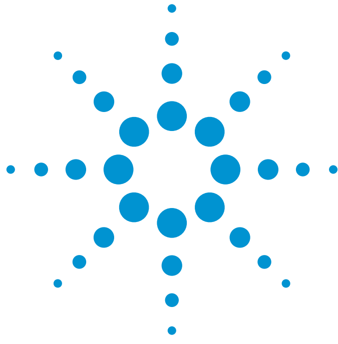


Agilent AdvanceBio Desalting-RP Cartridges for Online Desalting in 2D-LC/MS mAb Analysis

Application Note

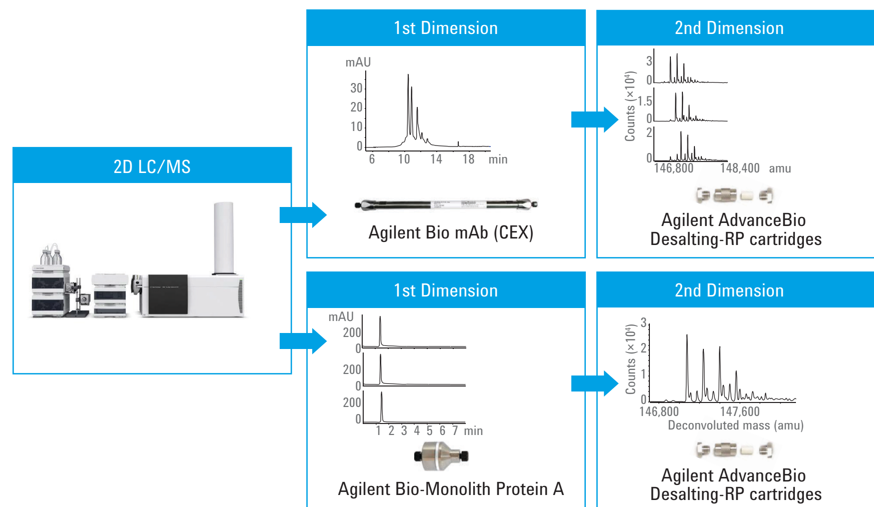
Biotherapeutics and Biologics

Authors

Suresh Babu C.V. and Ravindra Gudihal
Agilent Technologies, Inc.

Abstract

This Application Note describes the use of a desalting cartridge in the second dimension to desalt and characterize monoclonal antibodies (mAbs) using a 2D-LC/MS setup. Ion exchange (IEX) and affinity chromatography, which are primarily salt-based elution conditions, were used in the first dimensional separation. In the second dimension, an Agilent AdvanceBio Desalting-RP cartridge was used to desalt fractions before MS analysis. The results demonstrate the excellent performance of the desalting cartridge by providing high-quality MS data.



Examples of 2D-LC/MS workflows used for the characterization of monoclonal antibodies

Agilent Technologies

Introduction

Monoclonal antibody (mAb) therapeutics are a constantly growing area of the pharmaceutical industry. These molecules are complex in nature, and undergo various enzymatic or chemical modifications during the development process and their lifetime. These modifications result in heterogeneity including fragmentation, post-translational modifications (PTMs), degradation, aggregation, and sequence variants. Therefore, thorough physicochemical characterization is necessary to ensure the safety, quality, and efficacy of the product. Multidimensional liquid chromatography (2D-LC) coupled to mass spectrometry (MS) is the preferred analytical technique to characterize different mAb variants. Techniques such as affinity, ion exchange (IEX), and size exclusion chromatography (SEC) are used in the first dimensional separation. The typical mobile phase used with these LC techniques is aqueous-based, and contains nonvolatile salts, which are not compatible with MS detection. To identify these chromatographically separated peaks by MS, an online desalting approach in the second dimension was employed.

This Application Note describes the use of AdvanceBio Desalting-RP cartridges as an online desalting column in the second dimension to analyze mAbs. For the first LC dimension, IEX was used to characterize the charge variants; in addition, affinity capture, using protein A columns, was used to analyze different mAb samples including an innovator and biosimilar. The results demonstrated the excellent performance of Agilent AdvanceBio Desalting-RP cartridges by providing enhanced MS data.

