Analysis of Antibody Drug Conjugate Through the Characterization of Drug Antibody Ratios

Using an Agilent 1290 Infinity Binary LC System Coupled to an Agilent 6550 iFunnel Q-TOF

Application Note

Biopharma

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Introduction

Antibody drug conjugates (ADCs) are monoclonal antibodies covalently conjugated to cytotoxic drugs through either lysine or cysteine residues (Figure 1). The antibody brings specificity to drug delivery by binding to epitopes on the surface of cells responsible for a disease state (for example, cancer). Once the ADC reaches its target, the conjugated drug is released creating a high local concentration of cytotoxic drug. ADCs are gaining market share due to their potential for improved efficacy and reduced side effects[1,2]. Due to the nature of the conjugation chemistry, ADCs are a mixture of antibodies that are conjugated to varying amounts of a drug. The drug to antibody ratio (DAR) is an important quality attribute of an ADC, as the DAR can significantly affect the efficacy of the ADC[3]; low drug loading reduces potency while high drug loading can negatively affect pharmacokinetics. This study describes the determination of the DAR for both intact and reduced lysine conjugated ADCs using state-of-the-art liquid chromatography/mass spectrometry and easy-to-use data analysis software.

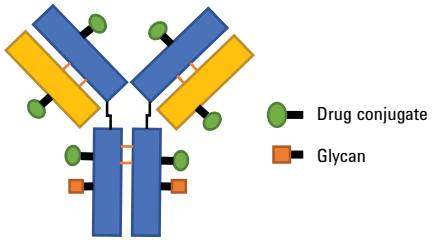

Figure 1. Schematic diagram of an ADC.

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Experimental

Materials

PNGase F was purchased from Sigma-Aldrich (St. Louis, MO). Rapid PNGase F was purchased from New England Biolabs (Ipswich, MA). All other Chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Workflow

To characterize the ADC and its subunits, the workflow shown in Figure 2 was followed. The workflow consisted of preparing glycosylated and deglycosylated ADCs in their intact and reduced forms and analyzing the samples with an Agilent 1290 Infinity LC coupled with an Agilent 6550 iFunnel Q-TOF LC/MS System. The resulting spectra was deconvoluted, and DAR calculations were performed with Agilent MassHunter BioConfirm Software and the Agilent DAR calculator, which rapidly and easily integrates all DAR peaks with minimal user input and reports final DAR values.

Sample preparation

Lyophilized ADCs were reconstituted in deionized (DI) water to 5 mg/mL, aliquoted, and stored at –80 °C until used.

For the intact ADC analysis, the reconstituted ADCs were diluted in 0.1 % formic acid in water to 1 mg/mL, just before the LC/MS analysis. Deglycosylation of the intact mAb was performed by adding 1 µL of 50 unit/µL PNGase F (Sigma-Aldrich) in 20 mM Tris-HCl buffer pH 8.2 to 100 µL of ADC (100 µg) and then incubated overnight at 37 °C. Five microliters of intact ADC was subjected to LC/MS analysis.

For reduced ADC analysis, ADCs were reduced by adding 10 µL of freshly dissolved dithiothreitol (DTT) (35 mM), 20 µL of 10 mM Tris buffer (pH = 7.5) to a 5 µL (5 µg) aliquot of ADCs followed by incubation at 50 °C for 10 minutes. For the reduced deglycosylated ADCs, ADCs were deglycosylated by adding 10 µL of freshly dissolved DTT (35 mM), 20 µL of PNGase F reagent (1:40 from original, New England Biolabs) in 10 mM Tris buffer (pH = 7.5), to a 5 µL (5 µg) aliquot of ADCs followed by incubation at 50 °C for 10 minutes. One microgram of each sample was subjected to LC/MS for analysis.

LC/MS Analyses

Instrumentation

LC/MS system

Agilent 1290 Infinity LC system including:

- Agilent 1290 Infinity Binary Pump G4220A
- Agilent 1290 Infinity TCC G1316C
- Agilent 1290 Infinity Sampler G4226A
- Agilent 1290 Infinity FC/ALSTherm G1330B

Agilent 6550 iFunnel Q-TOF LC/MS System including an Agilent Jet Stream ion source

Figure 2. Workflow for characterization of an ADC.

