: 0% B
2D-LC settings	
Flow	0.35 mL/min
2D-LC Operation Mode	Heart-cutting (time-based heart cut at 0.94 min; MHC; cut size: 21.6 s; 180 µL loop selected via 2D-LC dashboard)
Gradient Phases	Analysis: 35 min; equilibration: 0 min
Analytical Gradient	0 to 15 min: 0% B 15.1 to 35 min: 100% B
Stop Time	36.52 min
Multisampler	
Injection Volume	1 µL
Needle Wash	5 s in flush port; wash solvent:water
Draw Speed	40 µL/min
Eject Speed	100 µL/min
Offset	–1 mm
Vial Bottom Sensing	Checked
Thermostat	4 °C
Multicolumn Thermostats	
Column Temperatures	The trap column (AdvanceBio RP-mAb Diphenyl) was kept at 70 °C in the ^{1}D 1290 Infinity II Multicolumn Thermostat (G7116B), while the Bio-Monolith Recombinant Protein A and the AdvanceBio SEC columns were operated at 35 °C using the ^{2}D 1260 Infinity II Multicolumn Thermostat (G7116A).
VWD	
Wavelength	280 nm
Peak Width	>0.05 min (1 s response time) (10 Hz)

Results and discussion

Protein A affinity chromatography is the standard technique for purification of recombinant mAbs from matrix substances and can also be applied for mAb titer measurement. Therefore, a Bio-Monolith Recombinant Protein A column was used for the first chromatographic dimension (Figure 2). The column enables specific and reversible binding of the Fc region of antibodies to an immobilized Protein A ligand by application of a neutral binding buffer and an acidic elution buffer. The Protein A affinity chromatography resulted in excellent retention time precision showing a relative standard deviation (RSD) of 0.05% over seven consecutive injections of Trastuzumab.

To enable trapping and subsequent reduction of disulfide bonds, mAbs were eluted from the Protein A column and flushed onto the AdvanceBio RP-mAb Diphenyl column. As aqueous solvents were used in Protein A affinity chromatography, the mAb was retained by the reversed-phase column material. Trapping is driven by hydrophobic interactions between the amino acid moieties on the mAb surface with the reversed-phase column material. To reverse this interaction, application of nonpolar, organic solvent is needed. Selection of a 180 µL loop size via the 2D-LC dashboard resulted in a sampling time of 21.6 seconds. This time was sufficient to transfer the mAb peak eluting from the Protein A column to the reversed-phase trap column. If a larger volume than 180 µL is required to transfer a peak to the trap column, the desired volume can be adjusted by configuring a generic sampling loop via Agilent Lab Advisor software. After sampling the mAb peak by a time-based heart cut, the trap column was switched to the flow path of the second dimension using the 2D-LC valve. An aqueous TCEP solution enabled the reduction, while it also allowed the mAb to be retained on the reversed-phase trap column. TCEP is a reducing agent that is easily soluble and stable in aqueous solutions. To promote the disulfide bond reduction, the trap column was kept at 70 °C in a 1290 Infinity II Multicolumn Thermostat equipped with a Thermal Equilibration Device. Premixed acetonitrile:water (1:1) was used to elute the reduced mAb from the trap column and to separate the heavy and light chain on the SEC column. Due to the MS-friendly SEC conditions, the 2D-LC system could easily be coupled to the MS. To avoid contamination with salt transferred from the first chromatographic dimension and TCEP of the reduction step, the integrated diverter valve of the 6545XT AdvanceBio LC/Q-TOF was switched to the waste position in the beginning of the analysis and after elution of the heavy and light chain peaks (Figure 3). Application of the AdvanceBio SEC column in the second chromatographic dimension resulted in excellent retention time precision, as the heavy chain peak showed an RSD of 0.013% over seven consecutive injections and the light chain peak an RSD of 0.012%.

