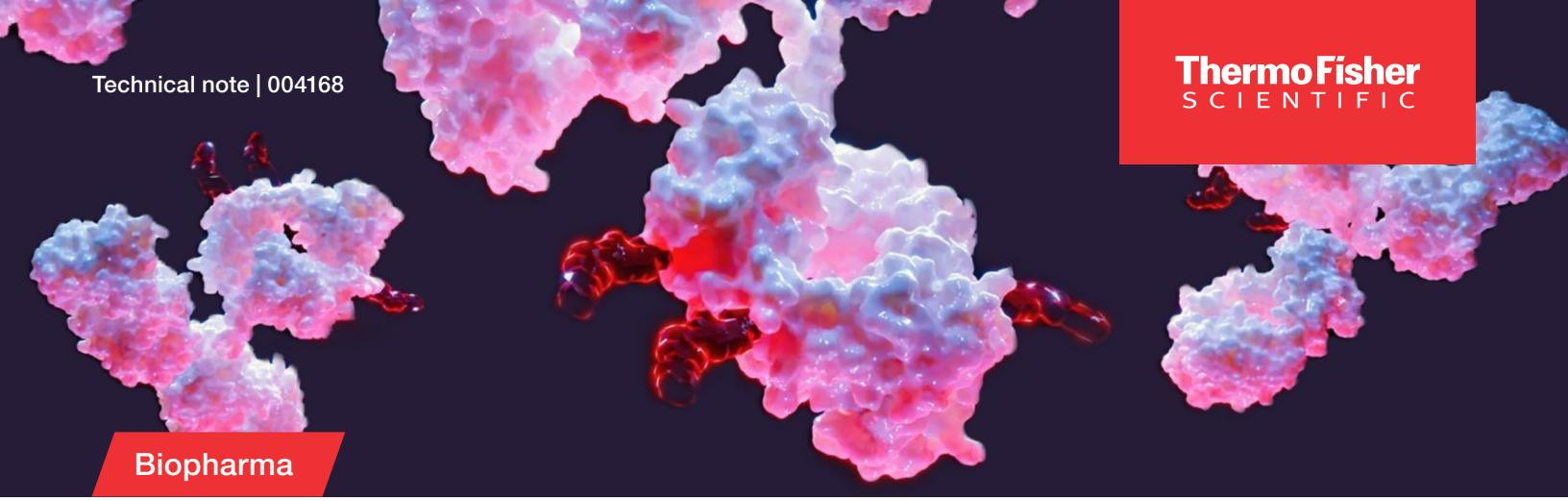


Technical note | 004168

Biopharma

In-depth characterization of KADCYLA on the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer

Using fast and sensitive electron-transfer/higher-energy collision dissociation (EThcD) fragmentation

Authors

Xiaoxi Zhang[1], Reiko Kiyonami[2], Hao Yang[3], Roberto Gamez[4], Cong Wang[5], Peter Krueger[5], Heiner Koch[5], Min Du[2]; [1]Thermo Fisher Scientific, Shanghai, China; [2]Thermo Fisher Scientific, Lexington, Massachusetts, USA; [3]Thermo Fisher Scientific, San Jose, California, USA; [4]Thermo Fisher Scientific, Texas, USA; [5]Thermo Fisher Scientific, Bremen, Germany

Keywords

Antibody-drug conjugates (ADC), native intact MS, peptide mapping, electron-transfer/higher-energy collision dissociation (EThcD), Orbitrap Excedion Pro Biopharma hybrid mass spectrometer, Proteome Discoverer software, BioPharma Finder software

Introduction

Over the last decade, antibody-drug conjugates (ADC) have evolved into promising and efficient therapeutic agents for targeted chemotherapy in cancers, with 14 ADCs currently approved by the U.S. Food and Drug Administration (FDA), and more than 100 ADCs in clinical studies as of April 2025.[1] ADCs are generated through the conjugation of monoclonal antibodies (mAbs) specifically targeting the tumor-associated antigens (TAAs) of the tumor cell with highly potent cytotoxic drug payloads via a cleavable or non-cleavable chemical linker.[2]

Antibody-drug conjugates (ADCs) have made significant strides in the field of oncology, starting with the approval of the first ADC, Mylotarg®, in 2000.[3] Mylotarg® targets the CD33 antigen and is used for the treatment of acute myeloid leukemia (AML). Over a decade later, in 2011, Adcetris® was approved for the treatment of Hodgkin lymphoma (HL) and anaplastic large cell lymphoma (ALCL).[4] Adcetris® targets the CD30 antigen. Following this, KADCYLA® (ado-trastuzumab emtansine) was approved in 2013 for the treatment of HER2-positive metastatic breast cancer, targeting the HER2 antigen.[4]

The significant efficacy and favorable safety profiles of these ADCs in patients with advanced cancers have led to their widespread recognition as a novel therapeutic approach. This recognition has come from patients, oncologists, and the biopharmaceutical industry. Consequently, ADCs have rapidly become an area of much interest in both the global biomedical academic community and the pharmaceutical industry. Due to their complex make-up and multiple components, analysis of their chemistry and functionality is challenging, requiring advanced techniques for comprehensive characterization to ensure their safety, efficacy, and quality.

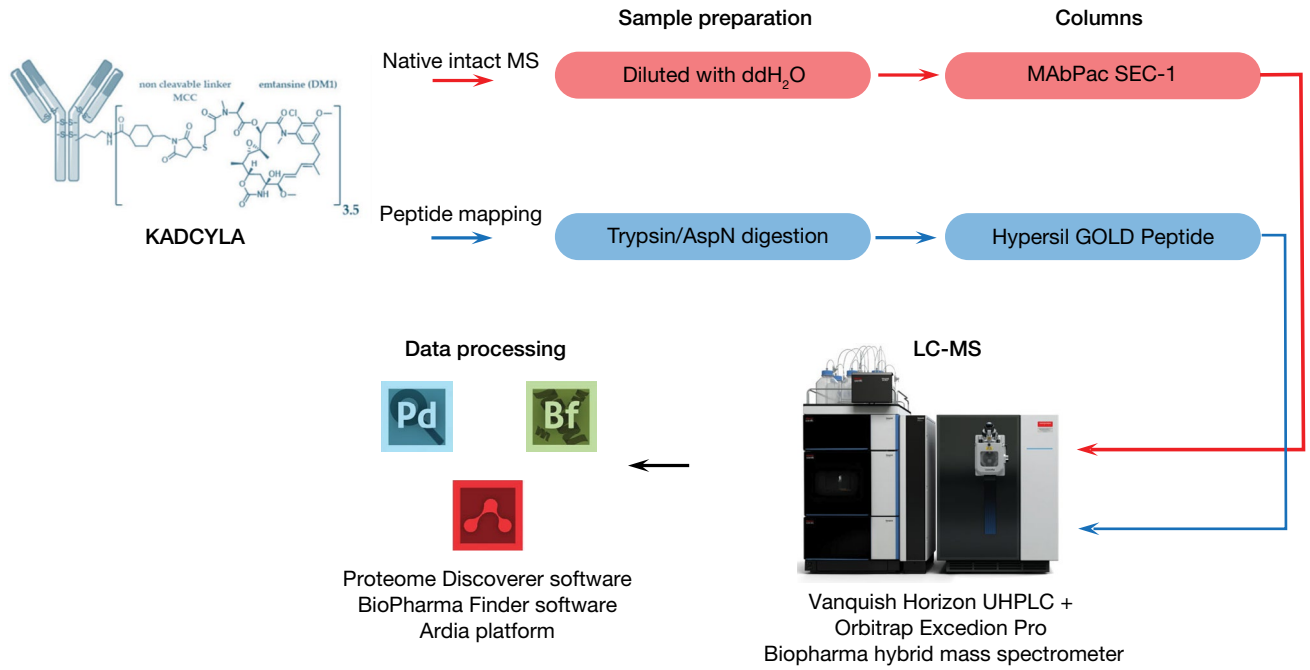


Figure 1. The UHPLC-HRAM MS characterization workflow for KADCYLA.

Here, we demonstrate the comprehensive characterization of KADCYLA, a lysine conjugated-ADC with a DAR distribution from 0–8, using a Thermo Scientific™ Vanquish™ Horizon UHPLC coupled to a Thermo Scientific™ Orbitrap™ Excedion™ Pro Biopharma Hybrid Mass Spectrometer with electron-transfer/higher-energy collision dissociation (EThcD) fragmentation (Figure 1).

Experimental

Sample and consumables

Sample

Commercially available KADCYLA was used. The schematic of KADCYLA is shown in Figure 2.

