Application Note

Biologics

SEC Coupled to High-Resolution Mass Spectrometry for Detailed Characterization of mAbs and ADCs

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Abstract

This Application Note describes how size exclusion chromatography (SEC) coupled to high-resolution mass spectrometry (HRMS) can be applied to the detailed characterization of monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs).

Introduction

Monoclonal antibodies (mAbs) are being developed at an explosive rate and have attracted great interest from both smaller biotech firms and big pharma companies. Developing mAbs and next-generation antibody drug conjugates (ADCs) is highly demanding in many ways. From an analytical perspective, handling mAbs and ADCs presents new challenges for chromatographers. As a result, the field of biochromatography is advancing rapidly as new and higher-resolution techniques for characterizing these biomolecules are evaluated and better understood[1,2].

The current contribution describes how size exclusion chromatography (SEC) with high-resolution mass spectrometry (HRMS) can be applied to the detailed characterization of mAbs and ADCs. These measurements provide insights into mAb identity and structural integrity, post-translational modifications (for example, glycosylation) next to drug distribution, and drug-to-antibody ratios (DARs). MS-compatible SEC conditions allowing separation and ionization in the denatured state are described.

Experimental

Materials

Acetonitrile and water were obtained from Biosolve (Valkenswaard, the Netherlands). Formic acid (FA), trifluoroacetic acid (TFA), dithiothreitol (DTT), sodium chloride, sodium phosphate, cysteine, EDTA, and papain were from Sigma-Aldrich (St. Louis, MO, USA). Immunoglobulin-degrading enzyme from *Streptococcus equi* ssp. *zooepidemicus* (IdeZ) was from Promega (Madison, WI, USA). Tris-HCl was acquired from Thermo Fisher (Waltham, MA, USA). Trastuzumab (Herceptin) and ado-trastuzumab emtansine (Kadcyla) were obtained from Roche (Basel, Switzerland). mAb Chinese hamster ovary (CHO) cell culture supernatant was kindly provided by a local biotechnology company.

Sample preparation

- **Intact mAb:** mAb was diluted to 2 mg/mL using 100 mM Tris pH 7.5.
- **IdeZ treatment:** The mAb or ADC was diluted to 0.5 mg/mL using 50 mM sodium phosphate, and 150 mM sodium chloride, pH 6.6. Then, IdeZ was added in an enzyme:protein ratio of 1:1 (U/µg), and digestion was performed at 37 °C for 60 minutes.
- **Papain treatment:** The mAb was diluted to 2 mg/mL using 50 mM sodium phosphate, and 1 mM EDTA, 10 mM cysteine, pH 7. After activation, the papain was added in an enzyme:protein ratio of 1:60 (µg/µg), and digestion was performed at 37 °C for two hours.
- **Reduction:** After dilution of the sample to 0.2 mg/mL using 100 mM Tris pH 8, DTT was added to a final concentration of 10 mM. Reduction was performed at 60 °C for one hour.

Before injection, all samples were centrifuged at 2,000 g for one minute.

Instrumentation

SEC/MS measurements were performed on the following instrument configuration:

- Agilent 1290 Infinity LC equipped with:
  - Agilent 1290 Infinity binary pump (G4220B)
  - Agilent 1290 Infinity autosampler (G4226A)
  - Agilent 1290 Infinity thermostat (G1330B)
  - Agilent 1290 Infinity thermostatted column compartment (G1316C)
  - Agilent 1290 Infinity diode array detector (G4212A)
- Agilent 6540 Q-TOF LC/MS (G6540A) equipped with Jet Stream ESI source

Software

- Agilent MassHunter Acquisition instrument control (B05.01)
- Agilent MassHunter Qualitative Analysis software (B07.00)
- Agilent MassHunter BioConfirm software (B07.00)

Results and discussion

SEC chromatography typically uses buffers containing nonvolatile salts such as a phosphate buffer. These conditions are not compatible with MS. Figure 1 compares the separations of the humanized mAb Herceptin on a Bio SEC-3 column using a nonvolatile buffer (150 mM phosphate pH 7.0) and an MS-compatible mobile phase (20 % ACN, 0.1 % FA, and 0.1 % TFA). When using a phosphate buffer, sharp peaks are obtained, and aggregates and fragments can be detected at levels as low as 0.1 %. Spraying a phosphate buffer into the MS, however, results in a dirty source and lack of MS signal. When using a mobile phase containing volatile constituents, proteins can be sprayed effectively into the MS, generating high-quality MS data.

