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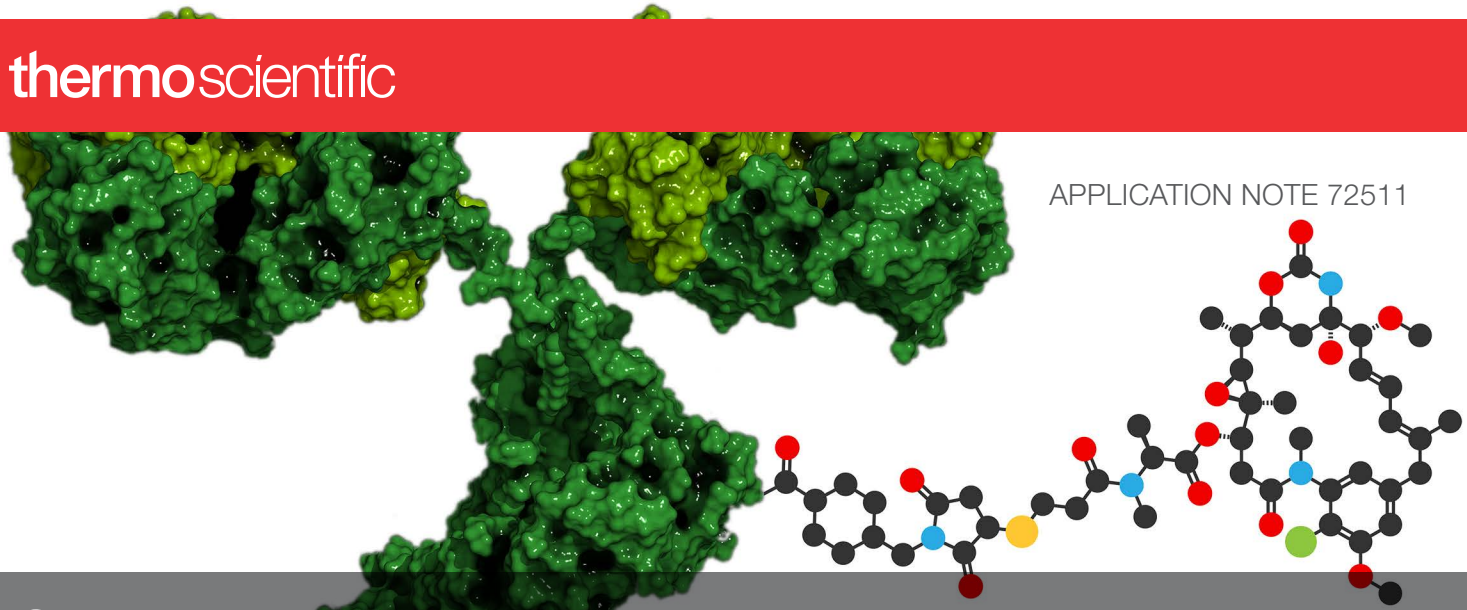

Complete characterization of a lysine-linked antibody drug conjugate by native LC/MS intact mass analysis and peptide mapping

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Keywords

Native MS, intact mass, peptide mapping, characterization, antibody drug conjugate, ADC, drug-to-antibody ratio, DAR, size exclusion, reversed phase, chromatography, mass spectrometry, Q Exactive, Orbitrap, biopharmaceutical, biomolecules, High Mass Range mode, BioPharma Option

Goal

To perform complete characterization of the lysine-linked antibody drug conjugate (ADC) trastuzumab emtansine using both intact mass and peptide mapping approaches with a single benchtop mass spectrometer. Critical quality attribute (CQA) measurements include drug-to-antibody ratio (DAR) and glycosylation pattern via intact mass analysis, and conjugation site localization via peptide mapping.

Introduction

Biotherapeutic compounds, also known as biologics, have been produced for almost four decades to provide new treatments for a variety of disease classes, including heart disease, arthritis, and several types of cancer. Monoclonal antibody (mAb)-based drugs have proven to be a powerful means for highly specific targeting of a therapeutic chemical activity to the site of a disease epitope. Such a strategy is employed in the design of antibody-drug conjugates (ADCs). ADCs are comprised of a disease-specific

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(usually cancer-specific) mAb that is decorated with one or more cytotoxic small molecules. Utilizing antibody-based targeting to deliver highly toxic compounds directly to cancer cells minimizes off-target activity and patient morbidity.

ADCs can be highly complex chemical structures. Trastuzumab emtansine is a lysine-linked ADC sold commercially as Kadcyla® (Genentech, Inc.). The manufacturing strategy for first generation lysine-linked ADCs involves conjugation of a linker moiety to free side chains of lysines on a mAb, followed by an additional round of conjugation of the mAb-linker to a cytotoxic drug. Two-stage conjugation results in a complex mixture of mAb-linker-drug compounds, which varies in terms of the number of conjugated drugs and/or linkers, and is further complicated by any chemical complexity already presented by the mAb. Such a high level of chemical complexity poses significant challenges for the analytical characterization of lysine-linked ADCs.

Complementary MS-based approaches of peptide mapping and intact mass analysis are needed to facilitate complete characterization of all complex biologics including ADCs. The use of 100% aqueous mobile phases at neutral physiological pH is known as native MS, or native LC/MS when coupled to separations. Native LC/MS analysis will allow proteins to retain physiologically similar structure. Compared to denaturing approaches such as reversed-phase liquid chromatography (RP LC) coupled to MS, performing size exclusion chromatography (SEC) using native, MS-friendly mobile phases produces spectra at higher mass-to-charge (*m/z*) and with reduced charge state values. Using native MS to improve mass separation of heterogeneous mixtures is a powerful strategy when considering the analysis of complex biotherapeutics such as random-lysine-linked ADCs. Here, a benchtop quadrupole-Orbitrap™ mass spectrometer has been utilized to perform both peptide mapping and high-resolution native LC/MS intact mass analysis. In this study, integrated characterization of a lysine-linked ADC, utilizing high mass range for native intact mass analysis and standard mode for peptide mapping, is demonstrated.

