

## LC-MS Analysis of Intact Lysine-Conjugated ADCs using the ACQUITY™ Premier UPLC™ and Xevo™ G3 QToF Mass Spectrometer

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Samantha Ippoliti, Ying Qing Yu

Waters Corporation

This is an Application Brief and does not contain a detailed Experimental section.

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### Abstract

This application brief demonstrates the use of the ACQUITY Premier UPLC System coupled to the Xevo G3 QToF Mass Spectrometer for the analysis of an intact lysine-conjugated Antibody-Drug Conjugate (ADC). MaxEnt1-deconvoluted data was used to calculate the drug-to-antibody ratio (DAR), a measurement key to the safety and efficacy of ADC therapeutics. Data acquisition and processing was performed using the waters\_connect™ UNIFI™ App Intact Mass Workflow, operated under the compliance-ready waters\_connect Informatics Platform.

### Benefits

- Streamlined ADC workflow employing custom calculations in the UNIFI App for automated DAR calculation and reduced risk of manual processing or reporting errors
- ACQUITY Premier UPLC System with high performance surfaces (HPS) for improved system uptimes and consistent recovery of analytes

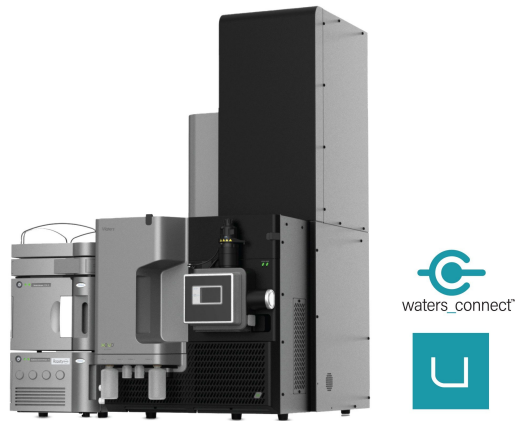
- High resolution benchtop Xevo G3 QToF Mass Spectrometer with sensitive and robust detection of biomolecules

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## Introduction

Antibody-drug-conjugates (ADCs) have gained significant momentum as a modality for improving cancer treatment in recent years. In fact, as of 2023, the FDA has approved eleven ADC therapeutics for a variety of cancer types.<sup>1-2</sup> An ADC is a monoclonal antibody (mAb) that has been conjugated to a drug toxin via a linker for targeted delivery to cancer cells. The linker-drug is generally conjugated through a chemical reaction with either lysine or cysteine amino acids in the mAb protein sequence, or through targeted site protein engineering. This application brief focuses on lysine-conjugated class of ADCs, using Kadcyła™ (ado-trastuzumab emtansine) as a model molecule for this case study. Lysine-conjugated ADCs tend to exhibit significant heterogeneity, as there are numerous surface accessible lysine residues in the sequence and the reaction is not specific to any given site. The result is a distribution of conjugation forms of linker-drug moieties to the mAb at varying levels.

To assess the global drug distribution and DAR, scientists have typically turned to intact mAb LCMS analysis for these heterogenous ADCs. This application brief demonstrates the capabilities of the ACQUITY Premier UPLC System coupled to the Xevo G3 QToF Mass Spectrometer for high quality and sensitive ADC analysis. The data was automatically acquired and analyzed using the UNIFI App within the compliant-ready waters\_connect Informatics Platform (Figure 1).



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*Figure 1. Acquisition was performed with the Xevo G3 QToF Mass Spectrometer coupled to ACQUITY Premier UPLC (BSM, FTN, TUV). Data was acquired, processed, and reported using the UNIFI App within the waters\_connect Informatics Platform.*

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## Results and Discussion

For this application brief, samples of trastuzumab and Kadcyla (ado-trastuzumab emtansine) ADC were treated with PNGaseF enzyme (removal of the N-glycosylation) to simplify the reversed phase LCMS analysis of the intact mAb and conjugated mAb structures. For method details about intact mAb RP-LCMS, see Application Note [720007635](#).<sup>3</sup> For this analysis, the Intelligent Data Capture (IDC) feature was enabled for real-time background noise reduction.