LC/MS parameters

Parameter	Agilent 1290 Infinity LC System	
	Intact	Reduced
Column	Agilent Poroshell 300SB-C8, 2.1 × 75 mm, 5 µm (p/n 660750-906)	Agilent PLRP-S 1000Å 2.1 × 150 mm, 8 µm (PL1912-3802)
Sample thermostat	5 °C	5 °C
Mobile phase A	0.1 % formic acid in water	0.1 % formic acid in water
Mobile phase B	70 % IPA/20 % ACN/ 10 % water with 0.1 % formic acid	99.9 % ACN with 0.1 % formic acid
Gradient (segmented)	At 0 minutes → 15 %B At 4 minutes → 20 %B At 5 minutes → 75 %B At 10 minutes → 100 %B At 10.1 minutes → 15 %B	At 0 minutes → 20 %B At 5 minutes → 20 %B At 6 minutes → 75 %B At 10 minutes → 90 %B
Post time	4 minutes	0 minutes
Column temperature	60 °C	85 °C
Flow rate	0.4 mL/min	0.4 mL/min
Parameter	**Agilent 6550 Q-TOF LC/MS System**	
	Intact	Reduced
Ion mode	Positive ion mode	Positive ion mode
Source	Agilent Dual Jet Stream	Agilent Dual Jet Stream
Drying gas temperature	290 °C	290 °C
Drying gas flow	14 L/min	14 L/min
Sheath gas temperature	400 °C	400 °C
Sheath gas flow	12 L/min	12 L/min
Nebulizer	45 psi	40 psi
Capillary voltage	5,500 V	4,500 V
Nozzle	2,000 V	1,500 V
Fragmentor voltage	400 V	250 V
Oct RF Vpp	750 V	750 V
Acquisition parameters MS mode	Data were acquired at 2 GHz, MS only mode, mass range 2,000–5,000 *m/z*.	Data were acquired at High (10,000 *m/z*) mass range, 2 GHz, MS only mode, mass range 800–4,000 *m/z*
Data analysis	The data obtained from LC/MS were analyzed using Agilent MassHunter Qualitative Analysis Software with BioConfirm Software. Deconvoluted spectrum of mAb was obtained using a Maximum Entropy deconvolution algorithm, and exported as a .csv file for DAR ratio calculation using the Agilent DAR calculator.	

Results and Discussion

Intact ADC analysis

Figure 3 shows the LC/MS analysis of intact ADC and the deglycosylated form of intact ADC. Total ion chromatogram with narrow peak width is achieved using IPA/ACN/H_2O solvent on an Agilent Poroshell 300SB-C8 column (panels A and D). Figure 3 also shows the charge state distribution envelope for both intact ADC and its deglycosylated form (panels B and E). The deconvoluted spectrum is shown in panel C.

The intact MS results showed that the ADC mass spectrum under study is heterogeneous with different populations of ADCs due to various glycosylation and drug loading. Due to this heterogeneity, the deconvoluted spectrum of intact ADCs is complex (Figure 3 panel C). To simplify the deconvoluted spectrum, the ADC was deglycosylated. The deglycosylated mass spectrum showed well resolved charge states which suggested the removal of the glycans from ADC leaving only the covalently attached drug molecules (Figure 3 panel F).

The deconvoluted spectrum showed eight major drug attachments (Figure 3 panel F). The DAR was calculated using the Agilent DAR calculator that automatically combines peak integration, and the calculation of DAR with minimal user intervention. Figure 4 shows the DAR calculator with the deglycosylated ADC.