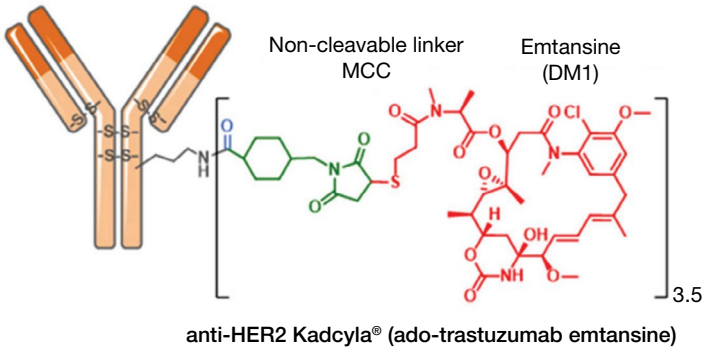


Figure 2. The schematic of KADCYLA.[4] MCC-DM1 represents the linker with the DM1 drug. Δm(MCC-DM1) = 956.3644 Da.

Consumables

- Thermo Scientific™ Water with 0.1% formic acid (v/v), Optima™ LC/MS grade, Cat. No. LS118-1
- Thermo Scientific™ Acetonitrile with 0.1% formic acid (v/v), Optima™ LC/MS grade, Cat. No. LS120-1
- Thermo Scientific™ Formic acid, LC-MS grade, Cat. No. 28905
- Sigma-Aldrich™ Guanidine hydrochloride, 8 M, pH 8.5, buffered aqueous solution, Cat. No. G7294-100ML
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5, Cat. No. 15567027
- Millipore™ Amicon™ Ultra Filter, sample volume 0.5 mL, regenerated cellulose membrane, MWCO 10 kDa, Cat. No. UFC501008
- Thermo Scientific™ Pierce™ Trypsin Protease, MS grade, Cat. No. 90058

- Promega™ rAsp-N, Mass Spec Grade, 1 × 10 µg, Cat. No. VA1160
- Invitrogen™ Ammonium acetate (5 M), Cat. No. AM9071
- Thermo Scientific™ Pierce™ DTT (Dithiothreitol), No-Weigh™ Format, Thermo Scientific, Cat. No. A39255
- Thermo Scientific™ Pierce™ Iodoacetamide, No-Weigh™ Format, Cat. No. A39271
- Thermo Scientific™ MAbPac™ SEC-1 Column, Cat. No. 075592
- Thermo Scientific™ Hypersil GOLD™ Peptide Column, Cat. No. 26002-152130

Sample preparation

Native intact mass analysis

Commercially available KADCYLA was diluted to 20 mg/mL using ddH_2O.

Peptide mapping

1. KADCYLA (200 µg) was diluted to 1 mg/mL using denaturing buffer (7 M guanidine hydrochloride, 50 mM Tris-HCl, pH=8.3).
2. The sample was reduced at 37°C for 30 minutes with 10 mM DTT, followed by 50 mM iodoacetamide alkylation at room temperature in the dark for 20 minutes. Then, the reaction was quenched by additional 10 mM DTT.
3. Buffer exchange was performed using an Amicon Ultra-0.5 centrifugal filter unit (10k) at 14,000 g with 10 min centrifugation, 4 times using 50 mM Tris-HCl (pH=7.5).
4. This solution was then aliquoted for trypsin digestion and AspN digestion. Trypsin digestion was performed at a 1:20 enzyme/protein ratio at 37°C for 4 hours. AspN digestion was performed at a 1:50 enzyme/protein ratio at 37°C for 5 hours.
5. The digested solutions were acidified with 1 µL of formic acid and then transferred to a sample vial for LC-MS analysis.

Chromatography

A Vanquish Horizon Binary UHPLC system, MAbPac SEC-1 column, and Hypersil GOLD Peptide column were employed for chromatographic separation.

For all experiments, the Vanquish Horizon UHPLC system was used, consisting of the following modules:

- Thermo Scientific™ System Base Vanquish™ Horizon/Flex, Cat. No. VF-S01-A-02
- Thermo Scientific™ Vanquish™ Binary Pump H, Cat. No. VH-P10-A-02
- Thermo Scientific™ Vanquish™ Split Sampler HT, Cat. No. VH-A10-A-02
- Thermo Scientific™ Vanquish™ Column Compartment H, Cat. No. VH-C10-A-03
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector VWD-F, Cat. No. VF-D40-A
- Thermo Scientific™ Viper™ MS Connection Kits for Vanquish™ LC System, Cat. No. 6720.0405

For native intact mass analysis, the column temperature was set at 30°C and the autosampler temperature at 10°C. Solvent A was 100 mM ammonium acetate. The flow rate was 200 µL/min and the injection volume was 1 µL (20 µg). An isobaric gradient of 100% A for 10 minutes was used for elution.

For peptide mapping, the UHPLC conditions are listed in Table 1.

Table 1. UHPLC conditions for peptide mapping.

Parameter	Value
Column	Hypersil GOLD Peptide column (Cat. No. 26002-152130)
Mobile phase A	Water with 0.1% formic acid (v/v)
Mobile phase B	Acetonitrile with 0.1% formic acid (v/v)
Flow rate	300 µL/min
Column temperature	50°C
Autosampler temperature	10°C
Sample injection volume	7 µL (10 µg)
Gradient	Linear
	Time (min) % B 0.0 1 5.0 1 6.0 10 70.0 60 72.0 90 77.0 90 79.0 1 82.0 1

Mass spectrometry

A Thermo Scientific Orbitrap Excedion Pro Biopharma hybrid mass spectrometer was used for data acquisition. The MS settings are found in Tables 2 and 3.

Data processing

Data analysis was performed using Thermo Scientific™ Biopharma Finder™ Software (version 5.4), Proteome Discoverer™ Software (version 3.2), and the Ardia™ Platform (version 1.1). The Biopharma Finder software data processing parameters for native intact MS are listed in Table 4, and parameters for peptide mapping sequence coverage and common modifications are listed in Table 5. Proteome Discoverer software parameters for conjugation sites identification can be found in Figure 3.

Table 2. MS settings for native intact analysis.

Parameter	Value
Source parameters	
Spray voltage (V)	+3,400
Sheath gas (Arb)	35
Aux gas (Arb)	5
Sweep gas (Arb)	1
Ion transfer tube temp. (°C)	250
Vaporizer temp (°C)	150
Application mode	Intact protein: high pressure
Full MS	
Orbitrap resolution	60,000
Scan range (*m/z*)	4,000–12,000
Maximum injection time (ms)	100
Microscans	10
AGC target	Custom
Normalized AGC target (%)	100
RF lens (%)	200
Maximum injection time mode	Custom
Data type	Profile
Source fragmentation (eV)	125

Table 3. MS settings for peptide mapping.

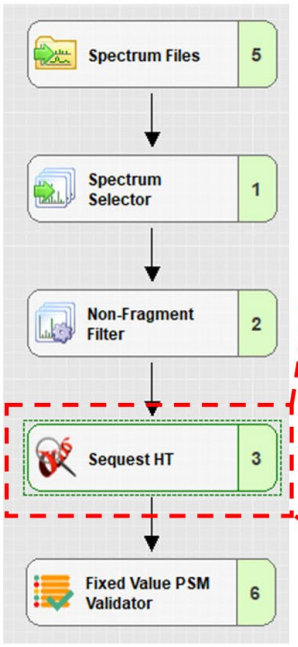
Parameter	Value
Source parameters	
Spray voltage (V)	+3,400
Sheath gas (Arb)	40
Aux gas (Arb)	10
Ion transfer tube temp. (°C)	320
Vaporizer temp (°C)	250
Application mode	Peptide
Full MS	
Orbitrap resolution	120,000
Scan range (*m/z*)	200–2,500
Maximum injection time (ms)	100
AGC target	Custom
Normalized AGC target (%)	300
Maximum injection time mode	Custom
RF lens (%)	40
Dynamic exclusion mode	Custom
Exclude after n times	1
Exclusion duration (s)	10
Excl. mass width	ppm
Mass tolerance high	10
Mass tolerance low	10
ddMS2	
Include charge states	2–8
Number of dependent scans	5
Isolation window (*m/z*)	2
Activation type	ETD
Use calibrated charge dependent ETD parameters	True
ETD supplemental activation	True
SA collision energy (%)	25
Orbitrap resolution	60,000
Scan range (*m/z*)	110–2,500
Maximum injection time (ms)	250
Microscans	1
AGC target	Custom
Normalized AGC target (%)	400
Maximum injection time mode	Custom
Data type	Profile

Table 4. Biopharma Finder software data processing parameters for native intact MS.