The resulting combined raw spectra mirror plot (Figure 2) highlights the added complexity of the deglycosylated ADC (red) compared to the deglycosylated unconjugated trastuzumab (blue). The Xevo G3 QToF Mass Spectrometer has the capability to detect these complex species using a 500-ng injection on column. The data was acquired and analyzed within the UNIFI App utilizing the intact protein workflow. Automated data processing was performed using MaxEnt1 deconvolution using the Manual peak width model, which uses manually measured full width and half maximum (FWHM) values for peaks in the higher and lower end of the

charge state envelope. In this case, values were both measured at 0.8  $m/z$ . The input  $m/z$  range was 2200–3400 and the output mass range was set between 140–155 kDa, with an output resolution of 1.0 Da. A maximum of twenty-five MaxEnt1 processing cycle iterations were performed for spectral deconvolution. The resulting deconvoluted masses (Figure 3) were mass matched against variable modification of 0–10 additions of linker-drug, with a 50-ppm mass error tolerance set as a 3x multiple of the average expected mass accuracy due to the complexity of the output spectra. The expected DAR species, as well as an additional linker-only series, were detected in the deconvoluted spectrum. These linker-only species are impurities which can be monitored using this method but are not factored into the DAR calculation. As shown in Figure 4, a drug distribution of 0–9 conjugation species was observed.

