Application Note

Biologics

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Efficient Monoclonal Antibody and Antibody Drug Conjugate Desalting Prior to Mass Spectrometry Using AdvanceBio Desalting-RP Cartridges

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Abstract

This Application Note describes the use of Agilent AdvanceBio Desalting-RP cartridges for the mass spectrometric analysis of salt-containing monoclonal antibody (mAb) and antibody drug conjugate (ADC) samples.

Introduction

Almost 30 years ago, the 2002 Nobel laureate, John Fenn, described for the first time an elegant way to present proteins to the mass spectrometer (MS)[1]. The principle known as electrospray ionization (ESI) revolutionized the analysis of proteins. In combination with high-resolution MS systems, ESI is now widely applied for the characterization of protein biopharmaceuticals such as monoclonal antibodies (mAbs) and antibody-drug conjugates (ADCs). The ESI process generates multiply charged ions from proteins, resulting in a characteristically charged envelope. This can readily be converted to the molecular mass by applying, for example, a maximum entropy algorithm. Such a deconvoluted spectrum provides information on molecular weight, structural integrity, and modifications. The success of ESI measurements largely depends on the sample composition. Samples encountered in biopharmaceutical analysis contain a high amount of nonvolatile salts, detergents, and so forth.This comes as a result of formulation or preceding chromatographic separations (for example, Protein A chromatography, cation-exchange chromatography, size exclusion chromatography, and hydrophobic interaction chromatography). Since ESI can only tolerate a certain amount of these nonvolatile constituents, desalting is required. This Application Note describes the use of the Agilent AdvanceBio Desalting-RP cartridges for the online reversed-phase (RP) desalting of mAbs and ADCs before time-of-flight (TOF) MS measurement.

Experimental

Materials

Acetonitrile, formic acid, and water were acquired from Biosolve (Valkenswaard, the Netherlands). Dithiothreitol, cysteine, EDTA, sodium phosphate, and papain were purchased from Sigma-Aldrich (St. Louis, MO, USA). Immunoglobulin-degrading enzyme from *Streptococcus equi* ssp. *zooepidemicus* (IdeZ) came from Promega (Madison, WI, USA). Tris-HCl was from Thermo Fisher Scientific (Waltham, MA, USA). Monoclonal antibody trastuzumab, marketed as Herceptin, and ado-trastuzumab emtansine, marketed as Kadcyla, were obtained from Roche (Basel, Switzerland).

Sample preparation

- **Reduction:** 100 mM DTT was added to the sample to a final concentration of 10 mM DTT following sample dilution to 0.21 mg/mL using 100 mM Tris pH 8.0. Reduction was performed at 60 °C for one hour.
- **Papain digest:** Herceptin was diluted to 2 mg/mL using papain digest buffer. Activated papain was added to the diluted sample at an enzyme:protein ratio of 1:60 (µg/µg). Digestion was performed at 37 °C for two hours.
- **IdeZ digest:** The proteins were diluted to 0.5 mg/mL using 50 mM sodium phosphate and 150 mM sodium chloride, pH 6.6. Subsequently, IdeZ was added in an enzyme:protein ratio of 1:1 (U/µg); digestion was performed at 37 °C for 60 minutes.
- **Intact measurement:** Proteins were diluted to 2 mg/mL using 100 mM Tris, pH 7.5.

Instrumentation

Online desalting MS measurements were performed with the following configurations:

- Agilent 1290 Infinity LC equipped with:
  - Agilent 1290 Infinity binary pump (G4220B)
  - Agilent 1290 Infinity autosampler (G4226A)
  - Agilent 1290 Infinity thermostat (G1330B), and
- Agilent 6540 Q-TOF LC/MS (G6540A) with a Jet Stream ESI source, or
- Agilent 6550 iFunnel Q-TOF LC/MS (G6550A) with Jet Stream ESI source, or
- Agilent 6560 ion mobility Q-TOF LC/MS (G6560A) 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)
- Agilent MassHunter DAR calculator software

Results and discussion

The AdvanceBio Desalting-RP cartridge, packed with wide-pore (1,000 Å) polymeric RP particles, is installed between the LC injector valve and the MS diverter valve. The latter directs the flow either to waste or the ESI needle. Following sample loading at low acetonitrile concentration, salts are diverted to waste for one minute. The diverter valve is subsequently switched to the ESI needle. The mAb or ADC is eluted at increased acetonitrile concentration and presented as gas-phase ions to the TOF mass analyzer.

Figures 1, 2, and 3 show the online desalting TIC chromatograms, with raw and deconvoluted spectra obtained from various Herceptin middle-down measurements. The data correspond to the MS analysis of DTT-reduced, papain-digested, and IdeZ-treated Herceptin. Reduction of the mAb with DTT generates the Lc and Hc, digestion with papain results in a Fab and Fc fragments, and IdeZ treatment gives rise to an $F(ab')_2$ and Fc/2 fragments. Although the aim of the cartridge is mainly to desalt proteins, partial separation is obtained, as shown by the different peak shapes (Figure 1) and the different spectra captured as a function of time (Figure 2). As shown in Table 1, samples contain a wide range of non-MS compatible chemicals at relatively high concentrations. Nevertheless, their presence does not prevent highly informative spectra that enable the determination of molecular weight, confirmation of the gene-derived sequence, and protein modifications such as glycosylation. Tables 2 and 3 also show that measurements using the AdvanceBio Desalting-RP cartridge are highly precise in terms of peak area and glycoform assessment.

