Drug-to-antibody ratio quantification using native SEC-MS analysis of antibody–drug conjugates

With thanks to:
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Summary

- This Application Note illustrates the use of native SEC-MS to reliably and accurately quantify the drug-to-antibody ratio (DAR) for interchain cysteine-linked ADCs, as demonstrated by Jones *et al*.[1]
- The ADC workflow within Byos® provides automated deconvolution, peak integration, and calculation of the DAR for ADCs
- The reporting template included in the workflow provides typical tables and plots required for reporting data from DAR analysis and can be fully configured for specific user requirements.

Introduction

As of the end of 2020, nine FDA-approved antibody-drug conjugates (ADCs) were available for targeted chemotherapy[2]. More than 80 additional ADCs were in clinical studies at that time. ADCs consist of a monoclonal antibody (mAb) attached to a potent cytotoxic drug via a cleavable linker. The ability of the mAb component of the ADC to recognize cancer antigens on the cell surface and thereby target the cytotoxic drug to cancer cells has long been recognized as an important development in cancer therapy.

The diversity of ADCs primarily comes from the types of linkers, drugs and the mAb attachment sites. Furthermore, the number of drug molecules attached to each antibody can vary, resulting in a distribution of the number of drug molecules attached a given mAb. The average antibody-to-drug ratio (DAR) is used as a quantitative assessment of the distribution of drug molecules and is also as a measure of the drug potency. Assays for measuring DAR should therefore be established in early development.

The DAR is dependent on the IgG subclass and conjugation strategy employed. There are currently two main conjugation strategies that do not require modification of the primary sequence. These are lysine conjugation and interchain cysteine conjugation.

In interchain cysteine-linked ADCs, some of the interchain disulfides have been reduced to accommodate covalent attachment of the drug linker. A consequence of this is that the associations between the chains in the ADC are a combination of covalent and non-covalent interactions. Analytical techniques therefore need to consider the nature of these associations, which is why native conditions are important in the analysis of cysteine-linked ADCs.

Strategies for measuring DAR have typically deployed hydrophobic interaction chromatography (HIC) with UV detection. Although the technique can provide the DAR, it does not provide any insight into the site(s) of drug attachment and may require long separation times, especially for highly heterogeneous ADCs. Reversed-phased chromatography with UV has also been deployed to measure both the DAR and obtain some chain-specific attachment site information. In this case, however, the ADC is reduced, and the drug distribution on the intact ADC cannot be determined.

MS-based methods provide several unique advantages. Firstly, DAR quantification using MS does not require chromatographically resolved LC peaks of the individual drug-loaded species and this can vastly reduce the method development and optimization time typically required for novel ADC molecules. Secondly, MS provides unambiguous identification by mass, which is critical to avoid mis-identification of peaks. Furthermore, it offers the potential for deployment of platform methods, which can be applied across modalities and chemotypes.

The MS methods for DAR quantification involve the following data processing steps, all of which are included in the preconfigured ADC workflow within Byos:

- The multiply charged mass spectrum of the ADC is deconvoluted into a zero charge mass spectrum
- The species observed in the mass spectrum are identified on the basis of agreement between theoretical and observed mass
- The relative levels of each of the individual drug-loaded species are inferred based on the apparent-relative height or area of that species.
- Calculation of the DAR based on the sum of all detected species is calculated.

In the work presented by Jones *et al.*[1], native SEC-MS was evaluated as a method to reliably and accurately quantify the DAR for interchain cysteine-linked ADCs. SEC-MS was compared to existing methodologies to determine whether it could be used as a standard method for interchain cysteine-linked ADCs, regardless of the target DAR range or chemotype. In this application note, we briefly discuss the results from the SEC-MS analyses. For full details refer to Jones *et al.*[1]

Experimental Conditions

A two-part investigational strategy was deployed.

1) Assess the suitability of the method for the determination of ADC DAR in a routine laboratory setting.
2) Assessment of the accuracy of the method for determining the DAR of interchain cysteine-linked ADCs.

To determine the method performance over three months, an interchain cysteine-linked maleimidocaproyl-valine-citrulline-p-aminobenzyloxycarbonyl MMAE (vcMMAE) ADC (herein called ADC-A), was injected no less than three times during every sample set, twice at the beginning and once at the end of the sample set.