Component detection	Value
Chromatogram and source spectra	
m/z range	5,000 to 7,000
Source spectra method	Sliding Windows
Sliding Windows definition	
RT range (min)	5.0 to 7.5
Target avg. spectrum width (min)	0.1
Target avg. spectrum offset	Scan offset = 1
Sliding Windows merging parameters	
Merge tolerance (ppm)	30
Merge scheme	Legacy merge scheme
Max RT gap (min)	1
Min. number of detected intervals	3
Deconvolution algorithm	
Deconvolution algorithm	ReSpect™
Deconvolution results filter	
Output mass range	147,000 to 160,000
Deconvoluted spectra display mode	Isotopic profile (new)
Charge state distribution	
Deconvolution mass tolerance (ppm)	15
Choice of peak model	Intact protein
Model mass range	147,000 to 160,000
Charge state range	5 to 100
Minimum adjacent charges	4 to 4
Rel. abundance threshold (%)	0
Quality score threshold	0
Target mass (Da)	160,000
Number of peak models	1
Left/right peak shape	Left = 2, Right = 2
Peak detection minimum significance measure	1 Standard deviation
Peak detection quality measure (%)	95
Peak model width factor	1
Intensity threshold scale	0.01
Noise compensation	TRUE
Charge carrier	H+
Identification	
Sequence matching mass tolerance (ppm)	20
Enable drug-to-antibody ratio	TRUE
Drug payload	MCC_DM1
Residue	K
Average mass	957.5242
Multiconsensus component merge	
Mass tolerance (ppm)	10
RT tolerance (min)	1
Minimum number of required occurrences	1
N-glycoforms	G0F/G1F/G2F

Table 5. Biopharma Finder software data processing parameters for peptide mapping sequence coverage and common modifications.

Component detection	Value
Select task to be performed	
Find all ions in the run	
Peak detection	
Absolute MS signal threshold (MS noise level * S/N threshold)	9.28E+05
MS noise level	11,600
S/N threshold	80
Beginning peak width (min)	0.52
Typical chromatographic peak width (min)	0.52
Ending peak width (min)	1.52
Maximum chromatographic peak width (min)	4.55
Relative MS signal threshold (% of base peak)	1
Width of Gaussian filter	3
Minimum valley to be considered as two chromatographic peaks (%)	80
Minimum MS peak width (Da)	1.2
Maximum MS peak width (Da)	4.2
Mass tolerance (ppm)	4
Ion alignment	
Maximum retention time shift (min)	2.63
Mass measurement	
Maximum mass (Da)	30,000
Mass centroiding cutoff (% from base)	15
Identification	
Peptide identification	
Search by Full MS only	No
Use MS/MS	Use all MS/MS
Maximum peptide mass	15,000
Mass accuracy (ppm)	5
Minimum confidence	0.8
Maximum number of modifications for a peptide	3
Advanced search	
Static modifications	Carbamidomethylation (C)
Variable modifications	• Gln→Pyro-Glu (Nterm) • Deamidation (NQ) • Double Oxidation (MWC) • NH_3 loss (NQ) • Oxidation (MW) • MCC_DM1(NTerm) • MCC_DM1(K) • Isomerization (D)
Glycosylation	CHO
Search for amino acid substitutions	None
Perform disulfide search	No
Protease	Trypsin or AspN
Specificity	Medium



1. Input Data	
Protein Database	Trastuzumab.fasta
Enzyme Name	Trypsin (Semi) or AspN (Full)
Max. Missed Cleavage Sites	3
Min. Peptide Length	4
Max. Peptide Length	144
2. Tolerances	
Precursor Mass Tolerance	15 ppm
Fragment Mass Tolerance	0.05 Da
Use Average Precursor Mass	False
Use Average Fragment Mass	False
3. Spectrum Matching	
Use Neutral Loss a Ions	True
Use Neutral Loss b Ions	True
Use Neutral Loss y Ions	True
Use Flanking Ions	True
Weight of a Ions	0
Weight of b Ions	1
Weight of c Ions	1
Weight of x Ions	0
Weight of y Ions	1
Weight of z Ions	1
4. Dynamic Modifications	
Max. Equal Modifications Per Peptide	3
1. Dynamic Modification	Oxidation / +15.995 Da (M)
2. Dynamic Modification	MCC_DM1 / +956.364 Da (K)
3. Dynamic Modification	Deamidated / +0.984 Da (N, Q)
4. Dynamic Modification	None
5. Dynamic Modification	None
6. Dynamic Modification	None
5. Dynamic Modifications (peptide terminus)	
1. N-Terminal Modification	None
2. N-Terminal Modification	None
3. N-Terminal Modification	None
1. C-Terminal Modification	None
2. C-Terminal Modification	None
3. C-Terminal Modification	None
6. Dynamic Modifications (protein terminus)	
1. N-Terminal Modification	MCC_DM1_N_term / +956.364 Da (N-Terminus)
2. N-Terminal Modification	None
3. N-Terminal Modification	None
1. C-Terminal Modification	None
2. C-Terminal Modification	None
3. C-Terminal Modification	None
7. Static Modifications	
Peptide N-Terminus	None
Peptide C-Terminus	None
1. Static Modification	Carbamidomethyl / +57.021 Da (C)

Figure 3. Proteome Discoverer software data processing parameters for conjugation sites identification.

Results

The design of the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer, as schematically shown in Figure 4, offers an extended mass range up to *m/z* 12,000 with "Intact Protein" application mode, which enables characterization of large molecules.

The implementation of fast and sensitive electron transfer dissociation (ETD) and electron-transfer/higher-energy collision dissociation (EThcD) provides an alternative fragmentation technique to higher-energy collision dissociation (HCD).

EThcD fragmentation can provide additional confidence in assignments and complementary sequence information, specifically for isomeric amino acid distinction as well as side chain modification identification and localization. The high sensitivity of this instrument also facilitates confident identification of low-level modifications.

Native intact MS analysis and DAR measurement

With conjugation at lysine residues, a distribution of DARs ranging from 0 to 8 drugs has been reported for KADCYLA,[4] indicating high structure heterogeneity. To reduce the interference from adjacent MS signals, intact MS under native condition is essential

