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Application Note

Analytical Scale Native SEC-MS for Antibody-Drug Conjugates (ADCs) Characterization

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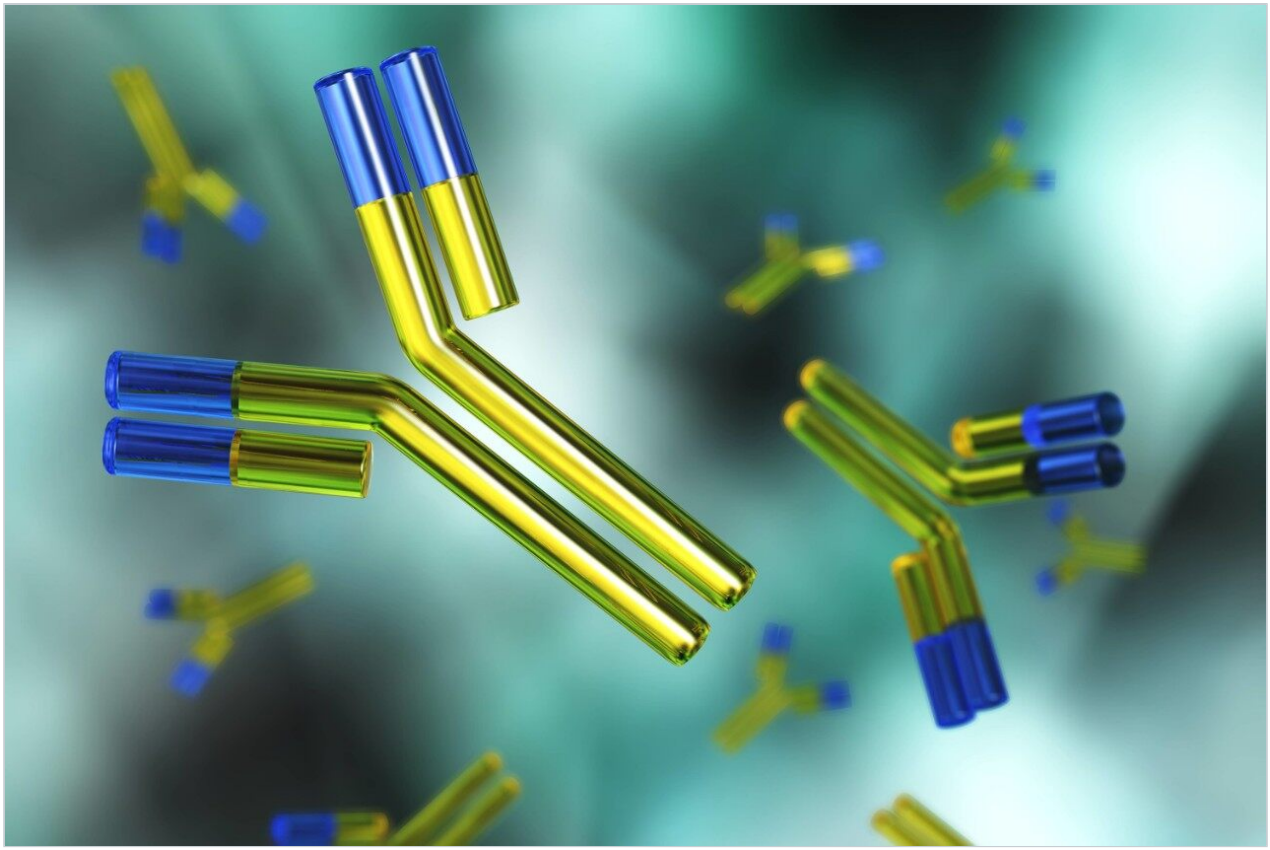

Abstract

This application note demonstrates the use of an analytical scale native SEC-MS analytical platform to examine both cysteine and lysine conjugated intact ADCs. Average DARs and drug loading distributions from the native SEC-MS analysis were compared with the values generated from HIC separation.

