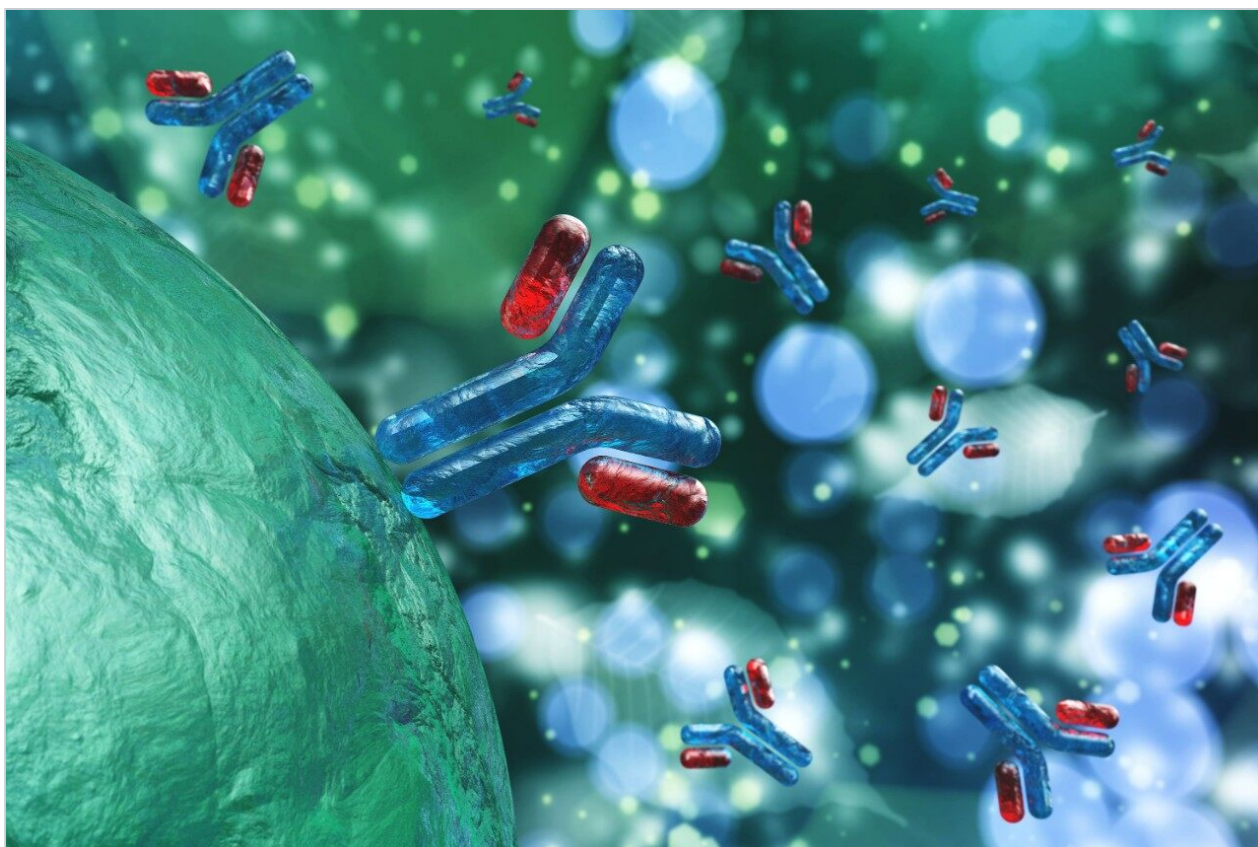


## Analysis of Cysteine-Conjugated Antibody Drug Conjugates (ADCs) Using a Native SEC LC-MS Workflow on the SYNAPT XS

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

## Abstract

Drug-to-Antibody Ratio (DAR) is a critical quality attribute (CQA) for antibody drug conjugates (ADCs) because it directly affects therapeutic efficacy and pharmacokinetics. Determination and monitoring of DAR is essential across the ADC development process and for commercial manufacturing and quality operations. For cysteine conjugated ADCs, native MS analysis is essential in order to keep noncovalent bonding intact between subunits of the antibody when ADC molecules are added across the bridging disulfide bonds. Native MS thus becomes an essential tool enabling correct measurement of the DAR by MS, as well as the drug loading distributions in the intact molecules. In this study, an analytical scale native SEC LC-MS analysis workflow (Figure 1), featuring native MS detection by the SYNAPT XS was employed for analysis of several cysteine-conjugated ADCs with variable drug load.