Application Note

Biopharma/Pharma

Native SEC/MS Analysis of mAbs on an Entirely Biocompatible Flow Path

Using the Agilent 1290 Infinity II Bio LC System and Agilent AdvanceBio SEC 200 Å 1.9 µm PEEK-lined column in mAbs analysis

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Abstract

Size exclusion chromatography (SEC) of recombinant therapeutic proteins, such as monoclonal antibodies (mAbs), is an essential analytical tool to detect the presence of size variants, which may have adverse effects on safety and efficacy. SEC is commonly carried out using nonvolatile phosphate buffers, which makes the separation incompatible with mass spectrometry (MS). For this reason, a desalting step (online or offline) is required for peak identification.

The use of volatile MS-compatible buffers, such as ammonium acetate, has been demonstrated to be significantly less effective in comparison with phosphate buffers when applied on classic stainless-steel instrument/column configurations due to nonspecific interactions. The latter can be counteracted by using biologically inert or biocompatible flow paths. This application note demonstrates the potential of the combination of a polyether ether ketone (PEEK)-lined Agilent AdvanceBio SEC column with the Agilent 1290 Infinity II Bio LC System for native SEC/MS analysis of mAbs.

Introduction

SEC, in which proteins elute based on hydrodynamic radius (size), is widely used for studying aggregation and fragmentation associated with mAbs.[1–4] SEC enables separation of size variants under nondenaturing aqueous conditions, often using a phosphate buffer at neutral pH and high ionic strength. It represents the only chromatographic technique that does not rely on interactions with the stationary phase and therefore does not require the application of a mobile phase gradient. Instead, molecules travel through the column at a speed dependent on particle pore accessibility. Very large molecules are excluded and elute first, while smaller molecules can penetrate pores to various degrees depending on size. Unfortunately, unwanted secondary interactions with the stationary phase can take place, drastically impacting separation by causing tailing peaks, poor recoveries, and shifted elution times. These interactions, commonly electrostatic or hydrophobic, can be suppressed by adapting the mobile phase composition (addition of salts, organic solvent, etc.).[5] More recently, however, novel and more inert SEC materials have been introduced that limit or prevent secondary interactions while using common mobile phase compositions. A powerful example is the AdvanceBio SEC column, which enables the analysis of the most challenging/problematic protein biopharmaceuticals by using silica particles coated with a hydrophilic polymer. In this way, potential active sites on the stationary phase are shielded.[6,7]

SEC is not compatible with MS due to the use of nonvolatile buffers and salts. Peak identification therefore requires fraction collection and desalting to allow successful electrospray ionization (ESI). Nowadays, these processes are typically performed in an automated manner using two-dimensional LC/MS (2D-LC/MS): first-dimension SEC peaks are collected in loops installed on a multiple heart-cutting valve and then desalted in an online manner using reversed-phase liquid chromatography (RPLC) or SEC in the second dimension.[8,9] In the last couple of years, however, there has been a trend to directly combine SEC with MS using volatile mobile phases such as ammonium acetate.[10] Interestingly, this trend has shed light on another type of nonspecific interaction: the interaction of mAbs with stainless-steel surfaces, which are omnipresent in the LC system, capillary tubing, and column hardware.[11,12] This effect is often masked when using a phosphate buffer at high ionic strength.

Successful native SEC/MS measurements of mAbs therefore demand fully biocompatible flow paths, which the 1290 Infinity II Bio LC System and PEEK-lined AdvanceBio SEC column provide. The 1290 Infinity II Bio LC System is a biocompatible instrument with an iron-free flow path that minimizes the risk of unwanted interactions.[13] To enable chromatography at very high pressures, the metal alloy MP35N is used throughout the system instead of stainless-steel. The stainless-steel surfaces in the PEEK-lined AdvanceBio SEC column are masked using PEEK. The combination of this system and column provides superior biocompatibility, chemical resistance, and mechanical and thermal stability.

This application note compares the performance of the 1290 Infinity II Bio LC System and PEEK-lined AdvanceBio SEC column with their stainless-steel counterparts for the native SEC/MS analysis of mAbs.