Experimental

Sample preparation

Consumables

- Thermo Scientific™ MAbPac™ SEC-1 column, 4.0 × 300 mm (P/N 075592)
- Thermo Scientific™ Acclaim™ Vanquish™ C18 column, 2.2 µm, 2.1 × 250 mm (P/N 074812-V)
- Thermo Scientific SMART Digest™ Kit (P/N 60109-101)
- Fisher Scientific™ LC/MS grade water (P/N W/011217)
- Fisher Scientific™ LC/MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ formic acid LC/MS grade (P/N 28905)
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ format (P/N 20291)
- Thermo Scientific™ Invitrogen™ UltraPure™ Tris hydrochloride (P/N 15506017)
- Honeywell Fluka™ TraceSELECT™ ammonium acetate, 99.9995% purity (metals basis)

Sample preparation for intact mass analysis

Powdered trastuzumab emtansine in formulation buffer was resuspended in water, yielding a final concentration of 5 mg/mL. A volume of 10 µL (50 µg total amount) sample in formulation buffer was autosampler injected on to a MAbPac SEC-1 column for LC/MS analysis.

Sample preparation for peptide mapping

Trastuzumab emtansine (50 µL) in formulation buffer (5 mg/mL) was added to a volume of 150 µL SMART Digest buffer (SMART Digest Kit) and vortexed for 10 s. The mixture was then transferred into a SMART Digest vial and incubated for 45 min at 70 °C with agitation (1,400 RPM, Eppendorf™ ThermoMixer™). Supernatant was transferred to a fresh 0.5 mL microcentrifuge tube. To reduce disulfide bonds, 50 µL of a 100 mM solution of DTT in water (final concentration of 20 mM) was added to the digested peptides and the solution was incubated for 45 minutes at 57 °C. The resulting peptide digest concentration was 0.5 µg/µL. A volume of 20 µL (10 µg total amount) was injected on to an Acclaim Vanquish C18 RP column.

Liquid chromatography and mass spectrometry

LC system configuration

- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)

MS system configuration

- Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer (P/N 0726030)
- BioPharma Option (P/N 0726055)

Native intact mass analysis LC/MS

SEC-LC conditions are described in Table 1. Settings for HMR mode MS tuning and method parameters are described in Table 2.

Table 1. Size exclusion LC conditions for native intact mass analysis.

LC Conditions	
Column:	MAbPac SEC-1 (4.0 × 150 mm)
Mobile Phase A:	50 mM ammonium acetate in water
Flow Rate:	0.300 mL/min
Column Temperature:	30 °C, forced air mode
Isocratic Elution:	100% mobile phase A for 10 min
	Time *% B*
	0 0
	10 0

Table 2. HMR mode settings for intact mass analysis.

Tune File	
Parameters for Ion Max Source with HESI-II Probe	**Setting**
Sheath Gas Pressure	20 psi
Auxiliary Gas Flow	5 arbitrary units
Probe Heater Temperature	225 °C
Source Voltage	4.0 kV
Capillary Temperature	325 °C
S-Lens RF Voltage	200 V
Mode	High Mass Range Mode
Trapping Gas Pressure	1.5 arbitrary units
Method File	
Full MS Parameters	**Setting**
Full MS Mass Range	*m/z* 2000–8000
Resolution Setting	70,000 (FWHM at *m/z* 200)
Microscans	10
AGC Target Value	3e6
Max Injection Time	500 ms
In-source CID	100 eV

Peptide mapping LC/MS

Reversed-phase LC gradient conditions are described in Table 3. Settings for Standard mode MS tuning and method parameters are described in Table 4. LC and MS parameters were not extensively optimized specifically for trastuzumab entansine peptide mapping.

Table 3. Reversed-phase LC conditions for peptide mapping analysis.

Column:	Acclaim RSLC 120 C18 (2.1 × 250 mm)
Mobile Phase A:	0.1 % formic acid in water
Mobile phase B:	0.1 % formic acid in acetonitrile
Flow Rate:	0.250 mL/min
Column Temperature:	60 °C, forced air mode
Gradient:	2–35% mobile phase B in 60 min
	Time *% B*
	0 2
	10 2
	70 35
	75 80
	77 80
	80 2
	90 2

Table 4. Q Exactive Plus MS settings for peptide mapping.

Tune File	
Parameters for Ion Max Source with HESI-II Probe	**Setting**
Sheath Gas Pressure	40 psi
Auxiliary Gas Flow	10 arbitrary units
Probe Heater Temperature	320 °C
Source Voltage	3.6 kV
Capillary Temperature	150 °C
S-Lens RF Voltage	60 V
Mode	Standard Mode
Trapping Gas Pressure	1.0 arbitrary units
Method File	
Full MS Parameters	**Setting**
Full MS Mass Range	*m/z* 200–2000
Resolution Setting	70,000 (FWHM at *m/z* 200)
Microscans	1
Target Value	1e6
Max Injection Time	100 ms
Default Charge State	2
In-source CID	0 eV
MS/MS Parameters	**Setting**
Resolution Setting	17,500 (FWHM at *m/z* 200)
Target Value	1e5
Max Injection Time	200 ms
Loop Count	5
Isolation Window	100 eV
Collision Energy	27% NCE
Intensity Threshold	2.0e4
Charge Exclusion	Unassigned
Dynamic Exclusion	10 s

Data analysis

Native intact LC/MS data analysis

Intact mass spectra were analyzed using the Intact Protein workflow in Thermo Scientific™ BioPharma Finder™ 2.0 integrated software, performing time-resolved deconvolution using the ReSpect™ algorithm in combination with Sliding Window integration. Detailed deconvolution method parameter settings are described in Table 5. Deconvolution spectra were annotated by entering four individual sequences into Protein Sequence Manager. To make each sequence, the amino acid sequence of trastuzumab, with a total of 16 disulfide bonds, fixed modifications (at amino acid position N300 on each heavy chain) corresponding to four possible glycan combinations (G0F/G0F, G0F/G1F, G1F/G1F, G1F/G2F), and a variable modification of MCC-DM1 (8 maximum) was used. An example of creating a protein sequence for intact mass analysis is shown in Figure 1. Masses for fixed and variable modifications are described in Table 6. DAR values were calculated by activating 'Enable Drug-To-Antibody Ratio' (in Identifications tab) and selecting MCC-DM1 as the variable modification.