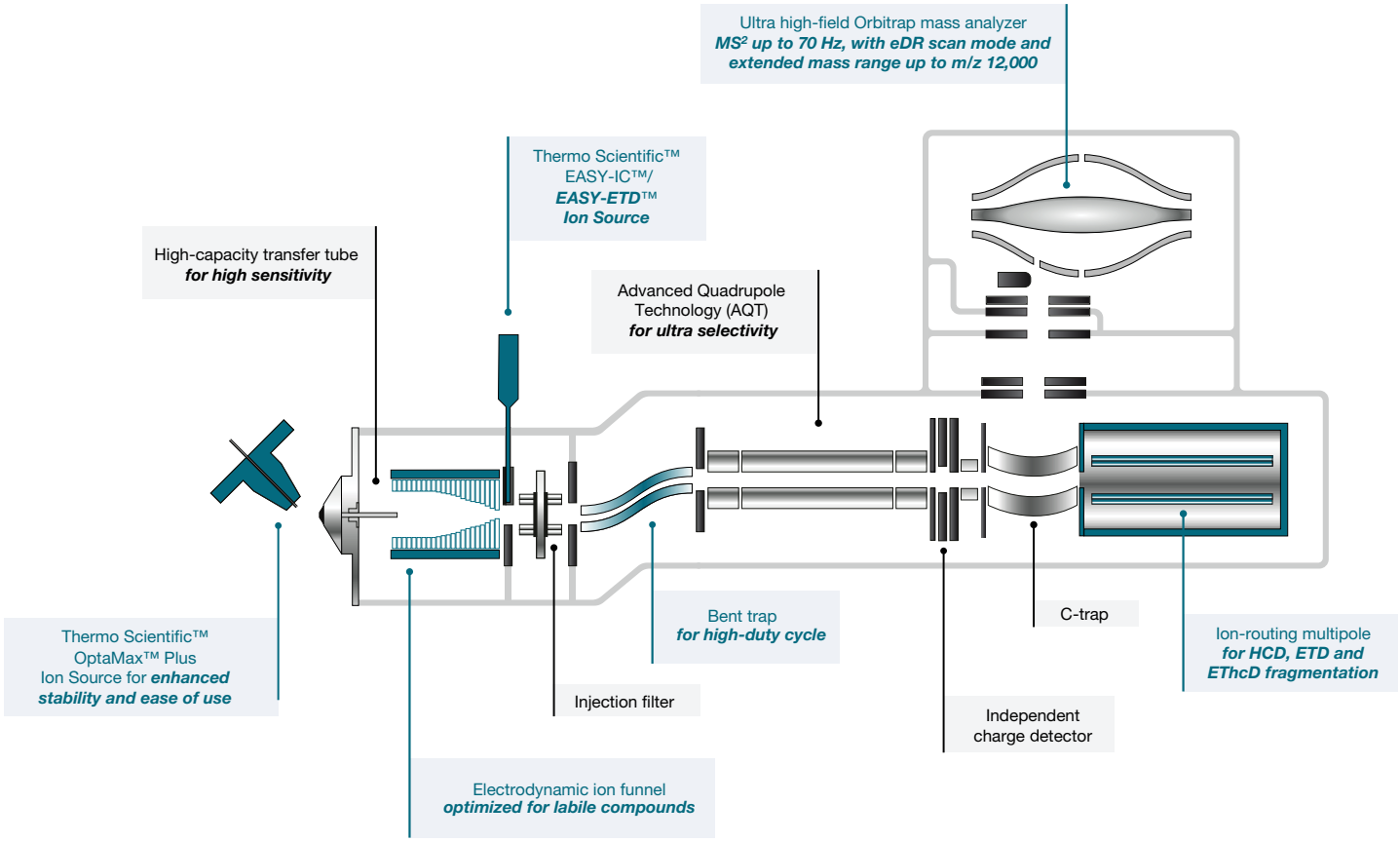


Figure 4. Schematics of the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer.

for accurate measurement of molecular weight and DAR at the intact ADC level. Native intact mass analysis was performed by isocratic separation using a MAbPac SEC-1 column with subsequent mass detection on the Orbitrap Excedion Pro BioPharma hybrid MS, which enables mass detection up to *m/z* 12,000. High-quality raw spectra were obtained for the KADCYLA sample without deglycosylation. The observed envelope for KADCYLA represents charge states from +22 to +29, detected over a mass range of *m/z* 5,000–7,000 (Figure 5A). Figure 5B displays the deconvoluted spectrum, where the ADC 0–8 DAR distribution can be observed. High-quality native intact mass data enables the detection of low levels of intact ADC D0 G0F/G1F, with relative abundances of 3.19%. The average DAR was automatically calculated as 3.46 by BioPharma Finder software, in agreement with previous publications[4].

Confident identification of conjugated peptides and precise localization of conjugation sites by peptide mapping using EThcD fragmentation

Peptide mapping is a widely used analytical method to characterize biopharmaceuticals, providing detailed information about sequence coverage, PTM identification, localization, relative quantification, sequence variants, and disulfide bonds mapping. In this study, 10 µg of trypsin- or AspN-digested KADCYLA was loaded, achieving 100% sequence coverage with MS/MS identification for both enzyme-digested samples (Figure 6A-B). Using two different enzymes provides complementary peptide identification information. Both HCD and EThcD were employed for peptide fragmentation. HCD generates rich b/y ions for peptide sequence identification, but it also fragments the conjugated payload, making conjugation site localization challenging, especially for peptides with multiple lysines. For EThcD on the Orbitrap Excedion Pro Biopharma hybrid mass spectrometer, c- and z*-ions are first formed by electron transfer dissociation using fluoranthene radical anions as reagent inside the ion routing multipole. The generated fragments are then transferred to the C-trap and accelerated back into the ion routing multipole for supplemental higher-energy collisional activation, further improving c- and z*-fragmentation efficiency and allowing the generation of additional HCD type ions (b/y). This design provides fast and sensitive EThcD fragmentation with short reaction times. EThcD on the Orbitrap Excedion Pro BioPharma hybrid mass spectrometer enables peptide fragmentation while keeping conjugated payloads intact, aiding in the localization of conjugation sites on peptides with multiple modification sites.

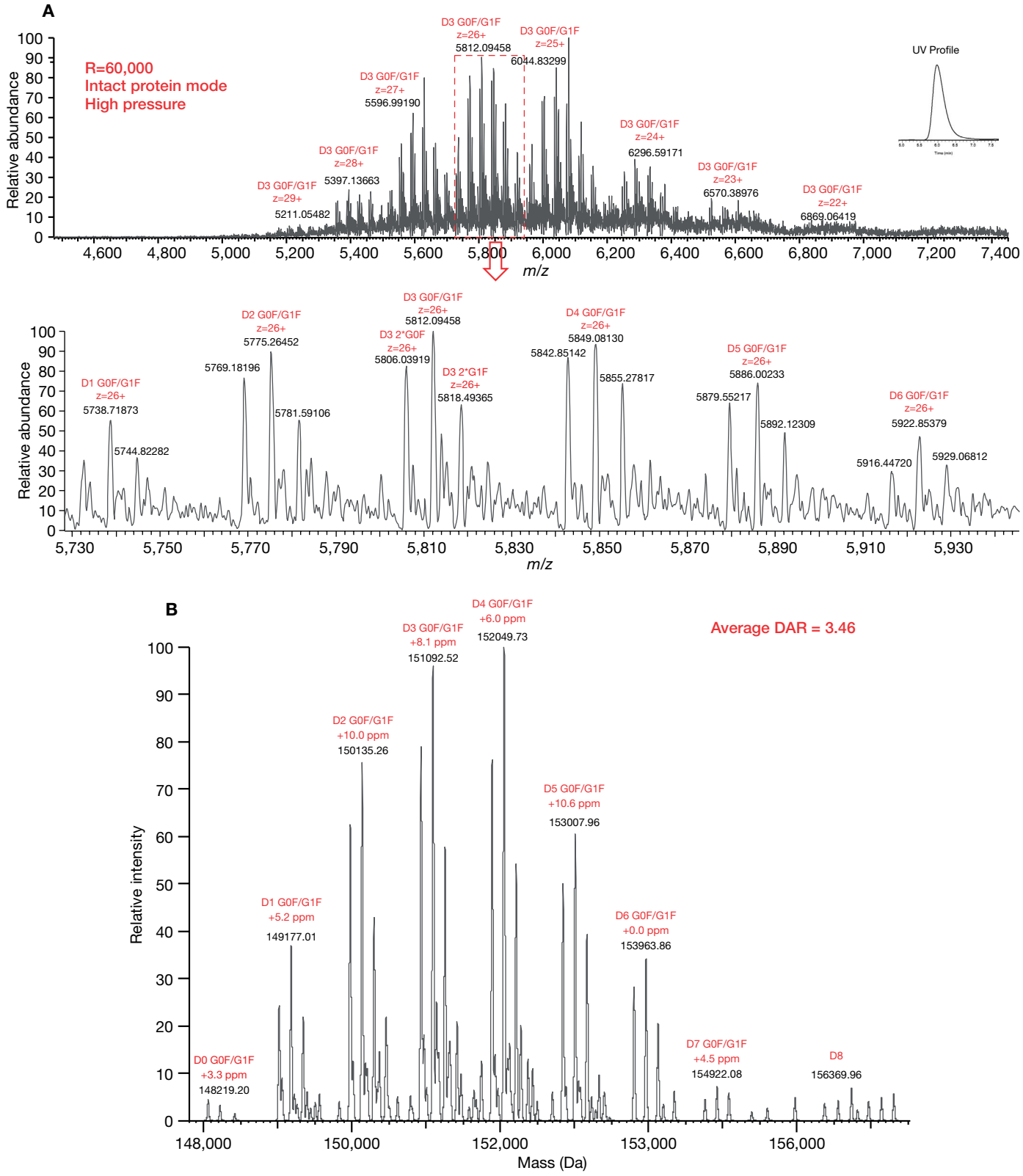


Figure 5. Native intact mass analysis of KADCYLA. (A) Full MS spectrum and zoom in spectrum of +26 charge envelope from intact KADCYLA under near native conditions. Data was acquired at a resolution setting of 60,000 at *m/z* 200 using intact protein application mode, high pressure. (B) Deconvoluted mass spectrum using the Sliding Windows and ReSpect algorithms. For G0F/G1F from D0 to D7, the mass accuracy was all less than 11 ppm, which proved the excellent mass accuracy of this platform for native intact mass analysis.