Benefits

- An improved native mass spectrometry with analytical scale Size Exclusion Chromatography (SEC) method is developed for characterization of cysteine-conjugated and lysine conjugated ADCs.
- This improved method offers increased sensitivity, robustness and simplified sample preparation process for DAR and drug load distribution of cysteine conjugated and lysine-conjugated ADCs
- UNIFI Scientific Information System automates the DAR and drug load distribution calculations and is well suited for streamlined critical quality attributes (CQAs) such as ADC DARs characterization

Introduction

Native mass spectrometry (MS) is widely used in academic and industrial labs for a variety of applications, such as protein folding,[1] protein-ligand, protein-protein interactions,[2] protein complex architecture,[3] small protein aggregation,[4] antibodies/antibody derivatives,[5,6] and antibody drug conjugates (ADCs).[7,8] Most of these native MS applications use static infusion from glass nano flow capillaries following extensive sample clean-up. The notable drawbacks for the infusion approach are the need of extensive sample clean-up before the analysis, as well as the need of highly skilled scientists to produce interpretable data. The effort of applying online native approaches, however, is still far from practical and routine. To address the challenges in performing native MS analysis, we developed an analytical scale native SEC-MS method that can be adopted for cysteine- and lysineconjugated ADC Drug-to-Antibody Ratio (DAR) measurements.

Figure 1. The ACQUITY UPLC H-Class Bio System (right) coupled to a bench top Vion IMS QTof Mass Spectrometer (left) controlled by a compliance-ready and workflow-driven software, UNIFI Scientific Information System, presents a single platform for robust native SEC-MS characterization of biotherapeutics (such as ADCs) with streamlined data acquisition, data processing, and reporting workflow.

Cysteine-conjugated ADCs use the intra chain thiol groups to conjugate with small cytotoxic drug molecules. This type of modification transforms the active mAbs from covalently linked tetramers to non-covalently linked complexes. If exposed to standard reversed-phase liquid chromatography (RPLC) mobile phases (e.g. organic solvent or acid), these non-covalently linked complexes will dissociate to smaller subunits. Therefore, native mass spectrometry with non-denaturing condition is used to keep the protein in its near-native state. The purpose of the analysis is to characterize DAR and drug loading distribution.

RPLC-MS is widely used for Lysine-conjugated ADC characterization. However, deglycosylation treatment is

usually needed to reduce the MS spectrum complexity for DAR calculations; as well as the link-only species formed in a two-step conjugation process. Compare to RP-MS, native SEC-MS extends the charge envelope to higher m/z mass window, therefore, it could improve mass spectrum quality for lysine-conjugated ADCs without the need for deglycosylation using PNGase F.

In this study, we describe a streamlined native SEC-MS methodology using an analytical scale SEC column (p/n:186008471) for ADCs analysis. The Waters Vion IMS QTof Mass Spectrometer, coupled with an ACQUITY UPLC H-Class Bio System and an ACQUITY UPLC Tunable Ultraviolet (TUV) Detector is used in the study. The workflow includes automated data acquisition, processing, and reporting. The SEC-MS results are compared to hydrophilic interaction chromatography (HIC) analysis,[9] the goal is to demonstrate the benefit of using analytical scale SEC-MS for routine ADC DAR characterization.