Peptide mapping data analysis

Peptide mapping data were searched using the Peptide Mapping workflow in BioPharma Finder 2.0 software. Detailed peptide mapping method parameter settings are described in Table 7. Variable modifications included N-glycan (CHO-derived), deamidation (N, Q), oxidation (M, W), and MCC-DM1. An example of creating a protein sequence for peptide mapping is shown in Figure 2, where there is only entry for the light and heavy chains.

Specific masses for fixed and variable modifications are described in Table 8. MCC-DM1 peptide identifications were manually validated by monitoring for the presence of a chromatographic doublet in an extracted ion chromatogram (5 ppm window) and a specific HCD fragmentation signature ion with a theoretical monoisotopic mass of 547.2211 Da, corresponding to fragmentation of DM1.

Table 5A. Intact protein analysis parameter settings in BioPharma Finder software.

Component Detection	
Chromatogram Parameters	**Setting**
m/z Range	4,000–8,000 *m/z*
Chromatogram Trace Type	BPC
Sensitivity	High
Rel. Intensity Threshold	1%
Source Spectra Method	**Setting**
Sliding Window	*On*
RT Range	3.0 to 5.5 min
Target Avg Spectrum Width	0.5 min
Target Avg Spectrum Offset	17%
Merge Tolerance	12 ppm
Max RT Gap	0.5 min
Min. Number of Detected Intervals	3

Table 5B. Intact protein analysis parameter settings in BioPharma Finder software.

Component Detection	
Deconvolution Parameters	**Setting**
Algorithm	ReSpect
Model Mass Range	148,000.00 to 157,000.00 Da
Mass Tolerance	12 ppm
Charge State Range	20 to 30
Minimum Adjacent Charges	3 to 3
Choice of Peak Model	Intact Protein
Target Mass	157,000.00 Da
Resolution at 400 *m/z*	Raw File Specific
Noise Rejection	95% Confidence
Rel. Abundance Threshold	1%
Identification	
Sequence Matching Mass Tolerance	20 ppm
Enable Drug-to-Antibody Ratio	On
Variable Modification	“MCC-DM1”

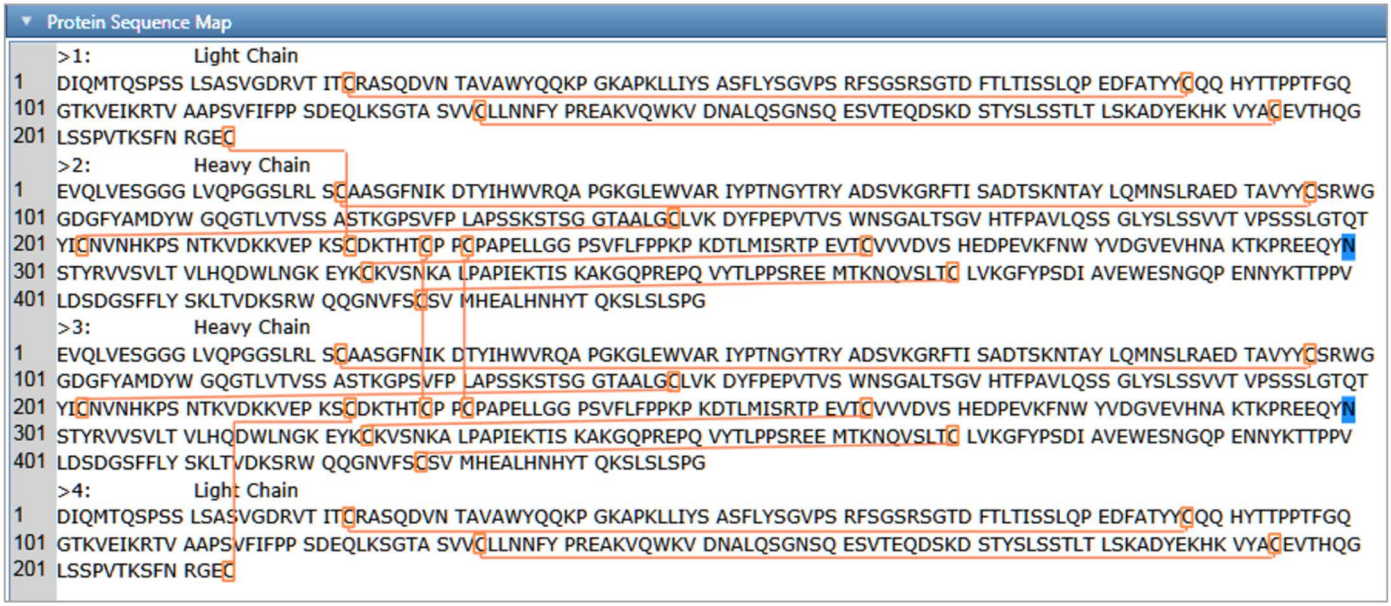


Figure 1. Setting up the Protein Sequence Map for intact protein analysis. For intact mass analysis, trastuzumab amino acid sequence is added as four separate polypeptide chains, with two copies each of light and heavy chains. Disulfide bonds were added by right-clicking at the site of a cysteine residue to create a linkage, then right-clicking at the site of a second cysteine residue to complete the linkage. The blue highlighted asparagine residues are N-glycan consensus sites (Nx[S/T]), which are known to become glycosylated in trastuzumab. A fixed modification at these sites was added by left double-clicking and selecting a specific glycoform for attachment.

Table 6. Modifications for intact protein analysis workflow.