The DAR calculator:

- Imports deconvoluted spectrum from BioConfirm Software
- Automatically picks peaks of different DAR values
- Calculates the relative peak areas of each DAR species
- Calculates an average DAR value and
- Reports out the results

Manual adjustment of the peaks to analyze is easily accomplished through the simple user interphase. The DAR calculator uses the following formula:

DAR = Σ(relative peak area × number of loaded drugs)/100

The DAR values were calculated using the relative peak area (%) of each peak and the corresponding number of drugs loaded from the deconvoluted spectrum. Then multiplying the relative peak area (%) by the corresponding number of loaded drugs to give weighted peak percentage, which measures the contribution of individual drug loaded species to the DAR (Figure 4). Manually calculated DAR and the DAR calculator calculated DAR are in complete agreement suggesting the reliability of the software. Figure 5 shows the DAR calculation for the intact ADC using the DAR calculator, which was difficult to interpret manually. The DAR calculator is a very useful tool to interpret and calculate the DAR in complex ADC samples.

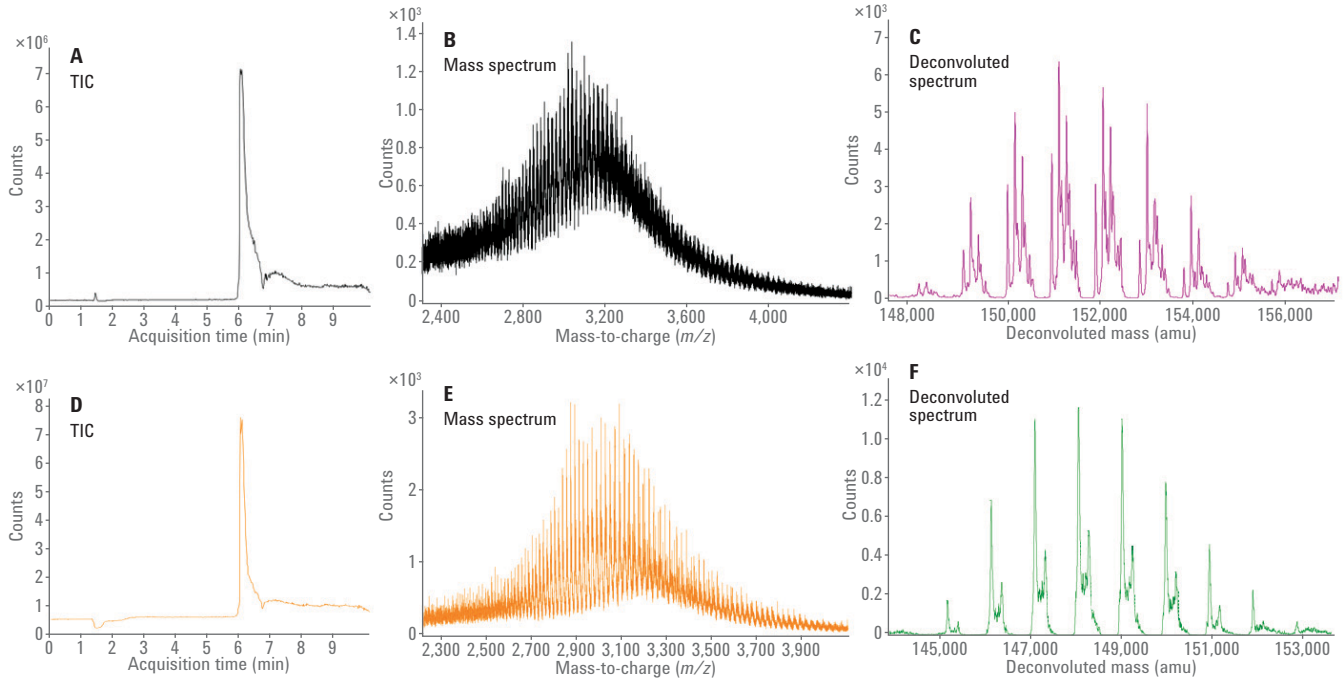

Figure 3. Total ion chromatogram (TIC), mass spectrum, and deconvoluted spectrum for intact (panels A, B, C) and deglycosylated ADC (panels D, E, F).

