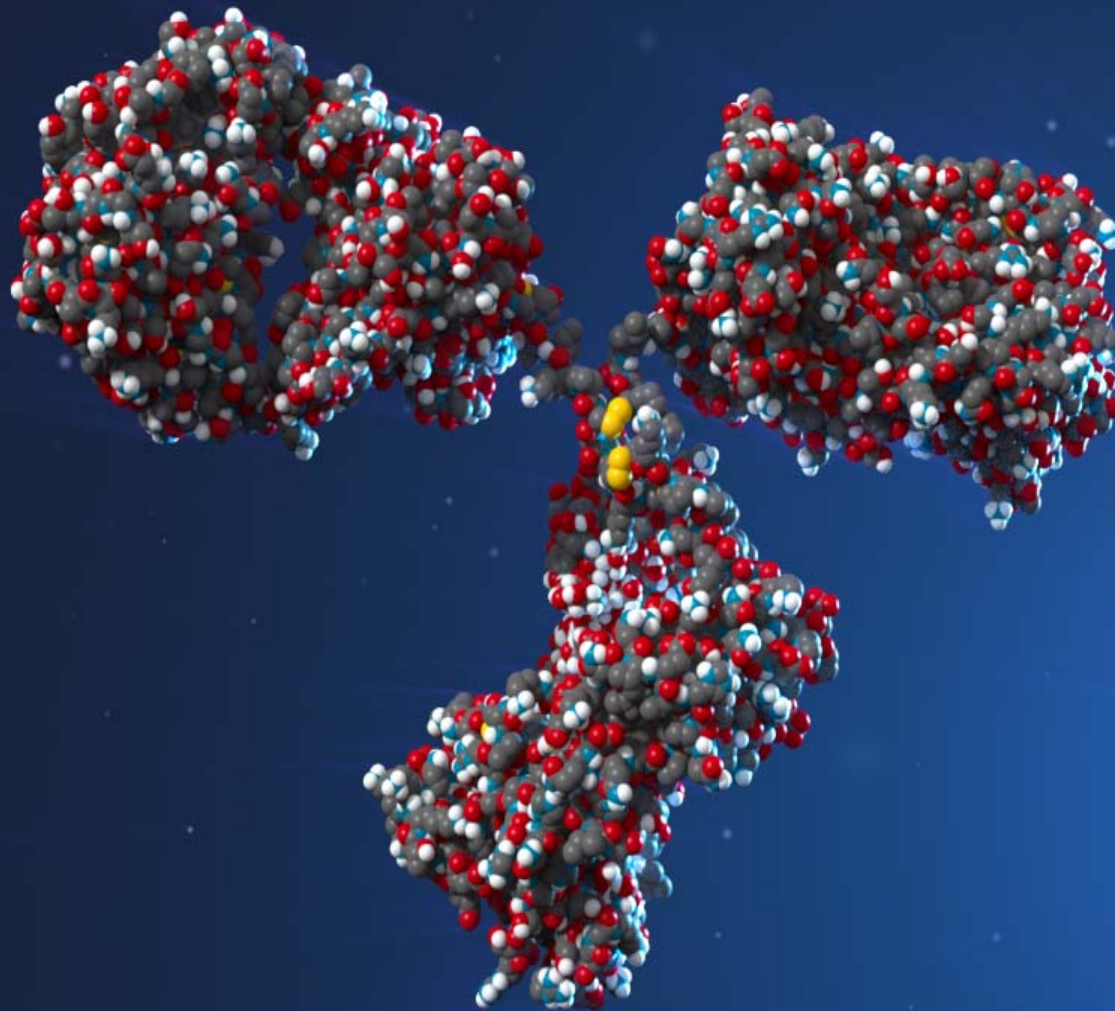
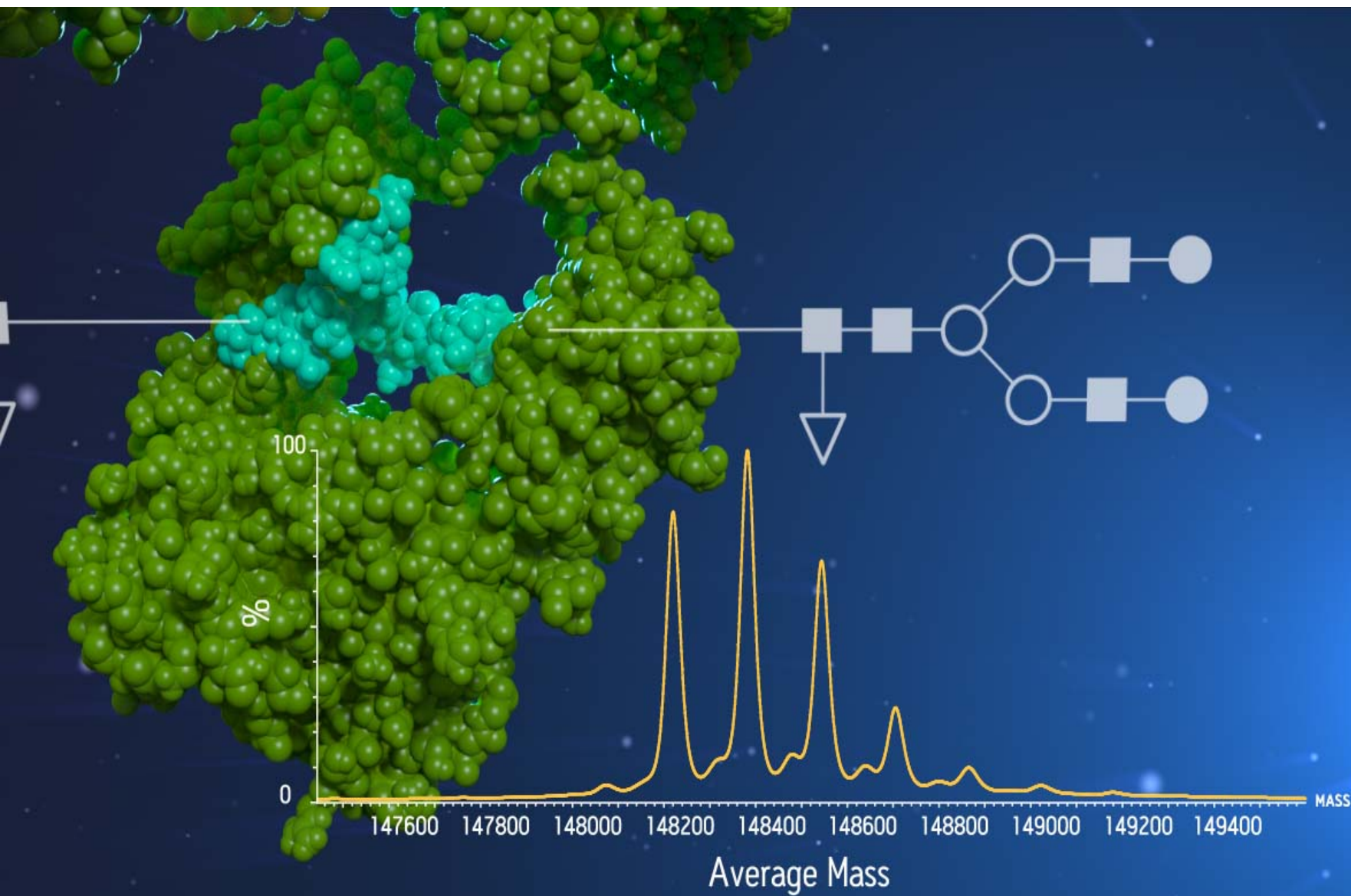