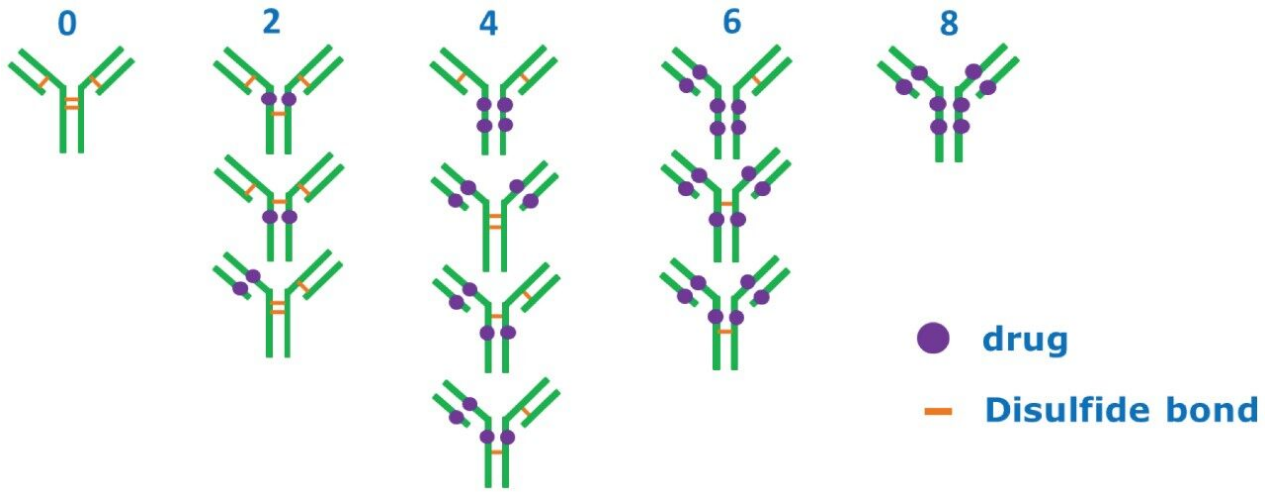

Figure 2. Cysteine-conjugated ADC molecules held together by non-covalent interactions between the light and heavy chains. The non-denaturing conditions are needed to preserve the non-covalent interactions and bonding. Possible combinations of ADC conjugation are shown in Figure 2.

Experimental

Reagents, solvents, and sample preparation

All of the ADCs used in this study were obtained from external collaborations. Samples were stored at -80 °C in the original buffer and were thawed and diluted to 2 µg/µL concentration with 50 mM ammonium acetate

(NH_4OAc) in H_2O before SEC-MS analysis. Lockmass compound [Glu1]-Fibrinopeptide B Standard (p/n: 700004729) was diluted to 320 fmol/µL in 50/50 H_2O/ACN with 0.1% Formic Acid (FA) and was used during the acquisition.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detector:	ACQUITY UPLC TUV, absorption wavelength: 280 nm
Column:	ACQUITY UPLC Protein BEH SEC 200Å, 1.7 µm, 2.1 mm × 150 mm
Column temp.:	25 °C
Sample temp.:	5 °C
Injection volume:	2 µL
Mobile phase:	50 mM ammonium acetate (NH_4OAc) in H_2O
Gradient:	Isocratic at 0.065 mL/min with total run time of 10 min

Gradient table:

Time (min)	Flow rate (mL/min)	50 mM NH_4OAc
Initial	0.065	100
10	0.065	100

Total run time = 10 min

MS conditions

MS system:	Vion IMS QTof
Acquisition range:	*m/z* 500–8,000 Da
Mode:	ESI+
Capillary voltage:	2.00 kV
Cone voltage:	140 V
Source offset:	80 V
Source temp.:	125 °C
Desolvation temp.:	250 °C
Desolvation gas low:	600 L/h
Lock mass:	[Glu1]-Fibrinopeptide B at 320 fmol/µL in 50/50 H_2O/ACN, 0.1% FA

Software for data acquisition and processing

UNIFI Scientific Information System 1.8.2

Vion IMS QTof driver pack 2.0

Software used for data collection and processing was UNIFI Version 1.8.2, which is configured using an intact protein analysis type that defines the automated processing for DAR calculation, please see reference [9] for detailed description of the automatic DAR calculation steps in UNIFI.