<sup>1-3</sup> The results were then compared to those produced from the previous generation Q-ToF systems to demonstrate the robustness of the native SEC LC-MS workflow (Figure 1) across time and these different systems.

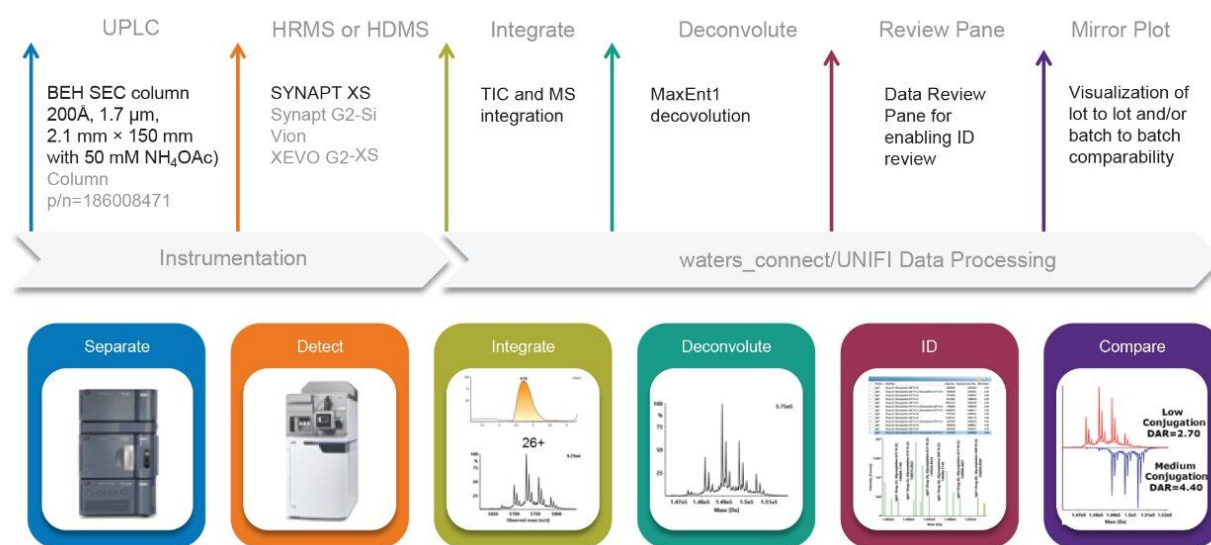


Figure 1. The analytical scale native SEC LC-MS analysis workflow. Biomolecules are loaded to a SEC column (ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 2.1 mm x 150 mm, p/n: 186008471) for desalting/separation under non-denaturing conditions before Q-ToF analysis (Xevo G2-XS, Vion, or SYNAPT G2-Si or XS). The raw mass spectrum obtained by combining the scans under the TIC peak were deconvoluted and centroided for quantification. DAR values were calculated by an intensity averaged custom calculation using the detected components within each sample.