**Advanced Characterization
of Antibody Drug Conjugates (ADCs)
by Liquid Chromatography
and Mass Spectrometry (LC/MS)**

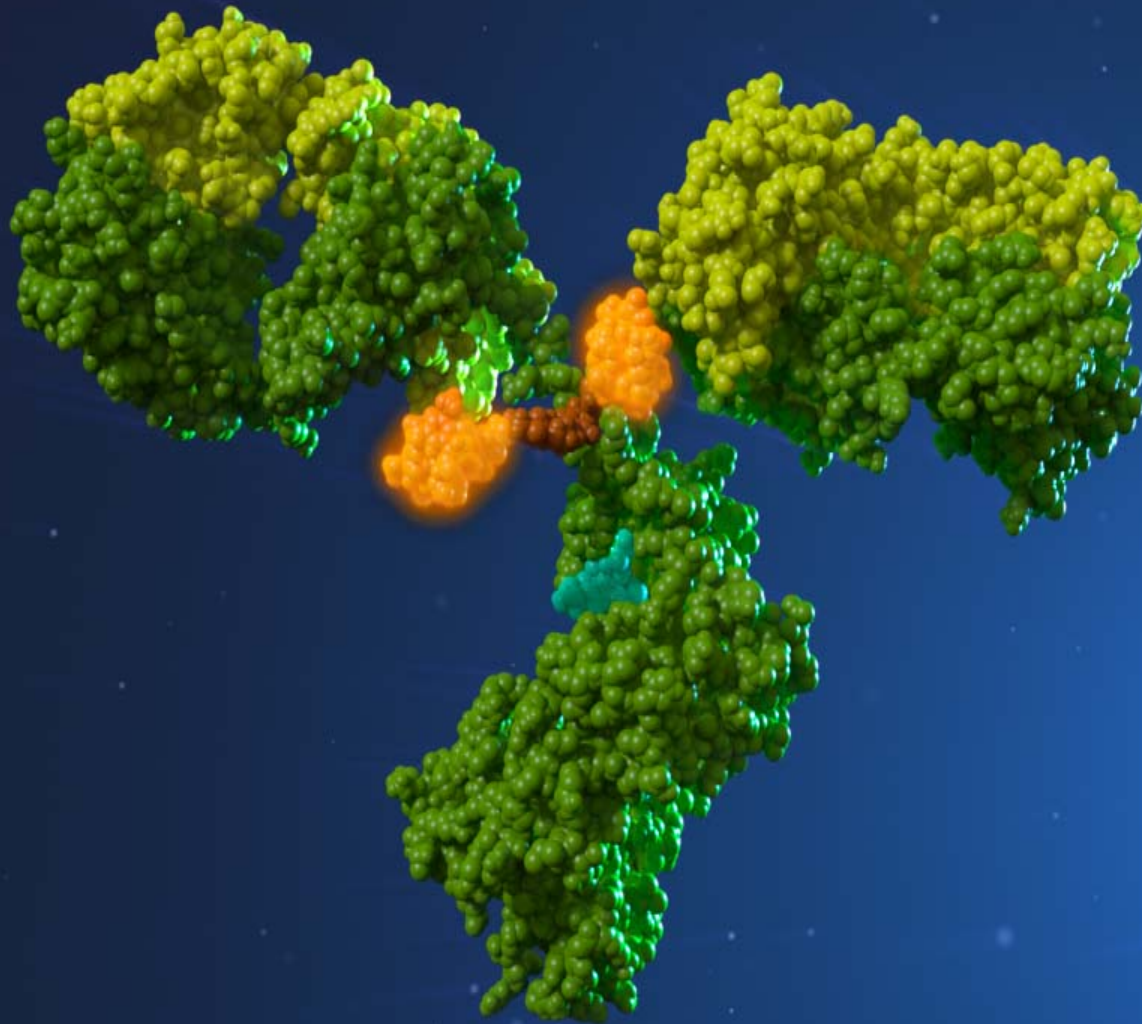
John Gebler, Ph.D.
Director



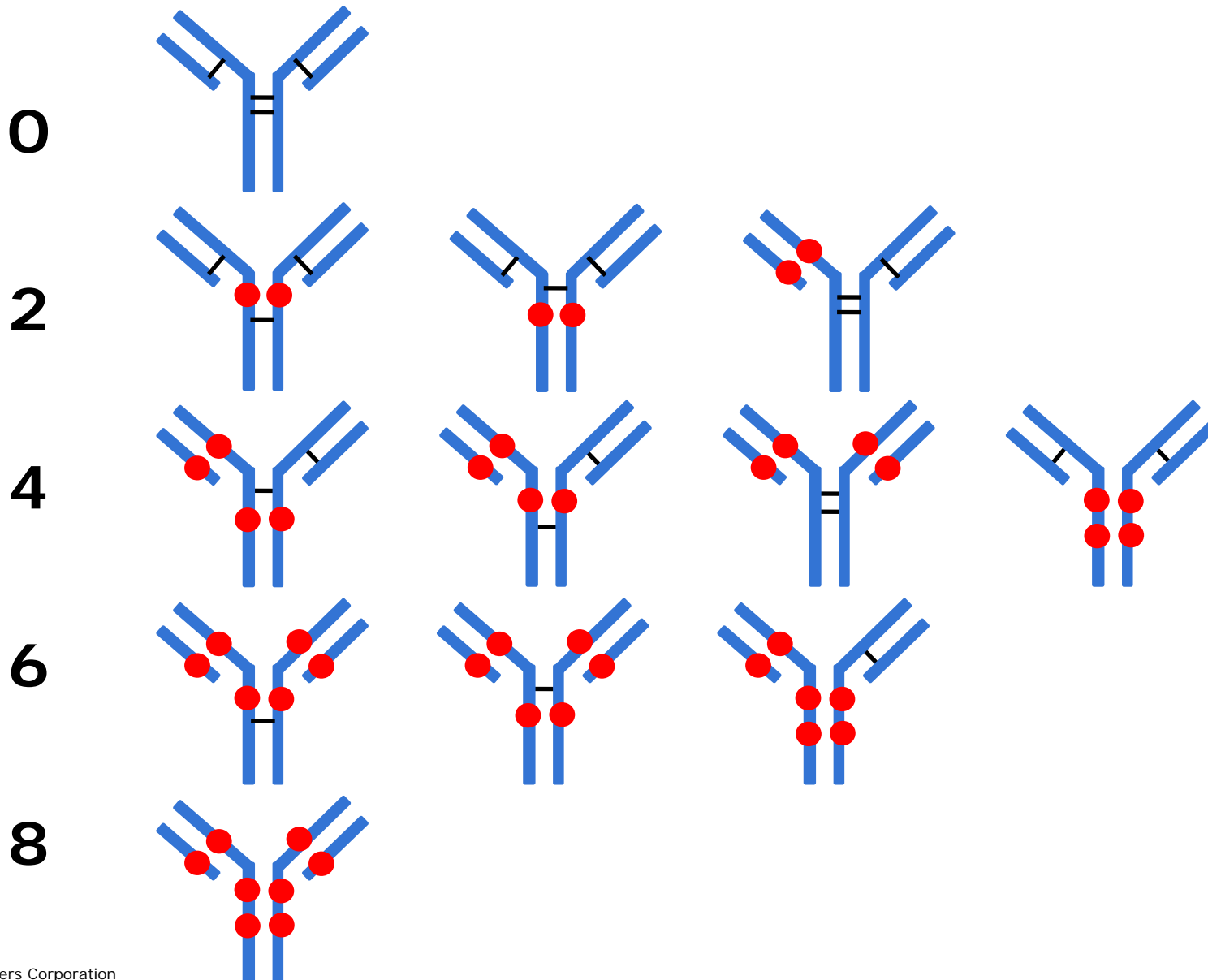


Intact ADC

Waters
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Cysteine Conjugation Location Possibilities "Positional Isomers"