Online desalting LC/MS method

Parameter	Value
Cartridge	Agilent AdvanceBio Desalting-RP, 2.1 × 12.5 mm, 10 µm, 1,000 Å (p/n PL1612-1102)
Mobile phase A	0.1 % Formic acid in water (v/v)
Mobile phase B	0.1 % Formic acid in acetonitrile (v/v)
Flow rate	400 µL/min
Injection volume	Volume corresponding to 1 µg of material, unless otherwise mentioned
Needlewash solvent	60 % Acetonitrile, 35 % water, 5 % isopropanol
Autosampler temperature	7 °C
Gradient program	0 minutes, 5 %B 0.5 minutes, 5 %B 3 minutes, 80 %B 4 minutes, 80 %B 4.1 minutes, 5 %B 6 minutes, 5 %B
Q-TOF source	Positive ionization mode Drying gas temperature: 300 °C Drying gas flow: 8 L/min Nebulizer pressure: 35 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 (light chain (Lc), heavy chain (Hc), Fab, Fc, F(ab')2, Fc/2)
Q-TOF detection	Mass range: 3,200 amu Data acquisition range: *m/z* 500 to 3,200 High resolution mode: 4 GHz Data acquisition rate: One spectrum per second Profile acquisition
Diverter valve	0 minutes Waste 1 minute MS 4.5 minutes Waste

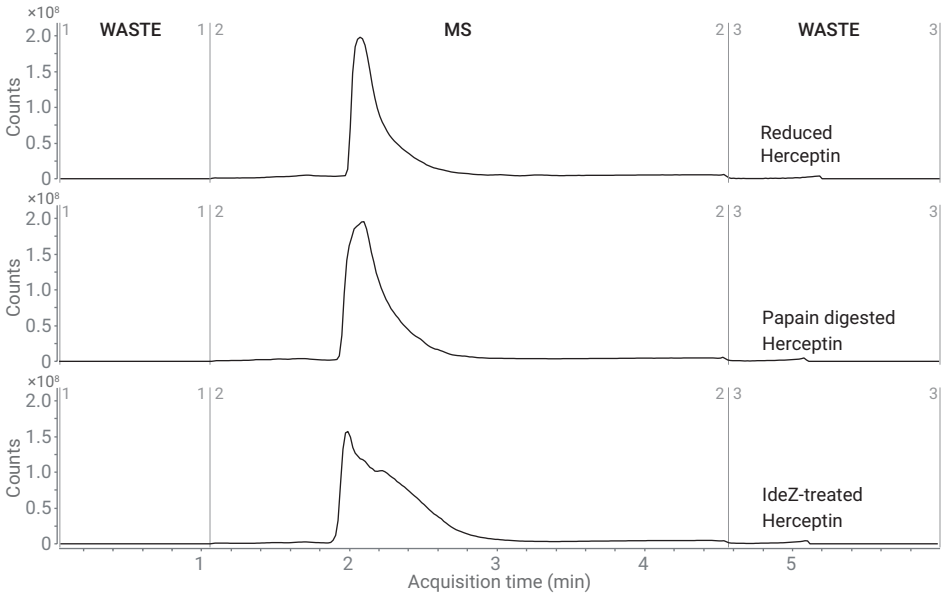

Figure 1. Online desalting TIC chromatograms obtained from the analysis of reduced, papain-digested, and IdeZ-treated Herceptin. Data were acquired on an Agilent 6540 Q-TOF LC/MS.