Experimental

Samples

Therapeutic monoclonal antibodies mAb1 (innovator), mAb2 (biosimilar), and mAb3 (innovator) were purchased from a local pharmacy, and stored according to the manufacturers' instructions. All solvents used were LC grade.

Instrumentation

An Agilent 1290 Infinity 2D-LC solution was used, comprising the following modules:

- Agilent 1260 Bio-inert quarternary pump (G5611A)
- Agilent 1290 Infinity binary pump (G4220A)
- Agilent 1290 Infinity autosampler (G4226A) with 1290 Infinity thermostat (G1330B)
- Agilent 1290 Infinity thermostatted column compartments (2X G1316C)
- Agilent 1290 Infinity valve drive (G1170A) with 2-position/4-port duo-valve (2D-LC valve head, G4236A)
- Agilent 1290 Infinity valve drives (2X G1170A) with multiple heart-cutting valves (2x G4242-64000) equipped with 40-µL loops
- Agilent 1260 Infinity diode array detectors (2x G1315C) with 10-mm flow cell
- Agilent 6530 accurate-mass Q-TOF LC/MS (G6530A)

Figure 1 shows the 2D-LC/MS configuration setup.

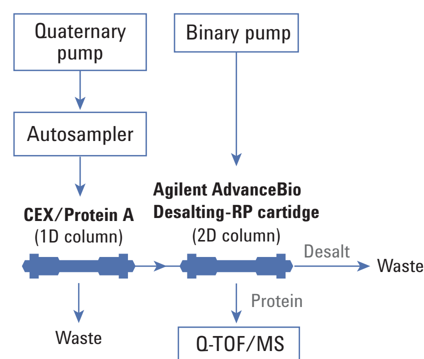


Figure 1. Schematics of 2D-LC/MS configuration.

Chromatographic conditions

Multiple heart-cutting 2D-LC/MS analysis (IEX → Desalting-RP; Affinity → Desalting-RP)	
First-dimension pump (IEX)	
Solvent A	Water
Solvent B	NaCl (850.0 mM)
Solvent C	NaH_2PO_4 (41.0 mM)
Solvent D	NaH_2PO_4 (55.0 mM)
Flow rate	0.75 mL/min
Gradient	Salt gradient calculated using Agilent Buffer Advisor (Buffer: 30 mM, pH: 6.3, and NaCl: 0–161.5 mM) 0 minutes: 30.3 %A, 0.0 %B, 59.6 %C, 10.1 %D 2 minutes: 26.0 %A, 5.0 %B, 56.9 %C, 12.1 %D 8 minutes: 21.5 %A, 10.0 %B, 54.9 %C, 13.6 %D 20 minutes: 13.3 %A, 19.0 %B, 51.9 %C, 15.8 %D 35 minutes: 30.3 %A, 0.0 %B, 59.6 %C, 10.1 %D
Post time	10 minutes