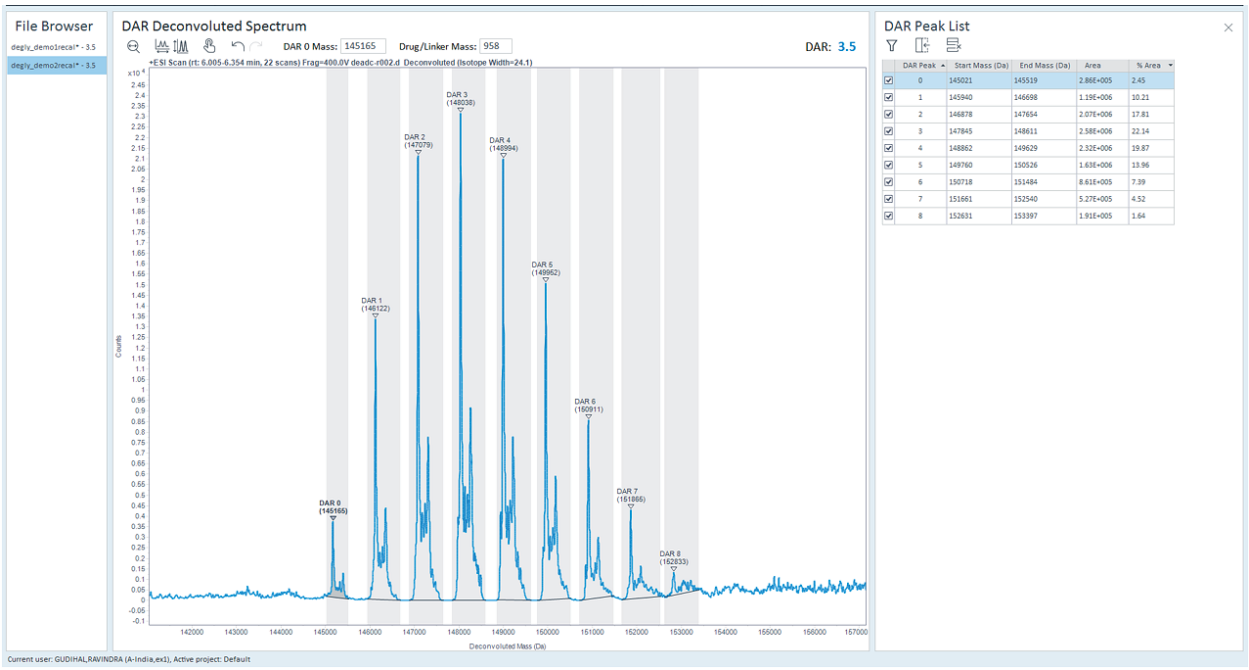

Figure 4. Calculation of the deglycosylated ADC using the Agilent DAR calculator. The DAR value of 3.4 is shown in the upper right of the deconvoluted spectrum.