## Benefits

- No pre-analysis sample preparation required
  - Automatic DAR and drug distribution measurements
  - Routine and robust native LC-MS solution
  - Analytical-scale SEC LC-MS analysis with streamlined workflow
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## Introduction

Native mass spectrometry has gained more and more popularity in the recent years, as it expands the capability of mass spectrometry to analyze complexes and other types of noncovalent interactions. One of the reasons for this advancement is the improvements for online SEC LC-MS native analysis.<sup>1-3</sup> Online SEC-MS minimizes the need for sample preparation and automates the analysis process, making the method more routine and robust. Under the denaturing conditions of reversed-phase LC-MS analysis, non-covalent linkages are broken in biological molecules. In order to access the higher order structural information a native LC-MS workflow can be employed using MS-compatible volatile buffers such as ammonium acetate and soft electrospray ionization MS conditions to maintain the biologic in its native or quasi-native state with noncovalent interactions kept intact. Since the functions of mAb-based biotherapeutics are typically accomplished by protein-protein or protein-ligand interactions, being able to directly measure such noncovalent interactions are very important in order to characterize this class of molecules. One example, cysteine-conjugated antibody drug conjugates (ADCs), have small drug molecules covalently attached to the host antibody via the thiol groups that typically participate in intra and inter-subunit linkages. These thiols are produced by the reduction of (primarily) inter-chain disulfide bonds that connect between the two heavy chains or between the light and the heavy chains. The loading of the small molecule drugs is ideally expected to occur in pairs of 2, 4, 6, or 8 attachment sites as shown in Figure 2 due to the release of two free thiols from each reduced disulfide bond. It is the residual noncovalent interactions of the subunits that holds the cysteine conjugated ADC molecule together once these disulfides are cleaved.

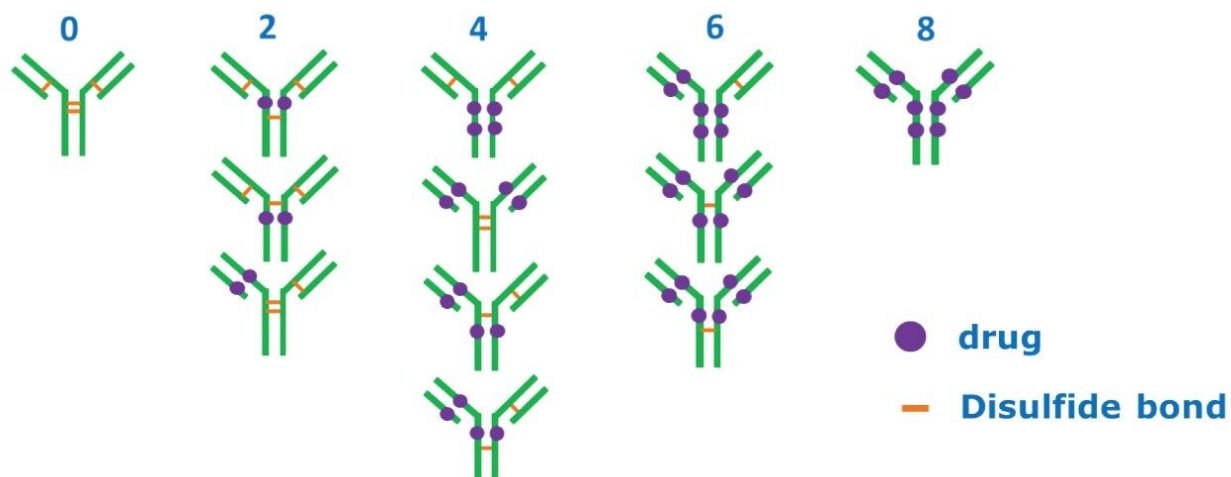


Figure 2. IgG1 antibody and its potential cysteine conjugated ADC drug loading distributions, including isomeric structures. Small drug molecules are attached to the host antibody via reduced disulfide linkages connecting the subunits, and typically observed in pairs of 2, 4, 6, or 8 drug molecules per antibody.