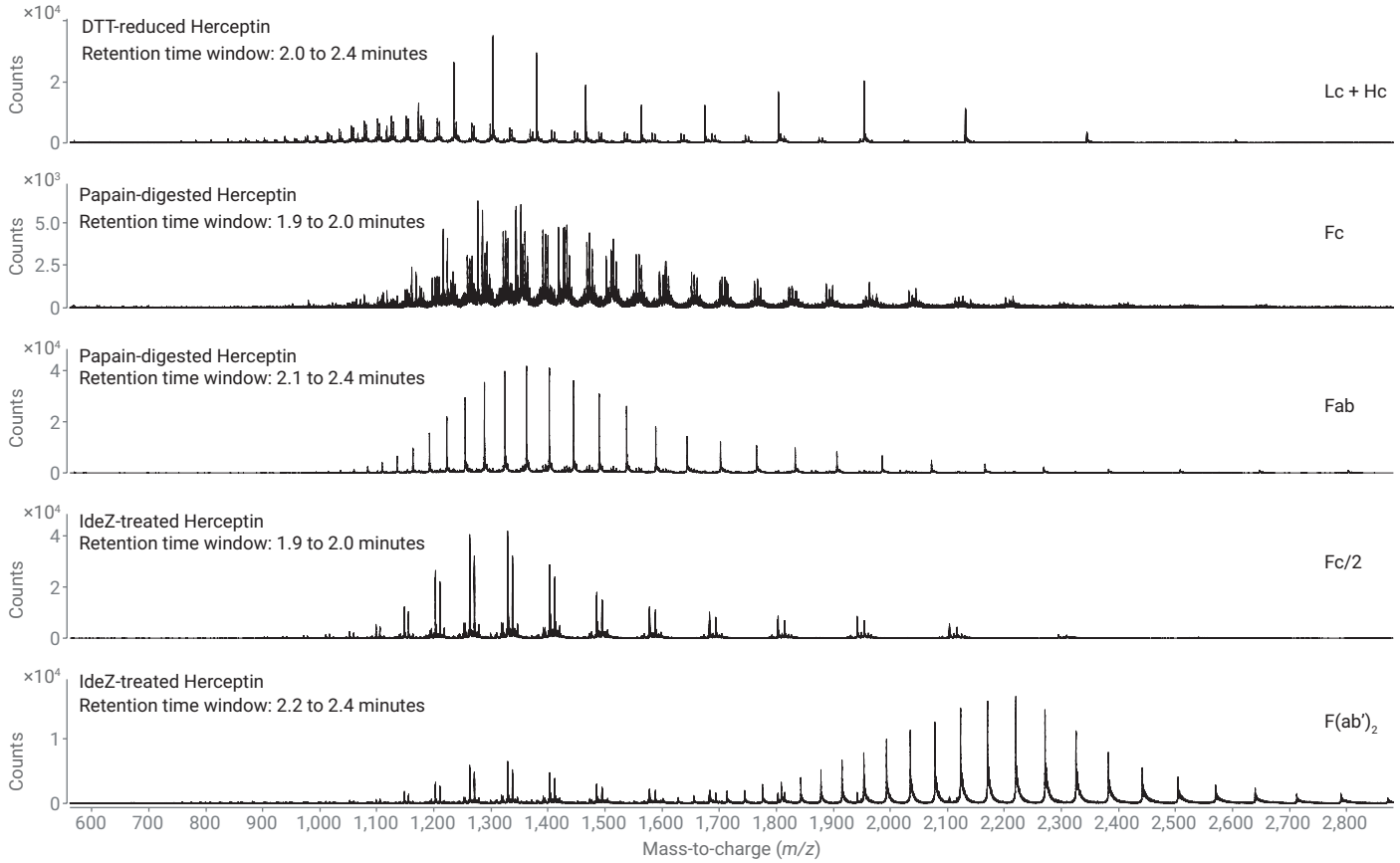

Figure 2. Raw spectra retrieved from the online desalting MS experiments of reduced, papain-digested, and IdeZ-treated Herceptin (Figure 1). Lc and Hc are not separated by the cartridge, Fc, Fab, Fc/2, and $F(ab')_2$ are partially separated, providing discrete spectra. Data were acquired on an Agilent 6540 Q-TOF LC/MS.

Table 1. Composition of injected samples (Figures 1–3).

	Reduced Herceptin	Papain-digested Herceptin	IdeZ-treated Herceptin
Formulation-derived			
mAb (mg/mL)	0.189	2	0.5
L-Histidine HCl (mg/mL)	0.004	0.045	0.011
L-Histidine (mg/mL)	0.003	0.029	0.007
Trehalose (mg/mL)	0.172	1.818	0.455
Polysorbate (mg/mL)	0.001	0.008	0.002
Sample preparation-derived			
Tris-HCl pH 8 (mM)	99	–	–
DTT (mM)	10	–	–
Papain (µg/µg)	–	0.0167	–
$Na_2HPO_4{\cdot}2H_2O$ (mM)	–	45.2	48.3
EDTA (mM)	–	0.9	–
Cysteine (mM)	–	9.0	–
IdeZ (unit/µg)	–	–	1
NaCl (mM)	–	–	145