For the second part of the strategy, a set of ADC samples with DAR values from ~2.5 to 8 was created by modulating the molar equivalents of reducing agent prior to adding the drug linker. All ADCs were conjugated to interchain cysteine residues using various drug linker chemotypes. The example data included in this application note is from an antibody conjugated with vcMMAE (ADC-A described above), and the DAR values for this ADC were from 2.8 to 5.7.

Parameters for SEC-MS data generation

For full experimental details see reference 1. Prior to mass spectrometric analysis, antibodies were deglycosylated using PNGase F. ADCs were separated by size exclusion chromatography and the resulting eluent was analyzed by ESI-MS over the m/z range 800-8000.

LC Column: PolyLC 2.1 mm × 200 mm, 5 µm, 300 Å, polyhydroxyethyl-A (PHEA) column
Mobile phase: 200mM ammonium acetate, pH7, 0.1mL/min
MS System: Bruker maXis II UHR-QTOF

Parameters for Automated Data Processing

Mass spectra were batch deconvoluted using the Byos Intact Mass algorithm incorporated in the ADC workflow. The DAR of ADCs was automatically calculated within the software.

Results and Discussion

Native SEC-MS Performance and Qualification

- Typical replicate injections of SEC-MS separation of an ADC-A sample are shown in Figure 1.
- The deconvoluted mass spectra clearly show differing numbers of drug molecules attached to the antibody (Figure 1A) and magnification of a specific region (Figure 1B) shows the evidence of lower levels of modified ADC forms.

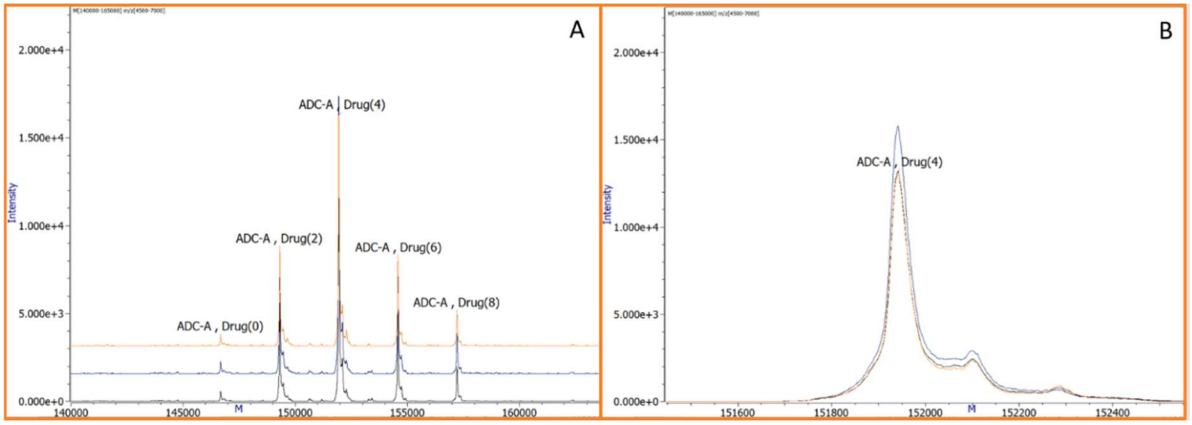

Figure 1. Replicate injections of the SEC-MS analysis of an ADC-A sample with an average calculated DAR of 4.13. 1A: Deconvoluted mass spectra from the three replicate injections. 1B: Zoomed portion of the mass spectrum showing the antibody with four drug molecules attached.

- Accurate deconvolution of multiply-charged ions is critical for reliable quantification of DAR and is made possible through Protein Metrics' Parsimonious charge deconvolution algorithm[4], which is embedded within the ADC workflow of Byos.
- Figure 2 shows a region of the multiply charged raw m/z mass spectrum prior to deconvolution (Figure 2A) and following deconvolution (Figure 2B).