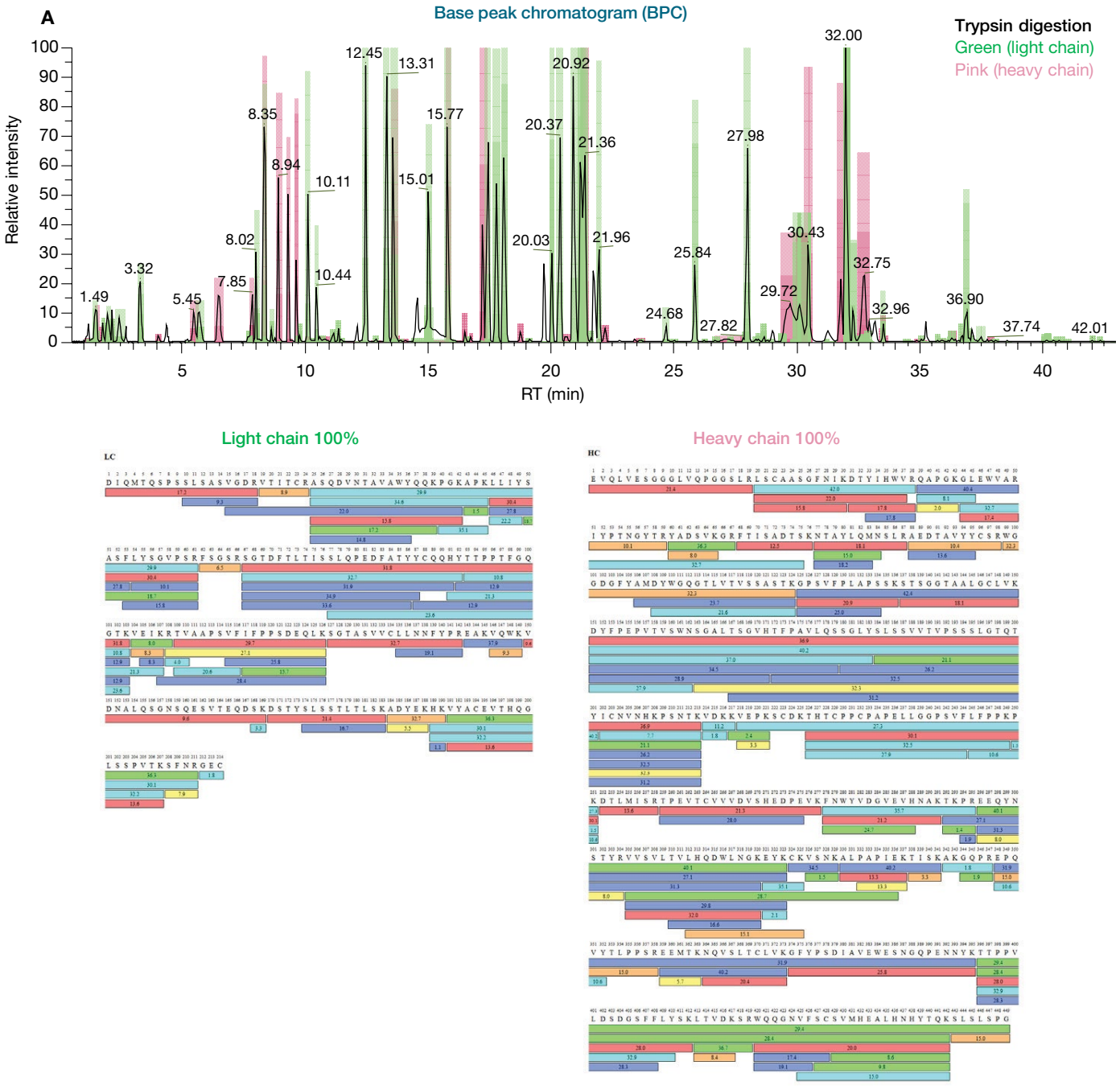


Figure 6A. Peptide mapping sequence coverage using a 65-minute gradient and EThcD fragmentation (supplemental activation energy = 25%). Full sequence coverage was achieved in a single injection for both enzymatic digestions. Base peak chromatograms and sequence coverage map of trypsin digestion are shown.

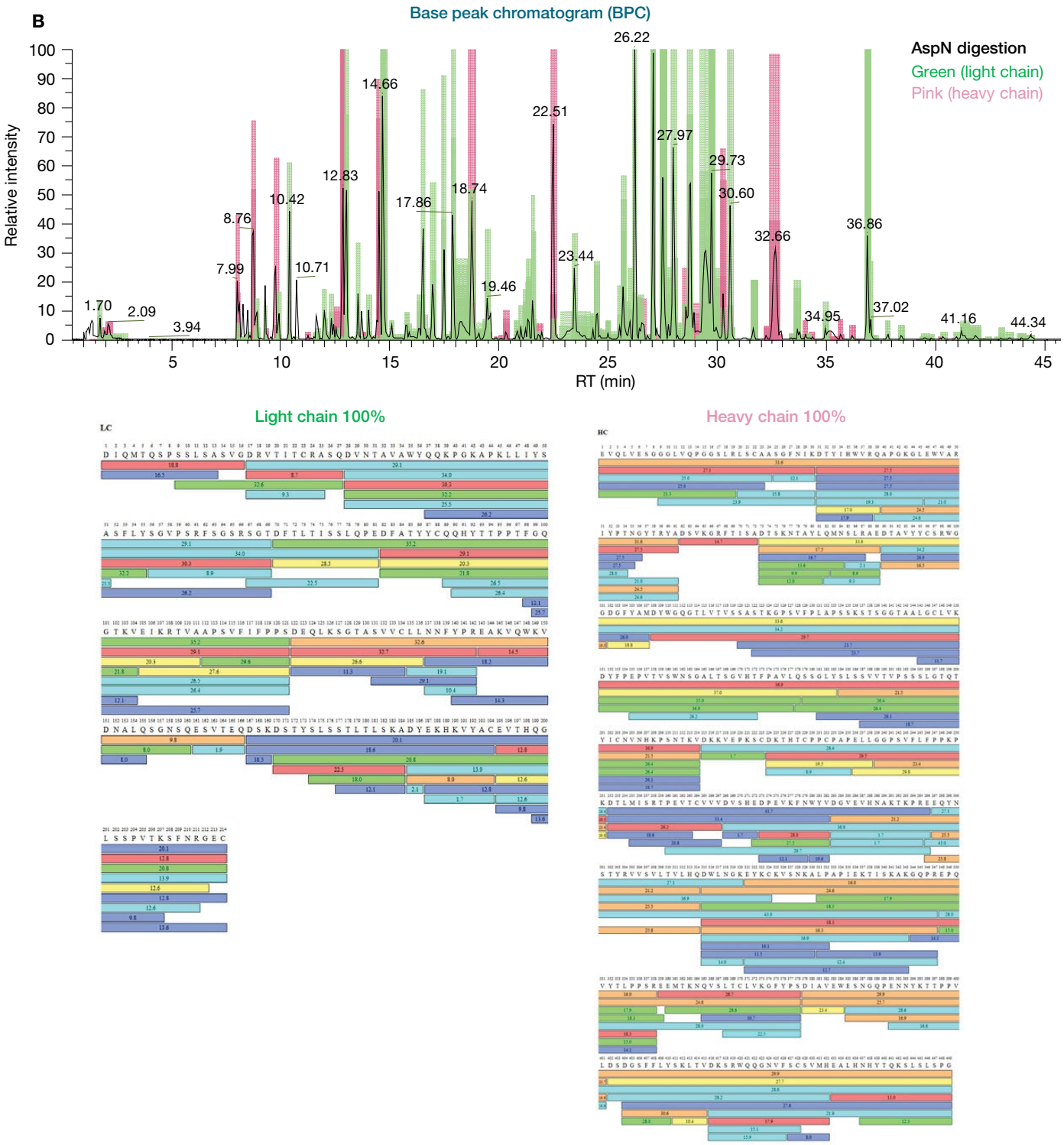


Figure 6B. Peptide mapping sequence coverage using a 65-minute gradient and EThcD fragmentation (supplemental activation energy = 25%). Full sequence coverage was achieved in a single injection for both enzymatic digestions. Base peak chromatograms and sequence coverage map of AspN digestion are shown.

There are 46 potential conjugation sites in total on the light chain (LC) and heavy chain (HC) of KADCYLA, including 44 lysines and two N-terminals, which means 92 conjugation sites for the intact molecule (Figure 7A). In this molecule, 43 out of 46 conjugation sites were identified using Proteome Discoverer software (Table 6). Figure 7B displays the EThcD MS2 spectrum of peptide ADYE*K(188)*HK*(190)* from the light chain, which contains two potential conjugation sites. EThcD fragmentation provided plenty of c/z ions for conjugation localization, as well as y ions. All these product ions can be used for peptide sequence identification and conjugation site confirmation. For this peptide, LC K188 was conjugated by linker-payload.

