

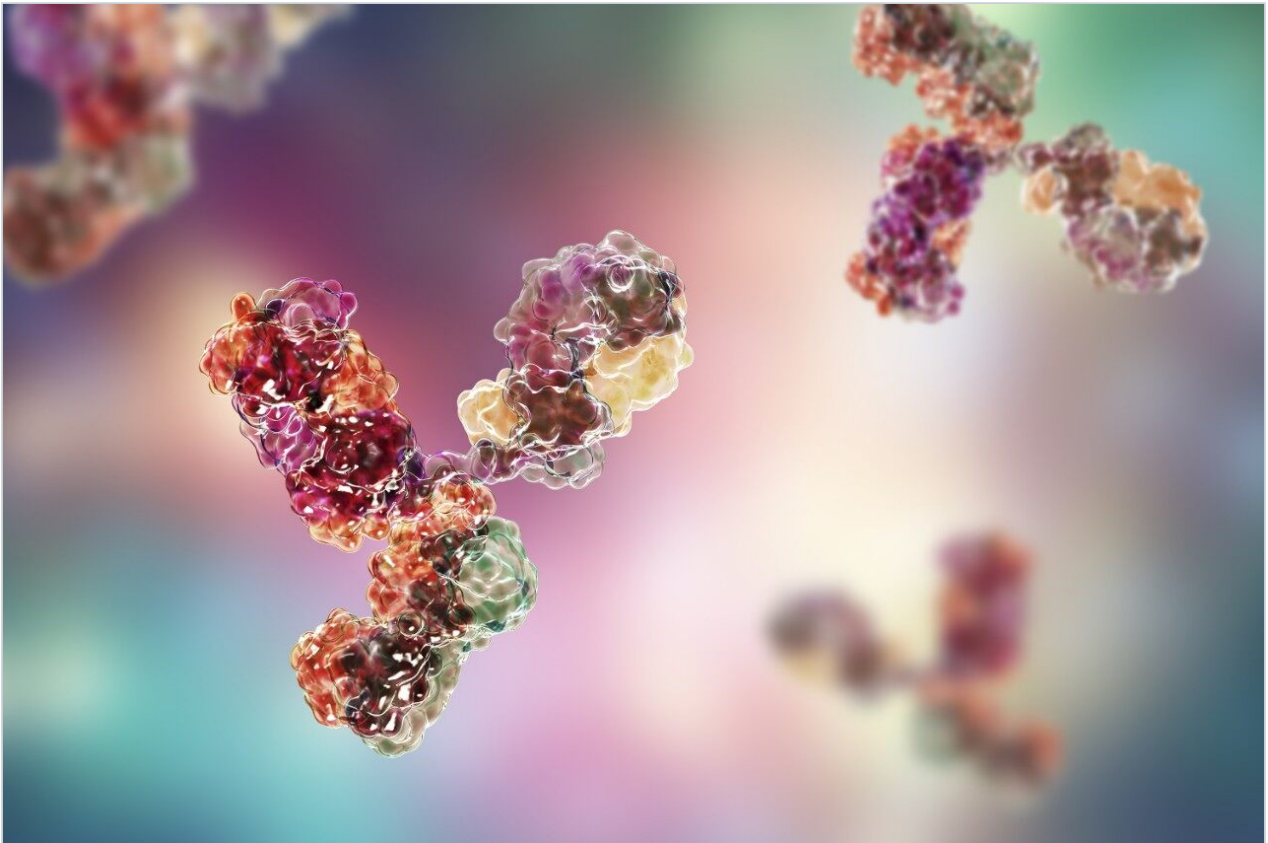
Application Note

## Development of Integrated Informatics Workflows for the Automated Assessment of Comparability for Antibody Drug Conjugates (ADCs) Using LC-UV and LC-UV/MS

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## Abstract

In this application note, we demonstrate the use of an integrated informatics workflow in UNIFI Software that streamlines data acquisition, processing and reporting for ADC analysis by LC-UV or LC-MS, so that information from multiple analytical techniques can be effectively integrated for rapid and quantitative assessment of the comparability of ADCs.

### Benefits

We demonstrate an integrated informatics workflow using UNIFI Software that streamlines data acquisition, processing, and reporting for ADC analysis by LC-UV or LC-MS, so that CQAs information, such as average DAR and drug load distribution for multiple batches of ADCs, from multiple analytical techniques can be effectively compiled for rapid quantitative assessment.

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## Introduction

Antibody Drug Conjugates (ADCs) have been emerging as a new class of anti-cancer therapeutics, combining the targeted delivery capability of a monoclonal antibody (mAb) with the potency of conjugated cytotoxic agents. By releasing the cytotoxic drugs inside target tumor cells, ADCs minimize exposure of non-target cells to toxic drug payloads and thereby reduce potential side effects of the treatment.

Liquid chromatography (LC) and mass spectrometry (MS) are among the most commonly used analytical techniques for characterizing ADCs.<sup>1,2</sup> Very often, a suite of techniques, such as hydrophobic interaction chromatography (HIC) LC-UV, SEC native LC-MS, and /or RP LC-MS, can be applied to achieve a more comprehensive characterization profile of an ADC.

Structural information that is derived from different analytical methodologies, especially when used to determine critical quality attributes (CQAs) such as drug-to-antibody ratio (DAR) and drug load distribution (which measures the homogeneity of the ADC population), is challenging to assemble to provide effective assessment.<sup>3</sup> Different informatics tools and manual calculations are usually needed for processing the complex datasets, leading to losses in productivity and time. With more than 60 ADCs currently in development,<sup>4</sup> more efficient informatics approaches, such as automated data acquisition, data processing and data reporting, are needed to support pharmaceutical researchers who are charged with characterizing

and quantifying ADCs.

In this work, we illustrate the benefits of applying an integrated informatics workflow that has been developed for UNIFI Software to streamline data acquisition, processing, and reporting for ADC analysis by LC-UV or LC-MS.

This workflow brings together information from multiple analytical techniques, so that it can be effectively used for rapid and quantitative assessment of the comparability of ADCs. DAR values and drug load distribution for multiple batches of ADCs are calculated automatically. We also perform a comparability study across analytical techniques based on the DAR values for different ADC samples.