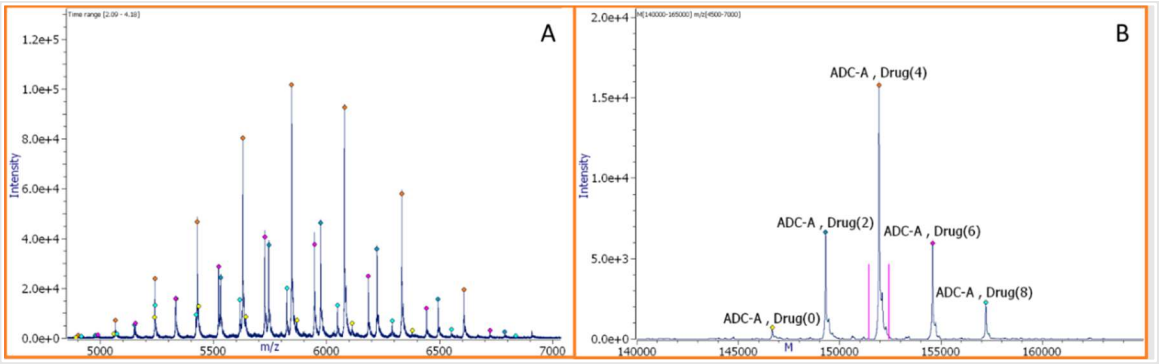

Figure 2. SEC-MS analysis of an ADC-A sample with an average calculated DAR of 4.13. 2A: Raw m/z spectrum 2B: Deconvoluted mass spectrum. Ions contributing to the deconvoluted mass spectrum are labeled with colored dots in Figure 2A and the corresponding deconvoluted mass is marked with and equivalent dot in the deconvoluted mass spectrum.

- The corresponding automatic calculation of the DAR for the replicate injections shown in Figure 1 are provided in Table 1 and presented as a bar chart in Figure 3.

PROTEIN METRICS

Protein name ↑	Drug Count	Peak # 1	2	3
ADC-A	0	0.00	0.00	0.00
	2	0.42	0.42	0.43
	4	2.00	2.01	2.00
	6	1.16	1.14	1.14
	8	0.60	0.58	0.60
	Level 1 Totals	**4.18**	**4.15**	**4.17**

Sum - DAR Fraction
Filter applied on: Drug Count, Protein name.

Table 1: Byos generated DAR values from the replicate injections of the sample shown in Figure 1. The table is included in the default ADC report template ('DAR Fraction' tab) within Byos.

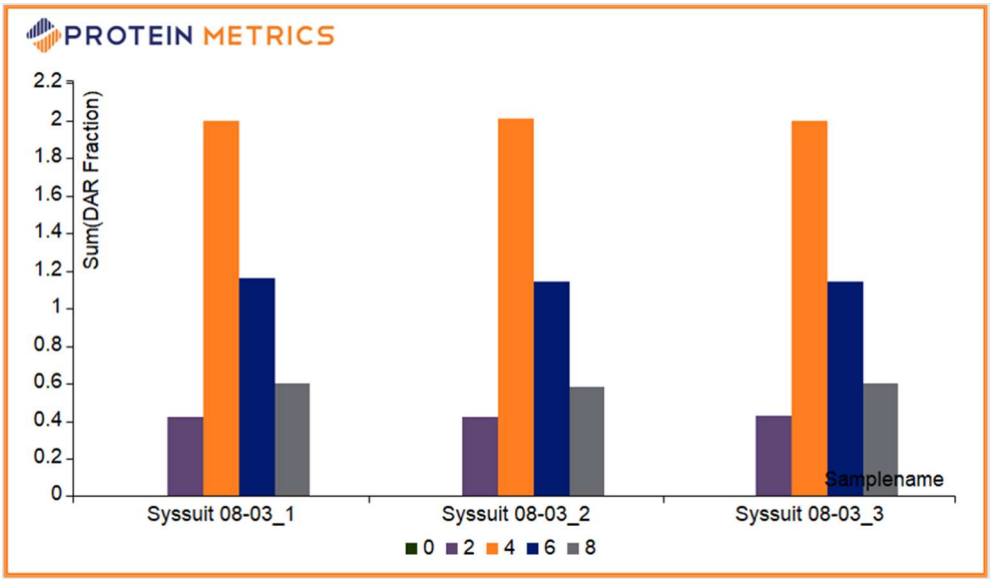

Figure 3: Automatically generated Drug Load Distribution Barchart from the replicate injections of the sample shown in Figure 1. The barchart is included in the default report template ('Drug Load Distribution – Barchart tab) from the ADC report template within Byos.