Intact ADC Characterization

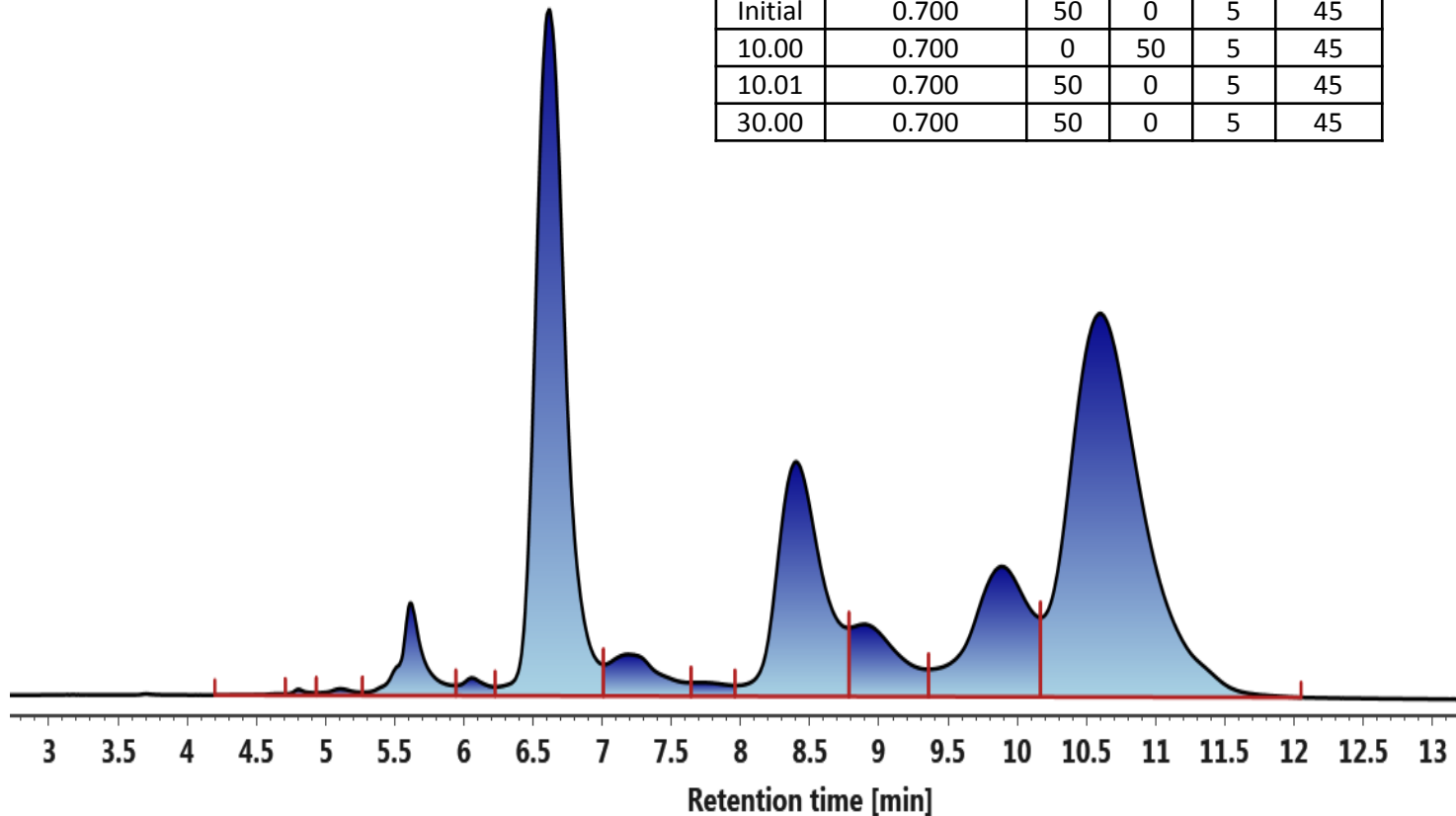
HIC LC/UV Characterization

HIC
4.6mm x 100 mm, 2.5 µm
Non-porous

A: 125 mM Phosphate buffer, pH 6.7 with 2.5 M (NH₄)₂SO₄
B: 125 mM phosphate buffer, pH 6.7
C: Isopropyl alcohol
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

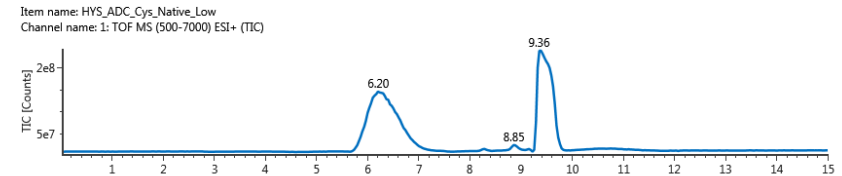
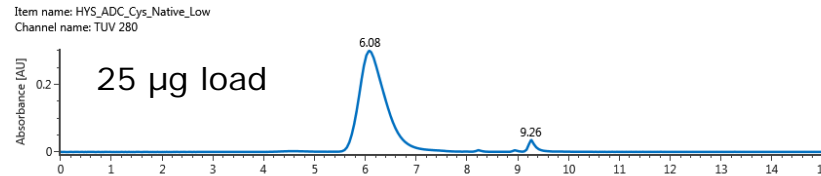


Cys ADC DAR via Intact Mass Analysis SEC-LC/Native MS (Deglycosylated)

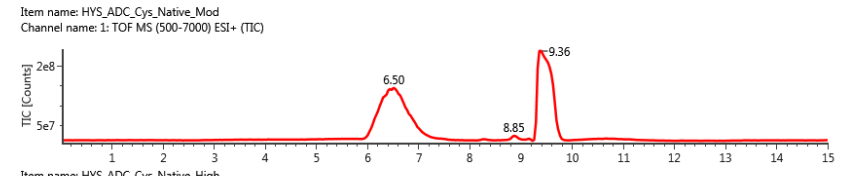
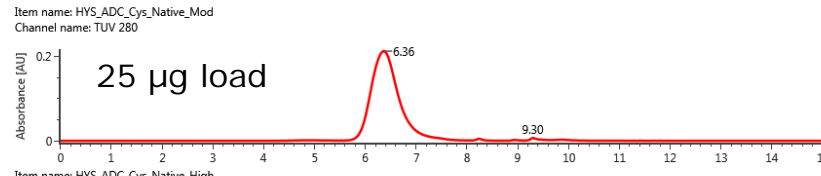
UV A280

QToF TIC

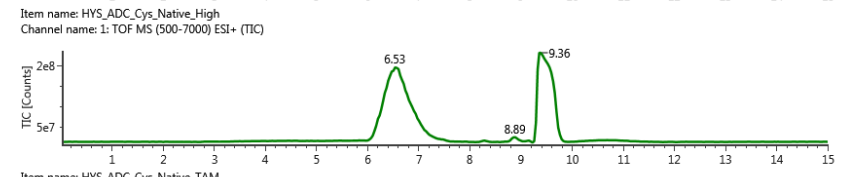
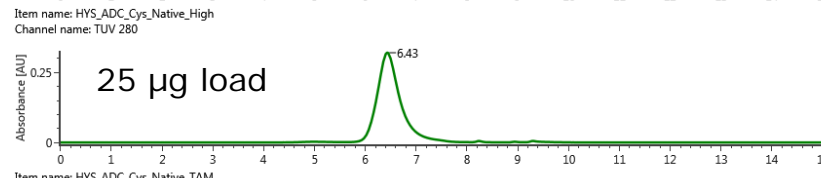
Cys-Low



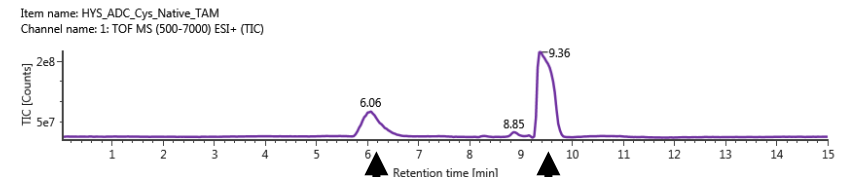
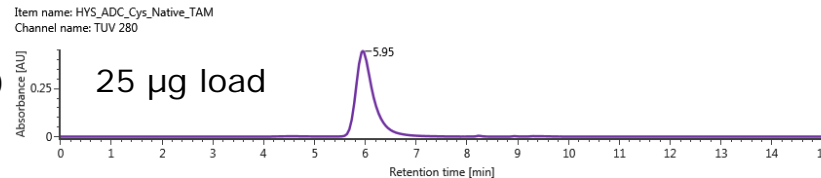
Cys-Med