Experimental

Materials

Sodium phosphate (NaH_2PO_4, Na_2HPO_4) and ammonium acetate were acquired from Sigma-Aldrich (St. Louis, MO, USA) and phosphate-buffered saline (PBS) was acquired from Thermo Fisher Scientific (Waltham, MA, USA). Type I water was produced from tap water by an Arium pro Ultrapure Lab Water System from Sartorius (Göttingen, Germany). Gel filtration standard, containing molecular weight markers thyroglobulin (670,000 Da), gamma-globulin (158,000 Da), ovalbumin (44,000 Da), myoglobin (17,000 Da), and vitamin B12 (1,350 Da), was obtained from Bio-Rad (Hercules, CA, USA). Trastuzumab and the antibody drug conjugate (ADC) brentuximab vedotin were provided by, respectively, Roche (Basel, Switzerland) and Seattle Genetics (Bothell, WA, USA). NISTmAb reference material (RM8671) was purchased from Agilent Technologies (Santa Clara, CA, USA).

Sample and mobile phase preparation

mAbs were diluted to 1 mg/mL in PBS. Both mAbs were slightly stressed (1 week at 50 °C) to increase high molecular weight (HMW) and low molecular weight (LMW) species. Gel filtration standard was diluted in water according to the manufacturer's instructions. Mobile phases were freshly prepared and filtered over a 0.2 µm bottle top filter before use.

Instrumentation

Two Agilent 1290 Infinity II LC Systems were used: the 1290 Infinity II LC (stainless-steel [SST] LC) and the 1290 Infinity II Bio LC (biocompatible). Both systems were equipped with a 75 µm internal diameter ultralow dispersion (ULD, also SST or biocompatible) kit to reduce peak broadening.

Configuration details

	Agilent Stainless-Steel LC System	Agilent Bio LC System
Pump	1290 Infinity II High-Speed Pump (G7120A)	1290 Infinity II Bio High-Speed Pump (G7132A)
Autosampler	1290 Infinity II Multisampler (G7167B) with integrated sample thermostat	1290 Infinity II Bio Multisampler (G7137A) with integrated sample thermostat
Column Compartment	1290 Infinity II Multicolumn Thermostat (G7116B) with ULD heat exchanger (G7116-60021)	1290 Infinity II Multicolumn Thermostat (G7116B) with Bio ULD heat exchanger (G7116-60091)
Detector	1290 Infinity II DAD (G7117B)	1290 Infinity II DAD (G7117B)
Flow Cell	Standard InfinityLab Max-Light Cartridge Cell, 10 mm (G4212-60008)	Max-Light Cartridge Cell LSS, 10 mm (G7117-60020)
Ultralow Dispersion Kit	Low dispersion kit for 1290 Infinity II LC (5067-5963)	Ultralow dispersion kit for 1290 Infinity II Bio LC (5004-0007)
MS System	6545 LC/Q-TOF with Agilent Jet Stream Technology	

Method

Two Agilent AdvanceBio SEC columns were evaluated: stainless-steel (SST) and PEEK-lined. SEC/UV data were acquired and processed in Agilent OpenLab CDS version 2.6. Native SEC/MS data were obtained in Agilent MassHunter for Data Acquisition (B.09.00) and analyzed using Agilent MassHunter Qualitative Analysis with BioConfirm add-on (B.07.00).

SEC conditions

	Stainless-Steel Column	PEEK-Lined Column
Column	Agilent AdvanceBio SEC 200 Å, 4.6 × 150 mm, 1.9 µm (p/n PL1580-3201)	Agilent AdvanceBio SEC 200 Å, PEEK-lined, 2.1 × 150 mm, 1.9 µm (p/n PL1980-3201PK)
Flow Rate	360 µL/min	75 µL/min
Injection Volume/Load UV	19 µL/19 µg	4 µL/4 µg
Injection Volume/Load MS		10 µL/50 µg
Mobile Phases	150 mM sodium phosphate, pH 7 100 mM ammonium acetate 50 mM ammonium acetate	
Column Temperature	25 °C	
Autosampler Temperature	6 °C	
Detection UV	280/4 nm, reference off, peak width >0.1 min	

Native MS parameters

Parameter	Value	
Ionization	Electrospray positive ionization	
MS Diverter Valve	Bypassed	
Source Parameters	Drying gas temperature	300 °C
	Drying gas flow	8 L/min
	Nebulizer pressure	35 psi
	Sheath gas temperature	300 °C
	Sheath gas flow	8 L/min
	Capillary voltage	3,500 V
	Nozzle voltage	1,000 V
	Fragmentor	350 V
	Skimmer	220 V
Acquisition Parameters	Mass range	Extended mass range (2 GHz)
		500 to 10,000 *m/z*
	Acquisition speed	1 spectrum/sec
	Mode	High resolution mode
		Profile acquisition

Results and discussion

Figure 1 shows the SEC UV 280 nm chromatograms of several mAbs in overlay with the gel filtration standard obtained on the 1290 Infinity II Bio LC and the AdvanceBio SEC 200 Å PEEK-lined column using 150 mM sodium phosphate (i.e., the golden standard mobile phase in SEC) and 100 mM ammonium acetate as the MS-compatible alternative. The successful use of the latter mobile phase opens opportunities for directly combining SEC with MS without the need for a desalting step.