Chromatographic conditions, continued

First-dimension pump (Protein A, affinity)		
Solvent A	20 mM sodium phosphate buffer, pH 7.4	
Solvent B	0.5 M acetic acid	
Flow rate	1 mL/min	
Gradient	0 to 0.5 minutes: 0 %B (binding) 0.6 to 1.7 minutes: 100 %B (elution) 1.8 to 3.5 minutes: 0 %B (regeneration)	
Second-dimension pump (Desalting-RP)		
Solvent A	0.1 % FA	
Solvent B	0.1 % FA in ACN	
Flow rate	0.4 mL/min	
Gradient	0 minutes: 5 %B 0.5 minutes: 5 %B 3.0 minutes: 80 %B 4.0 minutes: 80 %B 4.1 minutes: 5 %B 6.0 minutes: 5 %B	
2D gradient stop time	6.0	
2D cycle time	6.1	
Autosampler		
Injection volume	5 µL	
Sample temperature	5 °C	
Columns (IEX → Desalting-RP) (Affinity → Desalting-RP)		
First-dimension columns	Agilent Bio MAb NP5, 4.6 × 250 mm, 5 µm PEEK (p/n 5190–2407) Agilent Bio-Monolith Protein A, 4.95 × 5.2 mm, 5 µm (p/n 5069–3639)	
Second-dimension column	Agilent AdvanceBio Desalting-RP cartridge, 2.1 × 12.5 mm, 10 µm, 1,000 Å (p/n PL1612-1102)	
Thermostatted column compartment		
First-dimension column	Room temperature	
Second-dimension column	Room temperature	
Multiple heart-cutting		
Operation mode	Time-based, multiple heart-cutting was performed with the heart-cuts (2D time segments) set according to the first-dimension retention times. Heart-cutting of impurities was conducted with a sampling time of 0.04 minutes (loop filling of >200 %).	
Detection		
First dimension DAD parameters	Wavelength 280 nm/4 nm Ref. 360 nm/100 nm	
Second dimension MS parameters	Gas temperature Sheath gas temperature Gas flow Sheath gas flow Nebulizer Vcap Nozzle Fragmentor	350 °C 400 °C 8 L/min 11 L/min 35 psi 5,000 V 1,000 V 200 V
Timing of LC stream	Time segments with MS valve switching (waste/MS)	

Software

- Agilent OpenLAB CDS ChemStation Edition Software, version C.01.07 [27] with Agilent 1290 Infinity 2D-LC Acquisition Software, version A.01.02 [24]
- Agilent MassHunter Workstation Software, Version B.05.01, Build 4.0.479.0
- Agilent Buffer Advisor A.01.01 [009]

Results and Discussion

Due to heterogeneity of mAbs, 2D-LC with mass spectrometry detection is a method of choice for characterization of biotherapeutics. Chromatographic techniques such as IEX, SEC, and affinity chromatography are used to analyze critical quality attributes. In the present study, both affinity purification and IEX were selected in first dimensional separation. Followed with an RP desalting step in the second dimension to analyze mAb samples without the need for manual desalting of the fractions.

IEX → Desalting-RP

Weak cation exchange chromatography (WCX) using a salt gradient is commonly used for the separation of mAb charge heterogeneity. To further characterize these charge variant peaks, a desalting step is required before MS analysis. Figures 2 and 3 show the 2D-LC/MS charge variant results for innovator and biosimilar mAb. In the first dimension, an Agilent Bio MAb PEEK column was used to separate the charge species. Then, in the second dimension, an Agilent AdvanceBio Desalting-RP cartridge was used for desalting. The innovator mAb showed a single major peak, designated as the main peak, in addition to low levels of basic and acidic variants. Early and late-eluting peaks were called acidic and basic variants, respectively. The chromatogram of biosimilar mAb showed different separation profiles, indicating different degrees of basic and acidic variants compared to innovator mAb. The selected peaks of innovator and biosimilar mAb were transferred to a second-dimension desalting cartridge a using multiple heart-cutting method for MS analysis. References 1 and 2 provide more information on the multiple heart-cutting 2D-LC method. Figure 3 shows the deconvoluted MS results for these charge variant peaks. In WCX, the main peak of innovator and biosimilar mAbs were eluted at the same retention time, and respective deconvoluted mass measurements confirm that these peaks are of same identity. The MS analysis of peak 2 and peak 3 of biosimilar mAb reveals a 128 Da shift between peaks 1, 2, and 3. This shift corresponds to C-terminal lysine truncation (confirmed using carboxypeptidase B digestion, data not shown). This set of data demonstrates the effective online desalting approach using AdvanceBio Desalting-RP cartridges for identification of the mAb charge variants. For high resolution mAb separation, an Agilent AdvanceBio RP-mAb column can be used in the second dimension[3].