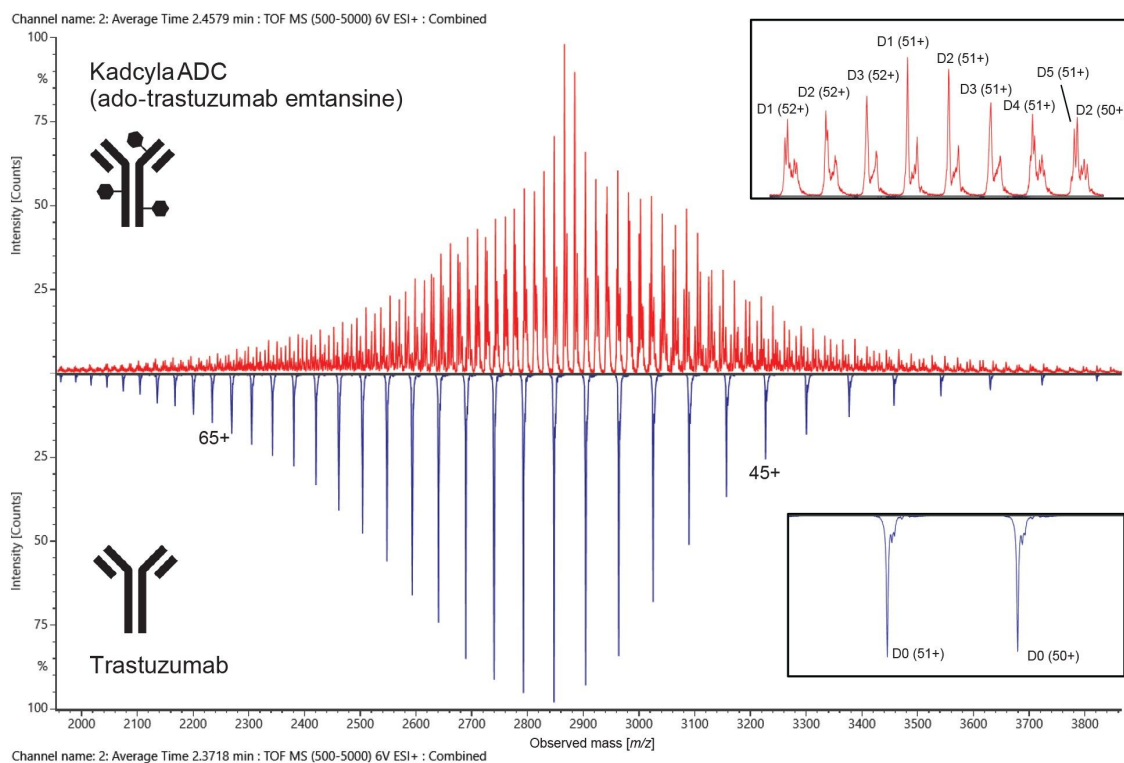


Figure 2. Mirror plot of combined raw  $m/z$  spectra for deglycosylated Kadcyła ADC (red) and an unconjugated trastuzumab antibody (blue), with zoomed sections inserted to the right of each to highlight the complexity of the conjugated sample.

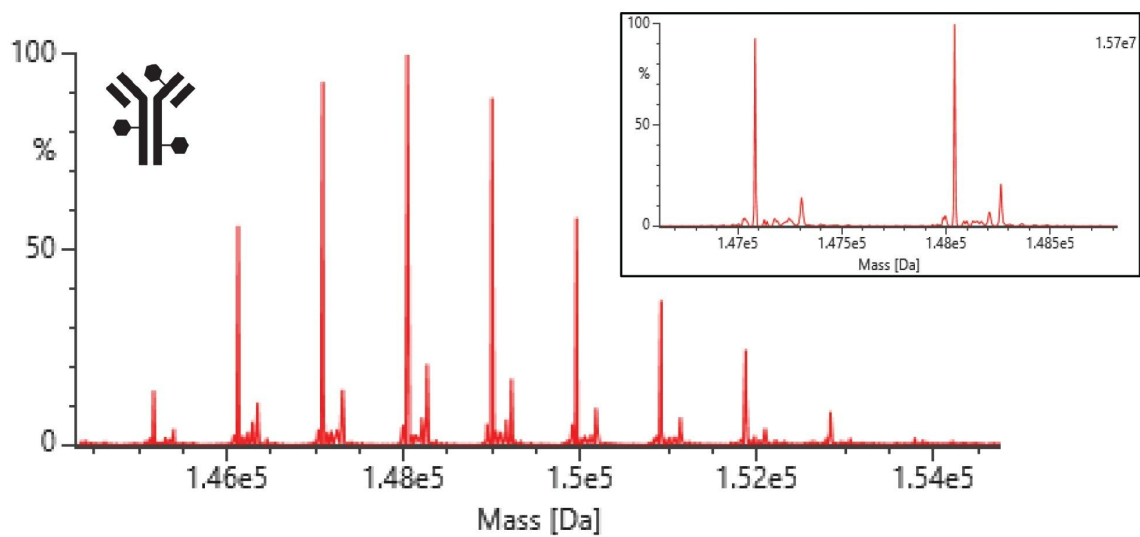


Figure 3. MaxEnt1 deconvoluted spectra for the deglycosylated Kadcylya ADC, with a zoomed section (inset) to highlight the resolution of minor species.