Note the satisfactory separation performance for all mAbs, including the ADC, which is facilitated by the hydrophilic polymeric coating of the AdvanceBio SEC particles. The hydrophobic nature associated with ADCs makes their analysis challenging on earlier-generation SEC particles due to secondary interactions with the stationary phase.

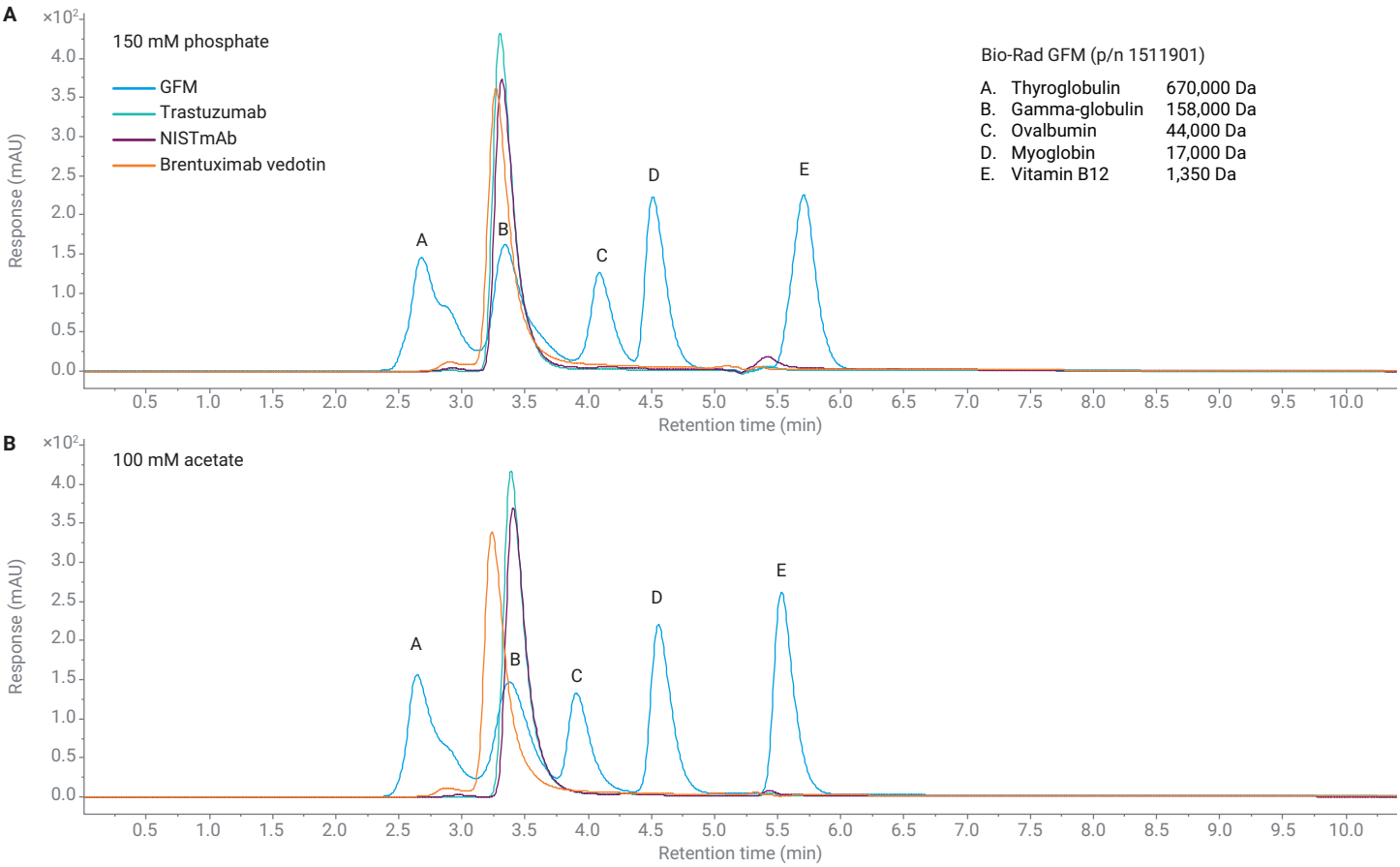

Figure 1. UV 280 nm SEC chromatograms of gel filtration standard in overlay with trastuzumab, NISTmAb, and brentuximab vedotin obtained on the Agilent 1290 Infinity II Bio LC and the Agilent AdvanceBio SEC 200 Å PEEK-lined column using (A) 150 mM sodium phosphate and (B) 100 mM ammonium acetate as mobile phase.