Drug-to-Antibody Ratio (DAR) is a critical quality attribute (CQA) for ADCs because it directly effects their therapeutic efficacy and pharmacokinetics. Successful ADC development and manufacturing rely on the determination of the DAR and its monitoring throughout the entire development and commercialization process. In a reversed-phase LC-MS experiment, the cysteine-conjugated ADCs will dissociate into half antibodies or composite light and heavy chain subunits due to the acidic mobile phase conditions and high organic compositions of the mobile phase used for elution. Therefore, native mass spectrometry conditions are essential in order to characterize the DAR for cysteine-conjugated ADCs in the intact molecules. In this study, analytical scale native SEC LC-MS analysis with the SYNAPT XS QToF System were employed for native MS analysis of a set of cysteine-conjugated ADCs with varying drug load.<sup>1-3</sup> The results are compared to those from previous generation Q-ToF systems to demonstrate the consistency of the native SEC LC-MS workflow (Figure 1) across these different systems.

## Results and Discussion

In this study, analytical scale SEC (ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 2.1 mm x 150 mm, p/n: 186008471 <<https://www.waters.com/nextgen/in/en/shop/columns/186008471-acquity-uplc-protein-beh-sec-column-200a-17--m-21-mm-x-150-mm-1-.html>> ) with 0.1 mL/min isocratic elution (50 mM

ammonium acetate) was conducted over a 10-minute runtime. The native SEC LC-MS system was comprised of an ACQUITY UPLC H-Class PLUS System coupled in-line to a SYNAPT XS. The system was operated with MassLynx (version 1.4.2) for data acquisition and the data were imported to the UNIFI App within the waters\_connect informatics platform (or to the UNIFI Scientific Informatics System, both of version 1.9.4) for streamlined data processing and reporting, including automated DAR calculation and drug distribution comparisons.

Compared to a reversed phase LC-MS analysis, biological samples under native MS analysis will carry fewer charged forms at higher mass-to-charge ratios because of compact native structure maintained under the native SEC-MS conditions. The combined raw spectral charge state envelope (Figure 3 left, centered around  $m/z=5,600$  with only 5 to 6 major charge states in the envelope), a zoomed-in single charge state of the (center), and the deconvoluted spectra (right) are shown for the naked antibody mAb, and three load levels (low, medium, and high) of conjugated ADCs. The glycosylation pattern displayed in the naked mAb spectrum is repeated for each conjugation form (0, 2, 4, 6, and 8) across all three levels of conjugated sample. The combined integrated peak areas of each of the glycoform from the deconvoluted spectra were used for the automated custom calculation of the total average DAR, and the drug loading distribution, within the waters\_connect/UNIFI data processing workflows, as previously described in detail.<sup>1</sup>