SEC/MS Method

Parameter	Value
Column	Agilent Bio SEC-3 (7.8 × 300 mm, 3 µm, p/n 5190–2511)
Mobile phase	20 % acetonitrile, 0.1 % FA, 0.1 % TFA in water
Flow rate	1 mL/min
Column temperature	24 °C (uncovered)
Injection volume	20 µL (unless noted)
Needlewash solvent	Water
Autosampler temperature	5 °C
DAD detection	214 and 280 nm
Q-TOF source	
	Jet Stream positive ionization mode
Drying gas temperature	350 °C
Drying gas flow	10 L/min
Nebulizer pressure	50 psig
Sheath gas temperature	350 °C
Sheath gas flow	11 L/min
Nozzle voltage	1,000 V
Capillary voltage	3,500 V
Fragmentor voltage	200 V (papain, reduced, IdeZ), 350 V (intact)
Q-TOF detection	Mass range: 3,200 amu Data acquisition range: *m/z* 1,000 to 3,200 High resolution mode (4 GHz) Data acquisition rate: One spectrum per second Profile acquisition
Diverter valve	Bypassed Column outlet directly connected to ESI needle via PEEK tubing (0.12 mm)

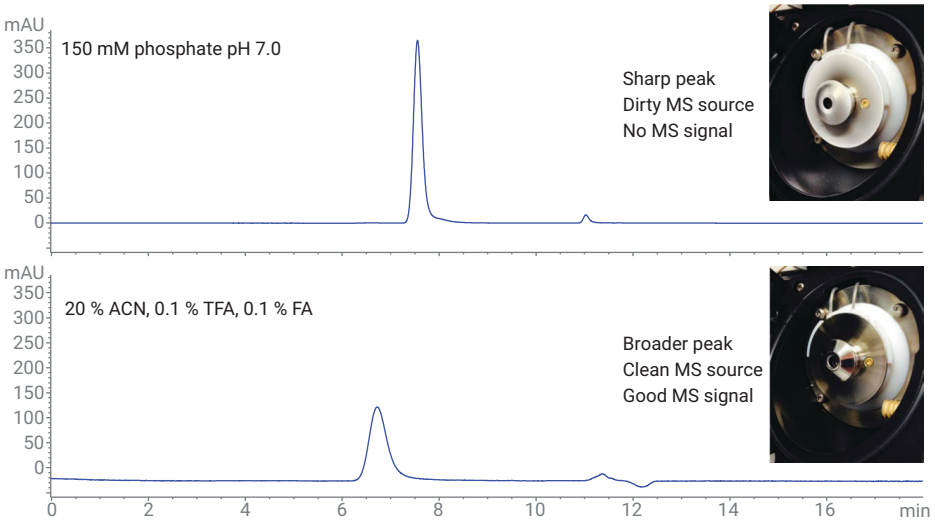

Figure 1. SEC/UV chromatograms (214 nm) of Herceptin obtained using different mobile phase compositions with an Agilent Bio SEC-3 column. To the right are pictures of the MS source. Injection volume: 2.5 µL.

SEC using a mobile phase containing 20 % ACN, 0.1 % FA, and 0.1 % TFA has successfully been applied on intact mAbs as well as fragments. These fragments originate from reduction (light and heavy chain (Lc and Hc)), papain digestion (Fab and Fc), or IdeZ digestion ($F(ab)'_2$ and Fc/2) (Figure 2).

The SEC/UV/MS analysis of papain-digested Herceptin is shown in Figure 3. UV and MS chromatograms are similar in terms of peak widths. The path from the UV detector to the MS system does not contribute substantially to peak dispersion where the MS diverter valve is bypassed. Deconvoluted MS data associated with the annotated peaks are shown in Figure 4. Fab, Fc, and smaller fragments are identified and glycosylation revealed. The measured molecular weight values are well below 0.005 % different from the theoretical molecular weight values. Peaks c, d, and e are resolved, though their molecular weights are similar. Therefore, the MS data of peaks c, d, and e show that the separation is not purely driven by size for these components.