To evaluate the impact of the absence or presence of stainless-steel components in the SEC flow path, mAb samples were analyzed on several system or column combinations. Figures 2 and 3 show the analysis of, respectively, trastuzumab and NISTmAb on a classic stainless-steel system and column (left), a stainless-steel system and PEEK-lined column (middle), and a biocompatible LC system and PEEK-lined column (right). All combinations were evaluated with a 150 mM phosphate mobile phase, and with 50 and 100 mM ammonium acetate phases as MS-compatible alternatives. Sodium phosphate gives satisfactory separation performance on all combinations tested, with good recovery of HMW species. When replacing sodium phosphate with ammonium acetate and reducing the mobile phase ionic strength, the quality of the separation is adversely affected where there are stainless-steel components in the flow path. The impact of the column hardware is such that HMW species are scarcely detected, if at all, when a stainless-steel column is used with ammonium acetate as mobile phase. HMW species reappear when a PEEK-lined column is installed and run with 100 mM ammonium acetate. With the 50 mM ammonium acetate mobile phase, which is the most beneficial in terms of MS sensitivity, only the biocompatible LC system and PEEK-lined column combination (bottom-right chromatograms) provides a useful result, with detection of the HMW impurities.

The impact of the instrument on peak width and tailing is further illustrated in Figure 4. Peak width is consistently smaller when the 1290 Infinity II Bio LC is applied. Peak tailing also does not increase upon replacing the phosphate mobile phase with MS-compatible acetate. On the other hand, both peak width and tailing deteriorate significantly when the mobile phase is altered on a stainless-steel LC system. Reducing the ionic strength of the mobile phase amplifies this effect.