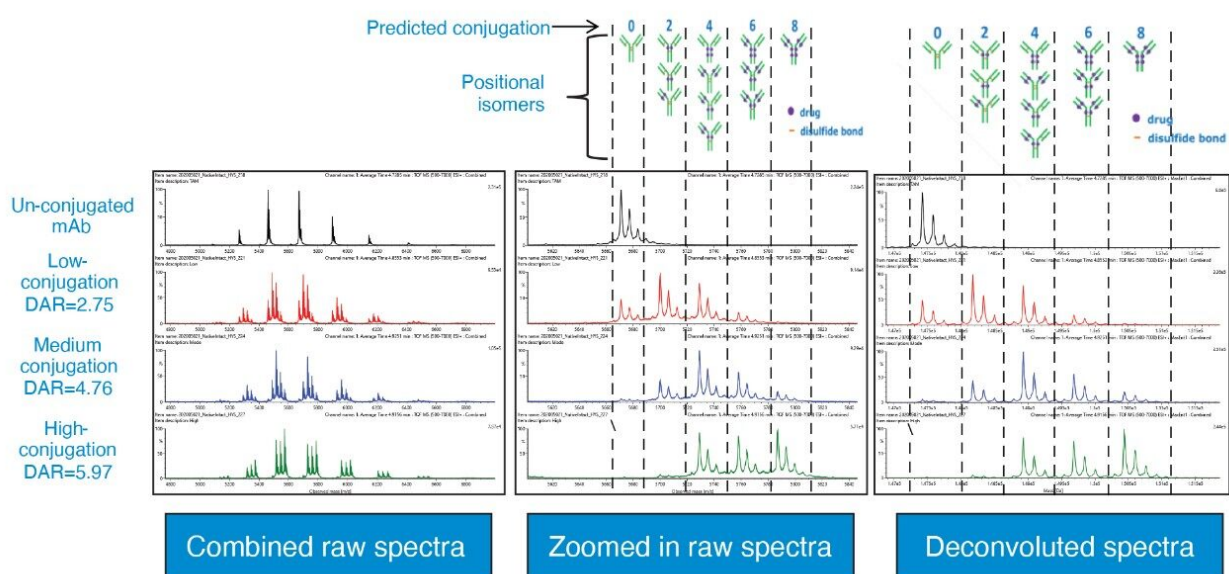


Figure 3. The combined multiple charge raw native mass spectra exhibit a narrow set of high  $m/z$  ions with peaks caused by glycoform and ADC heterogeneity (left), the zoomed-in region (of a single charge state) of the combined raw spectrum (center) and the deconvoluted spectra (right) are shown for the reference material (naked mAb), as well as the low, medium, and high conjugation level cysteine-conjugated ADC samples. Data were acquired on the SYNAPT XS HDMS System using native SEC LC-MS analysis.

Table 1 compares the calculated DAR and drug load distributions for three batches of ADCs among the HIC analysis data (from 2014),<sup>1</sup> and the native SEC LC-MS analysis data from a Xevo G2-S QToF system (from 2014), a Vion IMS QToF MS (from 2017) and the SYNAPT XS System (this study, 2020). The Xevo G2-S data was collected after the ADC was treated with PNGase F, while all other measurements were done using the glycosylated antibody. These experiment results indicated that MS-based DAR measurements can be performed consistently over time versus orthogonal approaches (HIC vs MS), across different Q-ToF MS platforms (Xevo G2-S, Vion IMS QToF MS, and SYNAPT XS Systems), and with and without sample deglycosylation. Overall, the analytical scale native SEC LC-MS analysis workflow results indicate that it is very effective in native analysis of ADCs to determine lot comparability for both DAR and drug load distribution studies.

Cysteine-conjugated ADCs drug loading distribution and DAR												
	Low				Medium				High			
	HIC	QTof1	QTof2	SYNAPT XS	HIC	QTof1	QTof2	SYNAPT XS	HIC	QTof1	QTof2	SYNAPT XS
ADC 2	0.81	0.74	0.64	0.62	0.38	0.41	0.35	0.37	0.07	0.09	0.05	0.04
ADC 4	1.14	1.17	1.37	1.32	1.67	1.57	1.81	1.80	1.23	1.11	1.19	1.16
ADC 6	0.75	0.60	0.64	0.61	1.61	1.45	1.51	1.46	1.72	1.72	1.86	1.81
ADC 8	0.12	0.21	0.05	0.12	0.78	0.97	0.70	0.71	2.95	3.05	2.98	1.94
DAR	2.83	2.72	2.70	2.75	4.44	4.40	4.37	4.36	5.97	5.97	6.07	5.94

QTof 1      deglycosylated samples, run on Xevo G2-S in 2014  
 QTof 2      non-deglycosylated samples, run on Vion in 2017  
 SYNAPT XS   non-deglycosylated samples, run on Synapt XS in 2020

Table 1. Total average DAR and drug distribution comparison across HIC (UV) and three native SEC LC-MS experiments show agreement across all three drug loading levels. The results indicated that DARs can be measured consistently using orthogonal approaches (HIC vs MS), or across different Q-ToF MS systems (Xevo G2-S, Vion IMS QTof MS, and SYNAPT XS).

## Conclusion

In this application brief, we have demonstrated an analytical scale native SEC LC-MS analysis workflow that can be applied across LC-MS systems for the analysis of cysteine conjugated ADCs. SEC-MS based workflows offer the efficiency benefits of automated sample operation over infusion based analysis, and removes the need for sample preparation (e.g. buffer exchange and deglycosylation) prior to MS analysis of DAR and drug distribution analysis for ADC molecules.

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