Cys-High



Cys-mAb



- Ammonium Acetate pH 7.0
 - 50-250 mM
- No acid - No organic

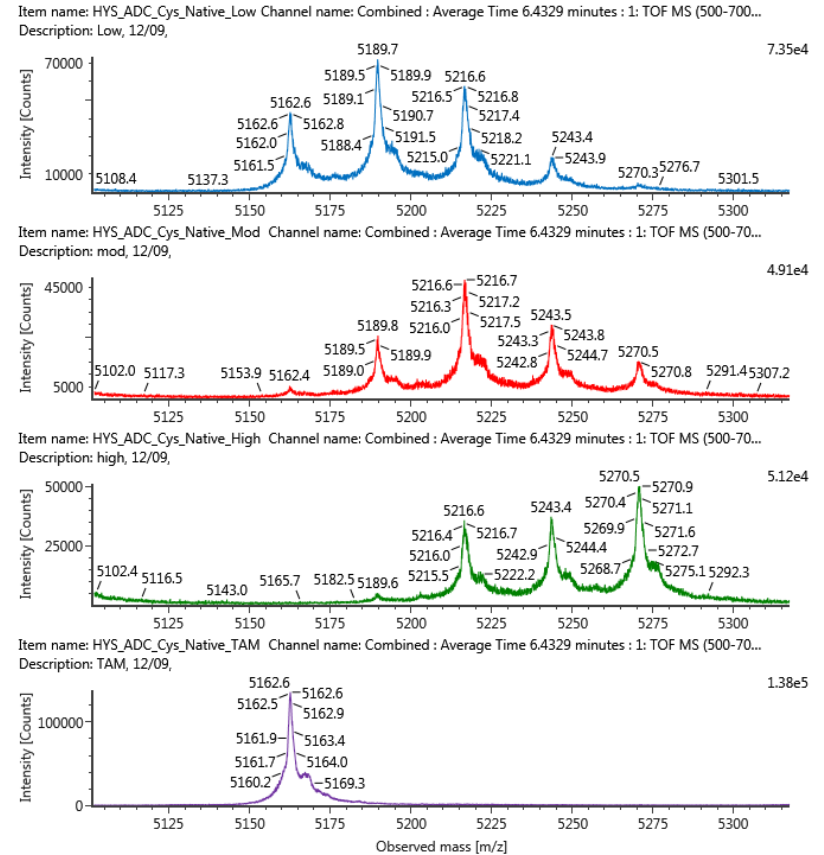
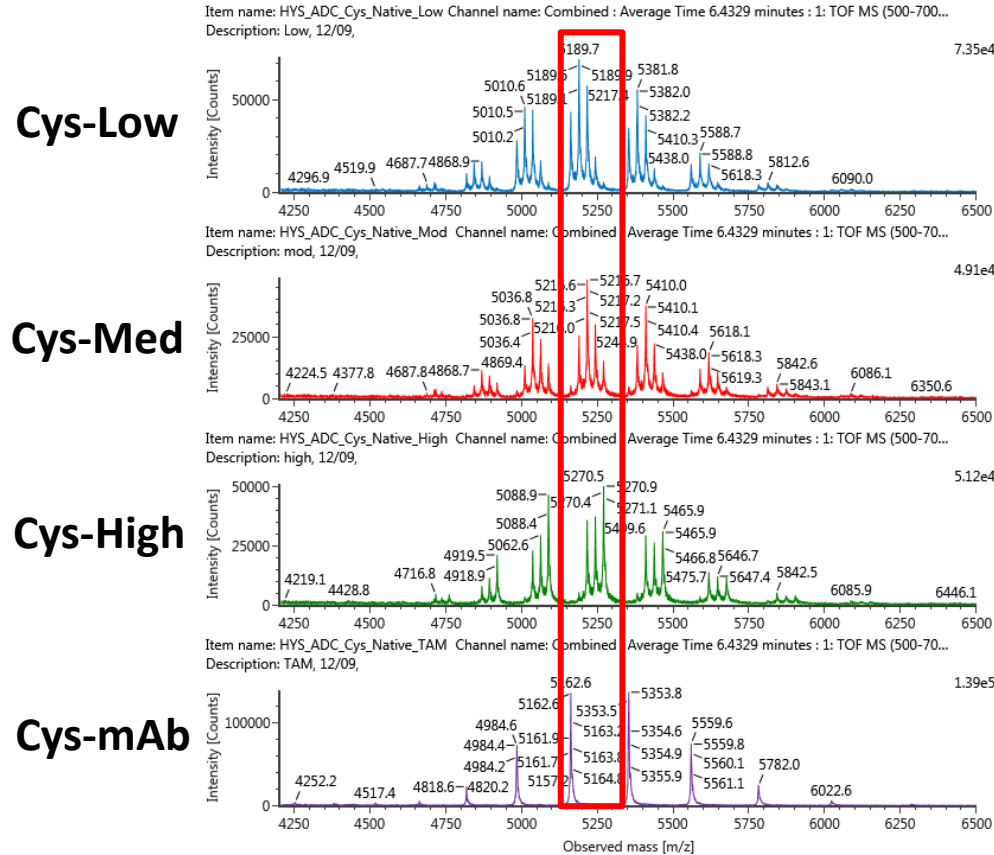
ADC Salt