MS parameters	
Time Segments	0 min: waste 20 min: MS 27 min: waste
Acquisition Mode	Positive, extended (*m/z* 10,000) mass range
Mass Range	*m/z* 900 to 3,000
Reference Mass Correction	Enabled, *m/z* 922.009798
Acquisition Rate	0.5 spectra/s
Quad Amu	*m/z* 400
Gas Temperature	350 °C
Drying Gas	12 L/min
Nebulizer	60 psi
Sheath Gas Temperature	400 °C
Sheath Gas Flow	11 L/min
Capillary Voltage	5,500 V
Nozzle Voltage	2,000 V
Fragmentor	380 V
Skimmer	140 V
Oct 1 RF Vpp	750 V

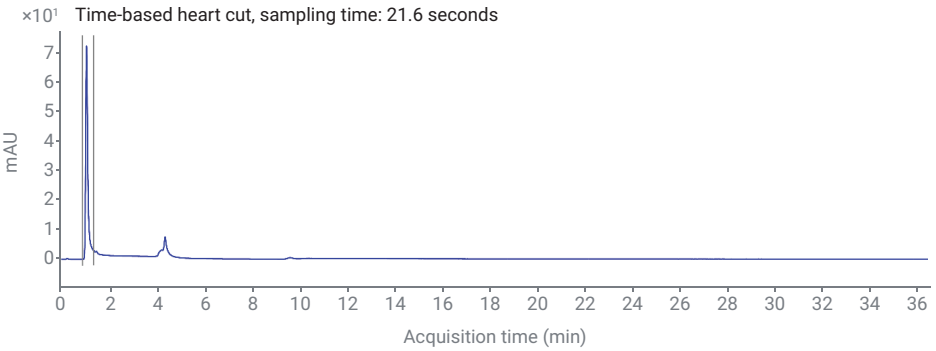

Figure 2. Protein A affinity chromatography of Trastuzumab in the first dimension showing the VWD signal at 280 nm.

MS data analysis was conducted by the MassHunter BioConfirm software. Maximum entropy algorithm and averaging of spectra across the top 25% of peak height over the mass range of *m/z* 1,000 to 3,000 were used for mass deconvolution. The deconvoluted masses were matched with usual mAb modifications and profiles as well as amino acid sequences of the heavy and light chain of Trastuzumab, which have been entered in MassHunter Sequence Manager. The mass spectrum resulting from the heavy chain of Trastuzumab as well as the different glycoforms identified in the corresponding deconvoluted spectrum are depicted in Figure 4.

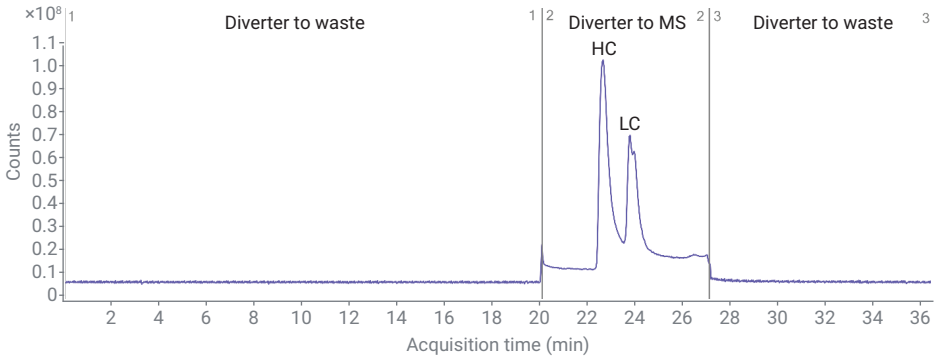

Figure 3. Online reduction of Trastuzumab by 2D-LC/MS showing the TIC of SEC in the second dimension separating the heavy chain (HC) and light chain (LC).