Variable Modifications				
Name	**Type**	**Mono. Mass**	**Avg. Mass**	**Residue(s)**
DM1	Side Chain	956.3644	957.53	K
Possible Static Modifications on Sequence				
Name	**Mono. Mass**	**Avg. Mass**	**Residue(s)**	**Location**
G0F	1,444.533	1,445.32	N	Heavy Chain N300
G1F	1,606.586	1,607.46	N	Heavy Chain N300
G2F	1,768.639	1,769.60	N	Heavy Chain N300

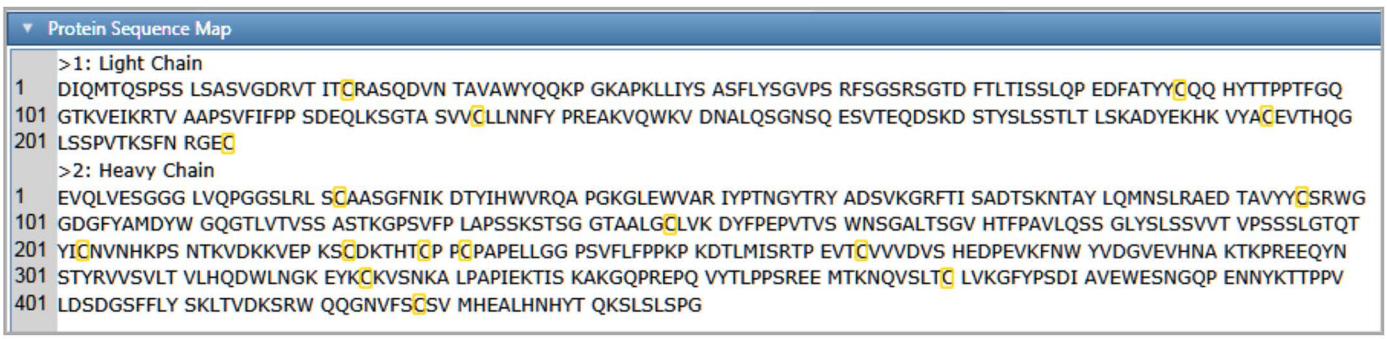


Figure 2. Setting up the Protein Sequence Map for peptide mapping analysis. For peptide mapping only one copy of each trastuzumab polypeptide chains is added.

Table 7. Peptide mapping analysis parameter settings in BioPharma Finder software.

Component Detection	
Task	Find All Ions in the Run
Peak Detection	**Setting**
Absolute MS Signal Threshold	4.00e4
MS Noise Level	2000.00
S/N Threshold	20.00
Typical Chromatographic Peak Width	0.40 min
Use Restricted Time	Off
Relative MS Signal Threshold	1.00%
Width of Gaussian Filter	3
Minimum Valley to be Considered	80%
Minimum MS Peak Width	1.20 Da
Maximum MS Peak Width	4.20 Da
Mass Tolerance	4 ppm
Ion Alignment	**Setting**
Maximum Retention Time Shift	2.33 min
Mass Measurement	Setting
Maximum Mass	30,000 Da
Mass Centroiding Cutoff	15%
Identification	
Peptide Identification	**Setting**
Search by Full MS Only	No
Use MS/MS	Use All MS/MS
Maximum Peptide Mass	15,000 Da
Mass Accuracy	5 ppm
Maximum Number of Modifications for a Peptide	1
Advanced Search	Setting
Enable Mass Search for Unspecified Modifications	Off
N-Glycosylation	CHO
Search for Amino-Acid Substitutions	None

Results and discussion

Native LC/MS analysis improves spectral separation of components in complex mixtures of intact proteins

The diversity of protein isoforms in an ADC sample is contingent upon the enzymatic or spontaneous chemical modifications that arise during the manufacturing process of the core antibody as well as the drug conjugation chemistry (Figure 3). Major forms of lysine-linked ADCs differ in N-glycan composition, the number of linker-drugs attached, as well as potential linker-only attachments.

The lysine-linked ADC, trastuzumab emtansine, is observed in intact mass analysis as a complex mixture. The broad mass range of components in ADC samples causes extensive *m/z* interferences when analyzed using denaturing approaches such as reversed-phase LC/MS. Compared to denaturing conditions, native LC/MS allows greater *m/z* separation of sequential charge state envelopes, and is ideal for resolving complexity of co-eluting protein species (Figure 4).[1]

The powerful Orbitrap high-resolution mass analyzer is ideal for studying very complex mixtures of intact proteins in great depth. Compared to conventional technologies, Orbitrap mass spectrometers can achieve much higher effective resolution of individual intact protein species, which allows an increased capacity to distinguish near-isobaric protein isoforms.[2] This characteristic of Orbitrap mass spectrometry allows users to approach ADC intact protein characterization in a manner which avoids sample pretreatment, such as spectral simplification methods like deglycosylation and digestion to subunits. This strategy precludes the potential of artifacts due to sample handling and adds the convenience of omitting the sample handling step. By analyzing trastuzumab emtansine ADC without any sample pretreatment, the LC/MS experiment reflects a true intact mass analysis.

Trastuzumab emtansine (5 mg/mL in formulation buffer) was desalted online using native SEC coupled directly to the mass spectrometer. Native SEC-MS was performed using a MAbPac SEC-1 size exclusion column with a sample injection volume of 10 µL (50 µg total amount). Mobile phase A (50 mM ammonium acetate) was delivered isocratically at a flow rate of 300 µL/min. The column eluent was coupled directly to an electrospray

Table 8. Modifications for peptide mapping workflow.

Variable Modifications				
Name	**Type**	**Mono. Mass**	**Avg. Mass**	**Residue(s)**
N-glycan	Side Chain	CHO library	CHO library	Nx(S/T)
Oxidation	Side Chain	15.9949	16.00	M, W
Deamidation	Side Chain	0.9840	0.99	N, Q
MCC-DM1	Side Chain	956.3644	957.53	K