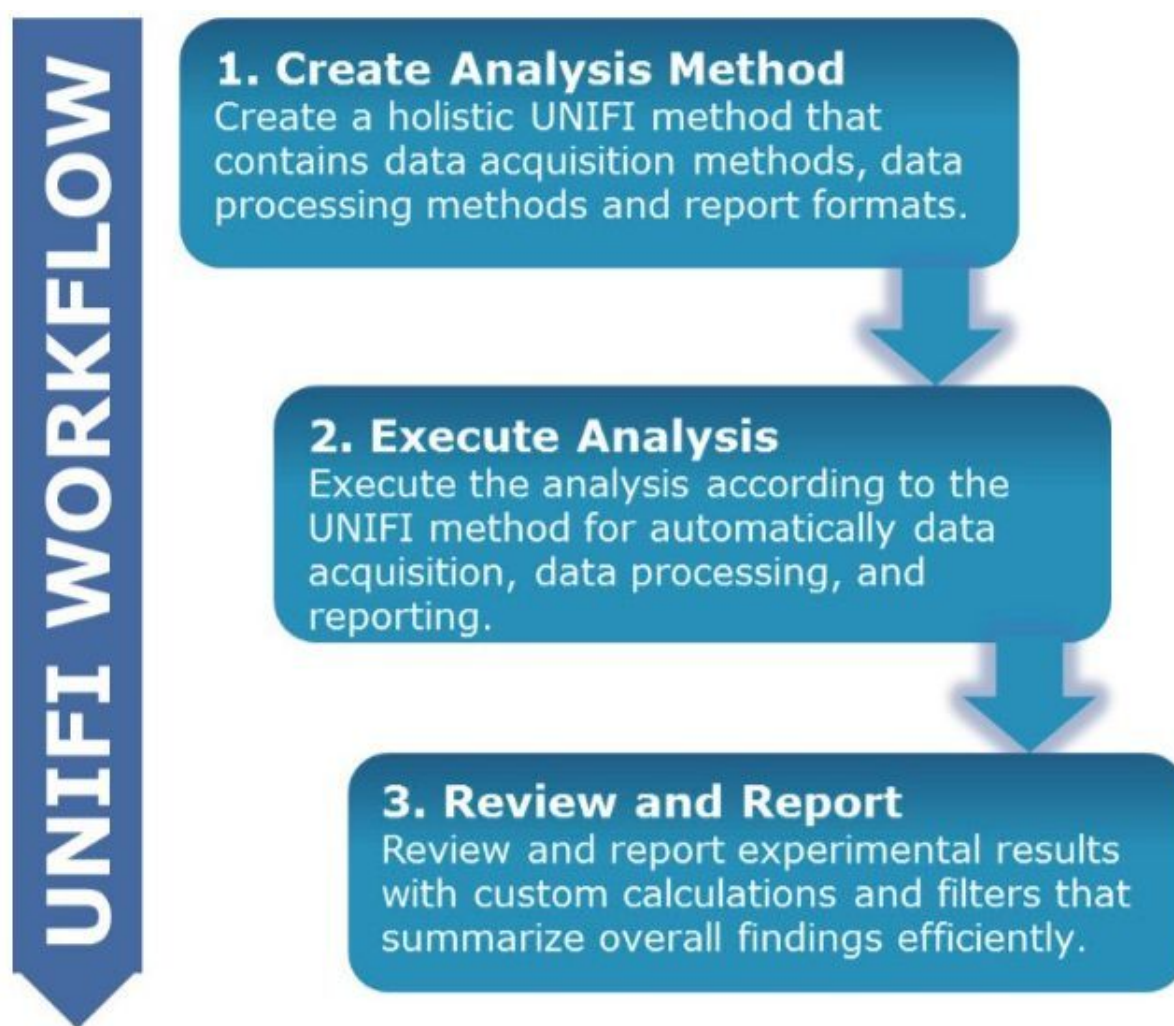


Figure 1. ADC analysis workflow with the UNIFI Scientific Information System.

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## Experimental

### LC (HIC) UV

The Waters Protein-Pak Hi Res HIC Column (4.6 x 100 mm, 2.5 µm) was conditioned prior to use as per the column care and use manual. Analysis samples were diluted to a concentration of 2 mg/mL in 1 M ammonium sulfate before injections.

### LC conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV Absorption
wavelength:	280 nm
Vials:	Total recovery vial, 12 x 32 mm glass, screw neck, cap, nonslit (p/n 600000750cv)
Column:	Protein-Pak Hi Res HIC, 2.5 µm, 4.6 mm x 100 mm, (p/n 176003576)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	10 µL
Mobile phase A:	125 mM phosphate buffer, pH 6.7 with 2.5 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>
Mobile phase B:	125 mM phosphate buffer, pH 6.7
Mobile phase C:	Isopropyl alcohol

Mobile phase D: Water

### Gradient table:

Time	Flow(mL/min)	%A	%B	%C	%D
Initial	0.700	50	0	5	45
10.00	0.700	0	50	5	45
10.01	0.700	50	0	5	45
30.00	0.700	50	0	5	45

### Native SEC QToF MS

Samples were diluted to a concentration of 0.5 mg/mL in 50 mM ammonium acetate before injection.

### LC conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Column:	ACQUITY UPLC Protein BEH SEC, 200Å, 1.7 µm, 4.6 mm x 150 mm (p/n 186005225)
Column temp.:	25 °C
Sample temp.:	4 °C
Injection vol.:	10 µL

Mobile phase: 50, 100, or 200 mM ammonium acetate in H<sub>2</sub>O

Gradient: Isocratic at 0.3 and 0.1 mL/min with total run time of 20 min

## Gradient

Time(min)	Flow(mL/min)	Comp.%A	Comp.%B	Comp.%C	Comp.%D	Curve
0.00	0.300	0.0	0.0	0.0	100.0	Initial
2.75	0.300	0.0	0.0	0.0	100.0	6
2.76	0.100	0.0	0.0	0.0	100.0	6
7.50	0.100	0.0	0.0	0.0	100.0	6
7.60	0.300	0.0	0.0	0.0	100.0	6
20.00	0.300	0.0	0.0	0.0	100.0	6

## MS conditions

MS system: Xevo G2-S QTof

Mode: ESI+ sensitivity

Capillary: 3.0 kV

Sampling cone: 150 V

Source offset: 80 V

Source temp.:	500 °C
Desolvation temp.:	500 °C
Cone gas flow:	300 L/h
Desolvation gas flow:	800 L/h

## RPLC QTof MS

Samples were diluted to a concentration of 1 mg/mL in 50 mM ammonium acetate before injection.