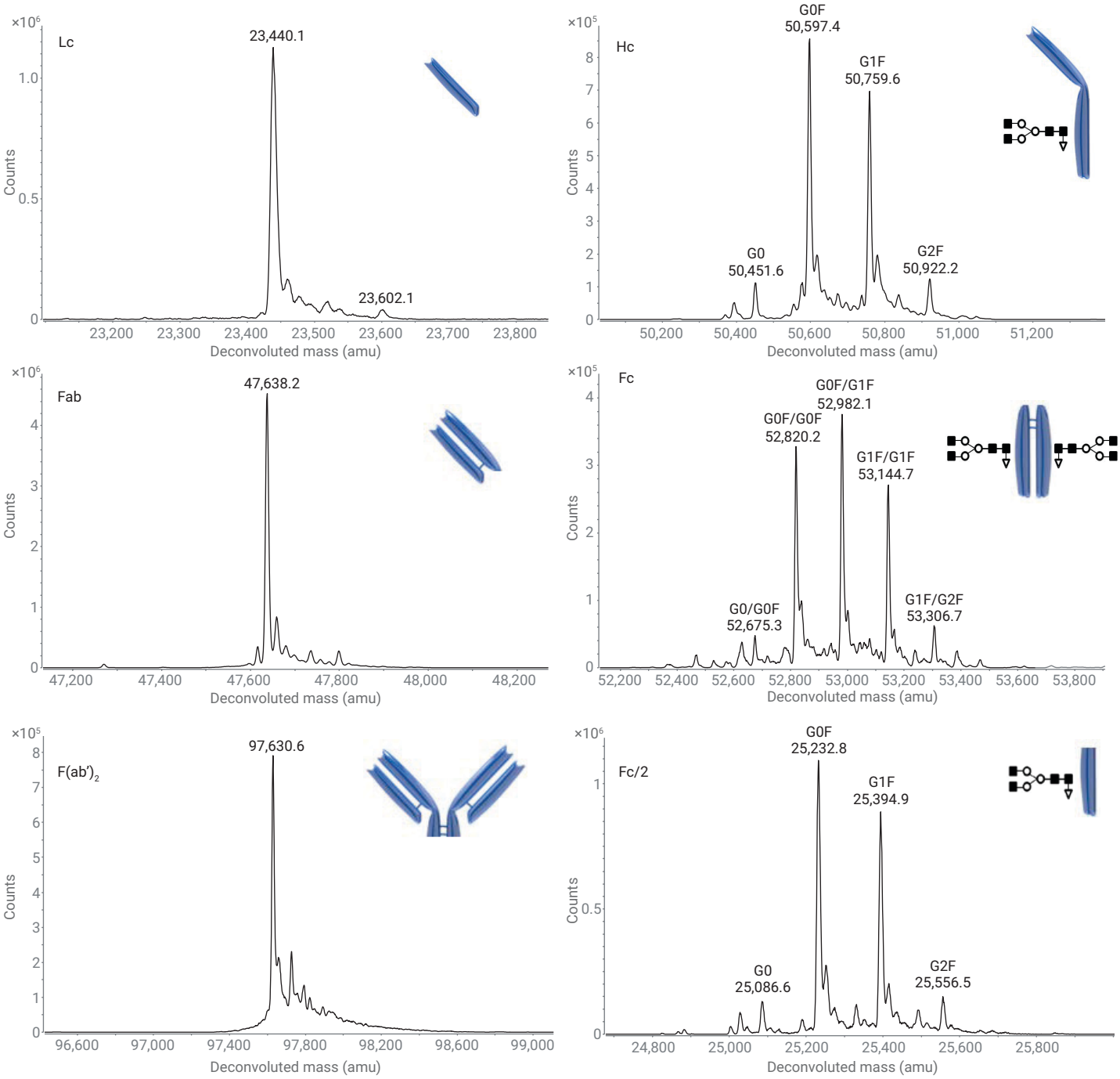

Figure 3. Deconvoluted spectra of the Herceptin Lc, Hc, Fab, Fc, F(ab')$_2$, and Fc/2 fragments desalted on the AdvanceBio Desalting-RP cartridge (Figure 1). Data were acquired on an Agilent 6540 Q-TOF LC/MS.

As demonstrated in Figure 4, the cartridge also successfully allows the measurement of mAbs at the intact protein level even in the presence of high salt concentrations. When combined with the 6550 iFunnel Q-TOF LC/MS, a 50-ng load on the cartridge still enables decent spectra (Figure 5).

Table 2. Peak area precision data obtained from the five-fold analysis of reduced, papain, and IdeZ-treated Herceptin. Extracted ion chromatograms were integrated to retrieve the peak area.

	DTT reduced Herceptin		Papain digested Herceptin		IdeZ treated Herceptin	
	Lc	Hc	Fab	Fc	$F(ab')_2$	Fc/2
Extracted ion	1,303.2	1,177.7	1,362.1	1,325.6	2,219.9	1,329.0
Peak area repl. 1	52,353,329	22,318,745	72,745,026	21,710,454	44,178,915	44,884,582
Peak area repl. 2	51,276,011	21,017,616	72,282,429	22,262,514	40,960,959	40,937,521
Peak area repl. 3	48,808,842	20,974,828	72,108,434	21,943,232	39,402,122	41,508,316
Peak area repl. 4	54,928,024	21,317,775	71,773,149	21,782,185	45,129,358	45,387,243
Peak area repl. 5	50,095,342	20,588,954	72,334,985	21,841,694	43,973,079	46,013,420
% RSD	4.5	3.1	0.5	1.0	5.7	5.4

Table 3. Glycoform precision data obtained from the five-fold analysis of reduced, papain, and IdeZ-treated Herceptin.