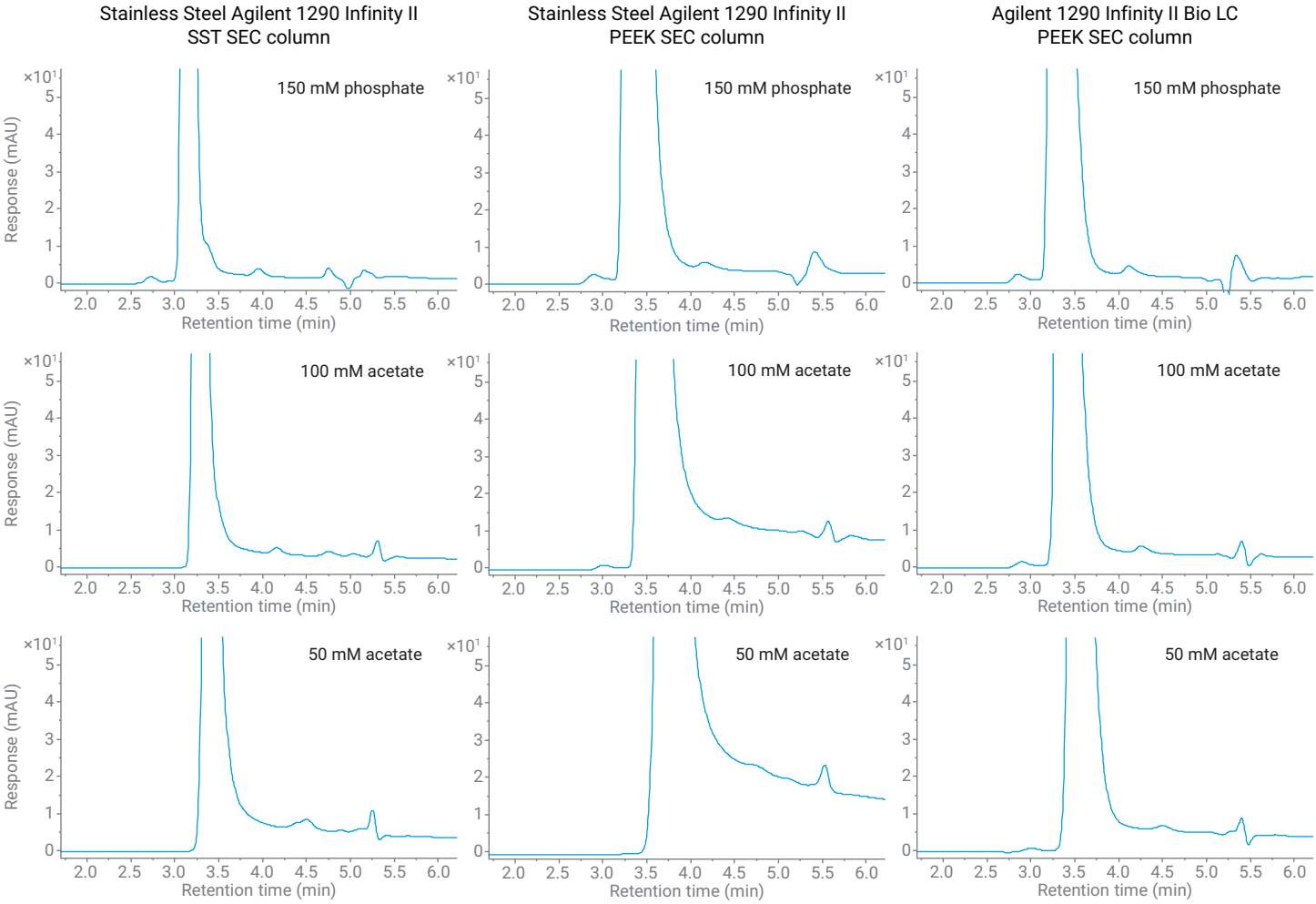

Figure 2. UV 280 nm SEC chromatograms of trastuzumab using different mobile phase compositions and different degrees of hardware inertness.