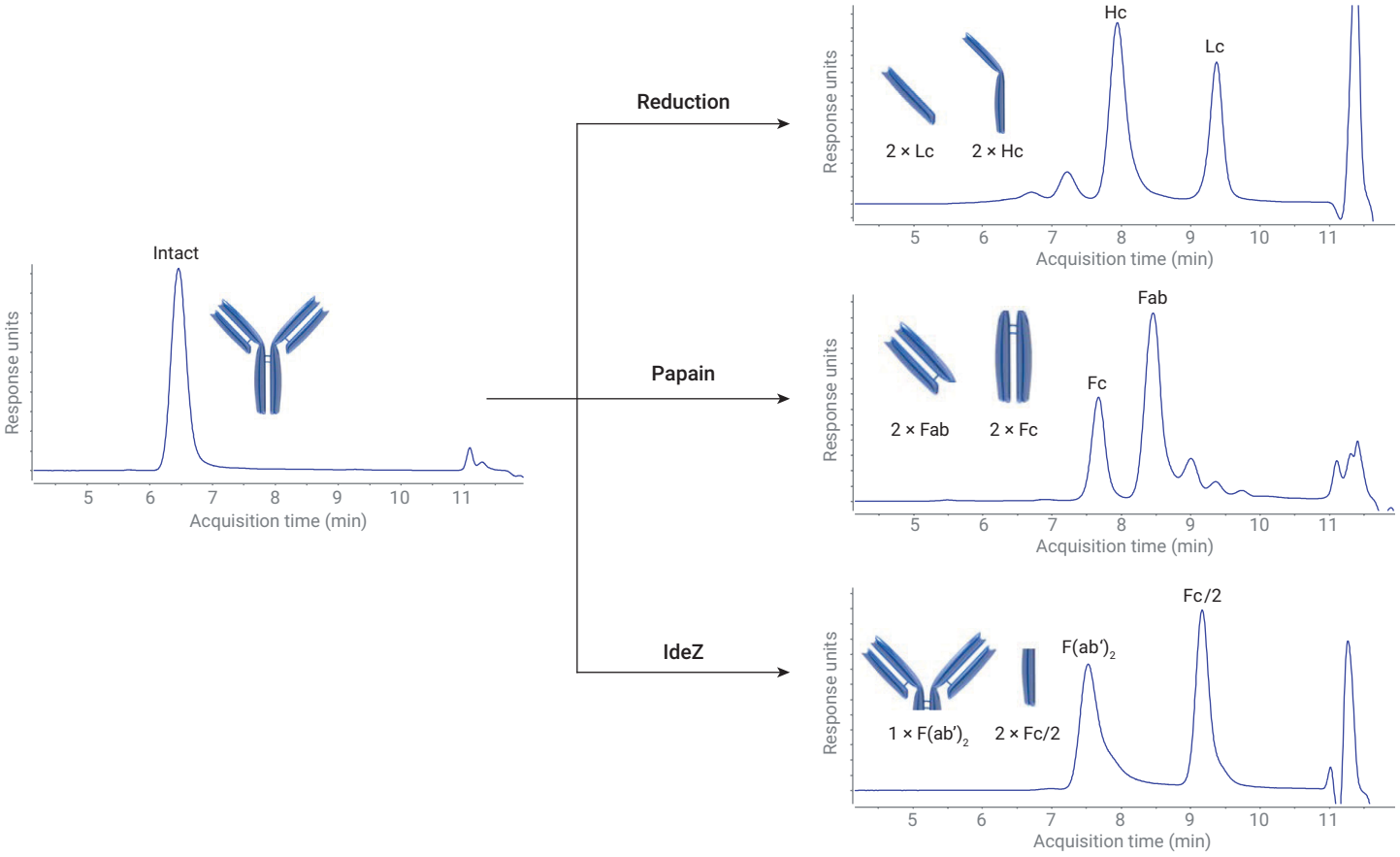

Figure 2. SEC/UV chromatograms (214 nm) of intact, DTT-reduced, papain-digested, and IdeZ-treated Herceptin. Mobile phase composition: 20 % ACN, 0.1 % FA, 0.1 % TFA. Column: Agilent Bio SEC-3. Injection volume: 20 µL except for intact mAb (5 µL).