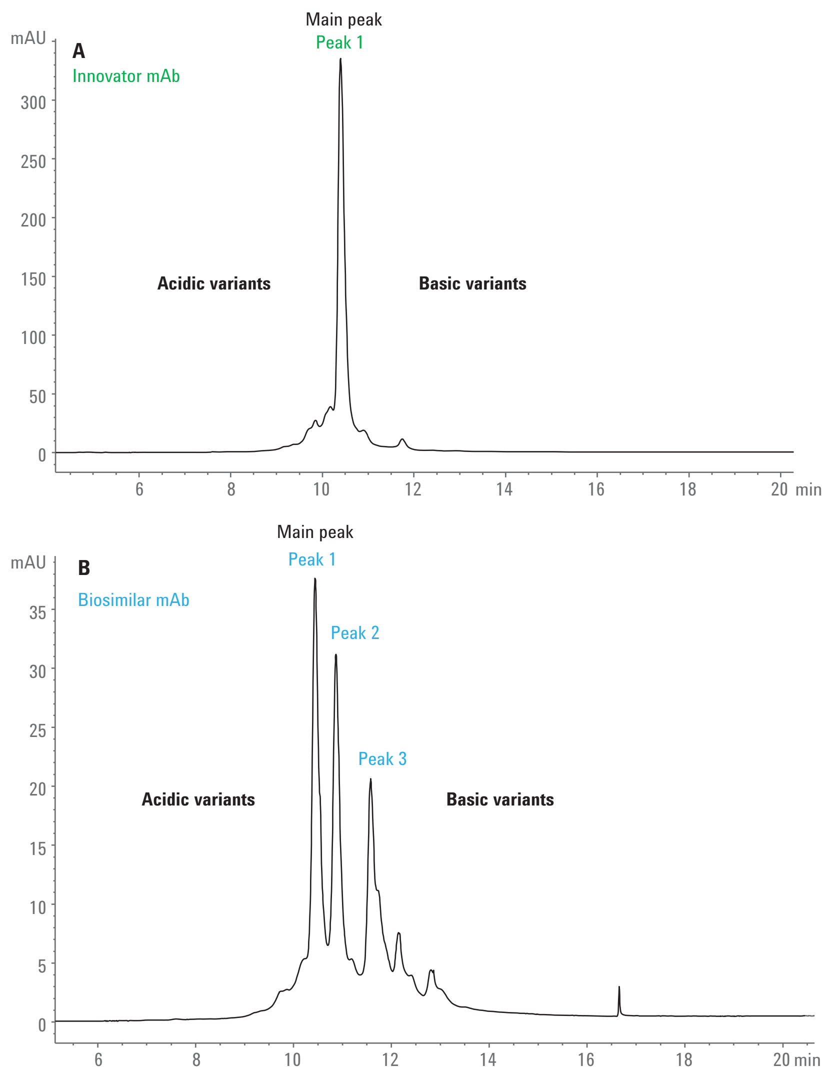


Figure 2. First-dimension charge variant profiles of innovator and biosimilar mAb using an Agilent Bio MAb 5 µm column. Peaks assigned 1, 2, and 3 were transferred to a second dimension (Agilent AdvanceBio Desalting-RP cartridges) for MS analysis.