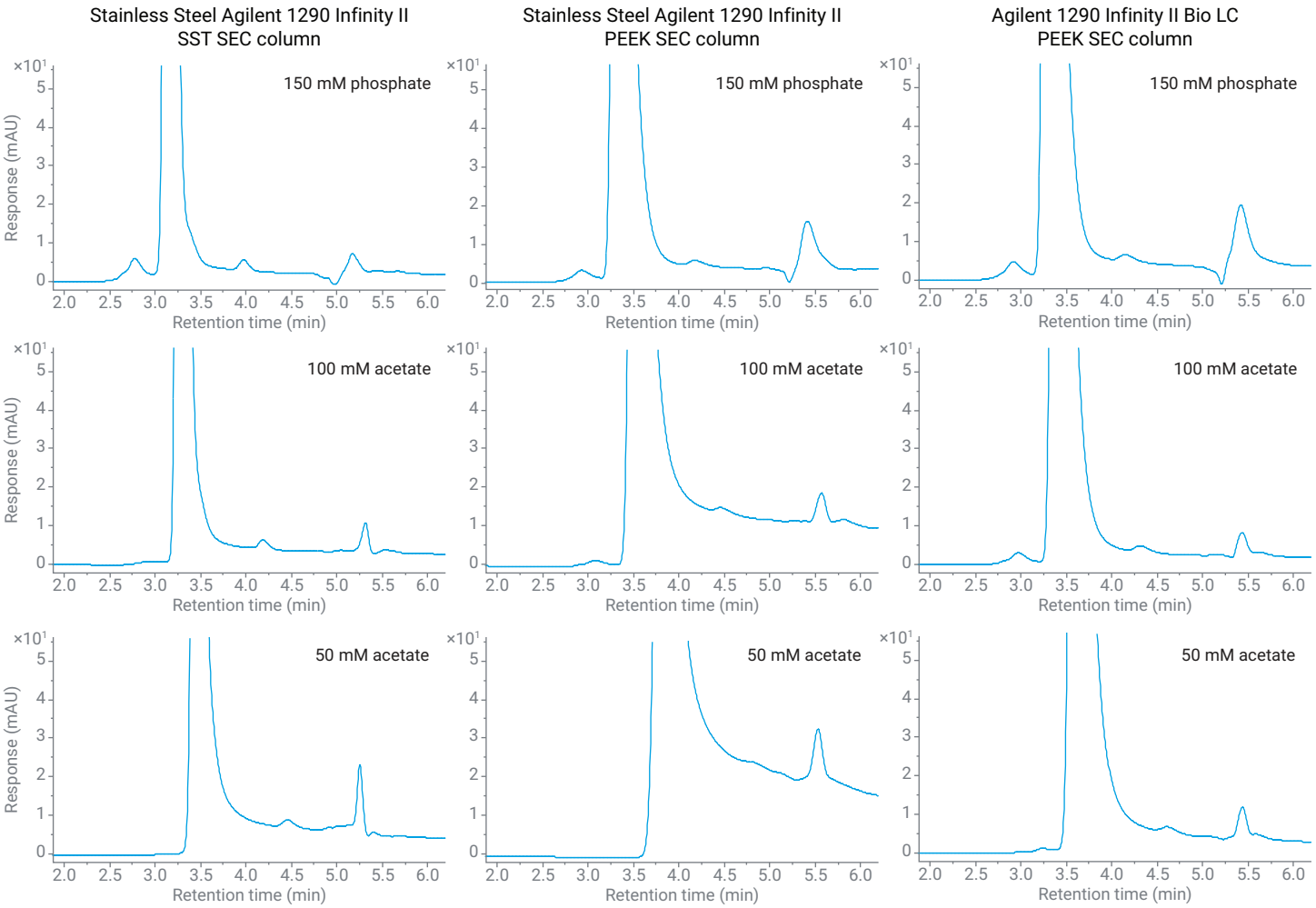

Figure 3. UV 280 nm SEC chromatograms of NISTmAb using different mobile phase compositions and different degrees of hardware inertness.

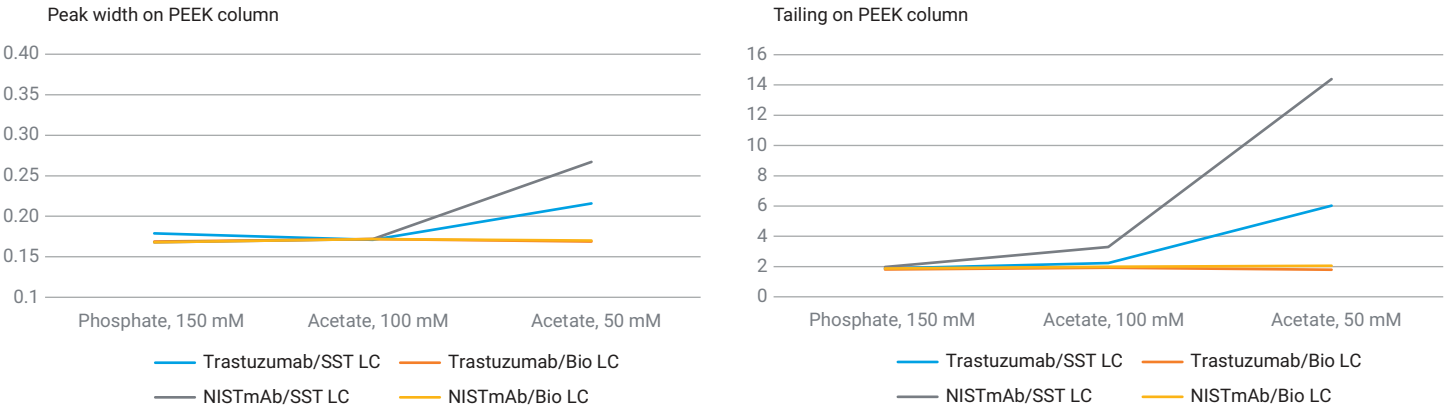

Figure 4. Peak width and tailing observed in the UV 280 nm SEC chromatograms of trastuzumab and NISTmAb obtained using the Agilent 1290 Infinity II LC (SST LC) and Agilent 1290 Infinity II Bio LC and different mobile phase compositions.

The excellent performance of the 1290 Infinity II Bio LC System combined with the PEEK-lined AdvanceBio SEC column (using ammonium acetate) opens opportunities for the direct hyphenation of SEC to native MS to support mAb characterization. As a proof-of-concept, mAbs were analyzed with this hardware and the Agilent 6545 LC/Q-TOF. The 100 mM ammonium acetate mobile phase was selected as a compromise between chromatographic performance and MS sensitivity.

The SEC/UV/MS analysis of the NISTmAb is presented in Figure 5. The UV 280 nm chromatogram shows HMW and LMW variants that could be identified as, respectively, mAb dimer and Fab fragments based on the deconvoluted MS spectra. The spectrum associated with the main peak highlights several glycoforms decorated with the bi-antennary complex N-glycans G0, G0F, G1F, and G2F. The spectrum also reveals the existence of mAb variants carrying C-terminal lysine.