- The clarity of the deconvoluted mass spectra provides unambiguous identification of the species in the DAR quantification. This is a key advantage of using SEC-MS compared to the aforementioned chromatographic methods.
- Methods that do not use MS may experience interference from aglycosylated higher-load species not being sufficiently separated from lower-load species. This can result in mis-assignment of peaks and over-reporting of low level species[1,5].
- The system suitability injections of ADC-A over the three-month period resulted in an average DAR of 4.195, with a range of 4.170 to 4.230 and a standard deviation of 0.015.
- The overall DAR range tested for ADC-A was 2.8 to 5.7. Typical mass spectra for low, medium and high DAR levels for ADC-A are shown in Figure 4. The corresponding automated DAR Table from Byos is shown in Table 2.
- As can be seen from Figure 4, the high drug load species are easily detected using MS. Mass detection appears to offer improved detection levels of high drug load species, which typically produce smaller, broader peaks (or no peaks) via HIC, resulting in no detection or poor quantification[1,5].

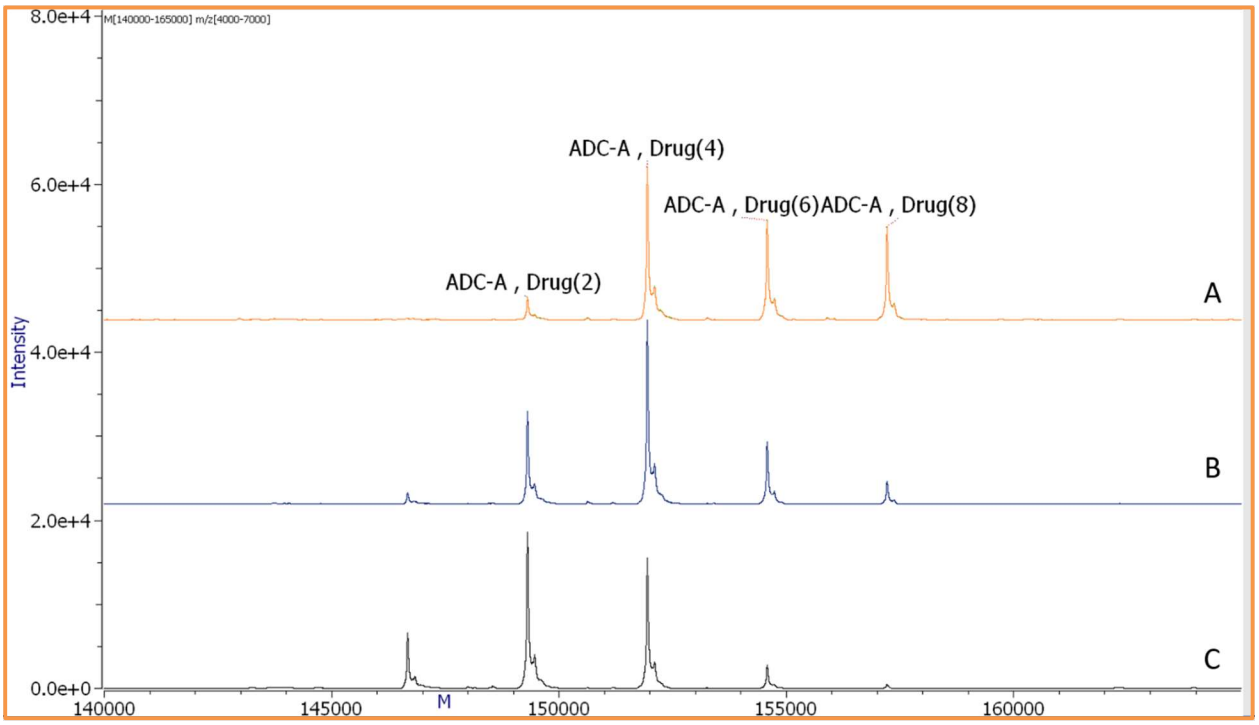

Figure 4. Example deconvoluted mass spectra from three ADC-A samples with different DAR values. The calculated DAR values were 5.42 (orange, A) 3.96 (blue, B) and 2.72 (black, C).