	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5	%RSD
Hc (DTT-reduced Herceptin)						
% G0	6.3	6.2	5.9	6.1	6.4	3.5
% G0F	46.7	47.1	47.4	47.5	46.5	0.9
% G1F	39.2	38.6	38.8	38.6	39.1	0.7
% G2F	7.8	8.1	8.0	7.8	8.0	1.4
Fc (Papain-digested Herceptin)						
% G0/G0F	5.1	5.2	4.9	4.9	5.3	3.4
% G0F/G0F	29.8	27.7	28.7	29.1	28.4	2.7
% G1F/G0F	34.0	35.5	36.3	34.4	35.4	2.6
% G1F/G1F or G2F/G0F	24.9	24.6	23.1	24.9	25.2	3.3
% G2F/G1F	6.2	7.0	7.0	6.6	5.7	8.4
Fc/2 (IdeZ-digested Herceptin)						
% G0	6.3	6.5	5.9	6.8	6.5	5.1
% G0F	47.6	47.8	51.0	48.0	47.5	3.0
% G1F	38.8	38.2	36.1	38.1	38.7	2.9
% G2F	7.2	7.5	7.0	7.2	7.3	2.5

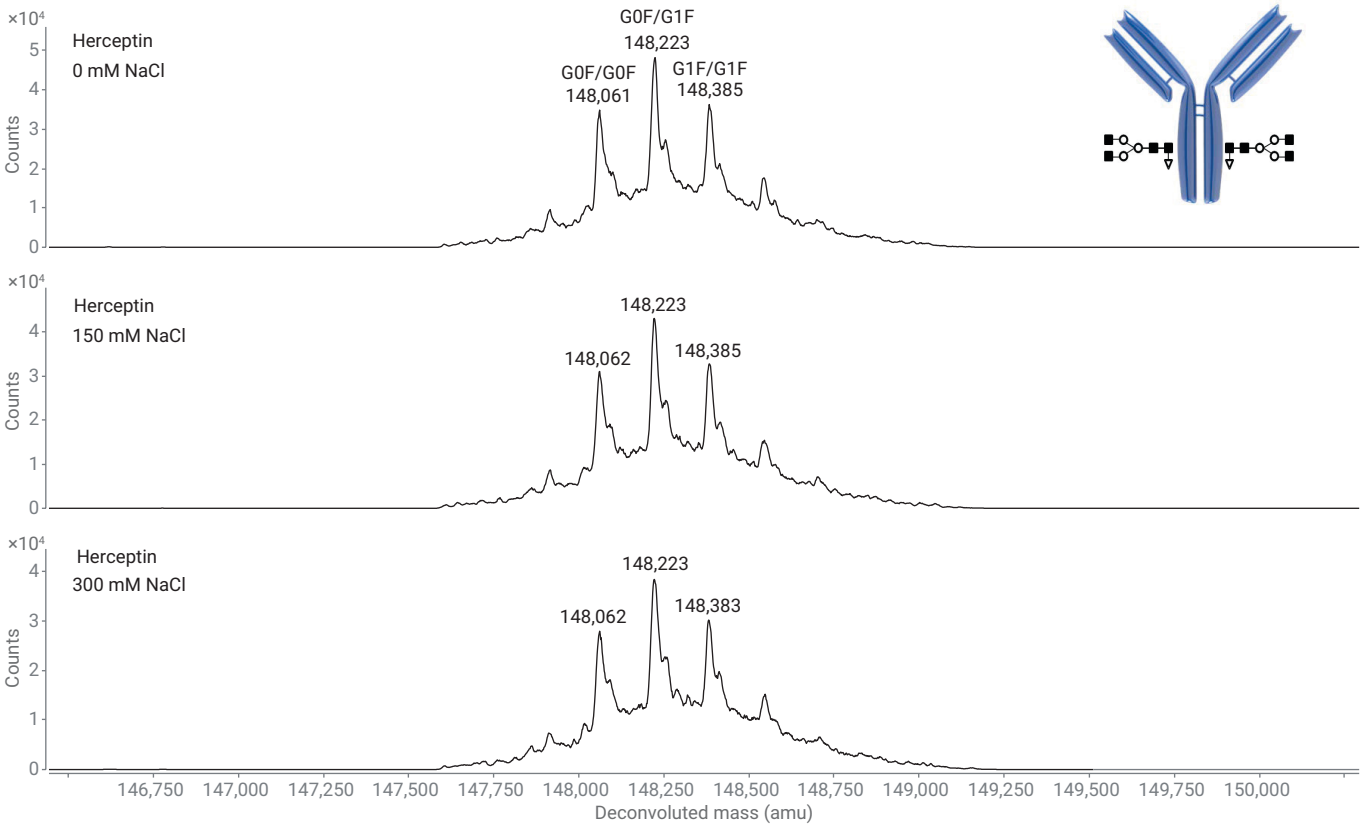

Figure 4. Deconvoluted spectra of intact Herceptin in the presence of 0, 150, and 300 mM NaCl, demonstrating the effectiveness of the AdvanceBio Desalting-RP cartridge (4 µg load). Data were acquired on an Agilent 6540 Q-TOF LC/MS.