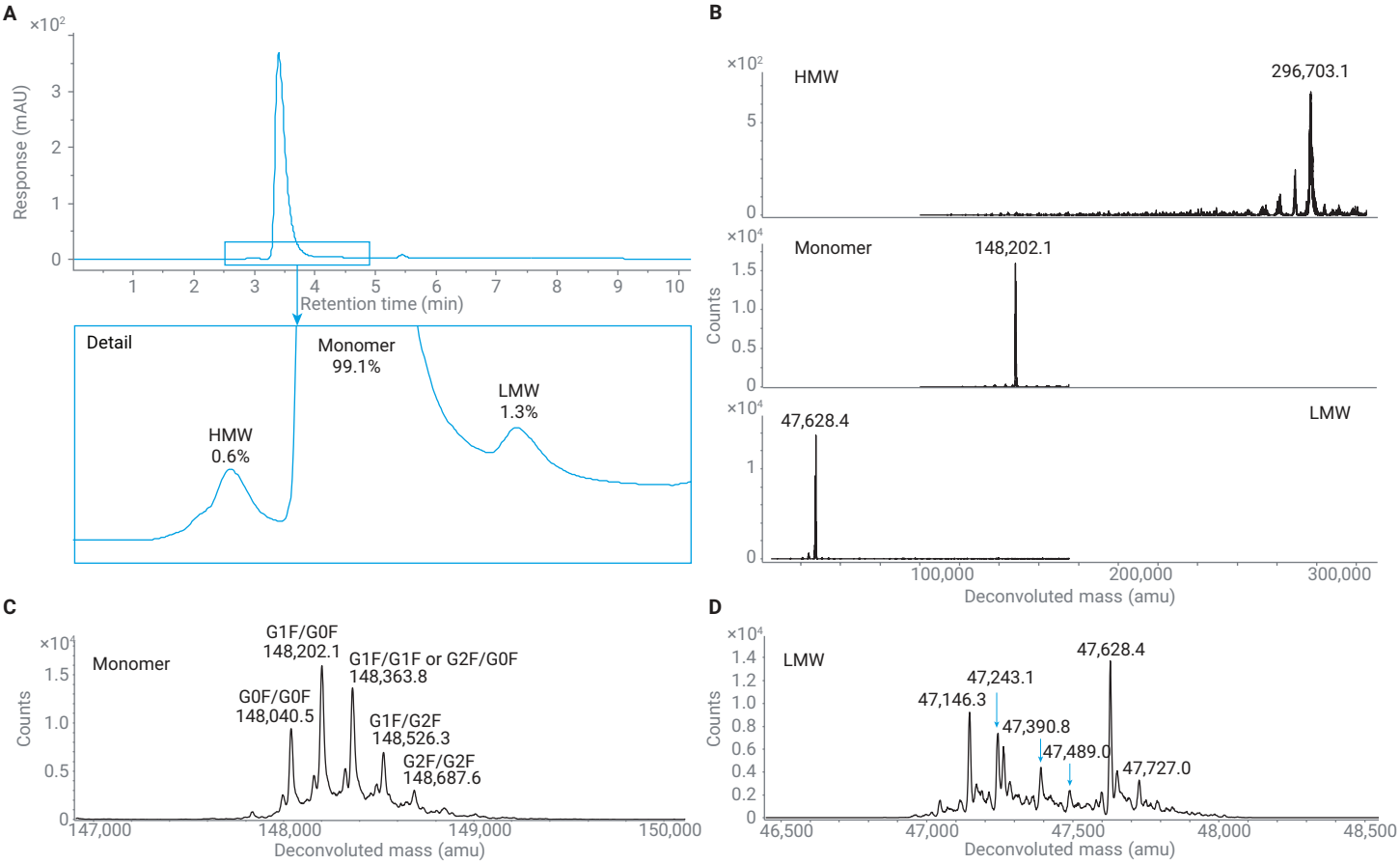

Figure 5. SEC/UV/MS of NISTmAb stressed at 50 °C for 1 week. (A) UV 280 nm chromatogram, (B) deconvoluted native MS spectra associated with peaks annotated as HMW, monomer, and LMW and zoom of the deconvoluted native MS spectra of monomer (C) and LMW (D).

Figure 6 presents the SEC/UV/MS analysis of brentuximab vedotin, an interchain cysteine-conjugated ADC. The measurement of this ADC clearly benefits from the nondenaturing SEC and MS conditions, as light and heavy chains are solely maintained by noncovalent interactions. This state results from the interchain disulfide bound reduction and subsequent conjugation of the free sulfhydryl groups.

The deconvoluted spectrum associated with the main peak highlights the drug distribution and drug-to-antibody ratio (DAR), a critical quality attribute (CQA) for ADCs. In addition, the glycosylation pattern is revealed as well as a subpopulation of DAR species carrying linker without cytotoxic drug.