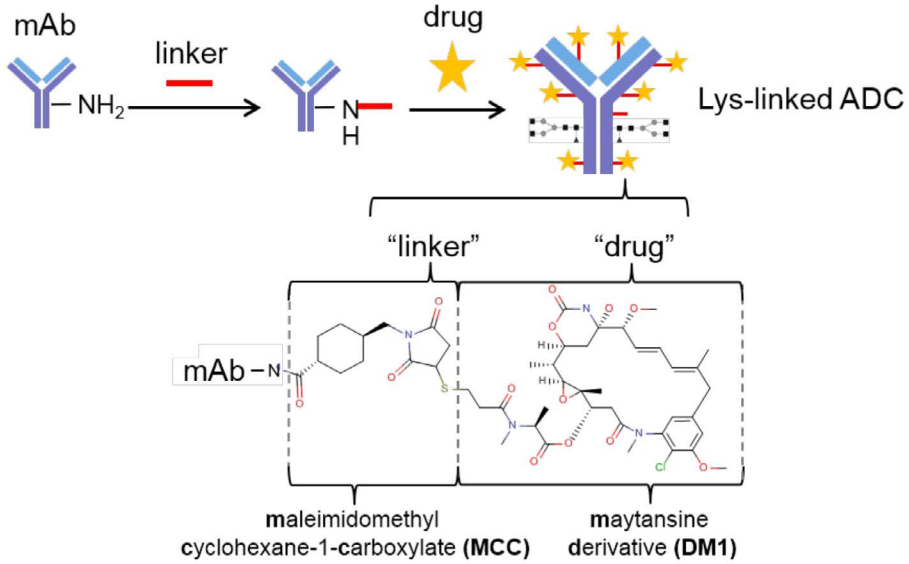


Figure 3. Multi-stage assembly of lysine-linked ADCs can result in a highly complex mixture. Trastuzumab emtansine is a lysine-linked ADC that is constructed using trastuzumab monoclonal antibody as a starting platform. ADC construction is a two-step conjugation process. The first step involves attachment of a hetero-bifunctional linker at the site of lysine side chains. In a second step, the remaining active site of the MCC linker becomes a conjugation site for a DM1 payload. Total ADC heterogeneity owes to both antibody growth and manufacturing as well as the linker-drug conjugation process.

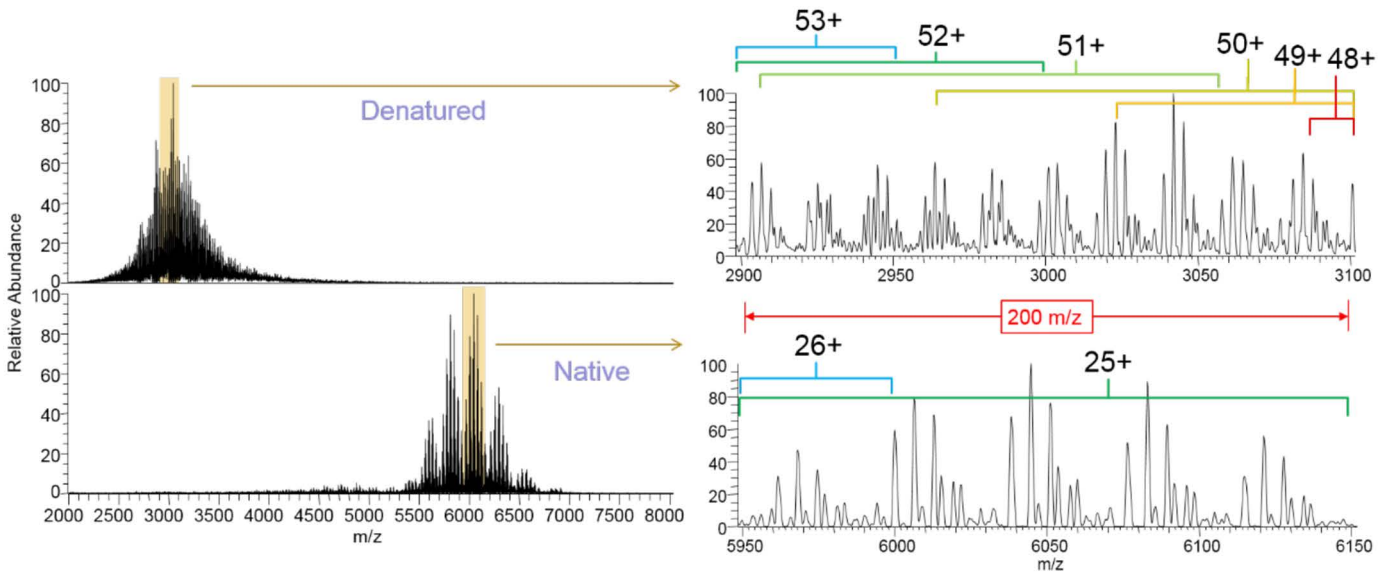


Figure 4. Native LC/MS analysis of intact proteins allows improved separation of mass peaks at higher *m/z* range. MS spectra acquired under denaturing conditions by reversed-phase LC are observed at lower *m/z* ranges while native MS spectra from online size exclusion LC are observed at higher *m/z* ranges. A detailed view (right side) shows that 2–3 sequential charge state envelopes overlap compared to an overlap of 0–1 charge state envelopes in the native MS spectrum.

ionization source on the inlet of a commercially available Q Exactive Plus mass spectrometer. The Q Exactive Plus MS was outfitted with the BioPharma Option and was operated in High Mass Range (HMR) mode to allow improved high mass transmission and scanning up to *m/z* 8000 for native intact analysis. MS1 spectra were collected using 10 microscans and a resolution setting of 70,000 FWHM.

The resulting base peak chromatogram showed one chromatographic peak (eluting 3.7–4.7 min), which corresponds to the desalted ADC and a later eluting chromatographic peak (eluting 4.8–5.7 min) corresponding to the formulation buffer salts (Figure 5A). The raw mass spectrum shows a distribution of protein ions in the range of approximately 5,500–7,000 *m/z*, corresponding to a charge state distribution of 23+ to 27+ (Figure 6A). Here, the baseline separation of the buffer salt peak was critical for preventing ion suppression during electrospray ionization of the ADC protein species. A major advantage of using SEC is efficient desalting, which is attained as a result of the size-based separation itself. This is analogous to the mandatory offline desalting step(s) performed in conventional native MS approaches that use centrifugal buffer exchange columns upstream of static nanospray infusion.[3]

Partial chromatographic resolution of the different drug loads was observed, with higher drug load isoforms eluting at slightly later retention times (Figure 5B-C). This phenomenon is presumably due to some weak secondary interactions of the chromatographic media with the linker-drug conjugations. In cases where secondary interactions appear to be a major influence on separations of a particular compound class, alternative choices for the SEC column should be considered. The Thermo Scientific™ Acclaim™ SEC-300 column has a hydrophilic surface and is thus an excellent alternative to the MAbPAC SEC-1 column. The partial separation of ADC isoforms can be accommodated by analyzing the raw data from the LC/MS intact mass analysis using the Sliding Window feature in BioPharma Finder software 2.0. Sliding Window