The structure of DM1 contains a stereocenter (Figure 7C). MCC-DM1 conjugation imparts a stereocenter and causes peptides to elute as a doublet in C18 reversed-phase LC. Figure 7C shows the XIC of peptide D*K(216)K(217)*VEP*K(221)* SC from the heavy chain, generated from AspN digestion. Stereocenter-caused doublet peak pairs elution can be observed in a narrow time range. The relative abundance of each isomeric peptide pair identified with the same linker-drug conjugation site was calculated using the combined diastereomers XIC peak area and are listed in the table. The high quality EThcD MS2 spectra provided sufficient information to pinpoint the different conjugation sites across these isomeric peptides. (Figure 7D).

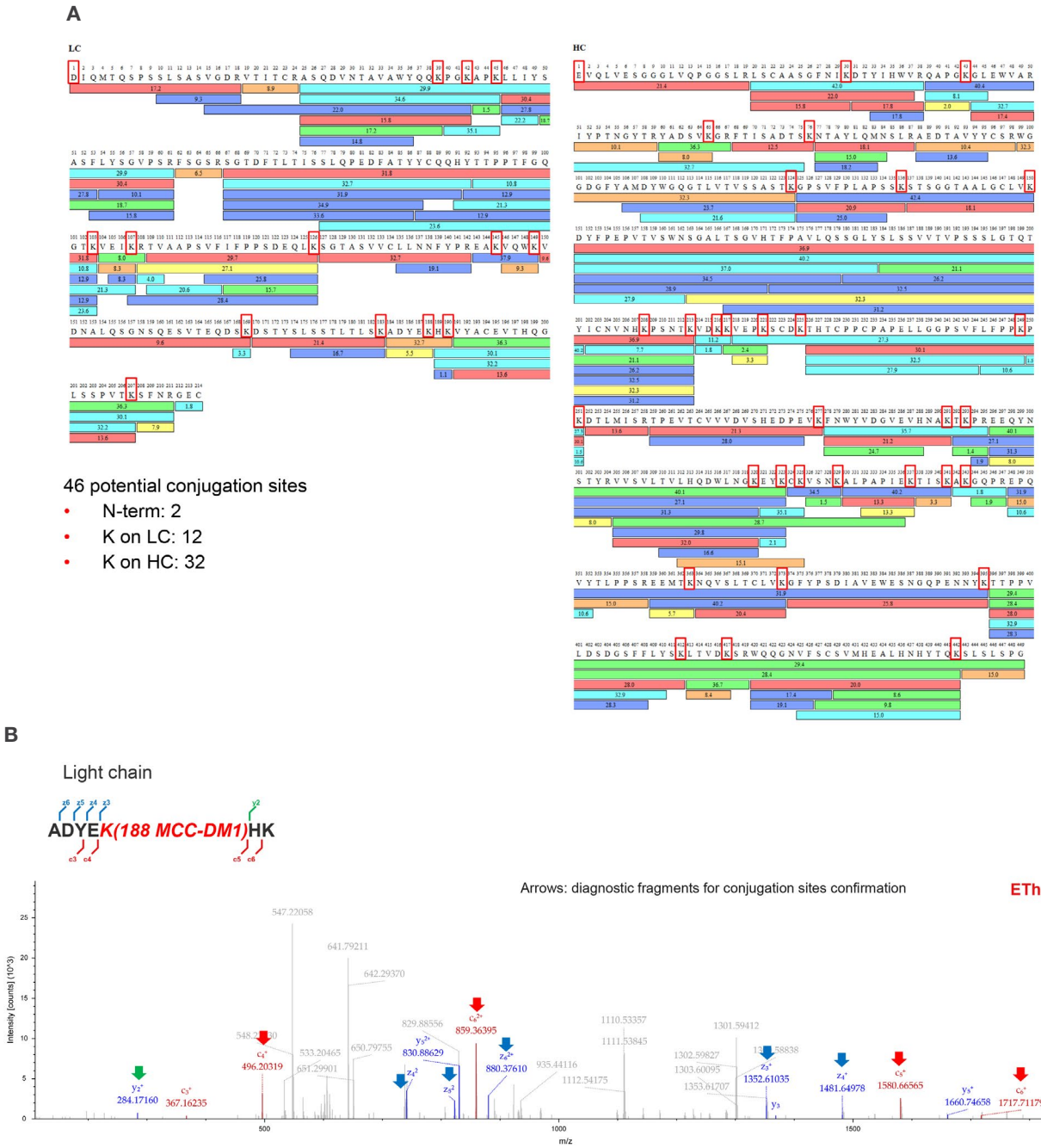
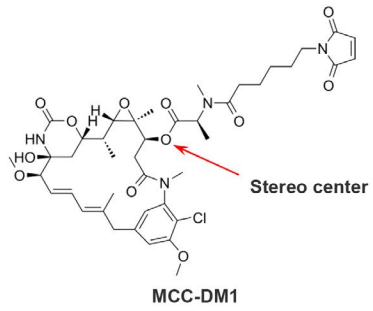
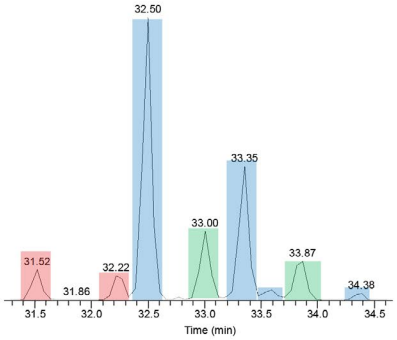


Figure 7A and B. Conjugated sites identification. (A) All potential conjugation sites in the protein sequence. (B) EThcD MS2 spectrum of peptide ADYE*K(188)HK*(190) from the light chain.