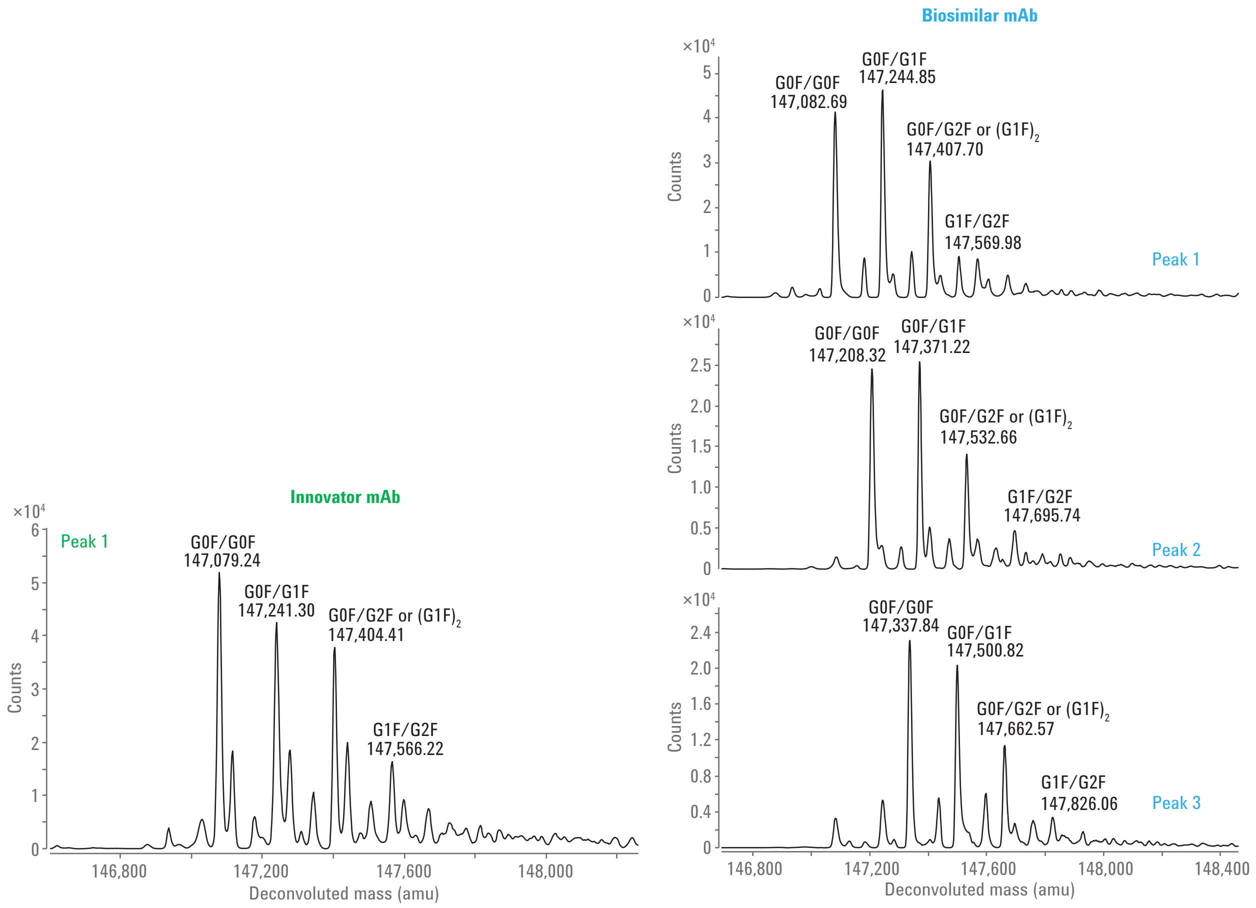


Figure 3. Deconvoluted mass spectra for peaks 1, 2, and 3 assigned in Figure 2.

Affinity → Desalting-RP

During mAb production and purification, it is important to ensure selection of the right clone, which produces the correct product candidate with high expression. Typically, affinity chromatography using Protein A and Protein G is applied to purify as well as determine the mAb concentration in the cell culture. Furthermore, the identity of the product peak can be confirmed using MS analysis. In this study, three pharmaceutical mAbs were analyzed using a 2D-LC/MS heart-cutting method. In the first dimension, an Agilent Bio-Monolith Protein A column was used to capture and enrich the mAb. An Agilent AdvanceBio Desalting-RP cartridge was used in the second dimension for desalting before mass spectrometry analysis. The affinity purified peaks from the Protein A column (Figure 4) were transferred using the heart-cutting method to the second-dimension desalting cartridge for MS analysis. Figure 4 shows the deconvoluted mass spectra for the three respective mAb samples. The analysis of mass measurements confirms the right quality of mAbs. This set of data demonstrates the quick online desalting approach using AdvanceBio Desalting-RP cartridges.