SEC-LC/MS Combined Raw Spectra

Sample

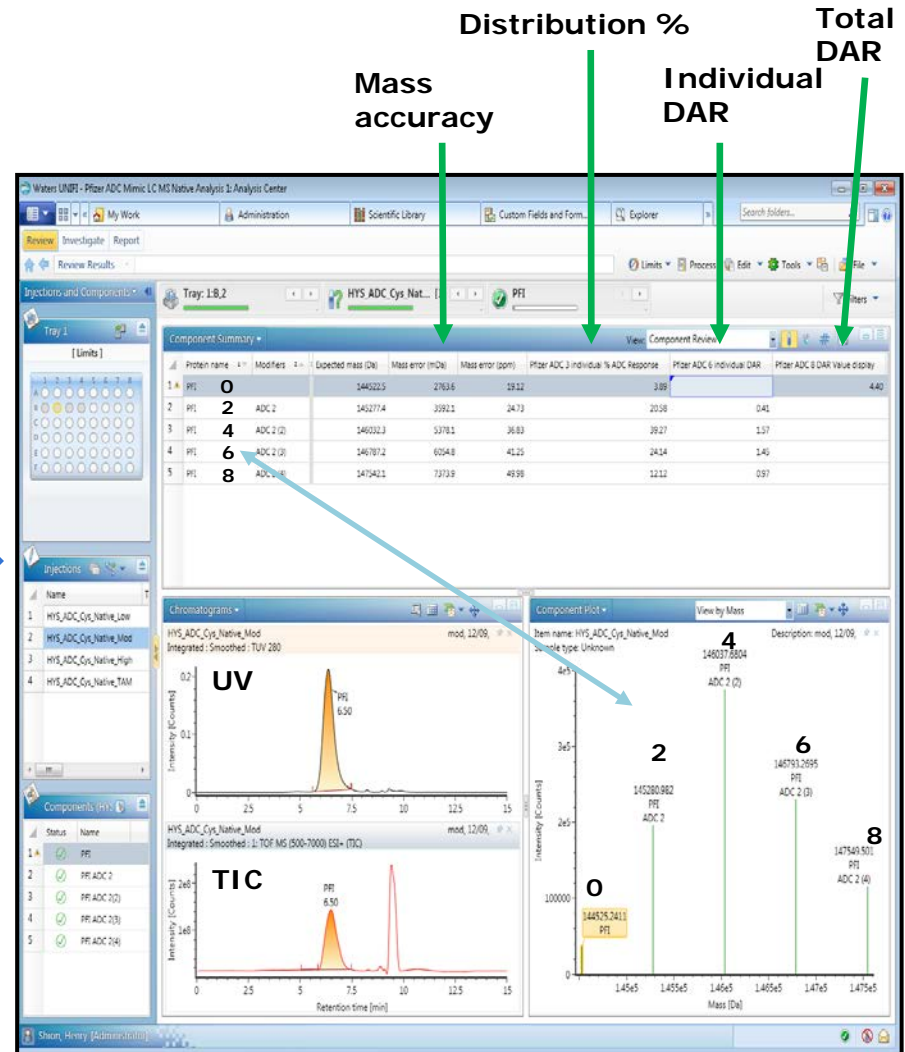
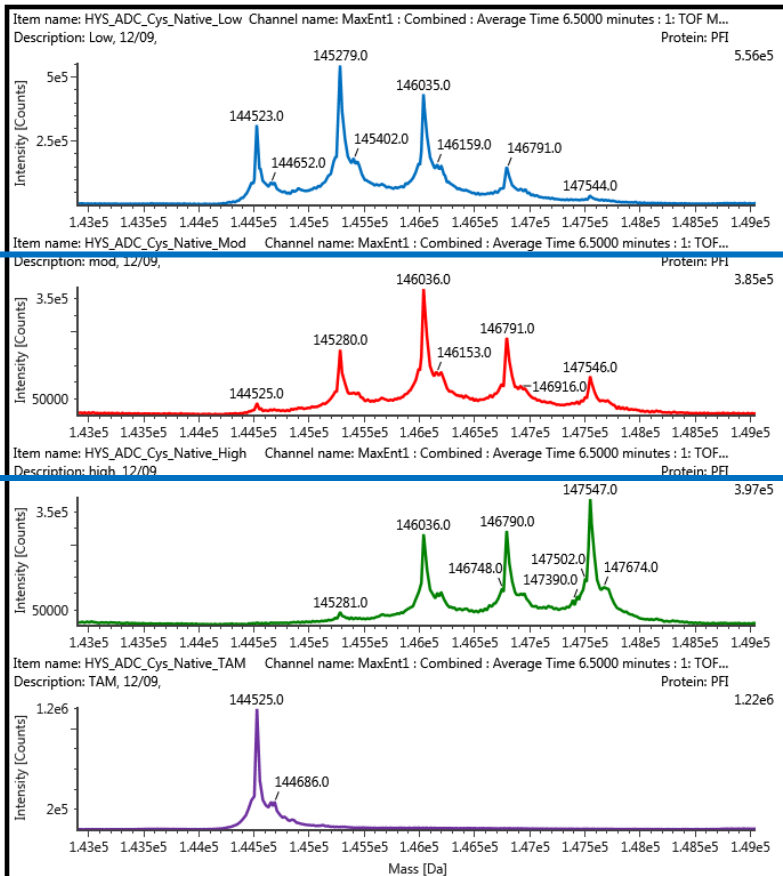
Combined Raw Spectra