## LC conditions

LC system:	ACQUITY UPLC H-Class
Detector:	ACQUITY UPLC TUV
Absorption wavelength:	280 nm
Column:	ACQUITY UPLC Protein BEH C <sub>4</sub> , 300Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186004495)
Column temp.:	80 °C
Sample temp.:	4 °C
Injection vol.:	10 µL
Mobile phase A:	Water
Mobile phase B:	Acetonitrile
Mobile phase C:	1% TFA (in water)
Mobile phase D:	1% FA (in water)

## Gradient:

Time (min)	Flow (mL/min)	Comp.%A	Comp.%B	Comp.%C	Comp.%D	Curve
0.00	0.400	85.0	5.0	10.0	0.0	Initial
1.00	0.400	85.0	5.0	10.0	0.0	6
2.00	0.400	60.0	40.0	0.0	0.0	6
2.10	0.200	60.0	40.0	0.0	0.0	6
8.00	0.200	25.0	75.0	0.0	0.0	6
9.00	0.400	5.0	85.0	10.0	0.0	6
10.00	0.400	5.0	85.0	10.0	0.0	6
11.00	0.400	85.0	5.0	10.0	0.0	6
15.00	0.400	85.0	5.0	10.0	0.0	6

## MS conditions

MS system: Xevo G2-S QTof

Mode: ESI+ sensitivity

Capillary: 3.0 kV

Sampling cone: 150 V

Source offset: 80 V

Source temp.: 500 °C



Desolvation temp.:	500 °C
Cone gas flow:	0 L/h
Desolvation gas flow:	800 L/h

## Data management

UNIFI Scientific Information System v1.7 for data acquisition, processing and reporting

UNIFI v1.7 was used for data collection and processing for all experiments, configured using an intact protein analysis type that defines the automated processing.

- Data processing first deconvolutes the mass spectra and searches for the theoretical mass values for the various antibody (protein) and drug entities that can occur within the samples
- It then matches them based upon specified tolerance values as a means to identify the components
- The components are identified using a syntax that includes the identification of the modifier, which, in this experiment, includes the antibody (protein) drug, and also includes the number of drug modifiers, which is the drug load
- Next, these identifiers are used in custom calculations to group components containing the drug by antibody and drug load
- Finally, the responses of the component and load values are utilized to calculate the DAR value. Custom fields containing the necessary custom calculations are included in the method such that they are automatically calculated, saved in the results, and available for viewing, trending, and reporting
- Both custom fields and analytical methods are protected by an administration and security framework via configurable user access controls, based upon roles that facilitate their use in routine analyses, particularly in regulated laboratories

## Methods

Analyses of ADC samples were performed using LC-UV and LC-UV/QToF-MS. Samples were analyzed at the intact protein level by LC(HIC)-UV and by LC-MS under either native (SEC-MS) or denatured conditions (reversed-phase LC-MS) with the control of a common informatics platform. The total run time for both HIC-LC and LC-MS experiments was 20 min. CQAs, such as the DAR value and drug load distribution, were automatically calculated based on either the HIC/UV chromatographic peak areas or the deconvoluted mass spectra using the informatics tool following automated data acquisition. Comparability assessment for

multiple batches of ADC samples were undertaken based on the DAR values and relative drug distribution. Both cysteine-conjugated and lysine-conjugated ADC mimics were prepared by a collaborator at a concentration of 10 mg/mL in formulation buffer.

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## Results and Discussion

### Analysis of cysteine-conjugated ADCs for determination of DAR value based on HIC LC-UV

HIC is a leading technique for the characterization of CQAs of ADCs, including DAR values and drug loading distribution. As a non-denaturing technique, HIC is often used to characterize cysteine-conjugated ADCs due to the nature of the conjugation chemistry. The intra-chain disulfide bonds that normally are present to maintain the linkages between the heavy and light chains of the mAb are occupied by drug conjugates. The cysteine-conjugated ADCs, when exposed to standard reversed-phase conditions (e.g., acetonitrile), would be reduced to sub-units that are dictated by which cysteine disulfide bridges remain intact after conjugation; characterization information such as drug distribution would be lost. However, non-covalent interactions such as hydrogen bonding and ionic pairing are sufficiently strong enough to maintain the ADC's tertiary structure when separated in non-denaturing conditions such as a salt gradient. This makes HIC ideal for determining CQAs such as drug distribution and DAR values for cysteine-conjugated ADCs.