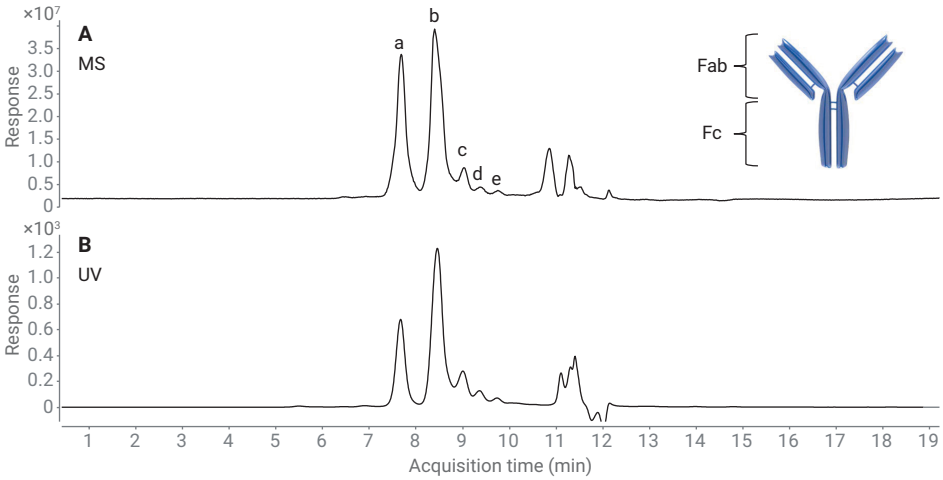

Figure 3. SEC/UV/MS chromatograms of papain-digested Herceptin. Mobile phase composition: 20 % ACN, 0.1 % FA, 0.1 % TFA; column: Agilent Bio SEC-3.

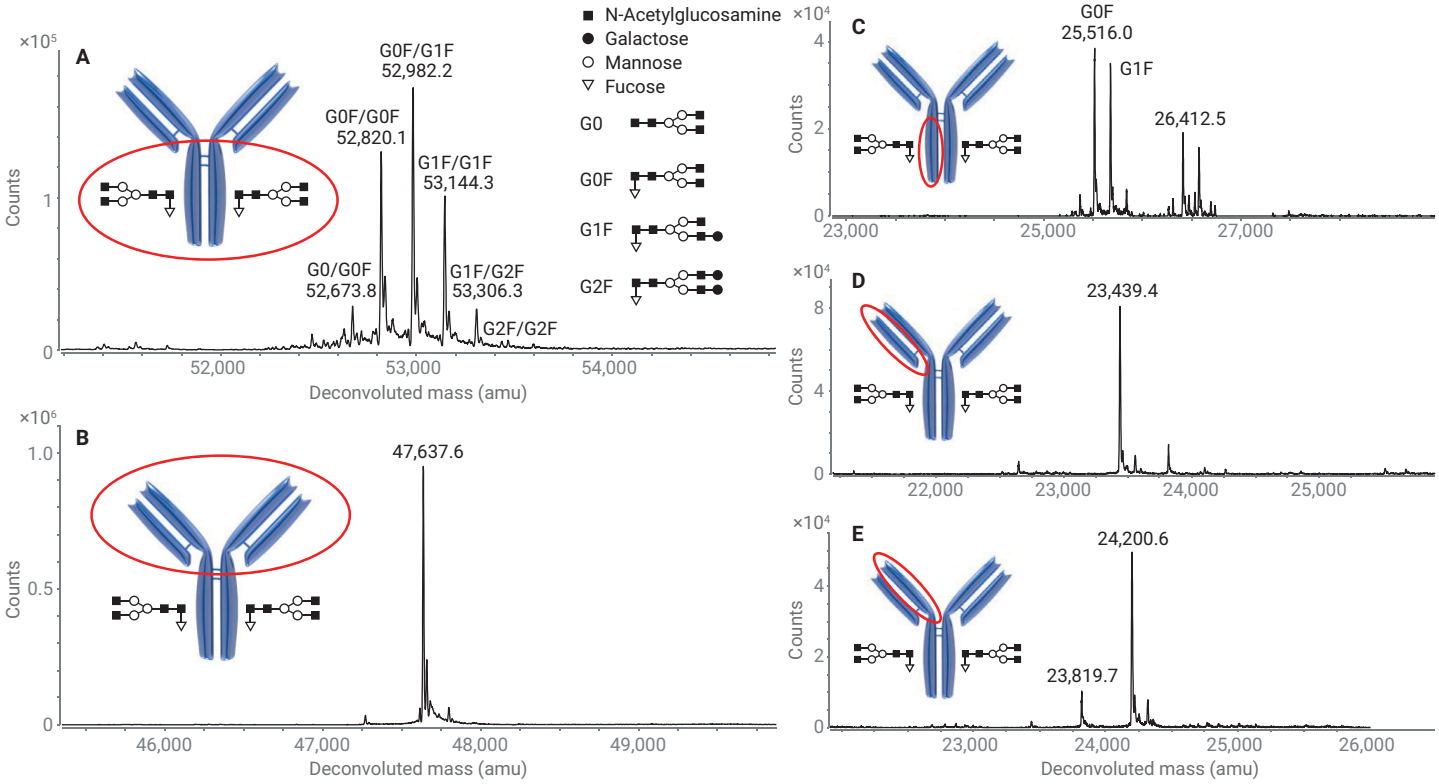

Figure 4. Deconvoluted Q-TOF MS spectra associated with the annotated peaks in Figure 3.