PROTEIN METRICS

		Peak # ←	1	2	3
Protein name ↑	Drug Count ↑				
ADC-A	0		0.00	0.00	
	2		0.84	0.50	0.12
	4		1.41	1.97	1.66
	6		0.38	1.00	1.62
	8		0.09	0.49	2.02
		Level 1 Totals	**2.72**	**3.96**	**5.42**

Sum - DAR Fraction
Filter applied on: Protein name.

Table 2: Byos generated DAR values from the three ADC-A samples shown in Figure 4. The table is included in the default report template ('DAR Fraction' tab) from the ADC workflow within Byos.

Determination of SEC-MS quantification limit

- An empirical quantification limit (QL) was determined by spiking unconjugated mAb into the ADC-A sample that had a DAR of 5.42 (Sample 3 from Table 2).
- This sample was selected for the QL evaluation because it did not contain a detectable level of unconjugated antibody.

- ADC-A samples containing unconjugated mAb at levels ranging from 0.5% to 2.5% by mass were analyzed in triplicate. The resulting total ion chromatograms for one replicate at each level are shown in Figure 5.

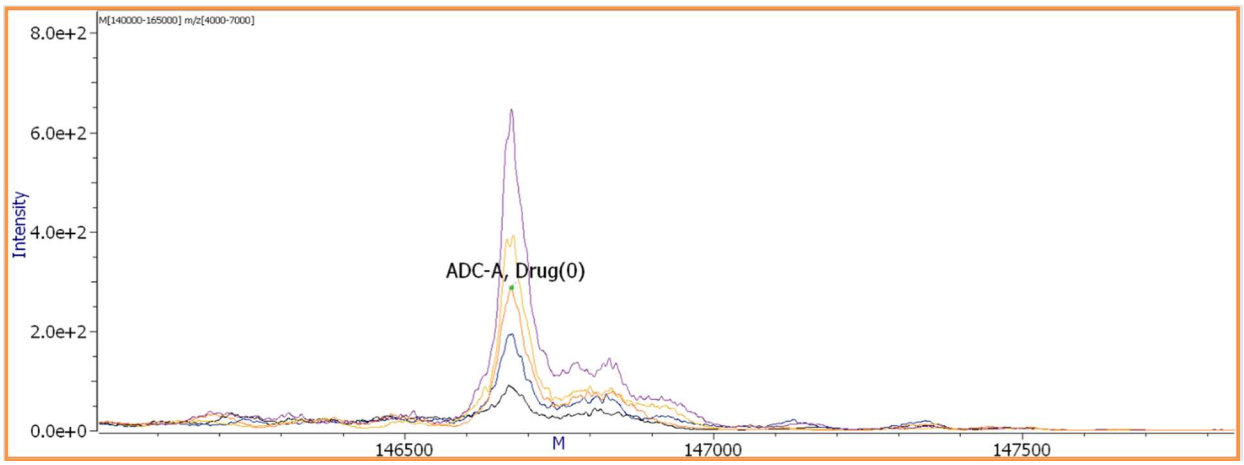

Figure 5. Overlay of the deconvoluted mass spectra from five different levels of unconjugated antibody spiked into the ADC-A sample with a DAR of 5.42. Levels are as follows: Black 0.5%; Blue 1%; Dark orange 1.5%; Light orange 2%; Purple 2.5%.

- The relative intensity of the unconjugated mAb was divided by the summed intensity of all forms of ADC-A.
- The result (in percent) was compared to the theoretical (spiked) level of unconjugated mAb (Table 3).
- The quantified level of unconjugated mAb was reproducibly recovered across all spike levels, thus supporting the establishment of a practical QL of 0.5%.
- Figure 6 shows the Byos plot of the measured relative population of the unconjugated mAb (%) versus the known amount of the spiked species, demonstrating that the MS signal response was linear.

PROTEIN METRICS

		Peak Comment	0.5 (%)	1 (%)	1.5 (%)	2 (%)	2.5 (%)
Protein name ↑	Drug Count						
ADC-A	0		0.59	1.1	1.5	2	2.8

Normalize Column - Intensity, Level 0
Normalize type - Sum
Filter applied on: Drug Count, Protein name.
Hidden values in: Drug Count.