Results and Discussion

Native mass spectrometry (MS) is widely used in academic and industrial labs for a variety of applications. In this method, protein samples are usually introduced to the MS via static infusion from glass nanoflow capillaries. Some of the benefits of this approach are high desolvation and ionization efficiency of the protein molecules due to the very low volume of aqueous mobile phase and lower temperature of the MS source conditions that are required. However, there are notable drawbacks associated with this approach, such as the need for extensive sample clean-up before the analysis, as well as the need for highly skilled scientists to produce interpretable data. In addition, the static infusion would not be very useful for complex samples without pre-separation. In a previous study,[9] in an attempt to address some of the challenges in performing native MS analysis, we applied online native SEC-MS approach, using a Waters ACQUITY UPLC Protein BEH SEC Column (p/n: 186005225) (with an ACQUITY UPLC H-Class Bio QSM and TUV coupled to a Xevo G2-S MS System) to analyze a set of cysteine-conjugated ADC samples after deglycosylation treatment. The experiment results show very good agreement with the data from an orthogonal method using Hydrophobic Interaction Chromatography (HIC) for the average DARs and drug load distributions. In this current study, we employed a Waters ACQUITY UPLC Protein BEH SEC Column (p/n: 186008471) with an ACQUITY UPLC H-Class Bio QSM and TUV coupled to a Vion IMS QTof MS System to analyze the same set of cysteineconjugated ADC samples, however, without deglycosylation sample treatment. The smaller diameter of the column (2.1 mm vs. 4.6 mm) enabled lower LC flow rate, while maintained a higher and stable LC system back pressure to retain high peak retention reproducibility form run to run. Lower flow rate also meant that less harsh MS source conditions can be applied to obtain high desolvation and ionization efficiency, therefore increasing the MS sensitivities. In general, a 5× increase in sensitivity was observed when switching from 4.6 mm to 2.1 mm I.D. column. In addition, the improved performance of the Vion IMS QTof MS System[10] with the new QuanTof 2 technology and enhanced TOF analyzer vacuum system resulted in better glycoforms mass resolution at the raw spectra level as shown in Figure 3. The advanced MS system facilitated the ability to analyze these ADC samples without deglycosylation treatment, therefore improved the sample analysis throughput.

Figure 3 shows the TUV and TIC chromatograms from a typical native SEC-MS experiment. The protein eluted at around 4.5 minutes, and the inorganic salts (buffers, etc.) in the injected sample eluted later at about 6.5 minutes. The total run time for the isocratic gradient is about 10 minutes. Raw MS spectrum can be generated by combining the MS scans of the TIC peak at 4.5 minutes.