Figure 5 shows the SEC/UV/MS data of intact Herceptin. Several peaks are annotated, and the corresponding deconvoluted spectra are shown in Figure 6. The intact mAb with a molecular weight of approximately 150 kDa is successfully measured and the glycosylation pattern is detailed. Fragments of the mAb are also highlighted.

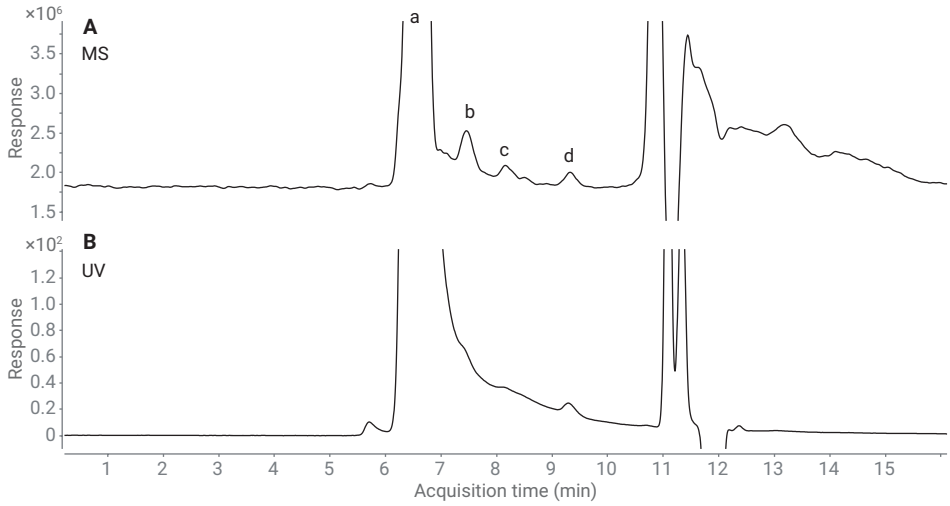

Figure 5. SEC/UV/MS chromatograms of intact Herceptin. Mobile phase: 20 % ACN, 0.1 % FA, 0.1 % TFA; column: Agilent Bio SEC-3.