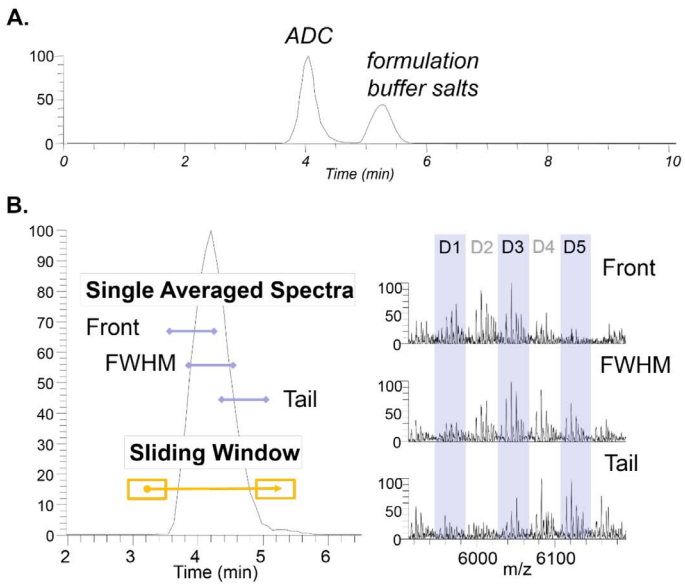


Figure 5. Native size exclusion chromatography (SEC)-MS of trastuzumab emtansine shows partial chromatographic separation of drug load isoforms. (A) Online SEC-MS allows automatic desalting of ADC by baseline separation of buffer salts that interfere with electrospray ionization. (B) A detailed analysis of the raw LC/MS data show that higher drug load isoforms (e.g., D1 compared to D5) are eluting at slightly later retention times. The deconvolution data analysis was performed using Sliding Window integration to accommodate this partial chromatographic separation to obtain good relative abundances necessary for correctly calculating a drug-to-antibody ratio.

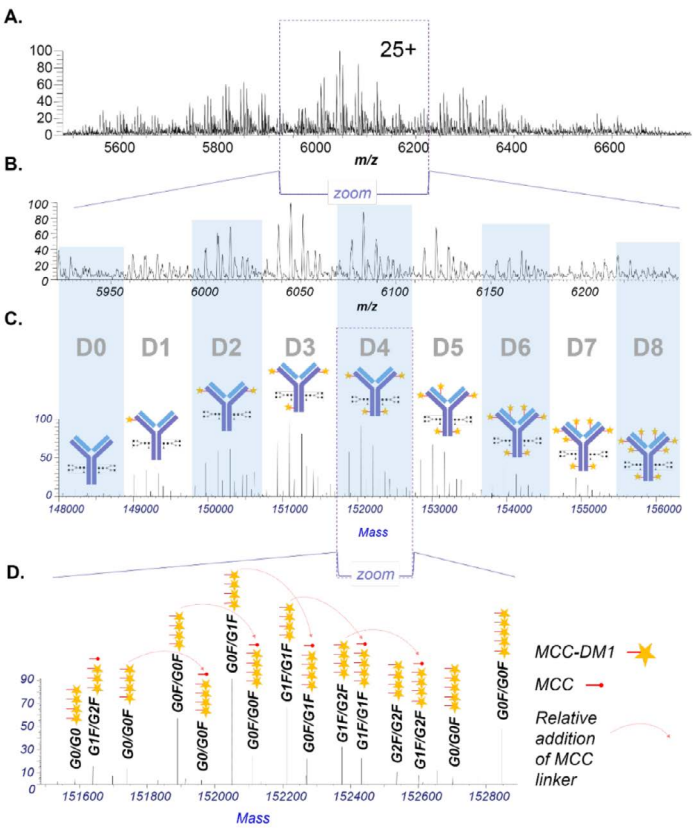


Figure 6. Native MS conditions allow excellent spectral separation of trastuzumab emtansine isoforms and enable high quality deconvolution. (A) Native intact Orbitrap MS spectra of trastuzumab emtansine acquired at R=70,000 setting. (B) A detailed view of the 25+ charge state. (C) The ReSpect / Sliding Window deconvolution results show a distribution of 0–8 MCC-DM1 linker-drug attachments. Native MS conditions yield excellent spectral separation of the ADC isoform masses, which can be easily comparted to deconvolution results for manual confirmation. (D) A detailed view of the D4 cluster shows the relative addition of an MCC linker-only attachment.

allows use of a deconvolution algorithm (e.g., ReSpect) continuously throughout a chromatographic run, or in this case through the entire SEC peak. Sliding Window is critical to the data analysis platform for correctly measuring the accurate relative quantities of the different drug load isoforms that become partially separated in chromatography. Good relative quantitation is a requirement for correctly calculating drug-to-antibody ratio.

Measuring a drug-to-antibody ratio (DAR) is a primary objective of ADC characterization, which seeks to estimate the drug potency. The true intact mass analysis of the untreated ADC showed a distribution of 0–8 linker-drug conjugations with each drug load 'cluster' showing an expected profile of N-glycoform masses (Figure 6B). A close inspection of the raw data compared to the deconvolved result showed a very high degree of similarity, which gave confidence in the accuracy of the deconvolution results (Figure 6C).

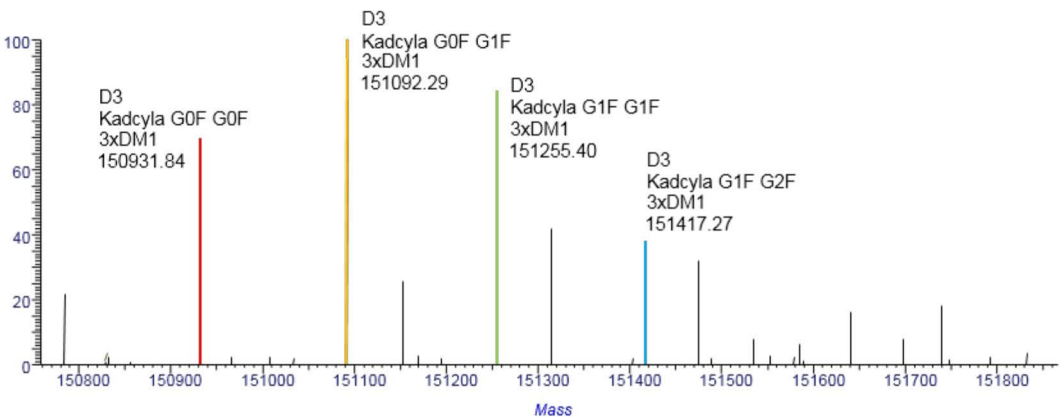
As an additional 'layer' of heterogeneity in the ADC sample, a low abundance distribution of isoforms which had become conjugated at lysines with MCC linker, but did not have a DM1 payload attached were observed (Figure 5E). These species, with a delta mass of approximately 219 Da, were recently reported to be a side product where during the step-wise conjugation process the active lysine-conjugated MCC linker becomes conjugated to another lysine on the ADC instead of the DM1 payload as intended. This results in an internally-crosslinked species.[4] These low-level linker-only species could be accurately identified within 20 ppm.