Table 3. Comparison of the theoretical spike level of unconjugated antibody with the calculated percentage from the relative intensity of the unconjugated mAb divided by the summed intensity of all forms of ADC-A. The default report from Byos was tailored to include this calculation for future automated quantification of the relative amount of unconjugated antibody.

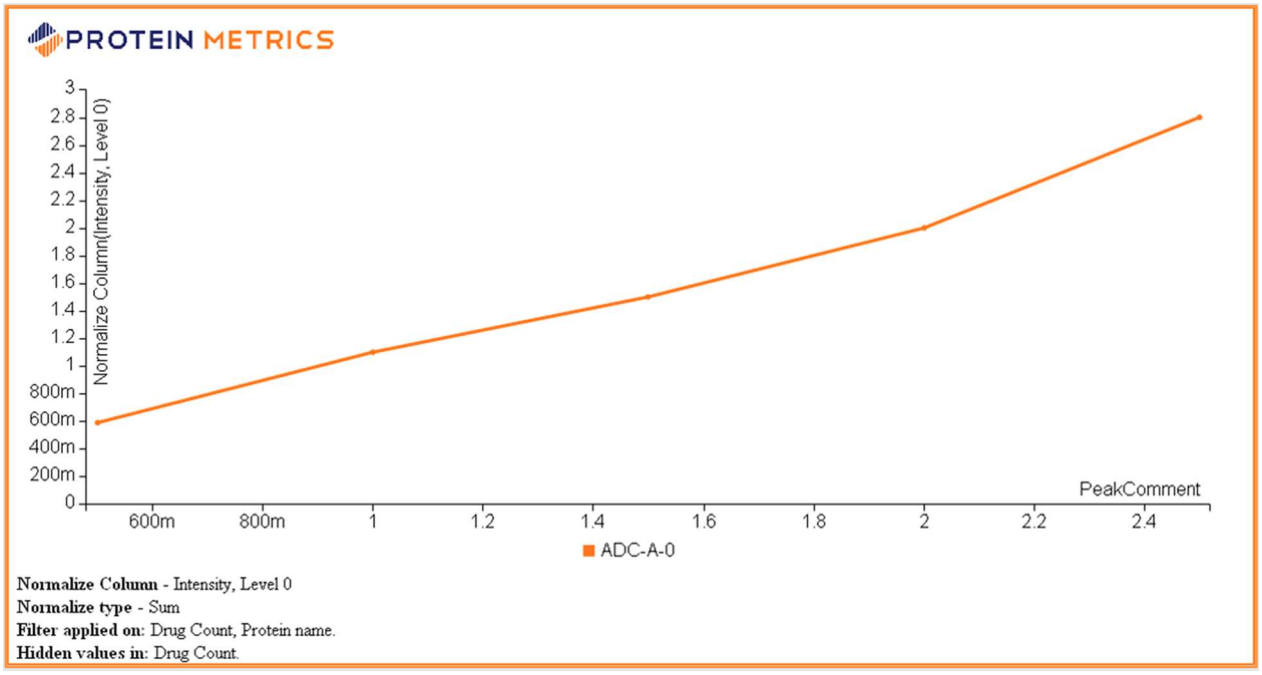

Figure 6. Relative signal intensity versus spiked amount of unconjugated mAb in the QL series

Conclusions

We have demonstrated that native SEC-MS can be used for quantitative assessment of DAR with cysteine interchain linked ADCs. Native SEC-MS provides quantitatively accurate measurements of the DAR of interchain cysteine-linked ADCs across a wide range of drug-loading. There is also the potential for other types of drug linkages, however appropriate validation studies would be required to determine sample pretreatment prior to analysis.

Automated processing and reporting using the ADC workflow within Byos improves the efficiency, and repeatability of data analysis. The accuracy of the embedded algorithm for deconvolution of overlapping multiply-charged ions ensures accuracy in both identification of the components and quantification of different drug loads.

For more resources and background on intact mass data analysis workflows, visit https://www.proteinmetrics.com/workflows/ *or contact the Customer Success Team at support@proteinmetrics.com.*

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info@proteinmetrics.com

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