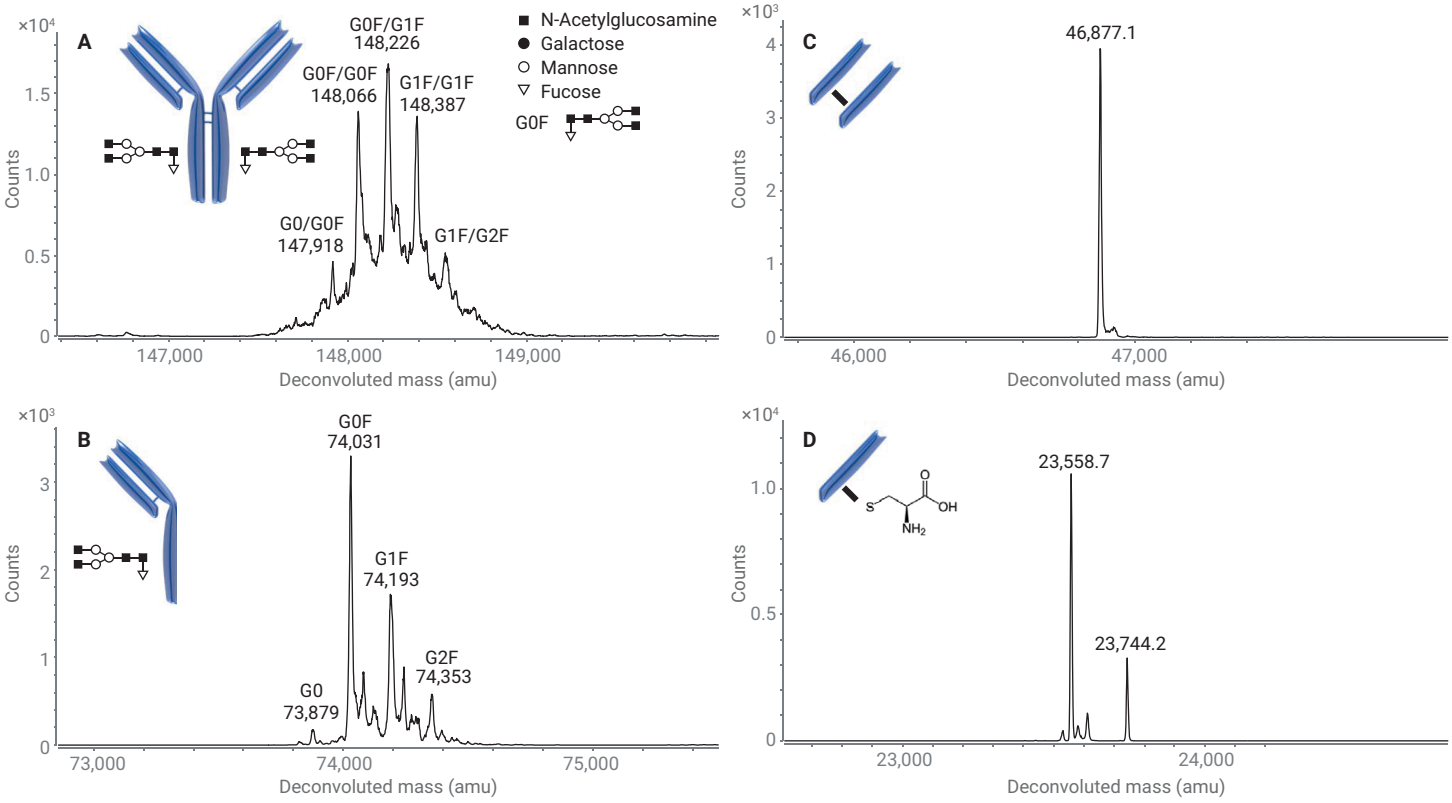

Figure 6. Deconvoluted Q-TOF MS spectra associated with the annotated peaks in Figure 5.

Figure 7 compares the SEC/UV/MS measurements of IdeZ-treated Herceptin and the lysine conjugated ADC Kadcyla. Deconvoluted spectra associated with the annotated peaks are shown in Figure 8. Insight into glycosylation, DAR, and drug distribution is provided.

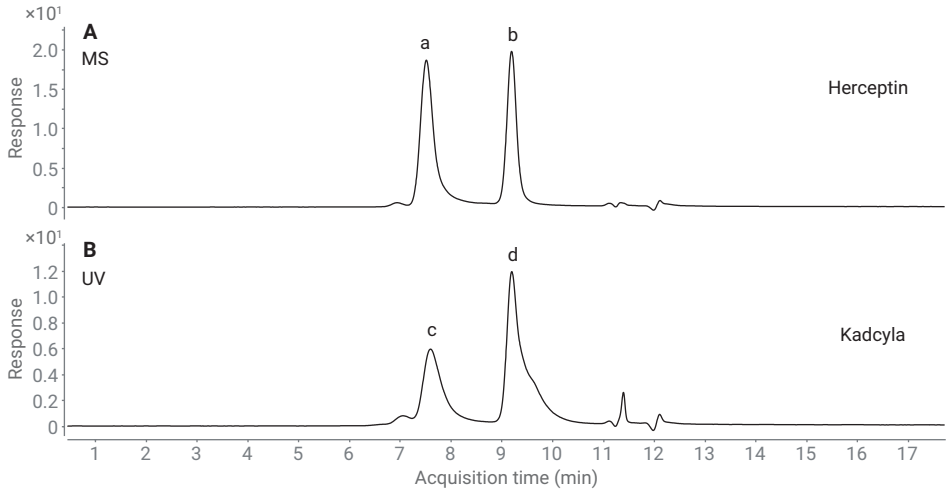

Figure 7. SEC/UV chromatograms (280 nm) of IdeZ-digested Herceptin and Kadcyla; column: Agilent Bio SEC-3.