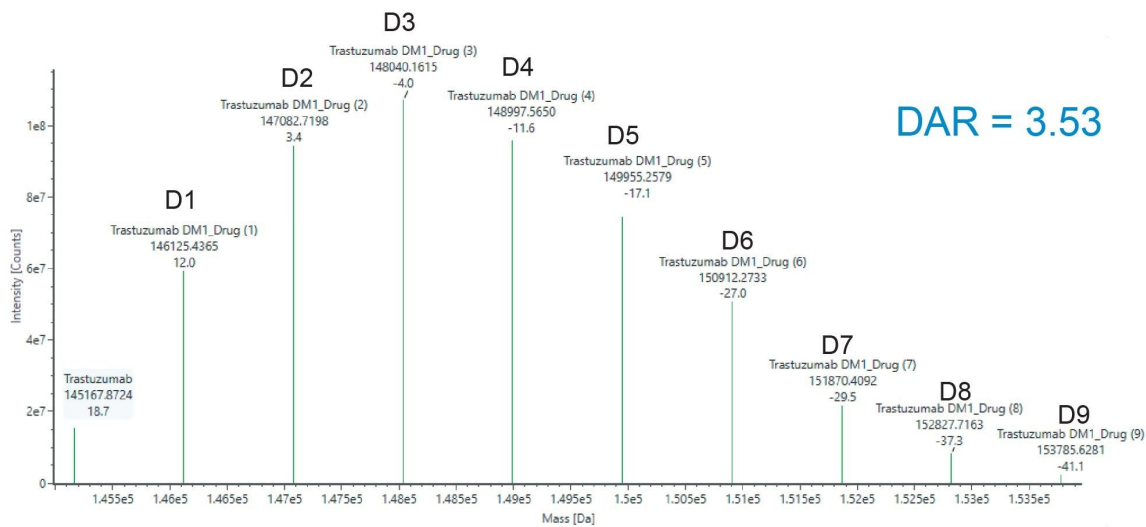


Figure 4. Deconvoluted mass spectrum (Centroid by Peak Area) of the Kadcyly ADC sample, showing a drug distribution of 0–9 drugs, which is used for the DAR (drug-to-antibody ratio) calculation. Mass accuracy values are shown for each species and are well below the 50 ppm mass matching tolerance threshold.

The DAR value is a weighted average of the responses for the unconjugated and conjugated species present and is commonly used as a metric to judge the conjugation process consistency and potential potency of the ADC. Automated calculation of the DAR value was made possible within the workflow using custom calculations. UNIFI automatically calculated the weighted average using drug load and MS response for each species. The calculated DAR value for this Kadcyly (ado-trastuzumab emtansine) sample was 3.53, which is comparable to previously published characterization studies.<sup>2</sup> A template with the automated DAR calculation is included in the default method deployed with waters\_connect, which enables scientists to quickly optimize the method for their ADC DAR calculations.

## Conclusion

The ACQUITY Premier UPLC System coupled to the Xevo G3 QTof Mass Spectrometer provided high quality intact mAb data for deglycosylated Kadcyła (ado-trastuzumab emtansine) ADC. The intact protein UNIFI workflow enabled data acquisition, automated MaxEnt1 deconvolution, mass matching, DAR calculation, and reporting, all within the compliance-ready architecture of the waters\_connect Informatics Platform. Kadcyła is a trademark of Genentech. Xevo, waters\_connect, UNIFI, and ACQUITY are trademarks of Waters Technologies Corporation.

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## References

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2. Chen L, Wang L, Shion H, Yu C, Yu YQ, Zhu L, Li M, Chen W, Gao K. In-Depth Structural Characterization of Kadcyła® (ado-trastuzumab emtansine) and its Biosimilar Candidate. *mAbs*. 2016. 8:7, 1210–1223.
3. Ippoliti S, Cornwell O, Reid L, Yu YQ, Harry E, Towers M. Comprehensive Biosimilar Comparability Assessment via Intact and Subunit RP-MS and IEX-UV-MS Using the Xevo G3 QTof System. Waters Application Note [720007635](#). 2022.

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[ACQUITY Premier System <https://www.waters.com/waters/nav.htm?cid=135077739>](https://www.waters.com/waters/nav.htm?cid=135077739)

[Xevo G3 QTof <https://www.waters.com/nextgen/global/products/mass-spectrometry-systems/xevo-g3-qtof.html>](https://www.waters.com/nextgen/global/products/mass-spectrometry-systems/xevo-g3-qtof.html)

[UNIFI Scientific Information System <https://www.waters.com/134801648>](https://www.waters.com/134801648)

[waters\\_connect <https://www.waters.com/waters/nav.htm?cid=135040165>](https://www.waters.com/waters/nav.htm?cid=135040165)

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