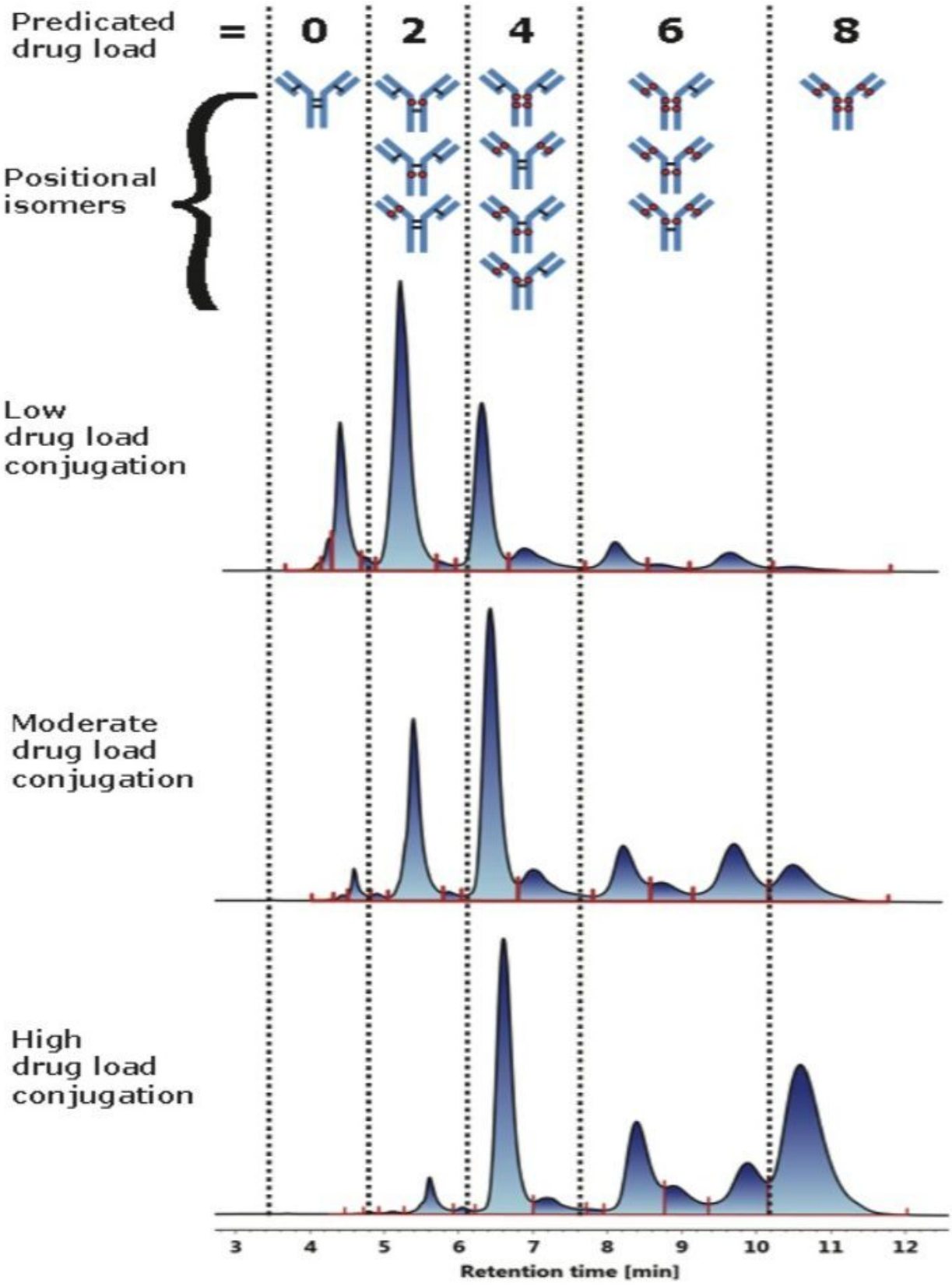


Figure 2. Cysteine conjugated ADC analysis using HIC. Drug distribution was determined for three different cysteine-conjugated ADC samples with increasing drug load.

In HIC chromatography, the separation mechanism applied is to increase the hydrophobicity of the protein by starting at a concentration of high salt to ion-pair with charge sites on the protein. With the charge sites masked, the hydrophobicity of the protein is increased, which allows the protein to bind to the low-retention surface of the HIC stationary phase (n-butyl surface). From the gradient listed, the salt concentration is decreased, thus exposing the charge sites of the protein, which increase the protein's hydrophilicity or affinity to the mobile phase, allowing it to elute. The drug conjugate, which is hydrophobic, increases the retention time of the ADC based upon its drug load. This behavior results in a HIC separation profile where the peaks are grouped by drug distribution (e.g., 0, 2, 4, 6, and 8 drug conjugates). DAR values can be readily calculated from the peak area of the drug profile distribution, as shown in Figure 3.

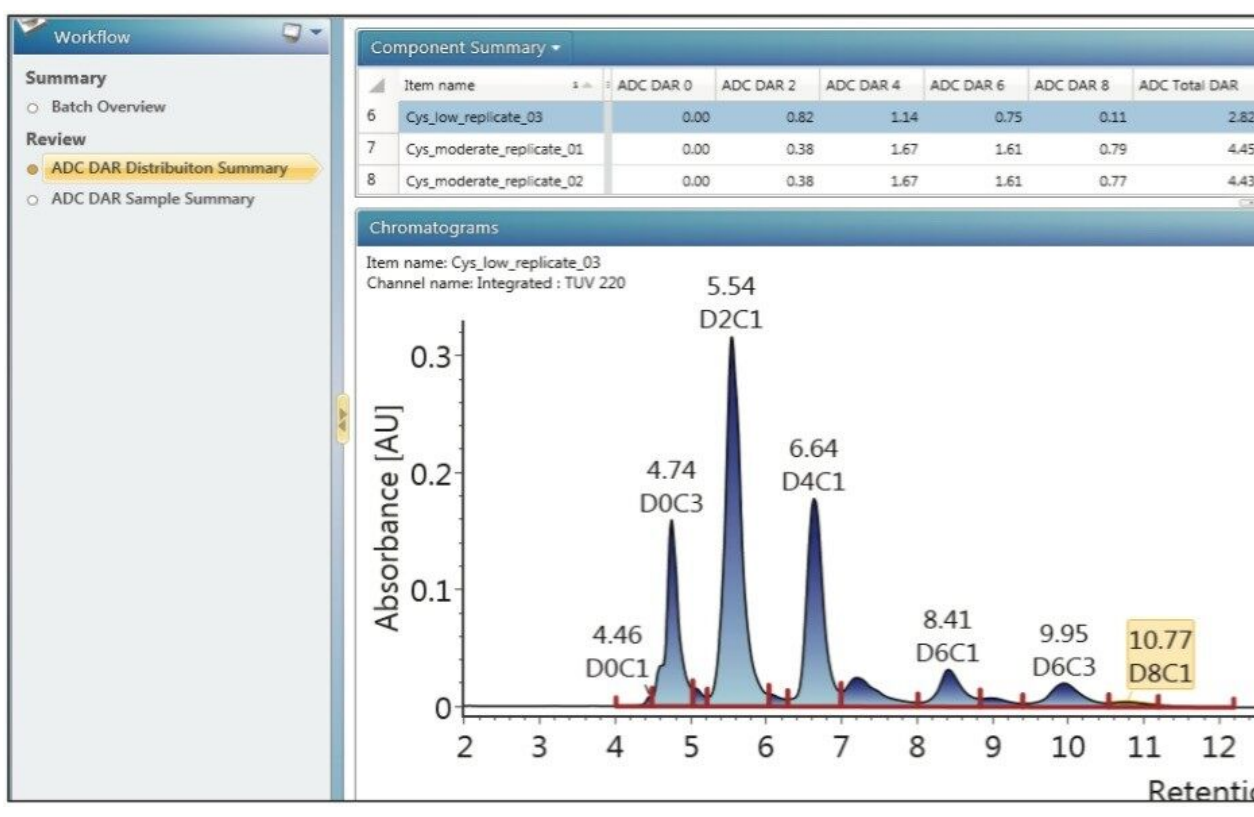
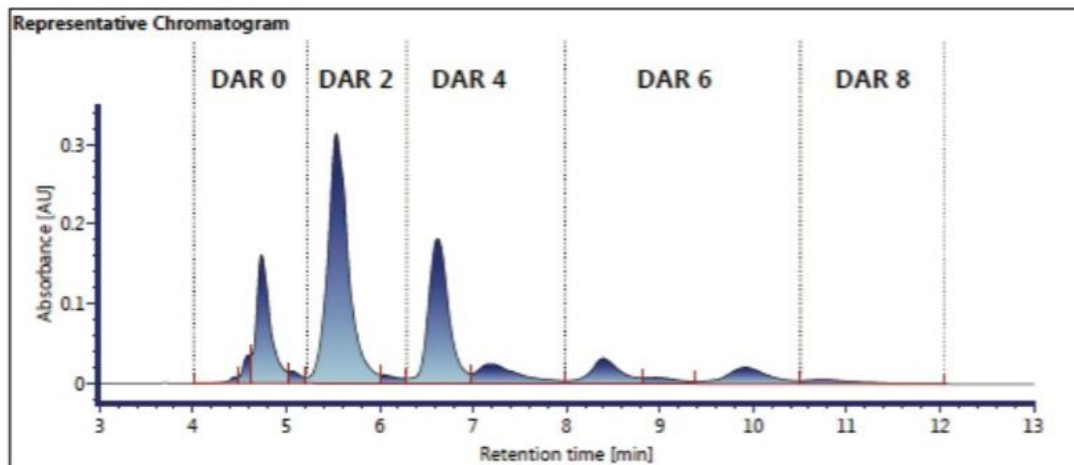


Figure 3. Integrated informatics for ADC analysis. Custom fields are used to calculate the DAR value using peak area and display the results in the component summary pane.