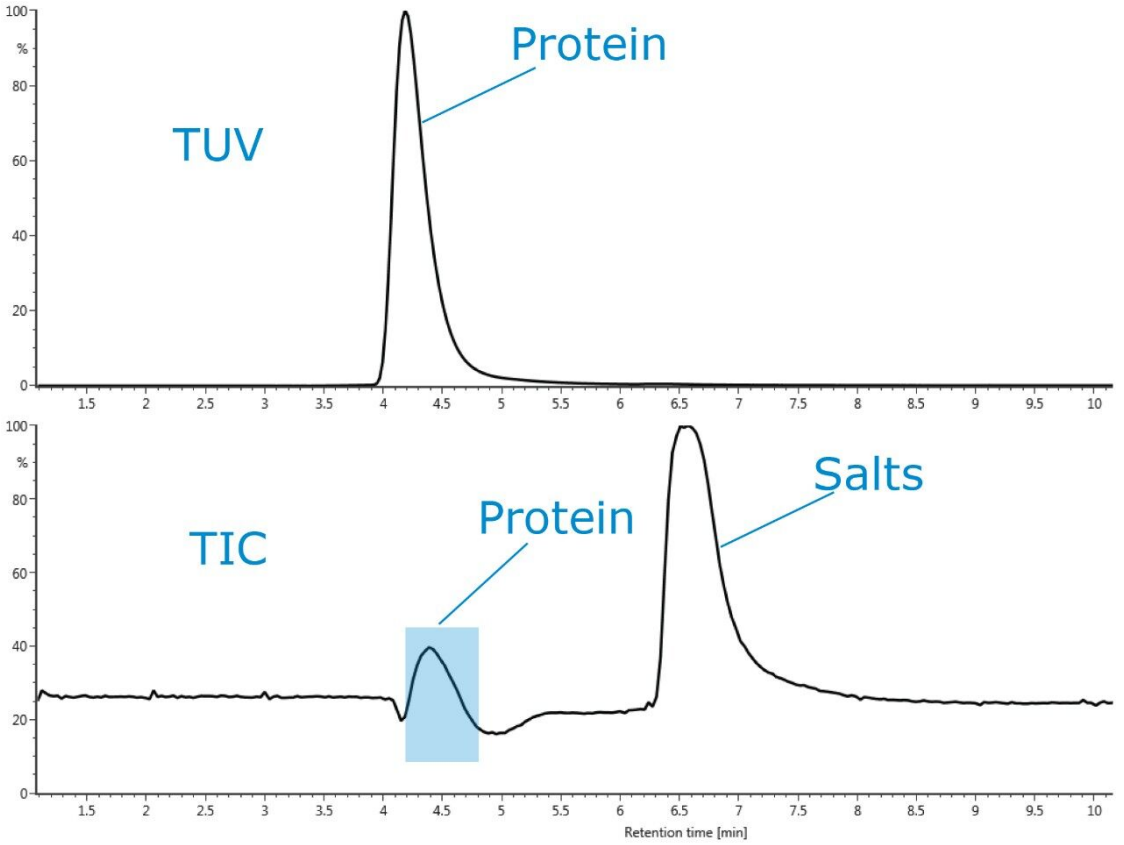

Figure 3. The TUV and TIC chromatograms from a typical native SEC-MS experiment.

Figure 4 shows the combined raw spectra multiple charge states envelope (left), the zoomed-in region (single charge state) of the combined raw spectra (center), and the deconvoluted spectra (right) of the reference materials (mAb), the low, moderate, and high conjugation level of cysteine-conjugated ADC samples without deglycosylation treatment from native SEC-MS analysis. The glycosylation pattern displayed in the reference mAb spectrum is repeated with good consistency in each conjugation form (0, 2, 4, 6, and 8) across all three levels of conjugated samples.

The integrated peak areas of each of the glycoform peaks from the deconvoluted spectra were used for automatic total average DAR and the drug loading distribution calculation within UNIFI as described in detail in reference [9].

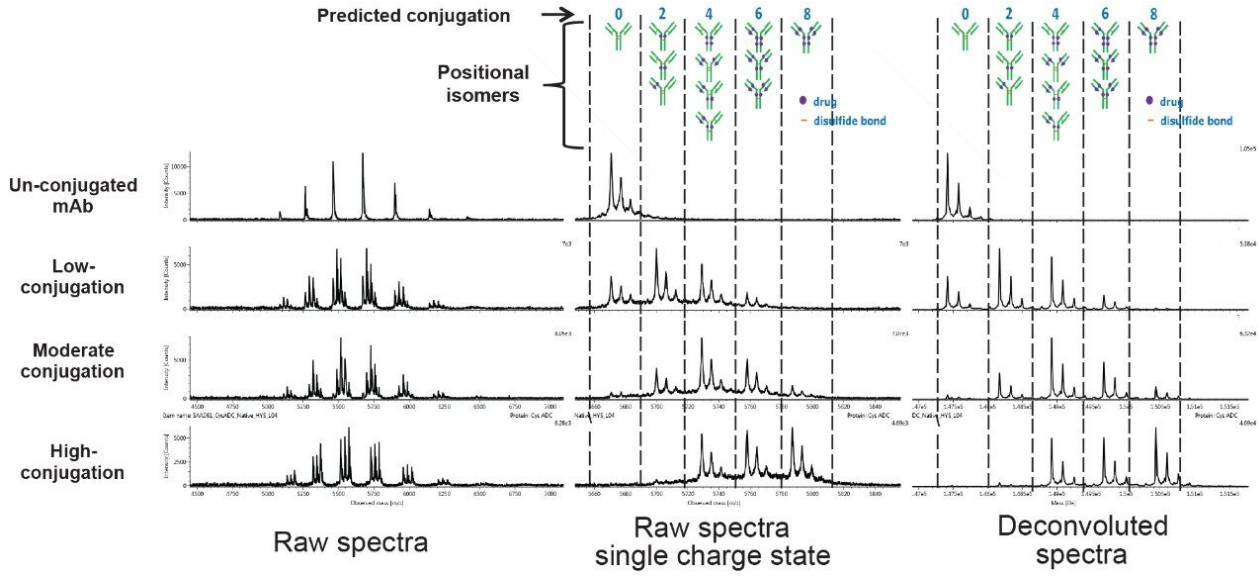

Figure 4. The combined raw spectra from multiple charge state envelope (left), the zoomed-in region (single charge state) of the combined raw spectra (center) and the deconvoluted spectra (right) of the reference materials (mAb), the low, moderate, and high conjugation level cysteine-conjugated ADC samples without deglycosylation treatment from native SEC-MS. Drug distribution was compared for three different cysteine-conjugated ADC samples with increasing drug load.