Zoomed Charge State

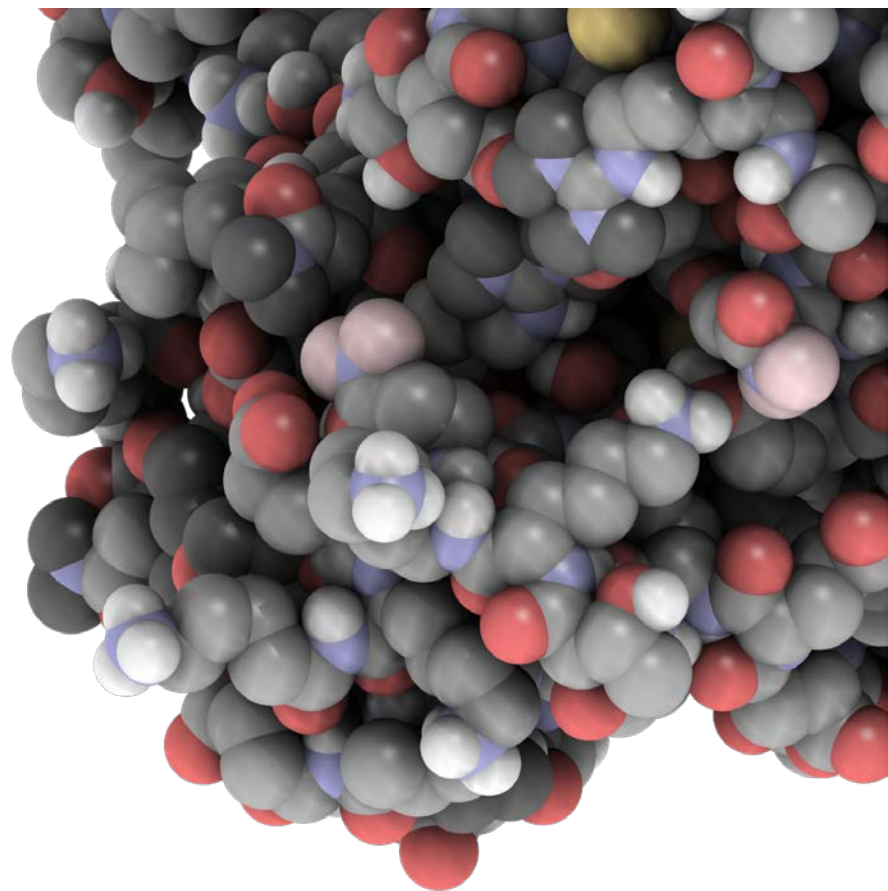
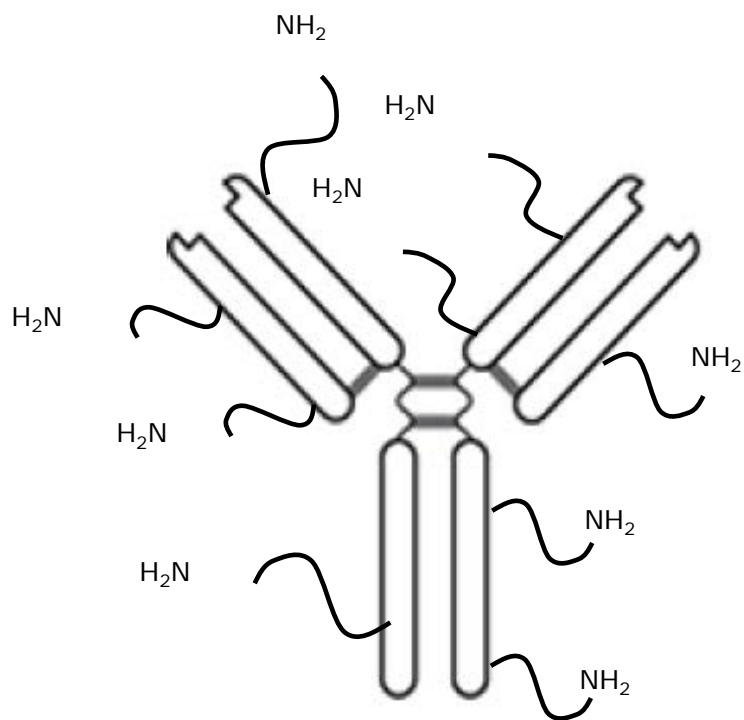


UNIFI Report of LC/MS (SEC)