Waters Biopharmaceutical Platform Solution with UNIFI offers the ability to automatically calculate the DAR values of the cysteine-conjugated ADCs. This is achieved by using a custom field option when setting up the processing method. As shown in the component summary pane of Figure 3, custom fields were programmed to calculate the individual DAR values for each drug distribution (e.g., 0, 2, 4, 6, and 8). A custom field was also designed to automatically calculate the total DAR value of the sample as seen in the last column of the component summary pane.

The Biopharmaceutical Platform Solution with UNIFI also features strong reporting functionality, with a workflow that gathers meaningful analytical measurements that can be presented as automated custom reports for the efficient communication and cataloging of analytical results. Report templates can be readily constructed and customized for the assessment of analysis results.

Figure 4 is an example of a report template designed for HIC characterization of biotherapeutics such as ADCs.



**Summary Report of Relative DAR Area**

	Item name	ADC DAR 0 % Area	ADC DAR 2 % Area	ADC DAR 4 % Area	ADC DAR 6 % Area	ADC DAR 8 % Area
1	Cys_low_replicate_01	16.67	40.61	28.49	12.61	1.62
2	Cys_low_replicate_02	16.75	40.73	28.62	12.49	1.42
3	Cys_low_replicate_03	16.76	40.79	28.59	12.52	1.34
	Mean	16.726	40.711	28.563	12.540	1.460
	% RSD	0.28	0.23	0.24	0.50	10.11
	Std dev	0.047	0.093	0.069	0.062	0.148

**Summary Report of Calculated DAR**

	Item name	ADC DAR 0	ADC DAR 2	ADC DAR 4	ADC DAR 6	ADC DAR 8	ADC Total DAR
1	Cys_low_replicate_01	0.00	0.81	1.14	0.76	0.13	2.84
2	Cys_low_replicate_02	0.00	0.81	1.14	0.75	0.11	2.82
3	Cys_low_replicate_03	0.00	0.82	1.14	0.75	0.11	2.82
	Mean	0.000	0.814	1.143	0.752	0.117	2.826
	% RSD		0.23	0.24	0.50	10.11	0.38
	Std dev	0.000	0.002	0.003	0.004	0.012	0.011

Figure 4. Automated reporting using UNIFI informatics. A report of peak area, DAR value, and associated statistics was automatically generated after data acquisition and analysis.

Using the results for the purified IgG sample from Figure 3, a summary report of the relative area and calculated DAR value based on that area, as well as the corresponding statistical evaluation (mean and %

RSD) are generated after data acquisition and processing. With this flexibility to design custom report templates based on analytical needs, the Biopharmaceutical Platform Solution with UNIFI serves as a powerful integrated system for the acquisition, processing, and reporting of results for ADC biotherapeutics.

### Analysis of cysteine-conjugated ADCs for the determination of DAR values based on native intact mass analysis (SEC LC-MS)

In a reversed-phase LC-MS experiment, the cysteine-conjugated ADCs will dissociate into the light chain and heavy chain subunits because of the acidic mobile phase conditions. Therefore, intact native mass analysis is performed using the non-denaturing conditions to keep the protein in its near-native state in the gas phase.

The commonly used buffer that maintains a protein in the native state is not suitable to ionization of the protein in mass spectrometry. Ideal buffers for MS intact analysis are ones that allow proteins to remain in their folded state, yet are volatile enough to enable sufficient ionization. Aqueous solutions of ammonium acetate (NH<sub>4</sub>OAc) are usually chosen for this purpose.

For our native SEC LC-MS experiments, we used a concentration of 50 mM NH<sub>4</sub>OAc. Because the protein is close to its native state in NH<sub>4</sub>OAc solution, the surface area is smaller than that of its denatured counterpart in the reversed-phase acidic mobile phases (usually with half organic and half water). Thus, proteins ionized in intact native mass analysis will have fewer charges than that in a reversed-phase experiment.

As shown in Figure 5, the narrower charge envelope distributions are centered around an  $m/z$  range of 5,200 with a 30+ charge state, rather than wider charge envelope centered around 2500  $m/z$  with a 60+ charge state for a reversed-phase denatured experiment.

Figure 5 displays the native SEC LC-MS raw spectra of the cysteine-conjugated ADC samples. The charge envelope distributions were compared for three different cysteine-conjugated ADC samples with increasing drug load, as well as with the unconjugated mAb as a control.