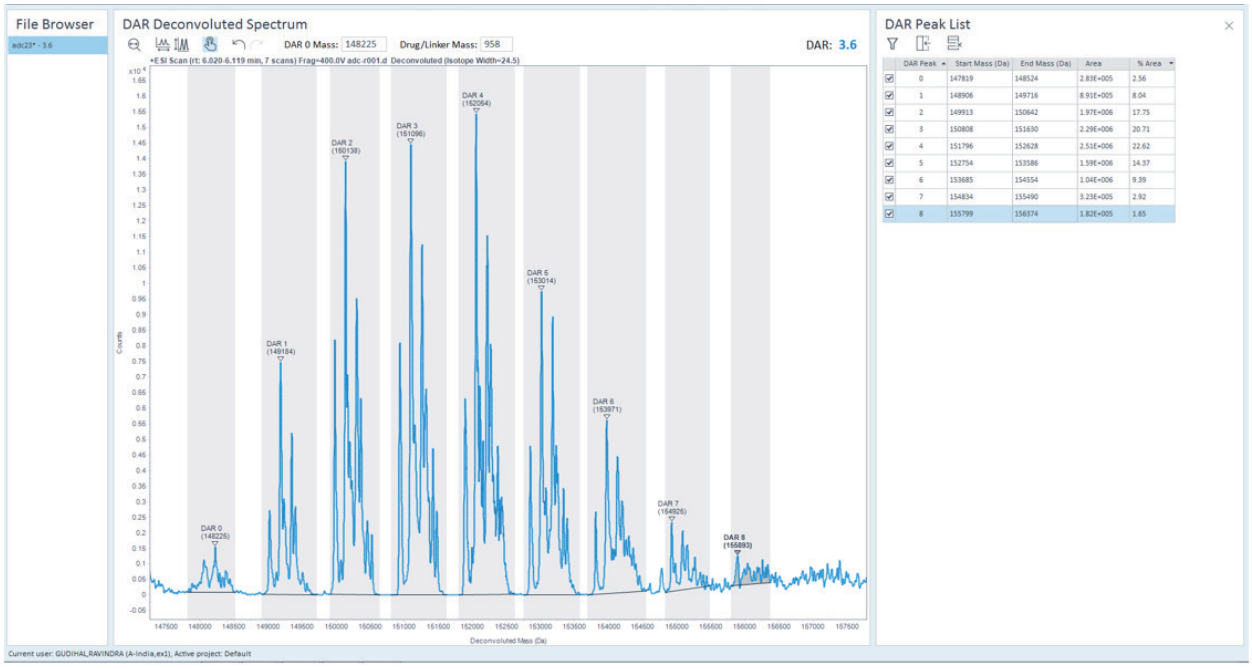

Figure 5. Calculation of the intact glycosylated ADC using the Agilent DAR calculator. The DAR value of 3.6 is shown in the upper right of the deconvoluted spectrum.

Reduced ADC Analyses

Figure 6 shows the LC/MS analyses of reduced glycosylated and reduced deglycosylated ADCs. The reduced ADCs consists of light and heavy chain drug conjugates with light chain D0 eluting out first and heavy chain D4 eluting out last (Figure 6 panels A and E). The mass envelope was between 600 and 3,500 (Figure 6 panels B and F). After deconvolution, four light chain species D0, D1, D2, and D3, and five heavy chain species D0, D1, D2, D3, and D4 were resolved (Figure 6 panels C, D, G, and H). Deglycosylation simplified the spectra of the heavy chain leaving a single dominant deglycosylated form of the ADC conjugated with different number of drugs, leading to higher signal intensity of each peak (Figure 6 panels D and H). Deglycosylation did not affect the deconvoluted mass spectra of the light chain associated drug conjugates (Figure 6 panels C and G), as the light chains are not N-glycosylated.

The exported deconvoluted spectra for both the light and heavy chain of the glycosylated and deglycosylated ADCs were subsequently loaded into the DAR calculator, where the peaks were labelled and integrated. The DAR ratio of the heavy and the light chain were calculated respectively with a combined DAR ratio reported.

Combined DAR =
2 × (Heavy Chain DAR + Light Chain DAR)

Figure 7 panels A and B show a representative report generated by the DAR calculator, including annotated deconvoluted spectra, the DAR ratio of the light and heavy chain respectively, the combined DAR ratio, and tables showing the theoretical and observed mass, peak area, and peak area percentage of ADCs carrying different number of drugs were reported. Table 1 shows the theoretical and observed mass, peak area, and peak area percentage of the reduced and deglycosylated ADCs carrying different number of drugs.