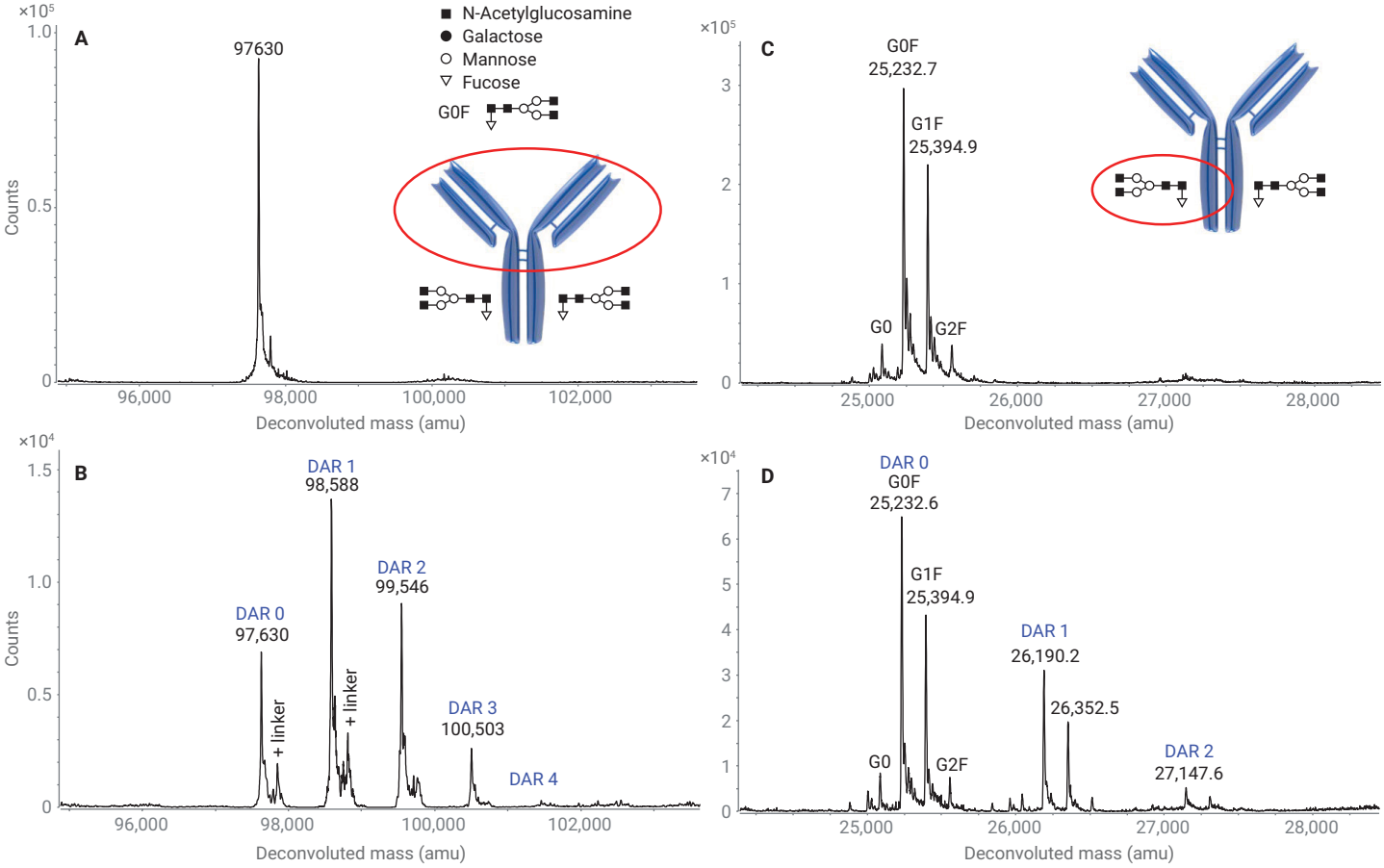

Figure 8. Deconvoluted Q-TOF MS spectra associated with the annotated peaks in Figure 7.

The SEC/UV/MS data of the supernatant of a mAb-producing CHO clone and the corresponding Protein A purified sample (intact and DTT reduced) are shown in Figure 9. Protein A binds the Fc region, and can be used to selectively enrich mAbs from cell culture supernatants. The corresponding MS data allow the annotation of the different peaks. Figure 10 presents the deconvoluted MS spectra associated with the Lc and Hc of the reduced Protein A purified sample. Insight into Hc glycosylation, Hc C-terminal lysine truncation, and Lc glycation is obtained. These data illustrate the level of detail of mAb characteristics that can be obtained with the described SEC/MS method.

Conclusion

SEC/UV/MS represents a powerful addition to the analytical toolbox and opens new possibilities for the detailed characterization of mAbs and ADCs. Intact, reduced, papain-digested, and IdeZ-treated mAbs and ADCs can successfully be analyzed. Conditions shown in this Application Note do not maintain mAbs and ADCs in their native state, and are expected to disturb noncovalent interactions.

Important note: All SEC/MS experiments were performed on an SEC column with an internal diameter of 7.8 mm operated at a flow rate of 1 mL/min. Sensitivity enhancement or reduced sample consumption is expected when performing these experiments on an SEC column with an internal diameter of 4.6 mm operated at a flow rate of 0.35 mL/min.