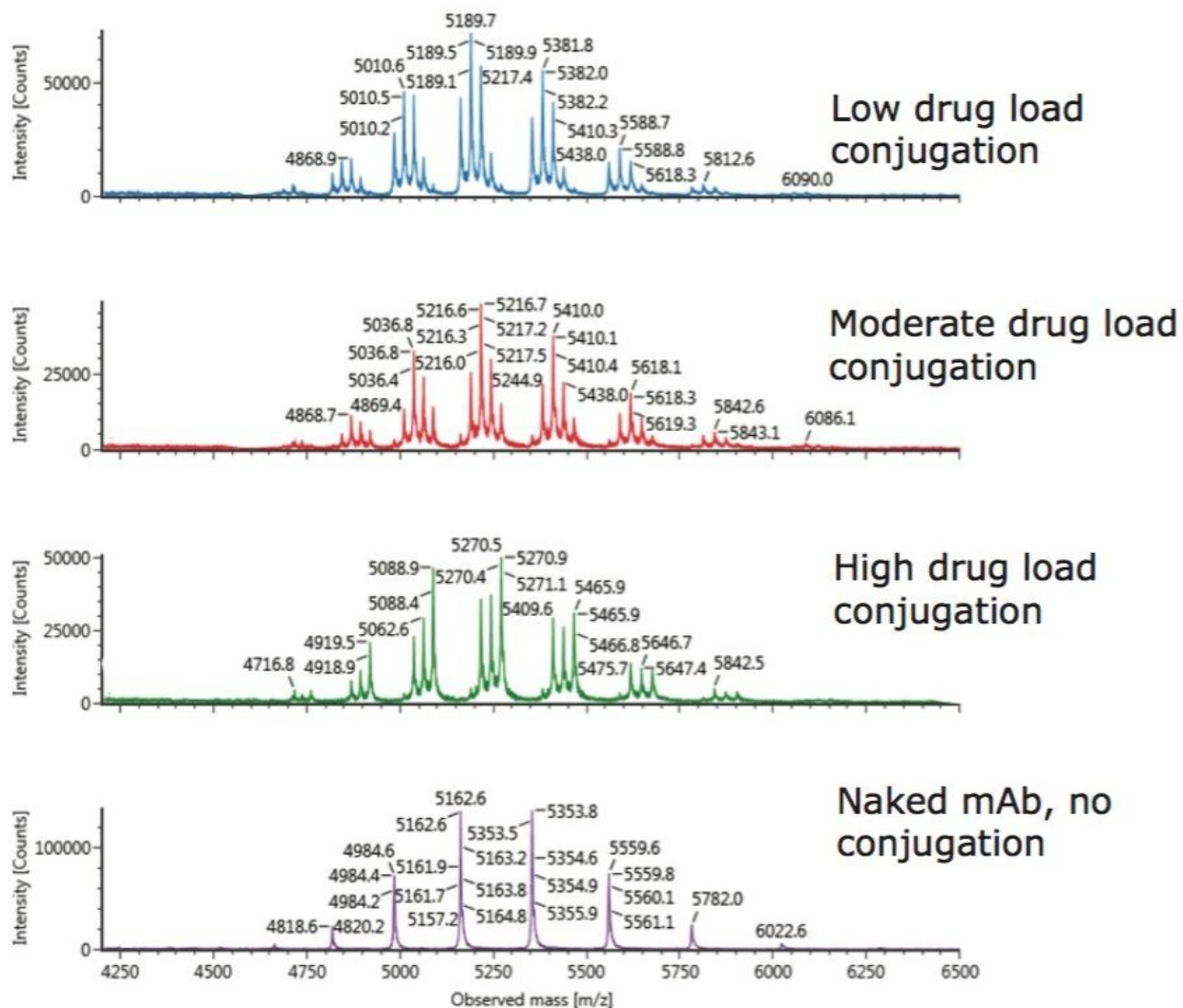


Figure 5. Native SEC LC-MS raw spectra of cysteine-conjugated ADC samples from different drug loading.

Figure 6 shows the deconvoluted intact mass spectra from the raw mass spectra for deglycosylated cysteine-conjugated ADCs from the native SEC LC-MS analysis shown in Figure 5. Drug distribution was compared for three different cysteine-conjugated ADC samples with increasing drug load, as well as with the unconjugated mAb.



Drug conjugates

=

0

2

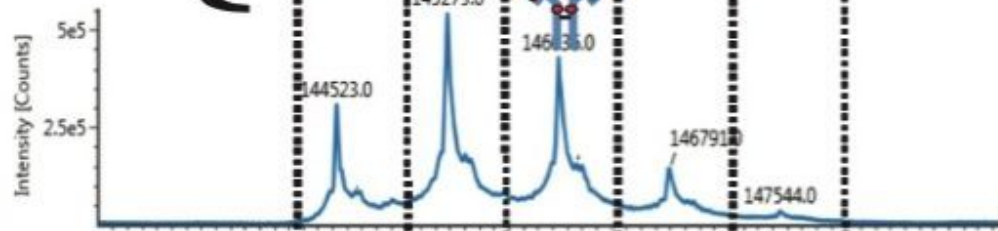
4

6

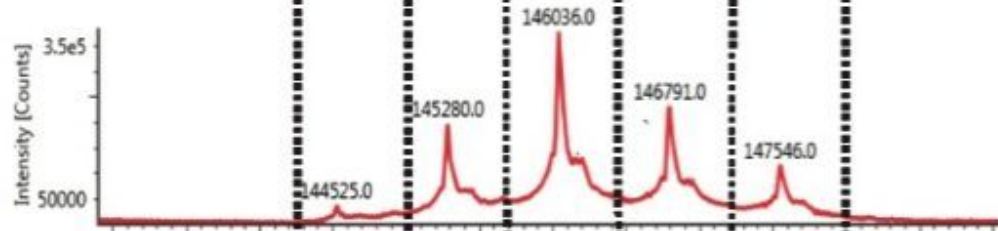
8

Positional isomers

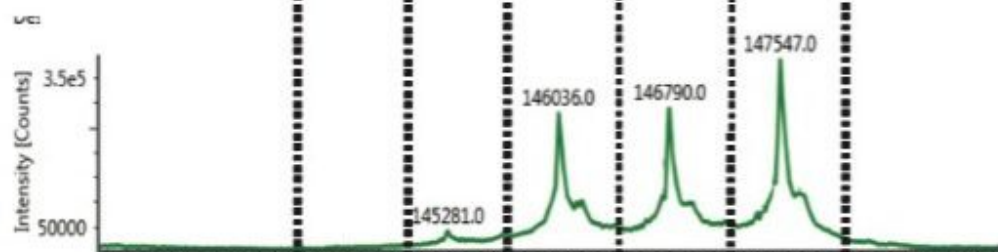
Low drug load conjugation



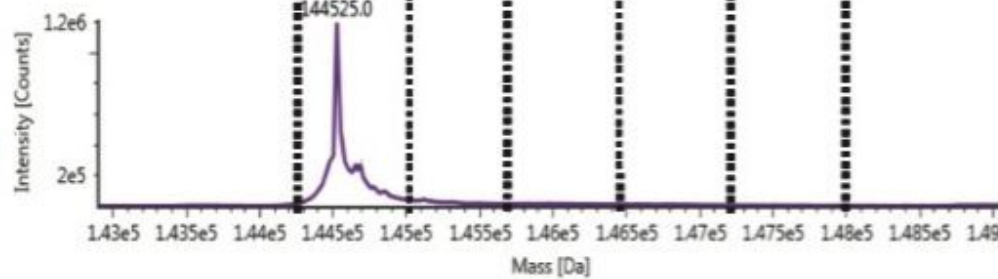
Moderate drug load conjugation



High drug load conjugation



Naked mAb  
(un-conjugated)



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*Figure 6. Deconvoluted intact mass spectra for cysteine-conjugated ADCs from native SEC-LC-MS after deglycosylation. Drug distribution was compared for three different cysteine-conjugated ADC samples with increasing drug load.*

As described in the previous section on HIC analysis, as well as in details about the informatics used in the Experimental section, the Biopharmaceutical Platform Solution with UNIFI can seamlessly and automatically acquire, process, and report data. Figure 7 is a screen capture of the UNIFI review pane that displays the processed and calculated data for DAR values from the native SEC LC-MS experiments.