At each DAR cluster, it was observed that the profile of N-glycoforms was consistent (Figure 7A). The automatic DAR calculator feature on BioPharma Finder software 2.0 was used to calculate an average DAR value based on the four most abundant glycoforms. Using the G0F/G0F, G0F/G1F, G1F/G1F, and G1F/G2F N-glycoform species, an average DAR value of 3.65 was calculated (Figure 7B). This value is consistent with the previously observed values of using static nanospray and native intact analysis.[1]

Peptide mapping of trastuzumab emtansine allows localization of conjugation sites

Peptide mapping was performed in triplicate using 90 min reversed-phase gradients for separation. The Q Exactive Plus mass spectrometer with BioPharma Option was operated in Standard mode at a resolution setting of 70,000 FWHM for MS and 17,500 FWHM for MS/MS. Tryptic peptide mapping analysis resulted in 100% sequence coverage (Figure 8).

A.



B.

Glycoform	G0F/G0F	G0F/G1F	G1F/G1F	G1F/G2F	Top 4 avg
DAR	3.58	3.71	3.64	3.65	3.65

Figure 7. Automatic calculation of Drug-to-Antibody (DAR) ratio using multiple glycoforms. (A) Detail of glycoform distribution shown with three conjugated drugs. (B) An average DAR of 3.65 was calculated using the average of values for four independently measured glycoforms.

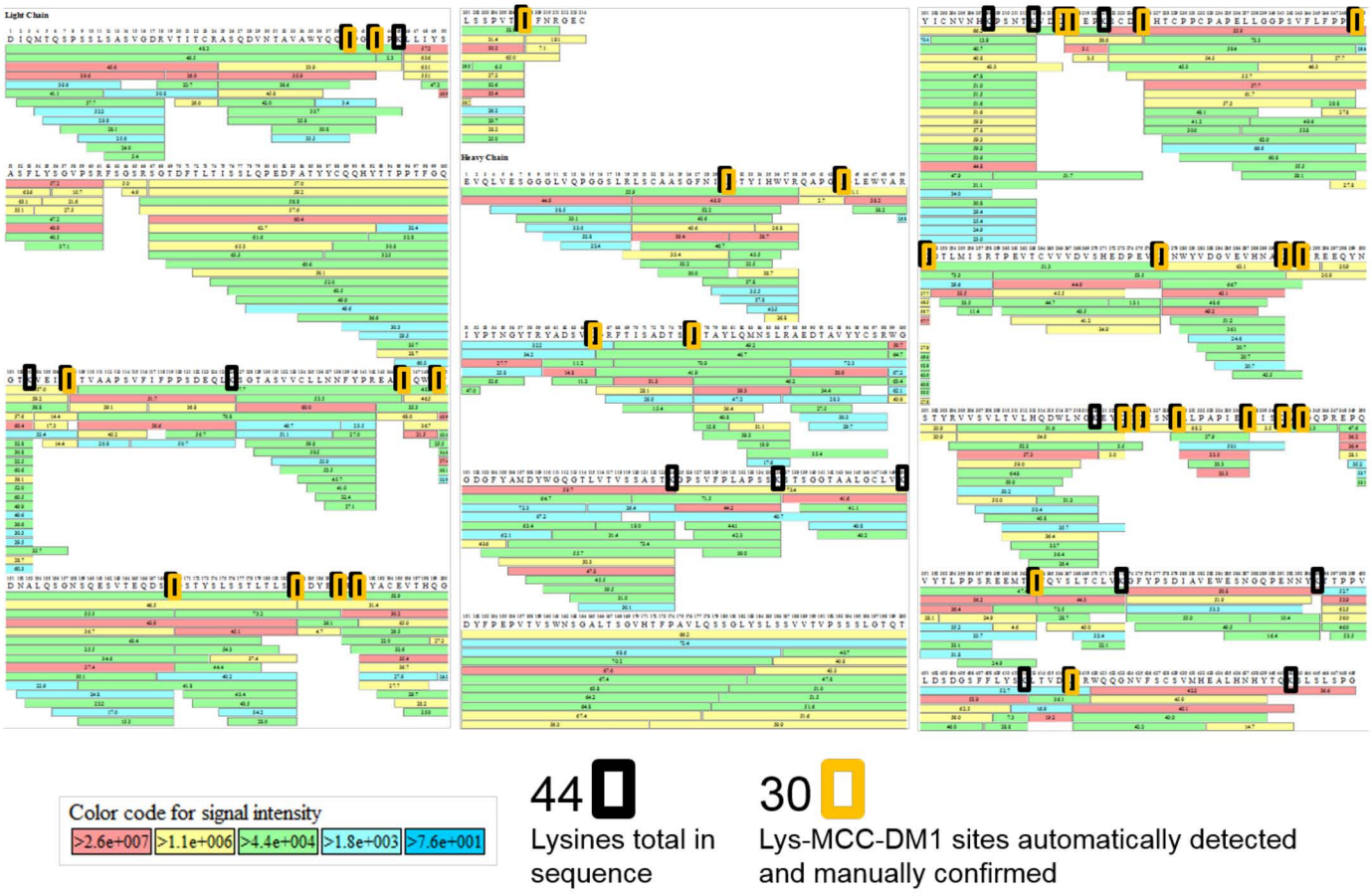


Figure 8. Trastuzumab emtansine peptide mapping results and lysine of MCC-DM1 conjugation sites. Peptides were automatically identified using BioPharma Finder software. MCC-DM1 linker-drug conjugated peptide identifications were manually confirmed and mapped onto the sequence coverage diagram.