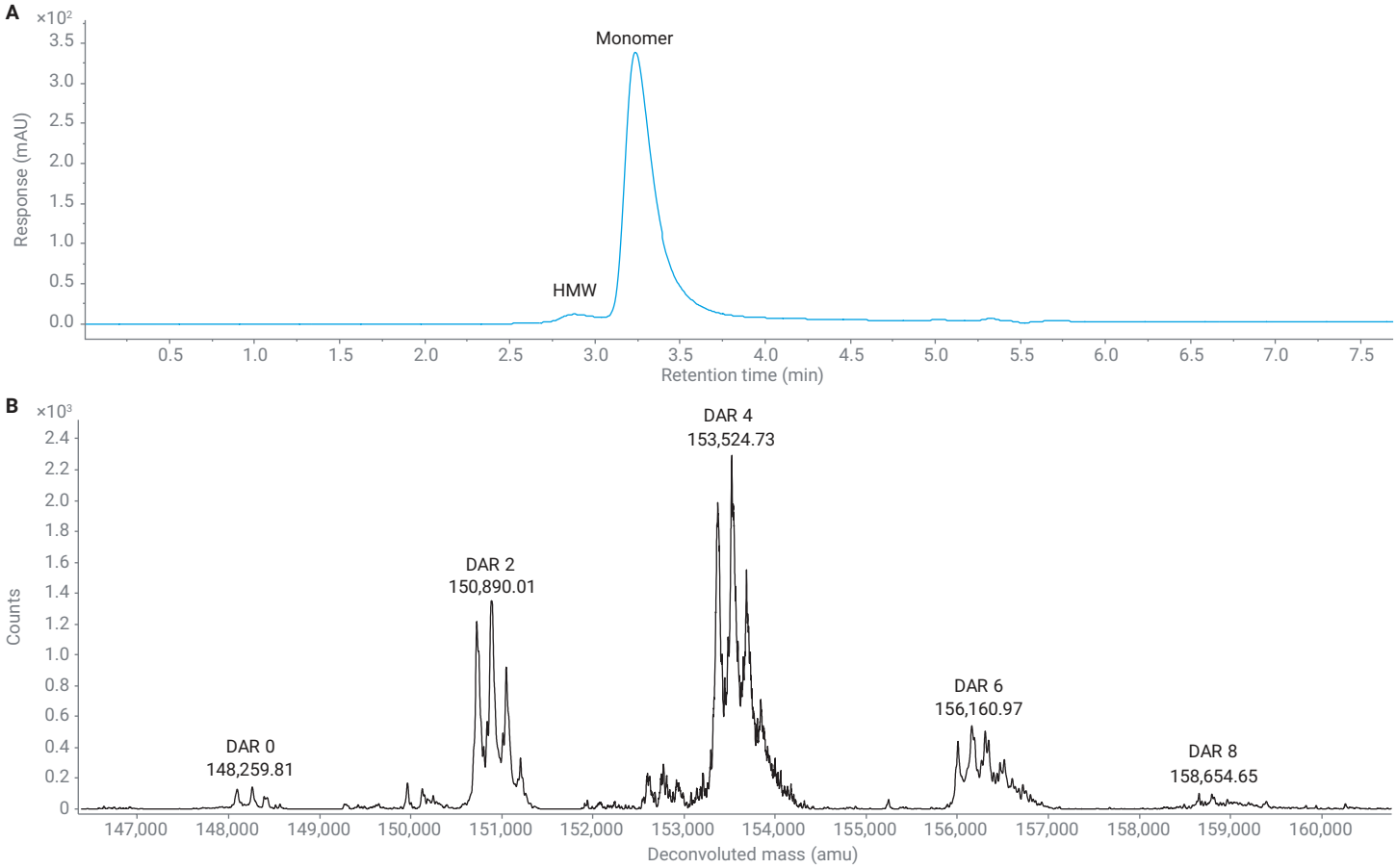

Figure 6. SEC/UV/MS of the ADC brentuximab vedotin. (A) UV 280 nm chromatogram and (B) deconvoluted native MS spectrum associated with the monomer peak, revealing a drug distribution from 0 to 8 and a DAR of 4.

Conclusion

The importance of LC instrument and column inertness cannot be underestimated when analyzing mAbs by native SEC/MS. The reduction of nonspecific interactions with stainless-steel components in the flow path is of utmost importance to guarantee adequate chromatographic performance and recovery of HMW species. The Agilent Infinity II Bio LC System with the PEEK-lined Agilent AdvanceBio SEC column is highly attractive in that respect. This application note demonstrates that conventional phosphate mobile phases can successfully be exchanged with MS-compatible ammonium acetate with limited impact on separation performance, resulting in excellent native MS measurements.

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