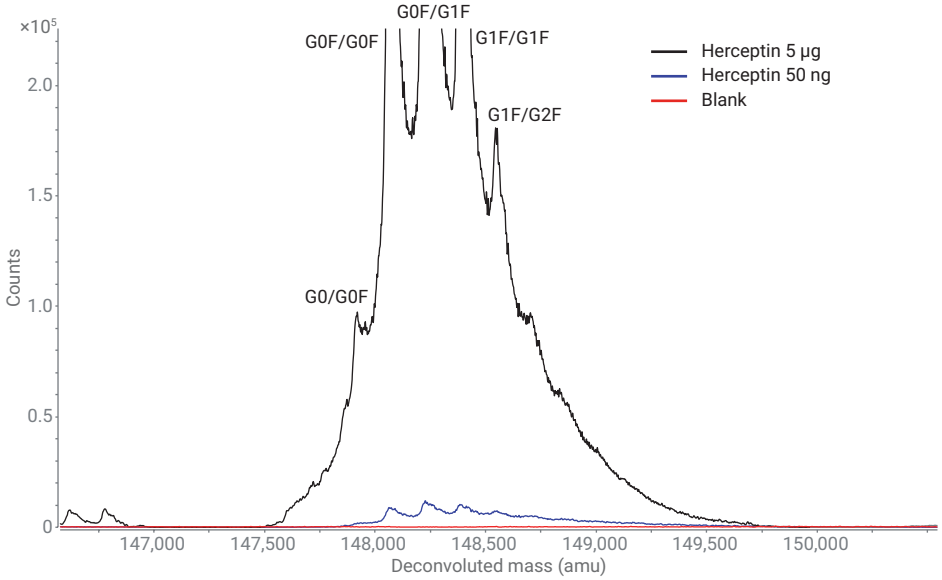

Figure 5. Deconvoluted spectra of intact Herceptin desalted on the AdvanceBio Desalting-RP cartridge at 5 µg (black) and 50 ng (blue) load. The red trace corresponds to a blank injection. Data were acquired on an Agilent 6550 iFunnel Q-TOF LC/MS.

The AdvanceBio Desalting-RP cartridge has also been used to measure the lysine-conjugated ADC Kadcyla at the intact level and following IdeZ treatment (Figures 6 and 7). Kadcyla combines the anti-HER2 antibody trastuzumab (Herceptin) with the cytotoxic microtubule-inhibiting maytansine derivative, DM1, by a nonreducible thioether linker (Figure 6). This measurement allows an accurate determination of the drug-to-antibody ratio (DAR), a critical quality attribute of ADCs, obtained using the MassHunter DAR calculator. A DAR of 3.6 is retrieved, which is in accordance with the DAR value reported in literature for this particular ADC[2].