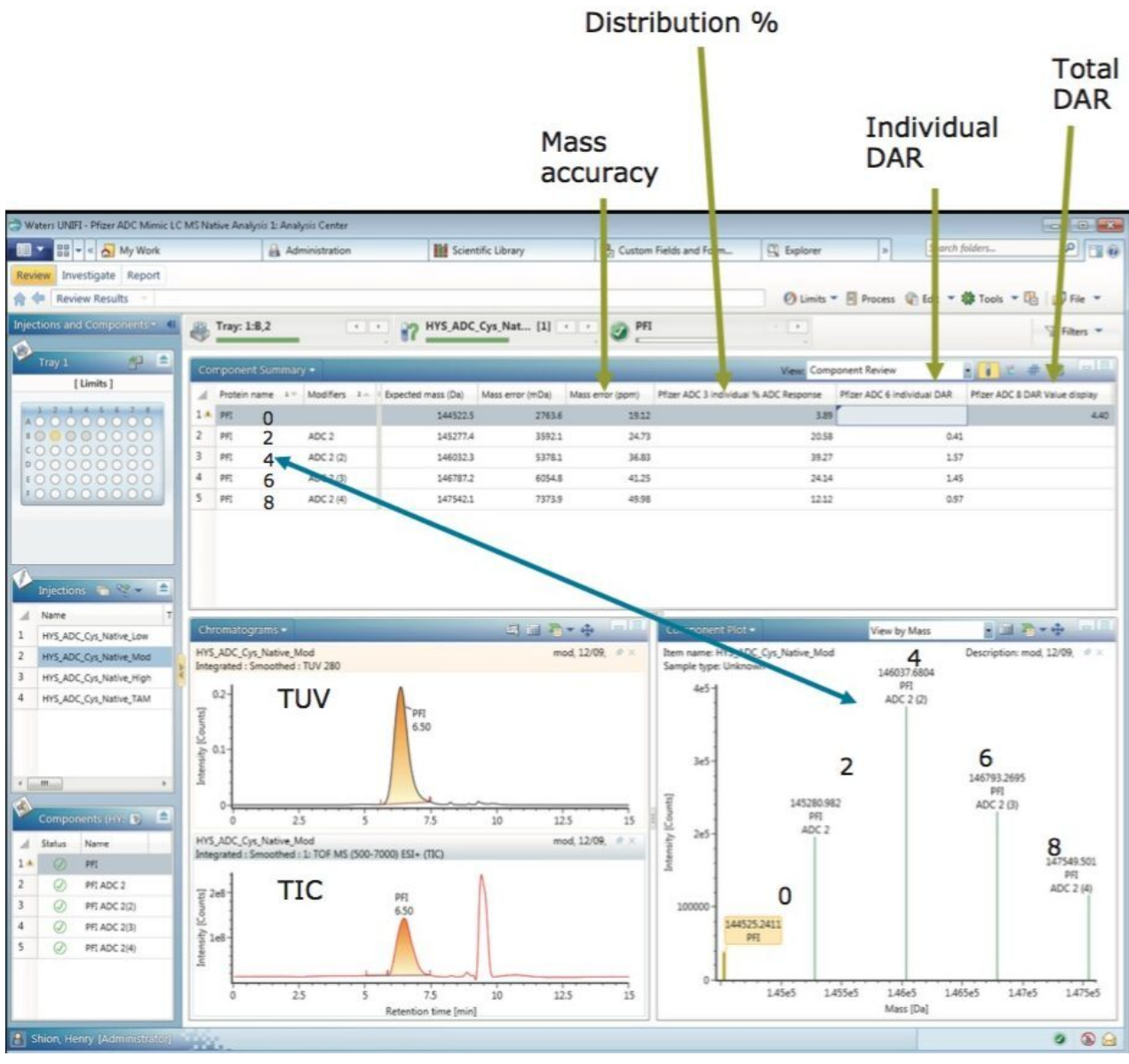


Figure 7. UNIFI review pane shows the automatically process data and calculated DARs from the native SEC-LC-MS experiments.

In this integrated workflow, the summed raw spectrum for each sample (as shown in Figure 5) obtained by combining the total ion chromatogram (TIC) peak (as shown in Figure 7) was deconvoluted using MaxEnt1 (as shown in Figure 6). The identified peak areas or the intensities in the deconvoluted spectrum were used to calculate the individual drug payload DAR and the total DAR for the sample, with the assistance of custom fields performing calculations in the background.

Figure 8 shows the CQAs of DAR and drug load distribution for three batches of ADCs, with a comparison

between the HIC and the native SEC LC-MS experiments in table (top) and 3D graph format (bottom). The experimental results from the two orthogonal methods show excellent agreement between the two methods for both the individual DARs, as well as the total average DARs for all three drug loading levels.

Drug loading distribution and DAR						
	Low		Mod		High	
	HIC	LC/MS	HIC	LC/MS	HIC	LC/MS
ADC 2	0.81	0.74	0.38	0.41	0.07	0.09
ADC 4	1.14	1.17	1.67	1.57	1.23	1.11
ADC 6	0.75	0.60	1.61	1.45	1.72	1.72
ADC 8	0.12	0.21	0.78	0.97	2.95	3.05
DAR	2.83	2.72	4.44	4.40	5.97	5.97

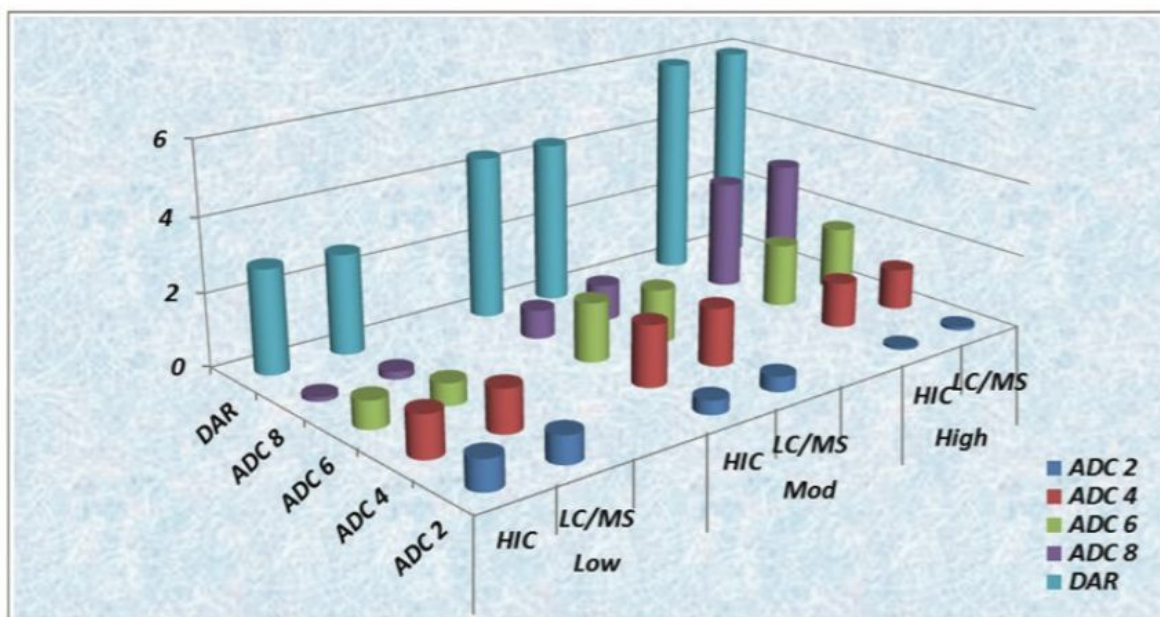


Figure 8. DARs comparison between HIC and native SEC-LC-MS experiments in table (top) and 3-D graph format (bottom) shown excellent agreement between the two methods for all three drug loading levels.