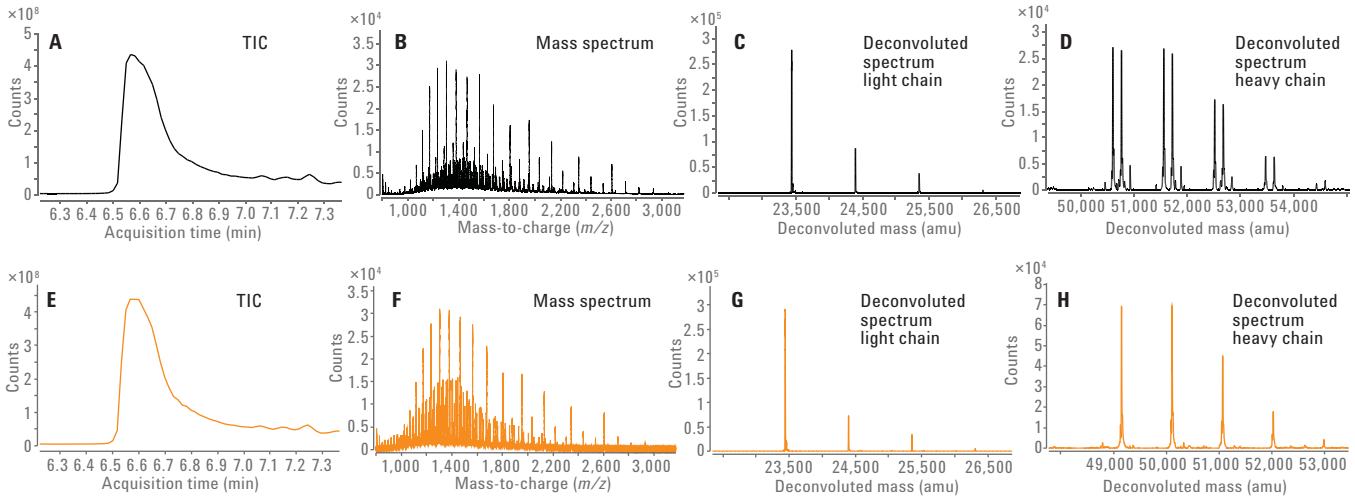

Figure 6. TIC, mass spectrum, and deconvoluted spectra for light and heavy chain of the reduced glycosylated (panels A–D) and deglycosylated ADC (panels E–H).

DAR Report

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DAR Calculation Results

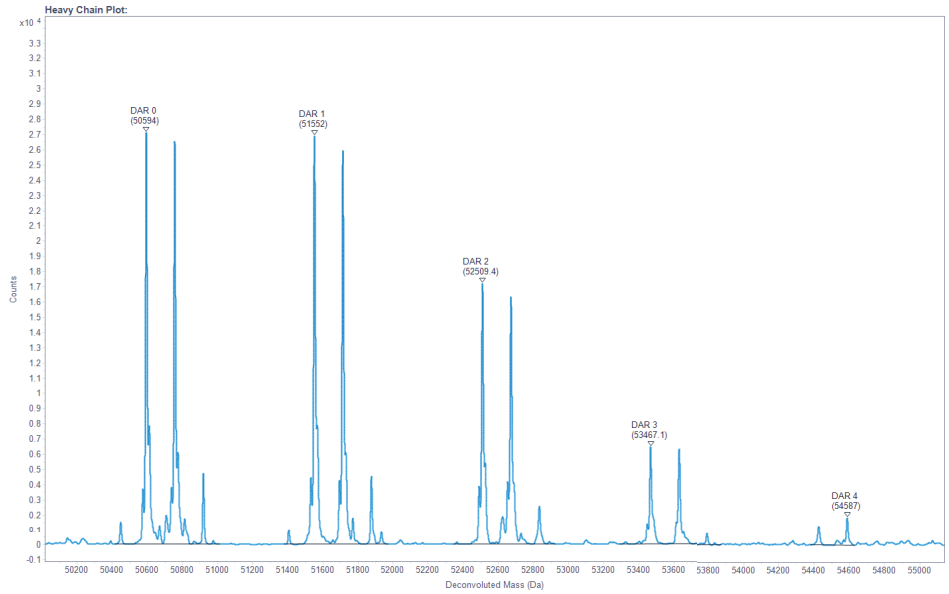
ADC Information

Data File	Reduced-ADC	**Combined DAR**	3.2
Processing Method	Reduced	**Heavy Chain DAR**	1.1
Report Time	9/16/2015 09:55 AM	**Light Chain DAR**	0.5

Property	Theoretical (Da)	Observed (Da)
Heavy Chain DAR 0 Mass	Not Given	50594
Light Chain DAR 0 Mass	Not Given	23439.3
Drug/Linker Mass	Not Given	979.8

Heavy Chain Results

Deconvoluted Spectrum



DAR Peak List

DAR Peak	Theoretical Mass (Da)	Observed Mass (Da)	Area	% Area
0	Not Given	50594	1.10E+006	33.41
1	Not Given	51552	1.08E+006	32.90
2	Not Given	52509.4	7.76E+005	23.57
3	Not Given	53467.1	2.77E+005	8.41
4	Not Given	54587	5.65E+004	1.72