Table 1 compares the calculated DAR and drug load distribution for three batches of ADCs among the HIC using data collected from 3 years apart, the original native SEC-MS analysis using Xevo G2-S QTof System[9] and the new native SEC-MS analysis with Vion IMS QTof MS. HIC data was also generated in 2014. The 2014 SEC-MS data was collected after the ADC was treated with PNGase F. The experiment results from the HIC methods shown good agreement among the methods for both the individual DARs as well as the total average DARs for all three drug loading levels. For example, the individual DARs with six drug payload for the three samples from HIC were 0.75, 1.61, and 1.72; for the 2014 native SEC-MS method they were 0.60, 1.45, and 1.72; and for the 2017 native LC-MS method they were 0.64, 1.51, and 1.86 respectively. The total DAR values from the HIC method were 2.83, 4.44, and 5.97 (low, moderate, and high); for the 2014 native SEC-MS method they are 2.72, 4.40, and 5.97, and for the 2017 native SEC-MS method, they were 2.70, 4.37, and 6.07 respectively. The results from this study indicated that the measured DARs for this set of cysteine-conjugated ADC samples were consistent from orthogonal approaches (HIC vs. MS) and from different QTof MS systems (Xevo G2-S vs. Vion) and sample preparations (with and without deglycosylation). The results therefore also validated the improved method with increased sensitivity and robustness, as well as simplied sample preparation. This could be very benefitial for lot-to-lot, batch-to-batch comparison studies.

Cysteine-conjugated ADCs drug loading distribution and DAR									
	Low			Mod			High		
	HIC	QTof 1	QTof 2	HIC	QTof 1	QTof 2	HIC	QTof 1	QTof 2
ADC 2	0.81	0.74	0.64	0.38	0.41	0.35	0.07	0.09	0.05
ADC 4	1.14	1.17	1.37	1.67	1.57	1.81	1.23	1.11	1.19
ADC 6	0.75	0.60	0.64	1.61	1.45	1.51	1.72	1.72	1.86
ADC 8	0.12	0.21	0.05	0.78	0.97	0.70	2.95	3.05	2.98
DAR	2.83	2.72	2.70	4.44	4.40	4.37	5.97	5.97	6.07

QTof 1 sample deglycosylated, run in 2014. QTof 2 sample non-deglycosylated, run in 2017.

Table 1. Total average DARs and drug distribution comparison amongst the HIC, the original and the new improved native SEC-MS experiments shown very good agreement of the three methods for all three drug loading levels.

Figure 5 shows the raw and deconvoluted spectra of SEC-MS analysis of lysine-conjugated ADC Kadcyla (Trastuzamab Entansine (T-DM1)) without deglycosylation. The number on top of the deconvoluted spectrum peaks represents the detected number of drugs that are conjugated to the mAb, trastuzumab, in the intact level. The automatically calculated DAR in UNIFI is 3.56 compared to the reported DAR 0f 3.50 from the manufacture Genentech.[11] For lysine-conjugated ADCs, characterization has been successfully performed using standard reversedphased (denaturing) LC separation/desalting conditions. However, pre-analysis sample treatment of deglycosylation is usually required in order to reduce the sample complexity, such as removing glycosylation to improve the sample heterogeneity, so that we can identify and quantify the distribution of different numbers of conjugated drugs, as well as the link-only species formed in the 2-steps conjugation process with less ambiguities. Compared to reversed-phased conditions, native SEC-MS raw spectrum shifts the charge envelope to higher *m/z* mass window separation of multiple charge species envelope, therefore, has the potential benefit of better resolving complexity of the lysine-conjugated ADCs without deglycosylation as demonstrated from the SEC-MS analysis of Kadcyla in here.