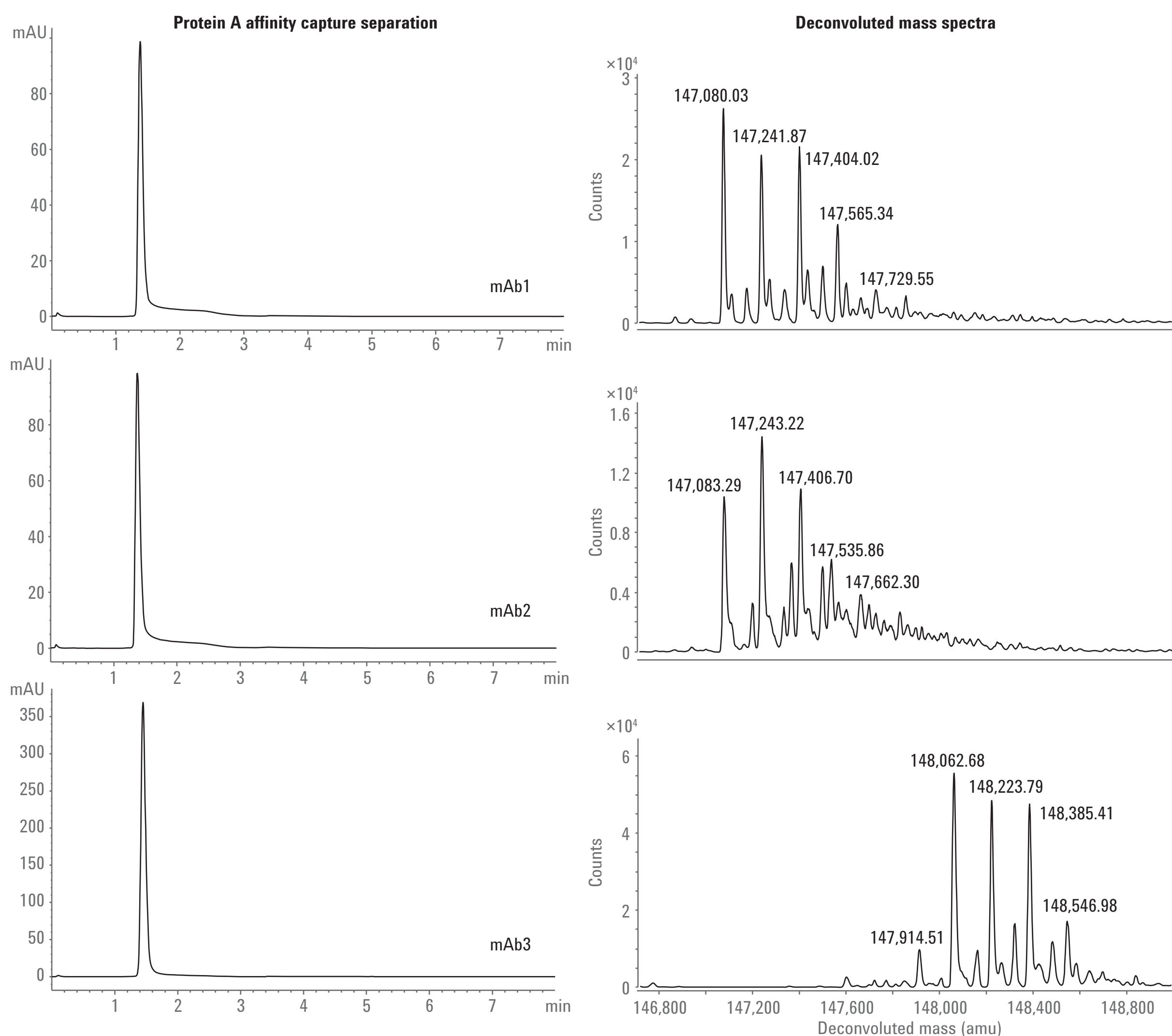


Figure 4. 2D-LC/MS of mAb profiles using Protein A as the first-dimension column and desalt cartridges as the second-dimension column. The selected main peak in the first-dimension chromatogram was captured and transferred to an Agilent AdvanceBio Desalting-RP cartridge, which effectively removed salts and provided high-quality MS results.

Conclusion

This study demonstrates a workflow solution for mAb characterization using an Agilent 2D-LC system coupled to Agilent biocolumns. This was achieved through the application of charge variant or protein affinity capture in the first-dimension separation, followed by desalting in the second dimension using an Agilent AdvanceBio Desalting-RP cartridge before MS analysis. The results demonstrate effective online desalting using AdvanceBio Desalting-RP cartridges.

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