BioPharma Finder software automatically identified 30 out of 44 lysine sites of MCC-DM1 linker-drug conjugation (Figure 8) (Table 9). The unique properties of the DM1 payload allowed for manual confirmation of the automatically identified conjugated peptides. The hydrophobic and stereocentric nature of the MCC-DM1 linker-drug moiety causes conjugated peptides to elute with increased retention times and as a doublet (Figure 9). Additionally, HCD spectra showed fragmentation of both the peptide as well as the linker-drug moiety. In this case, a signature fragment ion of 547 *m/z* was utilized to confirm the automatic identifications.[5]

Table 9. MCC-DM1 conjugated peptides identified in trastuzumab emtansine.

Peptide Sequence	Modification	Protein	Site	Delta Mass (ppm)	RT
ASQDVNTAVAWYQQKPGK	DM1	Light Chain	K39	-0.25	67.47
PGKAPK	DM1	Light Chain	K42	-0.31	63.21
VEIKR	DM1	Light Chain	K107	-0.46	65.96
EAKVQWK	DM1	Light Chain	K145	0.09	67.23
VQWKVDNALQSGNSQESVTEQDSK	DM1	Light Chain	K149	0.09	68.57
VDNALQSGNSQESVTEQDSKDSTYSLSSTLTLSK	DM1	Light Chain	K169	0.2	68.77
DSTYSLSSTLTLSKADYEK	DM1	Light Chain	K183	1.07	73.01
ADYEKHK	DM1	Light Chain	K188	-0.77	59.14
HKVYACEVTHQGLSSPVTK	DM1	Light Chain	K190	0.01	58.54
VYACEVTHQGLSSPVTKSFNR	DM1	Light Chain	K207	0.15	64.92
LSCAASGFNIKDTYIHWVR	DM1	Heavy Chain	K30	-2.37	73.12
QAPGKGLEWVAR	DM1	Heavy Chain	K43	-0.62	71.01
YADSVKGR	DM1	Heavy Chain	K65	-0.67	65.35
FTISADTSKNTAYLQMNSLR	DM1	Heavy Chain	K76	0.39	70.82
VDKK	DM1	Heavy Chain	K216	-0.74	63.99
KVEPK	DM1	Heavy Chain	K217	0.46	63.38
SCDKTHTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K225	-1.61	72.91
THTCPPCPAPELLGGPSVFLFPPKPK	DM1	Heavy Chain	K249	-0.4	73.5
PKDTLMISR	DM1	Heavy Chain	K251	0.01	70.58
TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAK	DM1	Heavy Chain	K277	-0.05	71.84
FNWYVDGVEVHNAKTK	DM1	Heavy Chain	K291	0.26	67.17
TKPR	DM1	Heavy Chain	K293	0.19	64.16
EYKCK	DM1	Heavy Chain	K323	-0.04	64.44
CKVSNK	DM1	Heavy Chain	K325	-0.47	62.93
VSNKALPAPIEK	DM1	Heavy Chain	K329	-0.38	68.18
ALPAPIEKTISK	DM1	Heavy Chain	K337	-0.66	71.07
TISKAK	DM1	Heavy Chain	K341	-0.25	64.06
AKGQPR	DM1	Heavy Chain	K343	-0.07	62.99
EEMTKNQVSLTCLVK	DM1	Heavy Chain	K363	-0.07	72.43
LTVDKSR	DM1	Heavy Chain	K417	-0.6	66.04

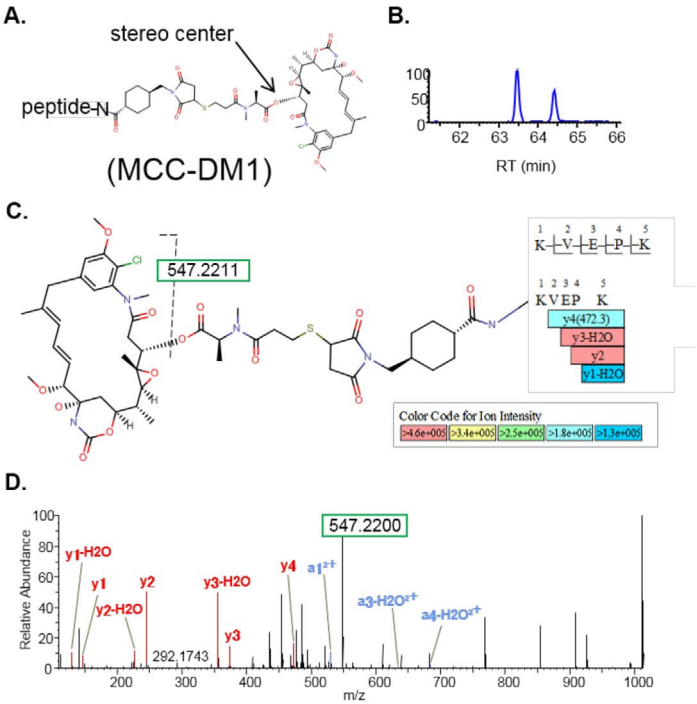


Figure 9. MCC-DM1 conjugation causes peptides to elute as a doublet and can be verified using HCD signature ion *m/z* 547. (A) The structure of DM1 contains a stereocenter. (B) MCC-DM1 conjugation imparts a stereocenter and causes peptides to elute as a doublet in C18 reversed-phase LC. A peptide KVEPK is shown as an extracted ion chromatogram (5 ppm window) with doublet behavior. (C) The DM1 stereocenter is labile in HCD conditions and produces a fragment with a calculated mass of 547.2211 Da. (D) The HCD fragmentation spectrum of KVEPK shows a high abundance ion at *m/z* = 547.2200, which matches the calculated signature ion with an accuracy of 2 ppm.

Conclusions

- Native LC/MS improves *m/z* separation of complex ADC spectra.
- Lysine-linked ADC mixtures may also include linker-only forms.
- Drug conjugation may change peptide behavior in peptide mapping.
- HCD fragmentation generates conjugate-specific signature ions.
- The Q Exactive BioPharma Option allows pre-optimized modes for peptide mapping and native intact mass analysis.

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