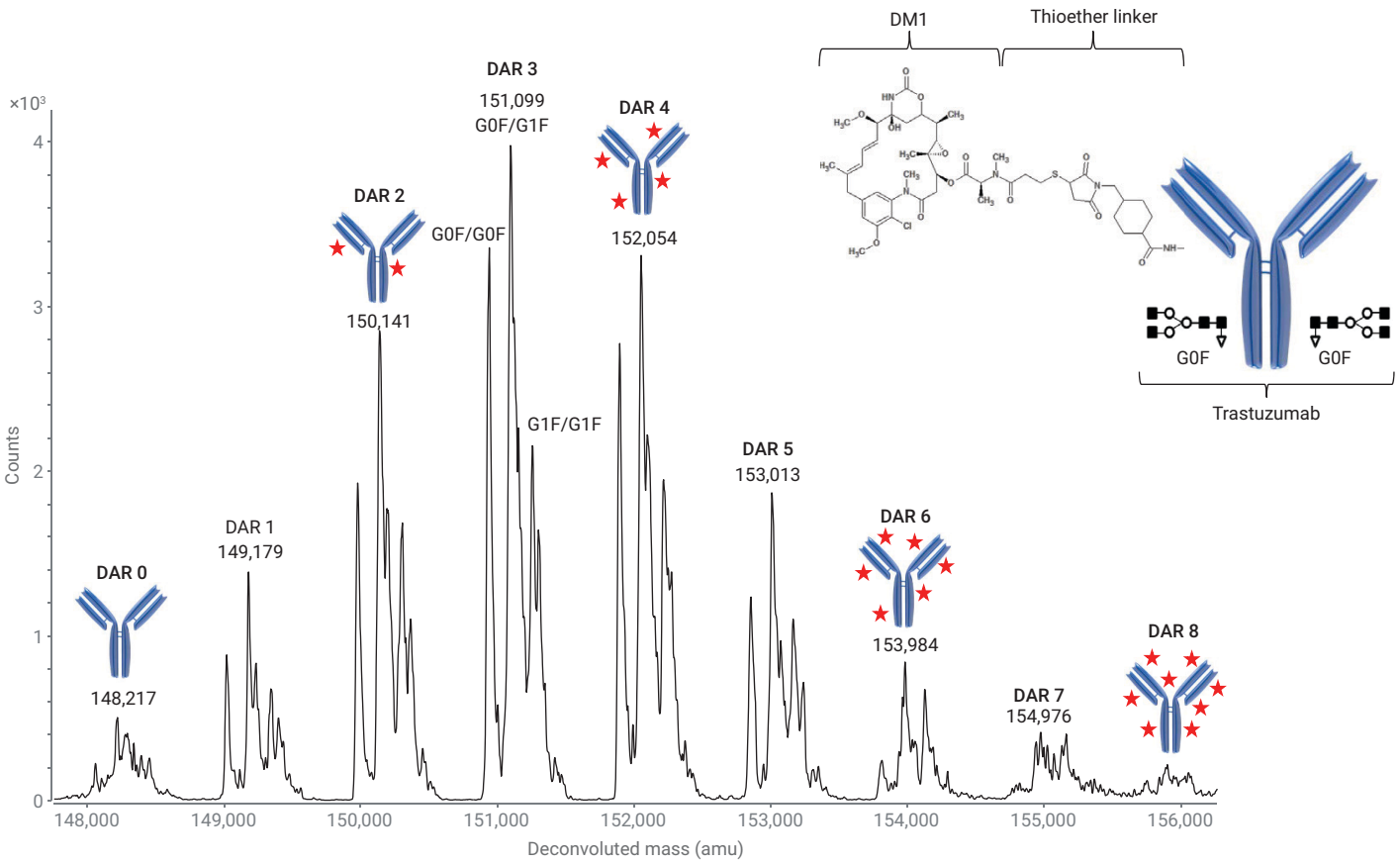

Figure 6. Deconvoluted spectrum of intact Kadcyla desalted on the AdvanceBio Desalting-RP cartridge. When combined with the MassHunter DAR calculator, a DAR of 3.6 is retrieved. Red stars correspond to the small molecule cytotoxic drug (4 µg load). Data were acquired on an Agilent 6540 Q-TOF LC/MS.

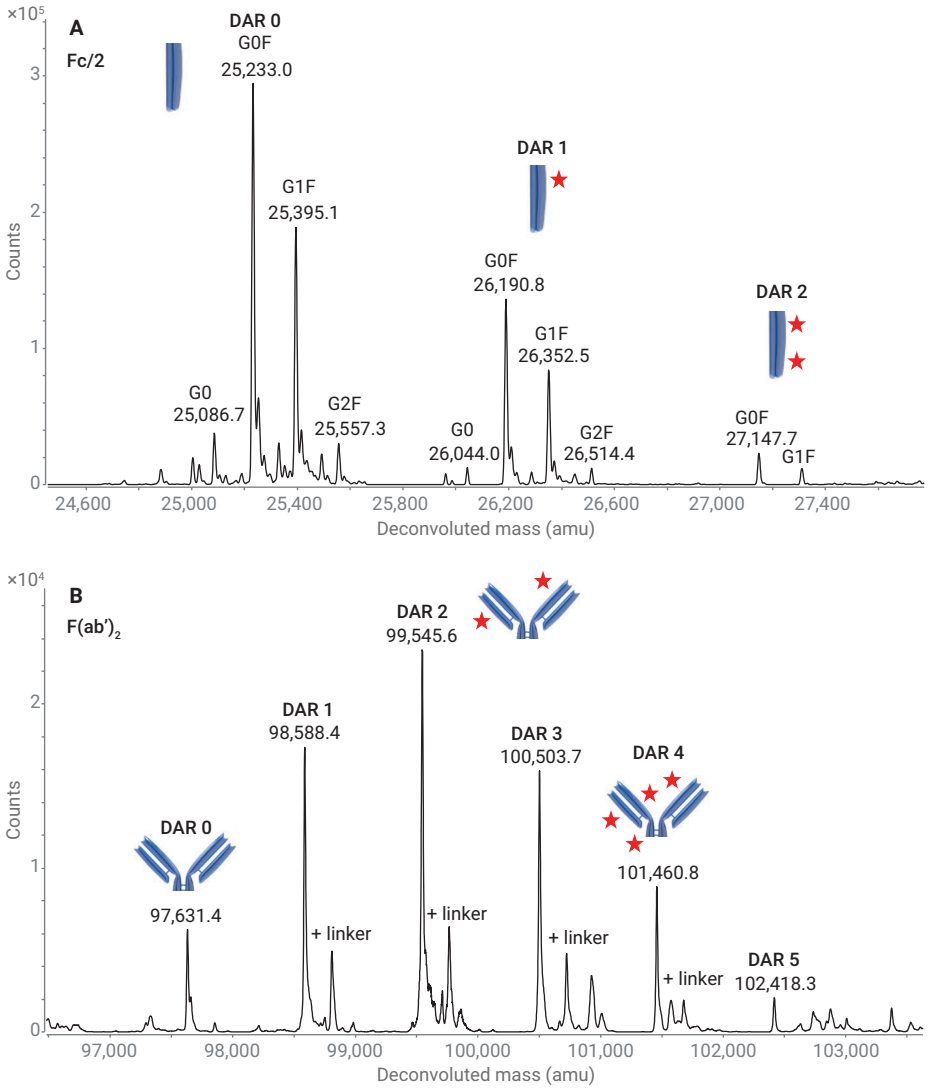

Figure 7. Deconvoluted spectra of IdeZ-treated Kadcyla desalted on the AdvanceBio Desalting-RP cartridge. Linker without drug is observed in the data as well, indicating that the reaction between the linker and the drug is not driven to completion (8 µg load). Data were acquired on an Agilent 6540 Q-TOF LC/MS.