C





K216 conjugated

K217 conjugated

K221 conjugated

Site	Relative abundance
K216	9.98%
K217	35.19%
K221	4.43%
Non-conjugated	50.40%

D

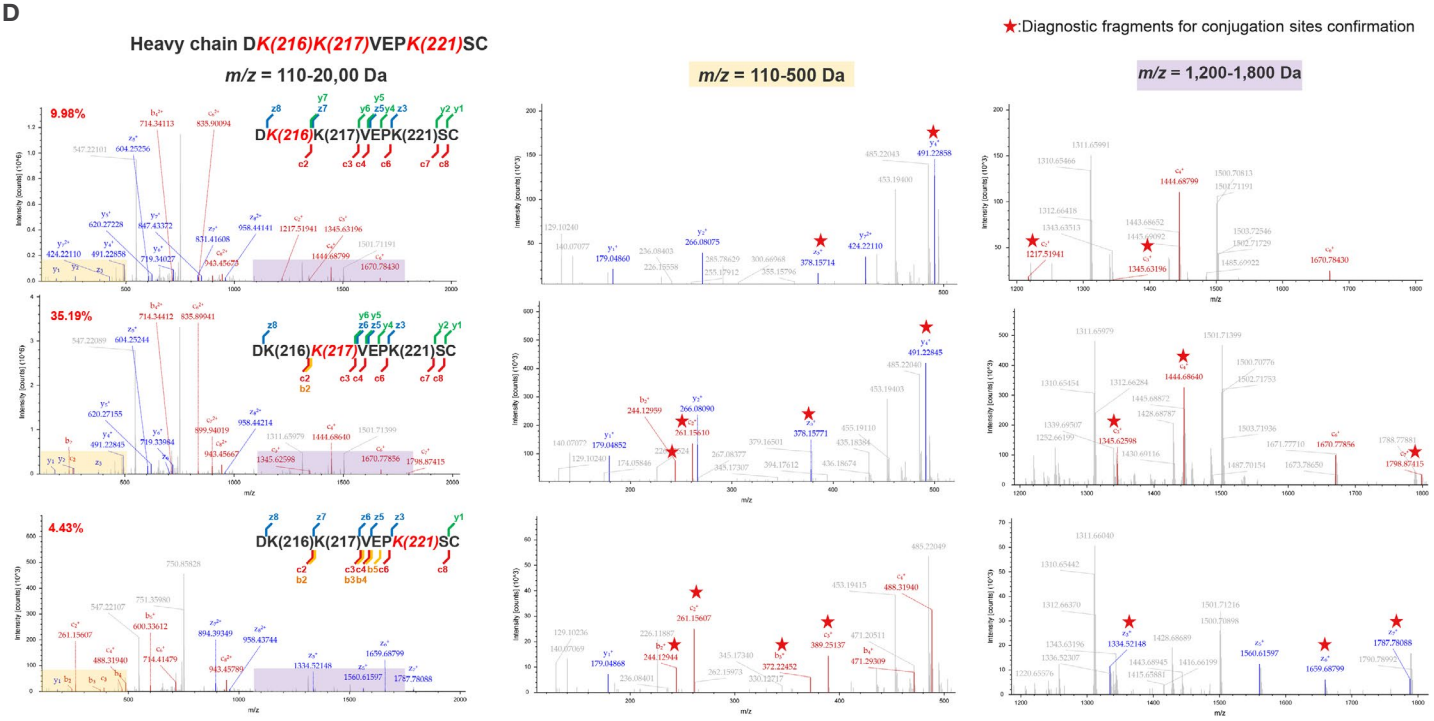
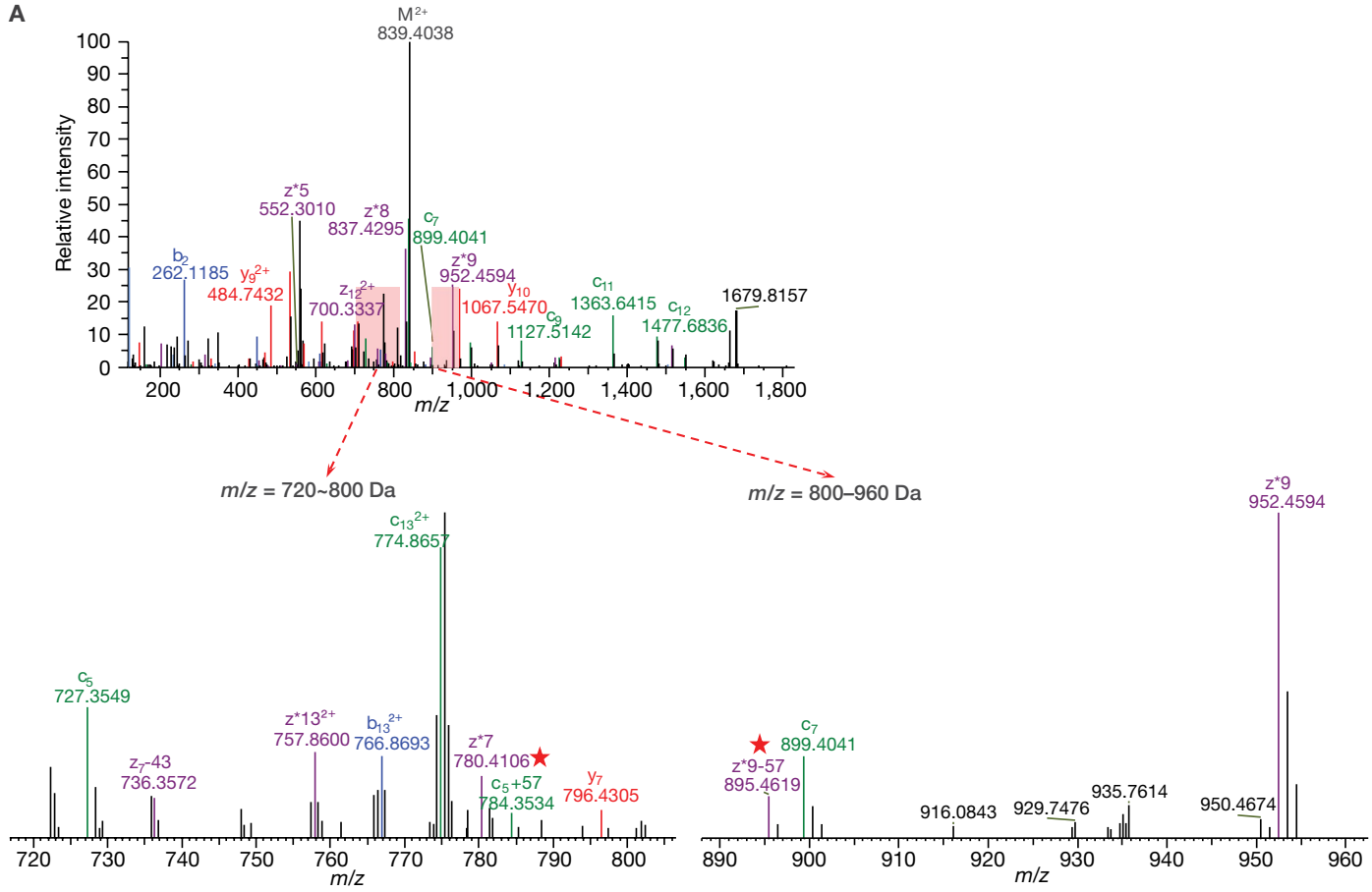


Figure 7C and D. Conjugated sites identification. (C) The structure of MCC-DM1, red arrow pointed to the stereocenter. The stereocenter-caused peak pairs were eluted in a 3-minute range, as labeled in the XIC. The relative abundance for the isomer pair was calculated using the XIC peak area. (D) EThcD MS2 spectra of peptide *DK(216)K(217)*VEP*K(221)*SC from the heavy chain, which has three conjugation sites. The EThcD MS2 spectra provided confident evidence for conjugation sites confirmation.

Table 6. Identified conjugation sites. "++" = characteristic fragments were identified for location. "+" = conjugation confirmed, lacking b/y ions coverage for conjugation sites. "-" = conjugation is not identified.

Trypsin				
Chain	Site	HCD MS2	EThcD MS2	Sequence
	N-term	-	++	*D*IQMTQSPSSLSASVGDR
LC	39	+	++	ASQDVNTAVAWYQQ*K*PGK
	42	+	++	ASQDVNTAVAWYQQKPG*K*APK
	45	+	++	AP*K*LLIYSASFLYSGVPSR
	103	+	++	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGT*K*VEIK
	107	+	++	VEI*K*R
	126	-	++	TVAAPSVFIFPPSDEQL*K*SGTASVVCLLNNFYPR
	145	+	++	EA*K*VQWK
	149	+	++	VQW*K*VDNALQSGNSQESVTEQDSK
	169	+	++	VDNALQSGNSQESVTEQDS*K*DSTYSLSSTLTLSK
	183	+	++	DSTYSLSSTLTLS*K*ADYEK
	188	+	++	ADYE*K*HK
	190	+	++	H*K*VYACEVTHQGLSSPVTK
	207	+	++	VYACEVTHQGLSSPVT*K*SFNR
HC	N-term	-	++	*E*VQLVESGGGLVQPGGSLR
	30	+	++	LSCAASGFNI*K*DTYIHWVR
	43	+	++	QAPG*K*GLEWVAR
	65	+	++	YADSV*K*GR
	76	+	++	FTISADTS*K*NTAYLQMNSLR
	124	+	++	WGGDGFYAMDYWGQGTLVTVSSAST*K*GPSVFPLAPSSK
	136	+	++	GPSVFPLAPSS*K*STSGGTAALGCLVK
	208	+	++	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNH*K*PSNTK
	213	+	++	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNT*K*VDK
	216	+	++	VD*K*K
	217	+	++	*K*VEPK
	221	+	++	VEP*K*SCDK
	225	+	++	SCD*K*THTCPPCPAPELLGGPSVFLFPPKPK
	249	+	++	THTCPPCPAPELLGGPSVFLFPP*K*PK
	251	-	++	THTCPPCPAPELLGGPSVFLFPPKP*K*DTLMISR
	277	+	++	TPEVTCVVVDVSHEDPEV*K*FNWYVDGVEVHNAK
	291	+	++	FNWYVDGVEVHNA*K*TKPR
	293	+	++	T*K*PR
	320	+	++	VVSVLTVLHQDWLNG*K*EYK
	323	+	++	EY*K*CK
	325	+	++	C*K*VSNK
	329	+	++	VSN*K*ALPAPIEK
	337	+	++	ALPAPIE*K*TISK
	341	-	++	TIS*K*AK
	343	+	++	A*K*GQPR
	363	+	++	EEMT*K*NQVSLTCLVK
	395	+	++	GFYPSDIAVEWESNGQPENNY*K*TTPPVLDSDGSFFLYSK
	417	+	++	LTVD*K*SR
	442	+	++	WQQGNVFSCSVMHEALHNHYTQ*K*SLSLSPG

As reported before,[5] EThcD fragmentation can provide c+57/z*-57 ion pairs for aspartic acid isomerization confirmation. Figure 8 displays the isomerization % of HC D283; the c+57/z*-57 ion pairs were automatically labeled by BioPharma Finder 5.4 software. Table 7 lists the isomerization % of HC D62, HC D283 and HC D404.