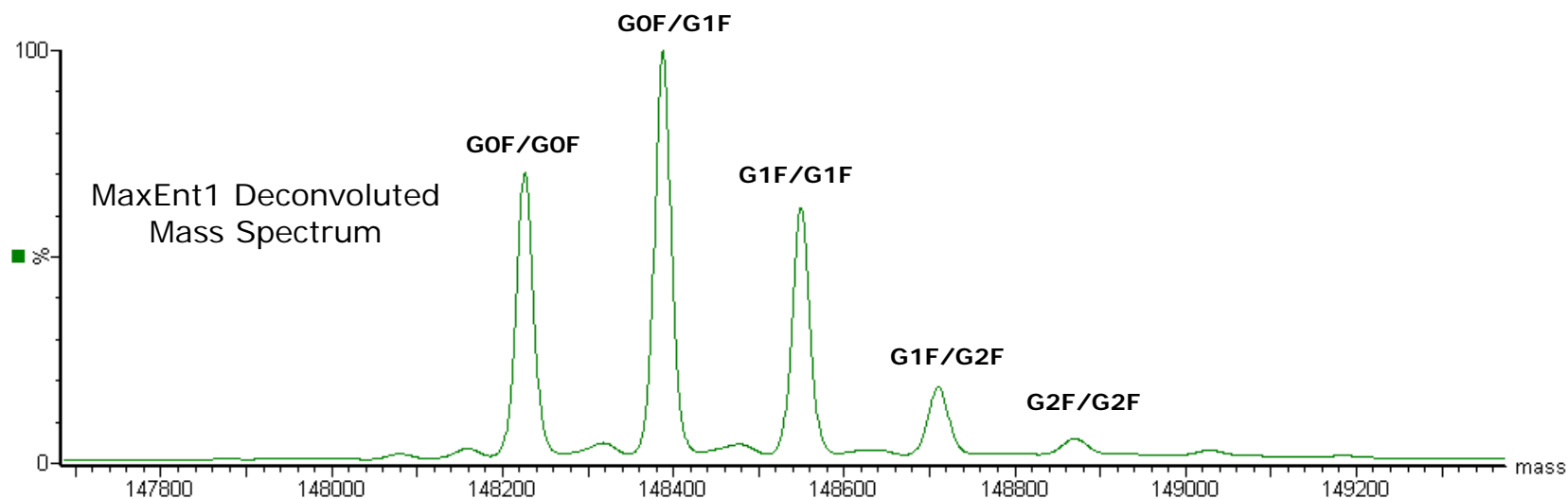
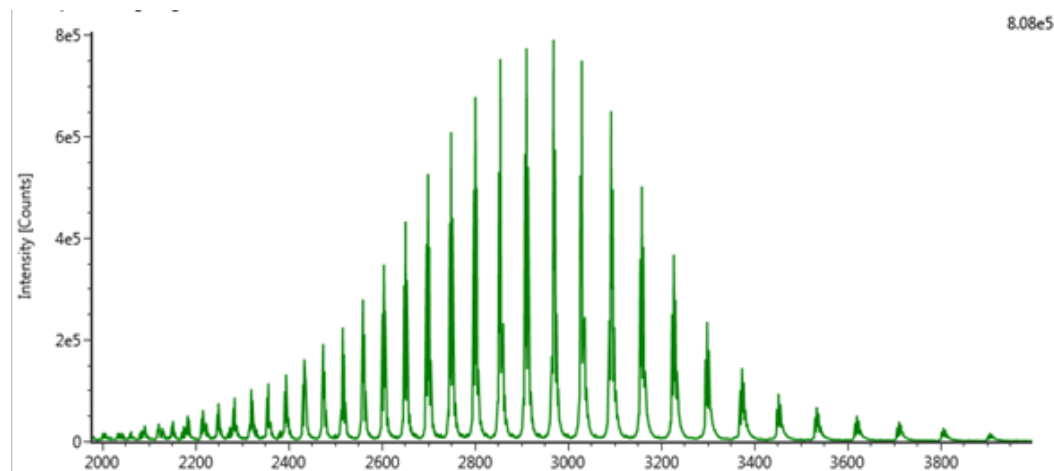
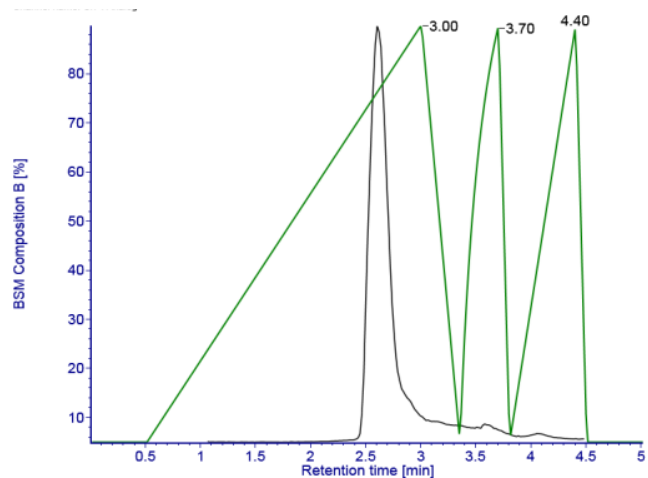
Deconvoluted Intact MS Spectra