The aim of the desalting cartridge is not to perform protein separations, for example, Lc and Hc. Nevertheless, when combining the online AdvanceBio Desalting-RP cartridge with a 6560 ion mobility Q-TOF LC/MS, which incorporates an ion mobility (IM) drift cell into the TOF analyzer, separation of the Lc and Hc can be governed at a millisecond timescale (Figure 8). This puts fewer constraints on the maximum entropy algorithm, where there are overlapping Lc and Hc *m/z* values.

Conclusion

The AdvanceBio Desalting-RP cartridge has successfully been implemented in our daily routine to allow the MS measurement of challenging molecules such as intact and fragmented mAbs and ADCs.

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References

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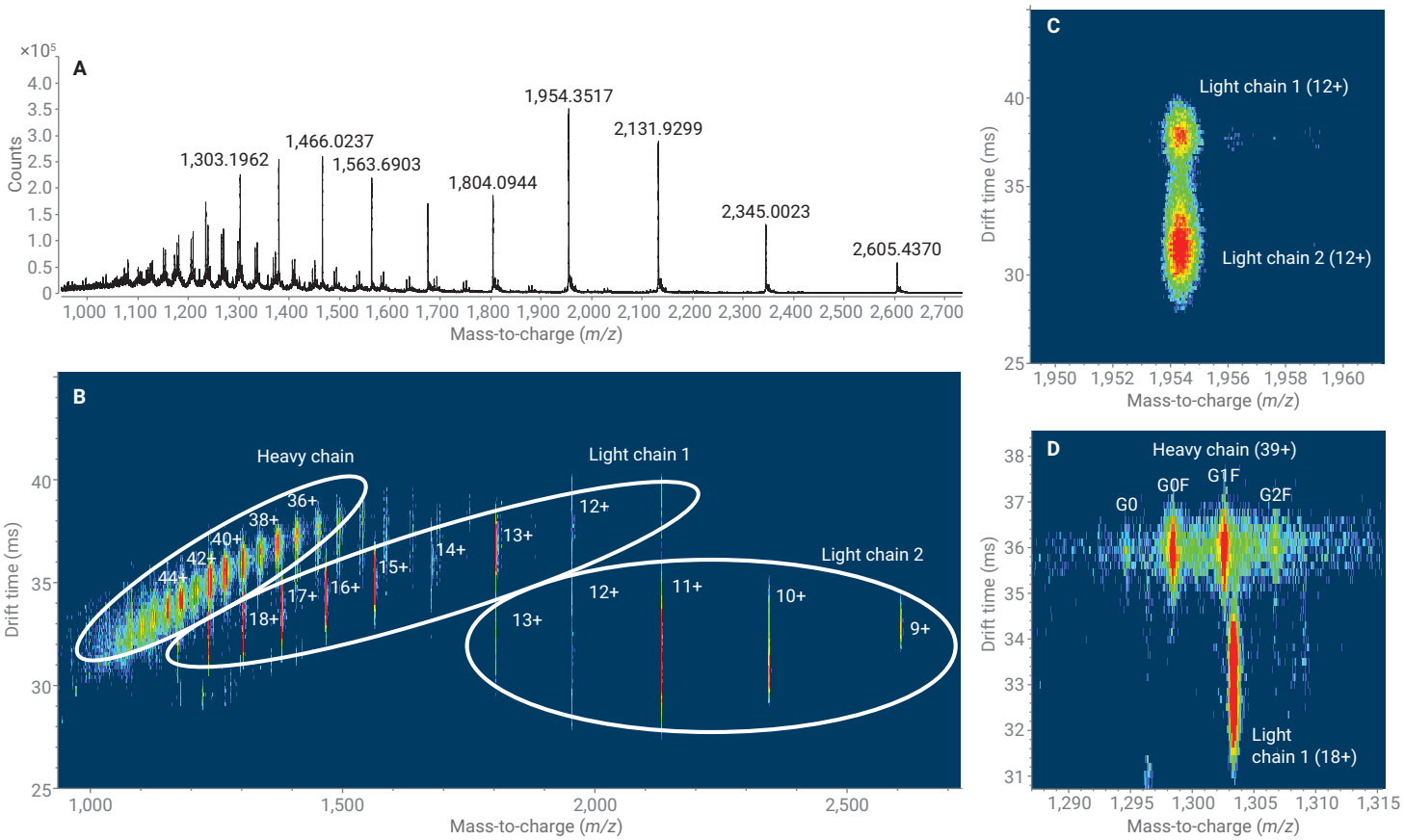

Figure 8. Raw MS spectrum (A) and IM-Q-TOF MS profile (B) of Herceptin Lc and Hc simultaneously desalted on the AdvanceBio Desalting-RP cartridge. Zoomed views are shown on the right. Two Lc conformations with identical *m/z* and molecular weight are also highlighted. Data were acquired on an Agilent 6560 ion mobility Q-TOF LC/MS.

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