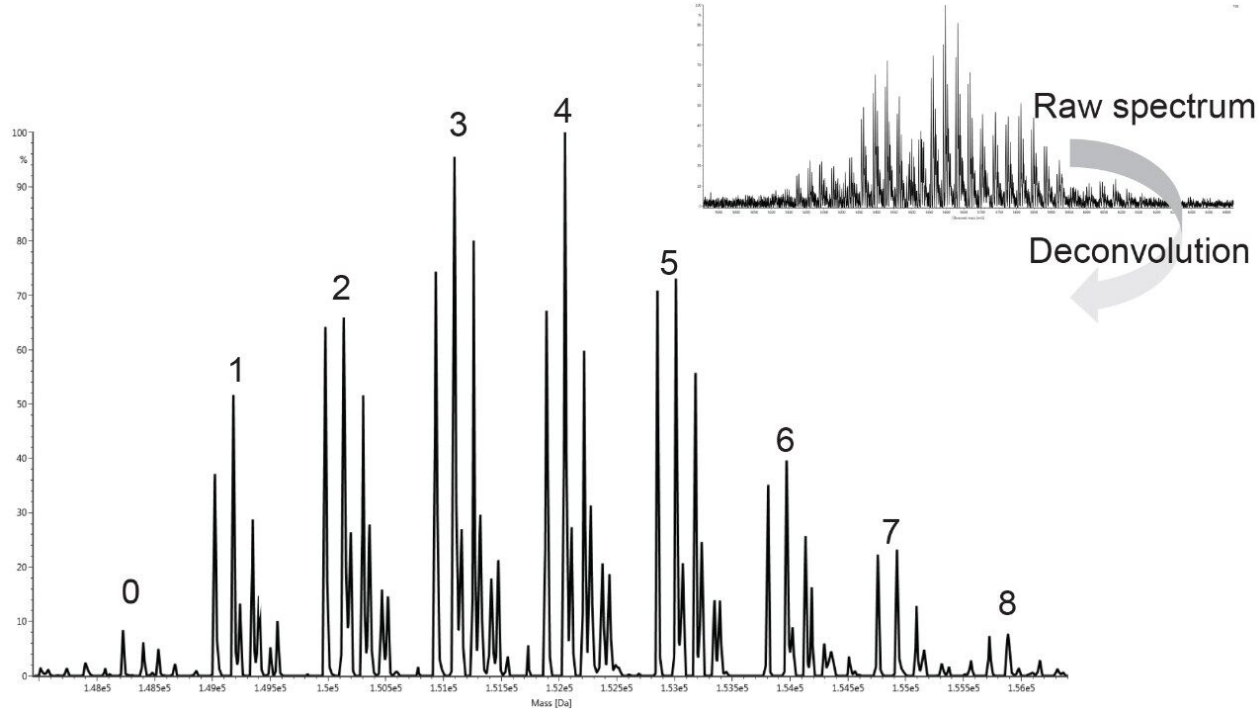

Figure 5. Raw and deconvoluted spectrum of SEC-MS analysis of Lysine conjugated ADC, Kadcyla (trastuzumab Entansine (T-DM1),) without deglycosylation. The number on top of the deconvoluted spectrum peaks represents the detected number of drugs that are conjugated to the protein Trastuzumab in the intact level. The calculated average DAR is 3.56, vs. the theoretical DAR of 3.50 [11].

Conclusion

We demonstrate the use of an analytical scale native SEC-MS analytical platform to examine both cysteine and lysine conjugated intact ADCs. Average DARs and drug loading distributions from the native SEC-MS analysis were compared with the values generated from HIC separation. We also compared the DARs value from two different QTof systems. Excellent agreement in DAR values was observed from HIC and SEC-MS studies. The current SEC-MS method has the benefit of increased sensitivity and robustness (higher system back pressure to ensure the peak retention reproducibility) and simplified sample preparation process (no need for sample deglycosylation). We believe that this native SEC-MS platform method adds benefit to 1) DAR and drug distribution calculations; 2) evaluation of new constructs and drug linker technologies; and 3) characterization of research molecules.

References

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