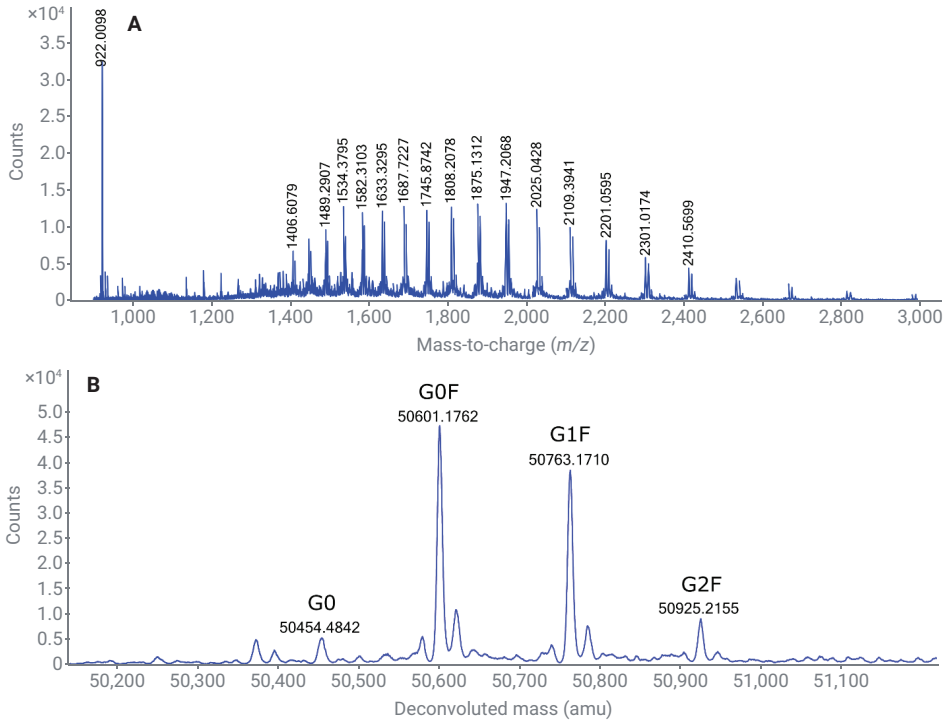

Figure 4. Extracted mass spectrum (A) and deconvoluted mass spectrum (B) of the heavy chain of Trastuzumab.

In Figure 5, the extracted mass spectrum and the deconvoluted mass spectrum of the light chain of Trastuzumab are depicted. The light chain was identified using the automated workflow for intact protein analysis and sequence matching functionality of the MassHunter BioConfirm software.

To further test the entire online reduction workflow, a second mAb sample was chosen for analysis. Therefore, 1 µL of Agilent-NISTmAb (part number 5191-5745) was used for injection and could be successfully reduced by application of the described system setup. MS data processing resulted in the deconvoluted spectra depicted in Figure 6. The deconvoluted masses could be matched with different glycoforms of the heavy chain as well as the amino acid sequence of the light chain of Agilent-NISTmAb.