B

Fragment coverage map

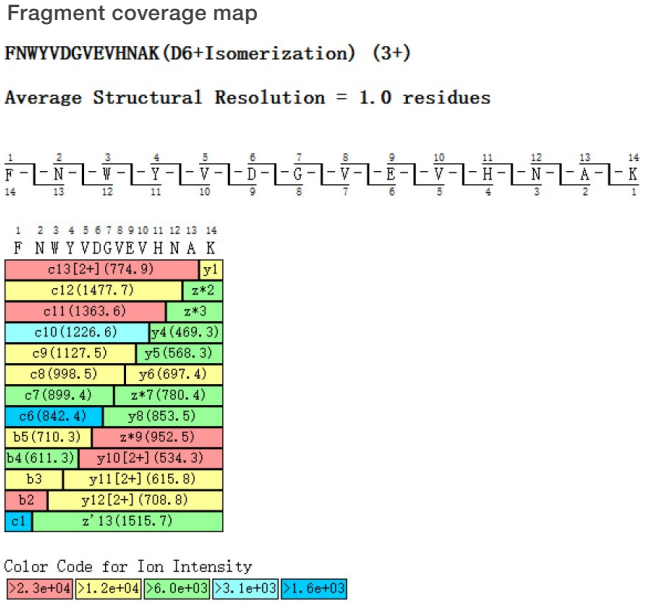


Table 7. Isomerization percentage for HC D62/D283/D404. All relative abundance percentages were calculated based on three replicate injections.

Residue #	Peptide sequence	Isomerization % avg.	CV
HC D62	YADSVK	0.06	24.12%
HC D283	FNWYVDGVEVHNAK	0.18	4.64%
HC D404	TTPPVLDSDGSFFLYSK	0.18	13.60%

Figure 8. Identification of low level isoaspartic acid. (A) EThcD MS2 spectrum for peptide FNWYV*D(283)*GVEVHNAK. The relative abundance of this isomerization is 0.06%. EThcD can generate b/y and c/z ions for peptide identification. The expanded view displayed c+57/z*-57 ions for isomerization confirmation. (B) Fragment coverage map for this peptide. For this low abundant modified peptide, the high quality MS2 spectrum provided evidence for confident identification results.

Other common modifications in biotherapeutic products, such as deamidation, succinimidation, and oxidation were identified and relatively quantified (Figure 9). The most abundant N-glycoform is A2G0F (51.02%). The lysine truncation% is 98.21%.

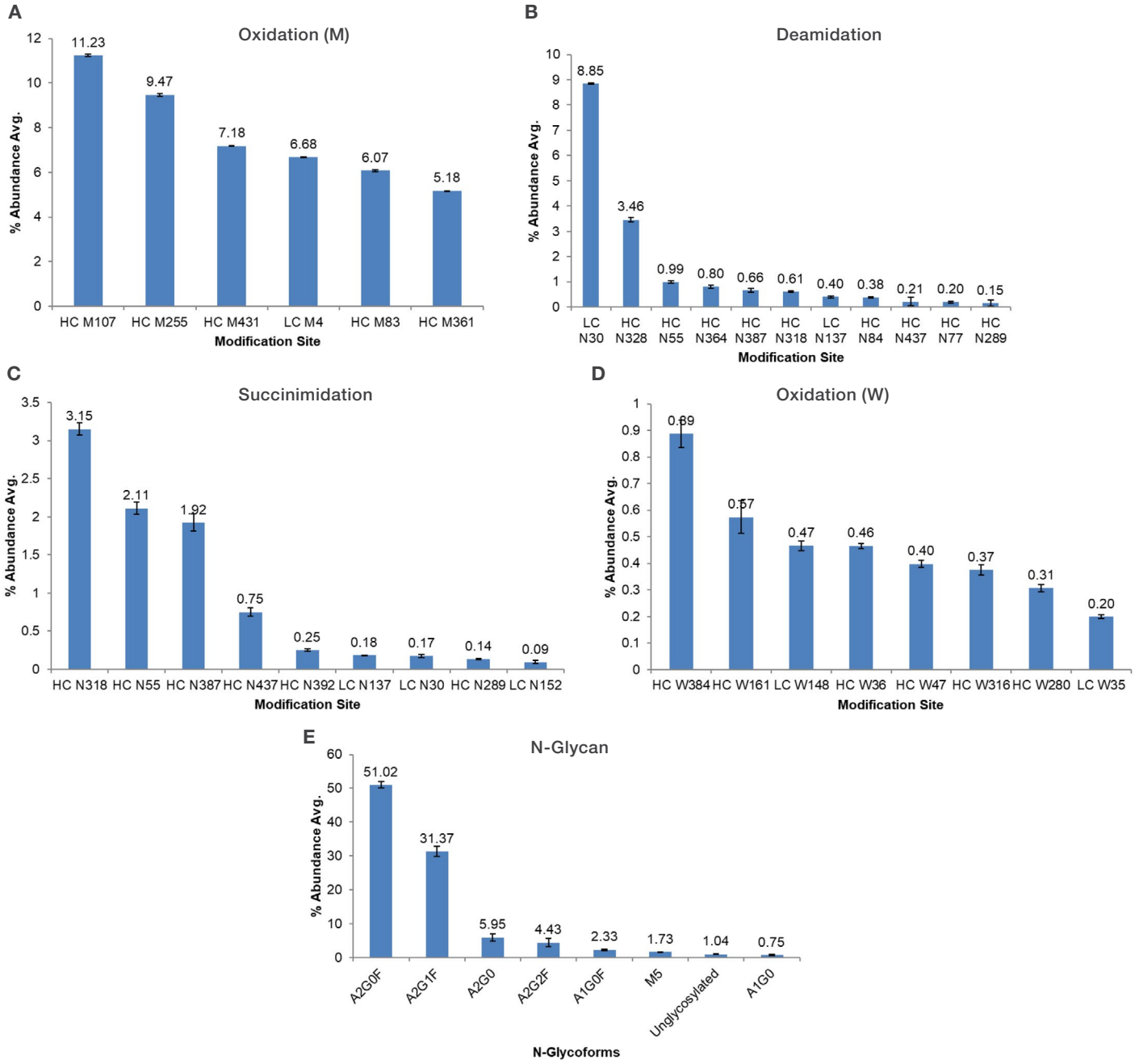


Figure 9. PTM report generated with the Ardia platform. A trypsin-digested KADCYLA sample, fragmented with EThcD was analyzed. (A) Methionine oxidation, (B) asparagine deamidation, (C) asparagine succinimidation, (D) tryptophan oxidation, (E) N-glycoforms on HC N300. All relative abundance percentages were calculated based on three replicate injections.

Conclusions

In this work, we demonstrated the outstanding performance of an Orbitrap Excedion Pro BioPharma hybrid mass spectrometer for in-depth characterization of KADCYLA, a lysine-linked antibody drug conjugate.

- High resolution and sensitivity benefit both native intact MS and peptide mapping analysis.
- Native intact mass allows accurate molecular weight (<11 ppm for main components) and DAR measurement.
- Both HCD and fast and sensitive EThcD fragmentation allow complete sequence coverage and characterization for multiple attributes in a single injection.
- HCD fragmentation provides confident sequence coverage and identification results for common modifications.
- Fast and sensitive EThcD fragmentation provides additional benefits for confident identification and localization of conjugation sites for peptides with multiple potential conjugation sites.
- In addition, fast and sensitive EThcD enables the differentiation of isomeric amino acids present at low level, as demonstrated in this study by confirming the identification of trace amounts of isoaspartic acid.
- Complementary and comprehensive N-glycoforms and PTMs identification results were obtained by combining HCD and fast and sensitive EThcD fragmentation results, illustrating the benefit of two orthogonal fragmentation modes on a single MS platform.

References

1. U.S. Food and Drug Administration Website. Drugs@FDA: FDA-Approved Drugs. (Accessed May 2025)
2. Fu, Z.; Li, S.; Han, S. et al. Antibody drug conjugate: The "biological missile" for targeted cancer therapy. *Signal Transduct. Target Ther.* **2022,** *7*(1), 93.
3. Ducry, L.; Stump, B. Antibody-drug conjugates: Linking cytotoxic payloads to monoclonal antibodies. *Bioconjug. Chem.* **2010,** *21*(1), 5.
4. Joubert, N.; Beck, A.; Dumontet, C.; Denevault-Sabourin, C. Antibody–drug conjugates: The last decade. *Pharmaceuticals* **2020,** *13,* 245.
5. Ni, W. et al. Analysis of isoaspartic acid by selective proteolysis with Asp-N and electron transfer dissociation mass spectrometry. *Anal. Chem.* **2010**, *82*(17), 7485.

Learn more at **thermofisher.com/proteintherapeutics**