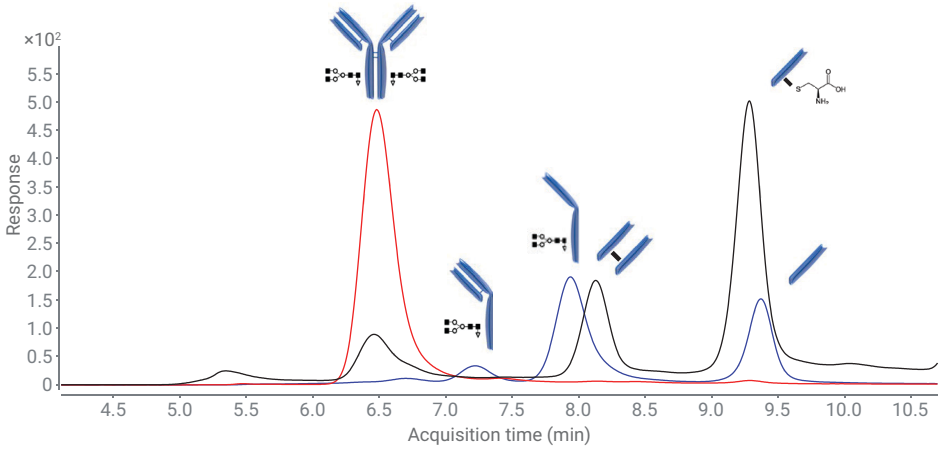

Figure 9. SEC/UV chromatograms (214 nm) of the supernatant of a mAb-producing CHO clone (black) and the corresponding Protein A purified sample (red: intact, blue: DTT reduced) produced using an Agilent Bio SEC-3 column.

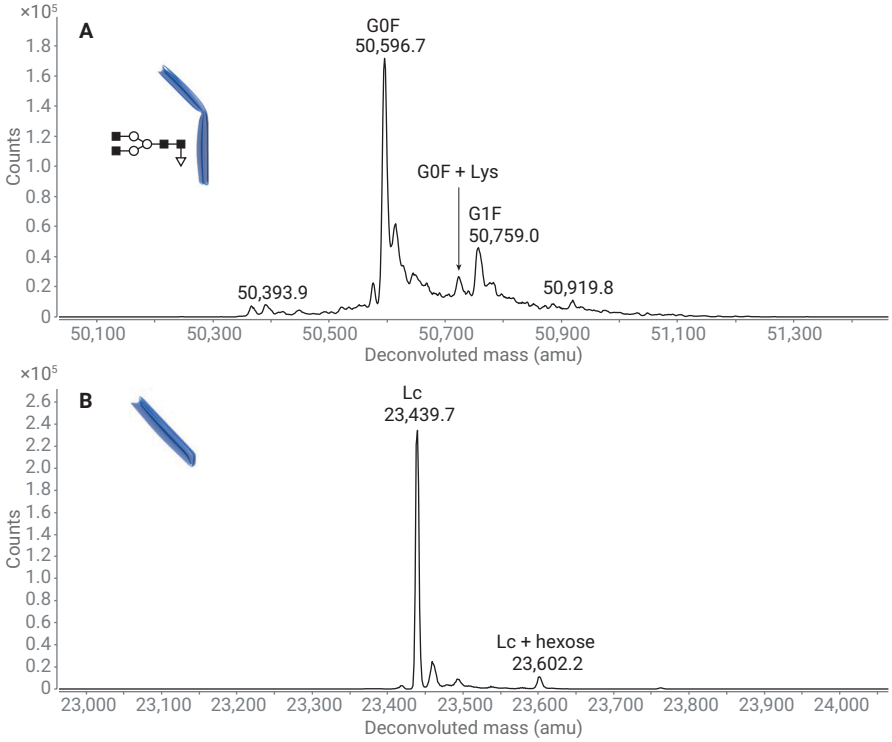

Figure 10. Deconvoluted Q-TOF MS spectra associated with the Hc and Lc peaks observed in the Protein A purified and reduced sample shown in Figure 9 (blue trace).

References

1. Sandra, K.; Vandenheede, I.; Sandra, P. Modern chromatographic and mass spectrometric techniques for protein biopharmaceutical characterization. *J. Chromatogr. A* **2014**, *1335*, 81–103.
2. Fekete, S.; *et al.* Chromatographic, Electrophoretic, and Mass Spectrometric Methods for the Analytical Characterization of Protein Biopharmaceuticals. *Anal. Chem.* **2016**, *88*, 480–507.

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