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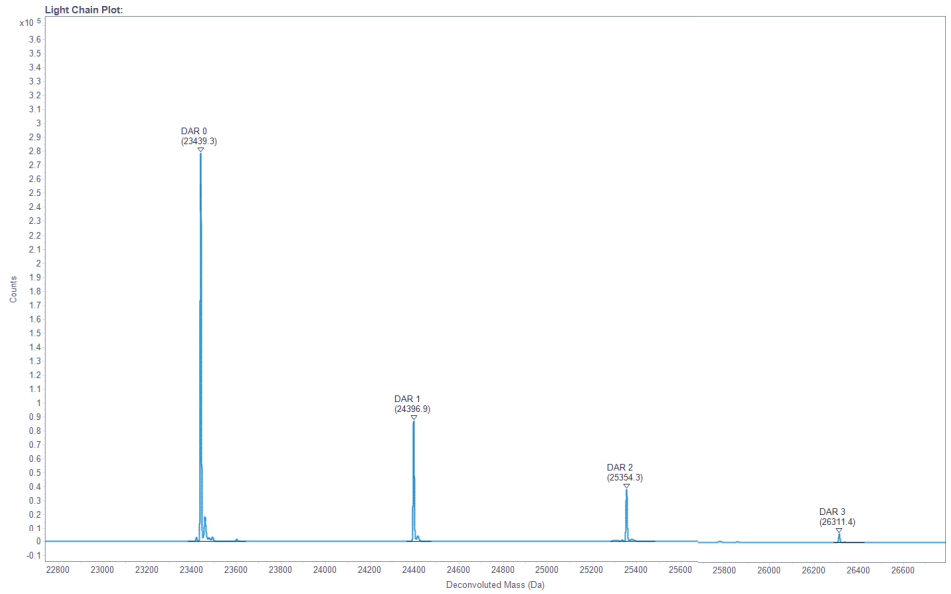
Figure 7A. A report (page 1) from the Agilent DAR calculator of a reduced glycosylated ADC.

Light Chain Results

Deconvoluted Spectrum



DAR Peak List

DAR Peak	Theoretical Mass (Da)	Observed Mass (Da)	Area	% Area
0	Not Given	23439.3	1.70E+006	65.01
1	Not Given	24396.9	5.60E+005	21.44
2	Not Given	25354.3	3.07E+005	11.74
3	Not Given	26311.4	4.71E+004	1.81

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Figure 7B. A report (page 2) from the Agilent DAR calculator of a reduced glycosylated ADC.

Conclusions

The reversed phase analysis of an ADC using an Agilent 1290 Infinity LC system coupled to an Agilent 6550 iFunnel Q-TOF with Agilent LC columns can be extensively used to reproducibly generate accurate DAR values on both intact and reduced ADCs with excellent performance.

Agilent MassHunter BioConfirm Software and DAR calculator can be used together to provide efficient data extraction, deconvolution, and an easy and intuitive approach to calculate and report DAR values for both intact and reduced ADCs.

Table 1. DAR peak list of a reduced deglycosylated ADC from Agilent DAR calculator.

DAR Peak	Theoretical mass (Da)	Observed mass (Da)	Area	% Area
Heavy chain results				
0	49148.65	49149.9	1.39E+006	31.87
1	50106.15	50107.7	1.41E+006	32.34
2	51063.65	51065.5	1.04E+006	24.00
3	52021.15	52023	4.29E+005	9.86
4	52978.65	52980.9	8.43E+004	1.94
Light chain results				
0	23439.1	23439.3	1.70E+006	70.48
1	24396.6	24396.8	4.57E+005	18.91
2	25354.1	25354.2	2.19E+005	9.08
3	26311.6	26311.2	3.71E+004	1.53

References

1. Chari, R. V.; Miller, M. L.; Widdison, W. C. Antibody-drug conjugates: an emerging concept in cancer therapy. *Angew Chem. Int. Ed. Engl.,* **2014**, *53(15)*, pp 3796-827.

2. Perez, H.L., *et al.*, Antibody-drug conjugates: current status and future directions. *Drug Discov Today,* 2014. **19**(7): p. 869-81.

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