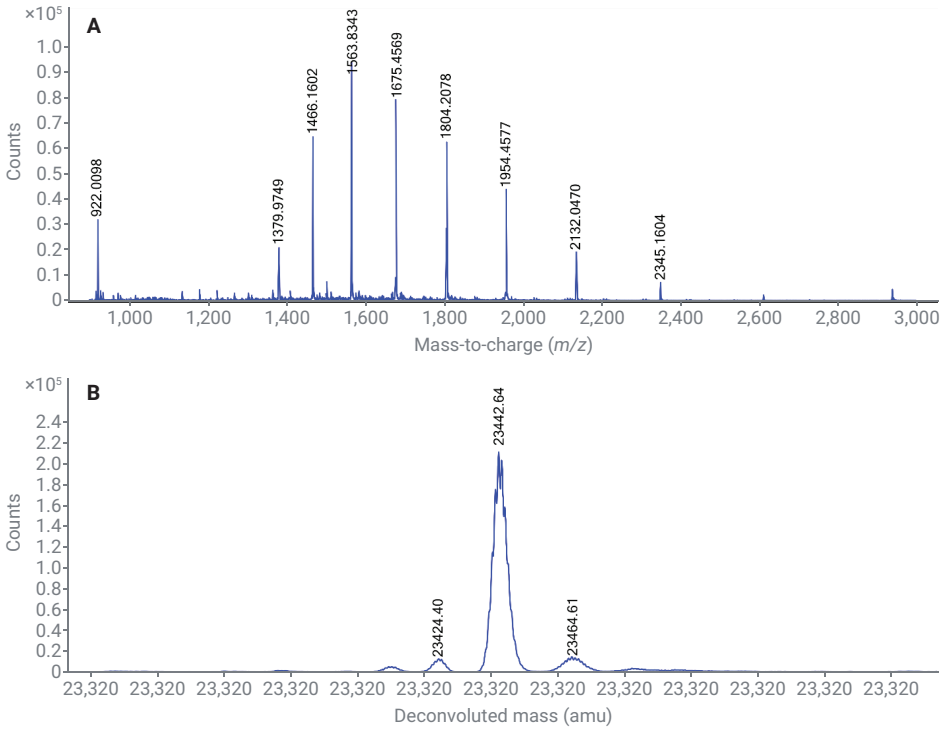

Figure 5. Extracted mass spectrum (A) and deconvoluted mass spectrum (B) of the light chain of Trastuzumab.

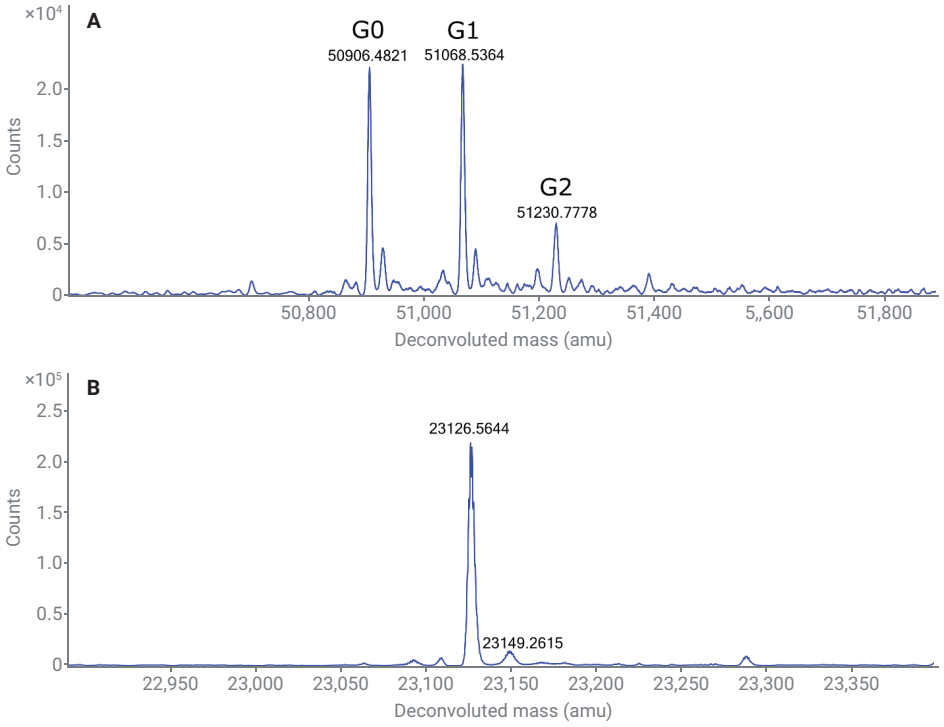

Figure 6. Deconvolution results of Agilent-NISTmAb (heavy chain: A; light chain: B).

Conclusion

Subunit analysis is a possible strategy to assess critical quality attributes in mAb products. This application note describes an online reduction workflow for mAbs using 2D-LC coupled to the Agilent 6545XT AdvanceBio LC/Q-TOF. Application of an Agilent AdvanceBio RP-mAb Diphenyl column instead of a 2D-LC sampling loop enabled trapping and subsequent reduction of two different mAbs. Reproducible purification of mAbs by Protein A affinity chromatography in the first dimension and separation of mAb subunits by SEC were achieved. 2D-LC was also used as a desalting tool to eliminate MS-incompatible solvent additives resulting from the first chromatographic dimension. Desalting allowed identification of mAb subunits by matching their deconvoluted mass spectrum with their amino acid sequences using the 6545XT AdvanceBio LC/Q-TOF system.

References

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