For instance, the individual DARs with six drug payloads for the three samples from the HIC method were 0.75, 1.61, and 1.72; while for the native LC-MS method they are 0.60, 1.45, and 1.72 respectively. The total DAR values from the HIC method were 2.83, 4.44, and 5.97; while for the native SEC LC-MS method, they are 2.72,

4.40, and 5.97 respectively. This is very important data for lot-to-lot and batch-to-batch comparison studies.

Considering that all of the information was obtained automatically, we believe that this integrated informatics workflow in UNIFI will be an enabling tool for increasing productivity during the ADC product development processes.

### Analysis of Lysine-Conjugated ADCs for the Determination of DAR Values Based on RP-LC/MS Intact Mass Analysis

Unlike cysteine-conjugated ADCs, the intra-chain disulfide bonds that maintain linkages between the heavy and light chains of the mAb for the lysine-conjugated ADCs are intact. Therefore, reversed-phase chromatography is suitable for the analysis of lysine-conjugated ADCs when linker chemistry is not labile at acidic pH.

Figure 9 is a screen capture of the UNIFI review pane that shows the experimental results of the reversed-phase LC-MS analysis for three batches of lysine-conjugated ADCs with increasing drug payload. The component summary table displays the identified drug load distribution based on the deconvoluted spectra peaks (Figure 10), with automatically calculated DARs, integrated chromatogram, and DAR values bar chart for the three level drug loading lysine-conjugated samples. The DARs determined here can be used for lot-to-lot comparison of lysine conjugates. However, calculated absolute DAR values need to be verified by an orthogonal technique.

The spectra in Figure 10 compare the difference in drug distribution for three batches of lysine-conjugated ADC preparation at various levels of drug loadings and can be automatically generated in the report. It was observed that as many as up to 12 drugs were bonded to the mAb of interest.



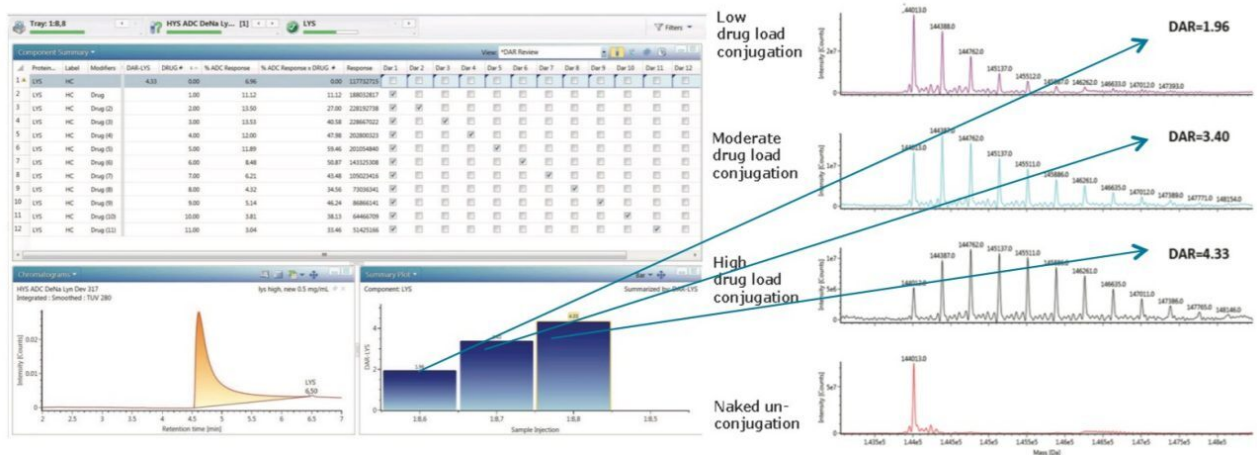


Figure 9. (Left) UNIFI review panel displays the experiment results from the automated analysis of lysine-conjugated ADCs to determine the DAR values by RP-LC-MS intact mass analysis.

Figure 10. (Right) The deconvoluted MS spectra for lysine-conjugated ADCs.

## Conclusion

In this application note, we have demonstrated the use of an integrated informatics workflow in UNIFI Software that streamlines data acquisition, processing and reporting for ADC analysis by LC-UV or LC-MS, so that information from multiple analytical techniques can be effectively integrated for rapid and quantitative assessment of the comparability of ADCs.

DAR values and drug loading distributions for cysteine-conjugated ADCs are automatically acquired from HIC LC-UV analysis and from native SEC LC-MS analysis, and the results show excellent agreement between these two methods. The workflow automatically produces DARs and drug loading distributions for lysine-conjugated ADCs from RP LC-MS analysis.

This automated workflow removes the necessity of manual data processing and reduces the associated human errors. The workflows in UNIFI can be saved and shared, enabling consistent high-quality data generation, processing, and reporting for complex ADC datasets with an unparalleled capability to aggregate and manage data.

Collectively, we believe this integrated informatics workflow will be an enabling tool for organizations working in the discovery or development of ADC biotherapeutics, providing increased confidence in results,

productivity, and financial gains.

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## References

1. Chen J, Yin S, Wu Y, Ouyang J. Development of a native nanoelectrospray mass spectrometry method for determination of the drug-to-antibody ratio of antibody-drug conjugates. *Anal Chem*. 2013 Feb 5;85(3):1699–704.
  2. Valliere-Douglass JF, McFee WA, Salas-Solano O. Native intact mass determination of antibodies conjugated with monomethyl Auristatin E and F at interchain cysteine residues. *Anal Chem*. 2012 Mar 20;84(6):2843–9.
  3. Dario Neri. Antibody-drug conjugates for cancer therapy. Nature Tech. Poster. 2013.
  4. ADC Review Website, ADC Drug Map.
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