Lysine-Conjugated ADCs



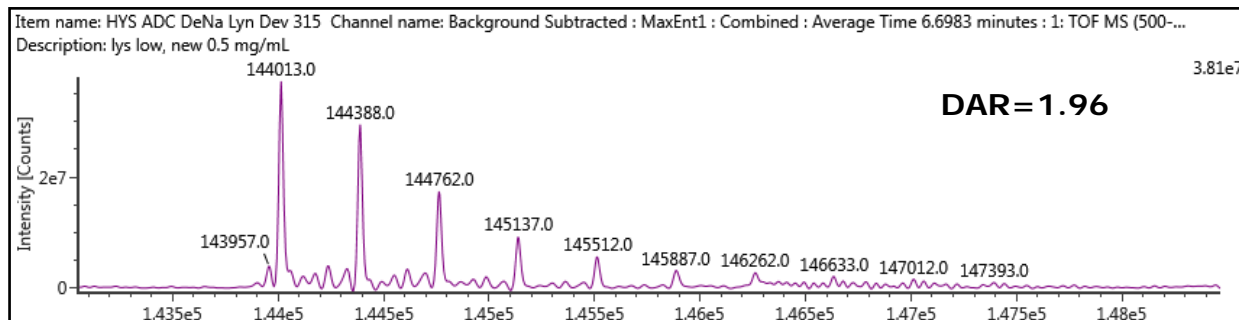
LC (RP)/MS Method



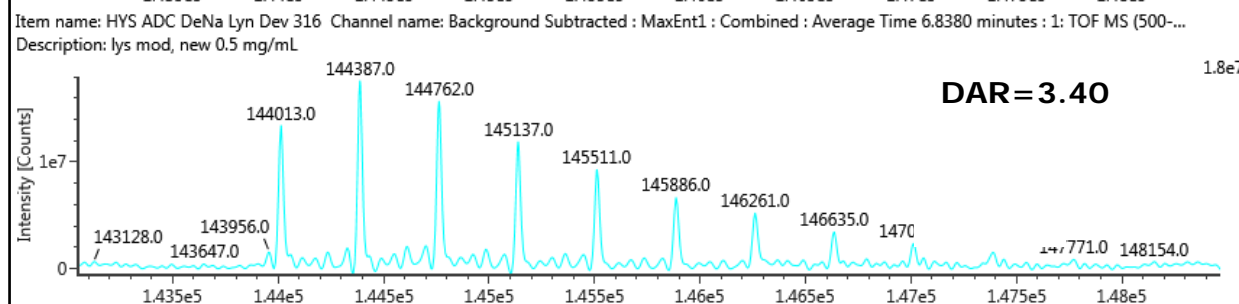
DAR Comparison of MS

Reversed-phase LC/MS by Waters

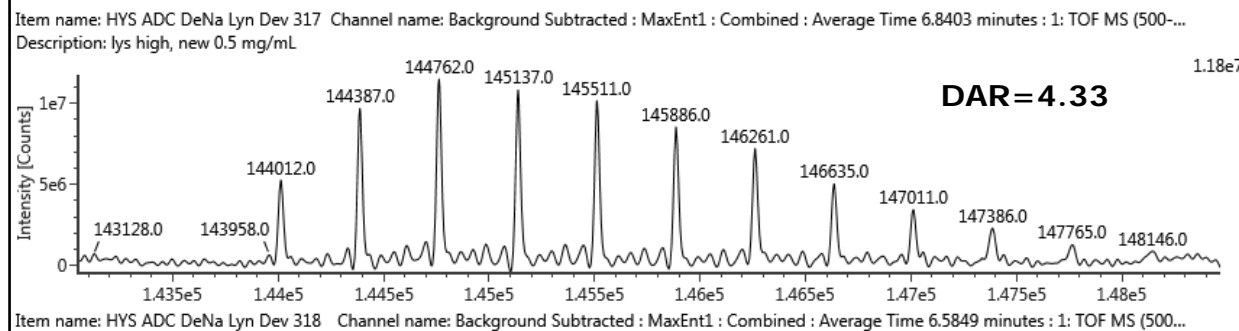
Lys-Low



Lys-Med



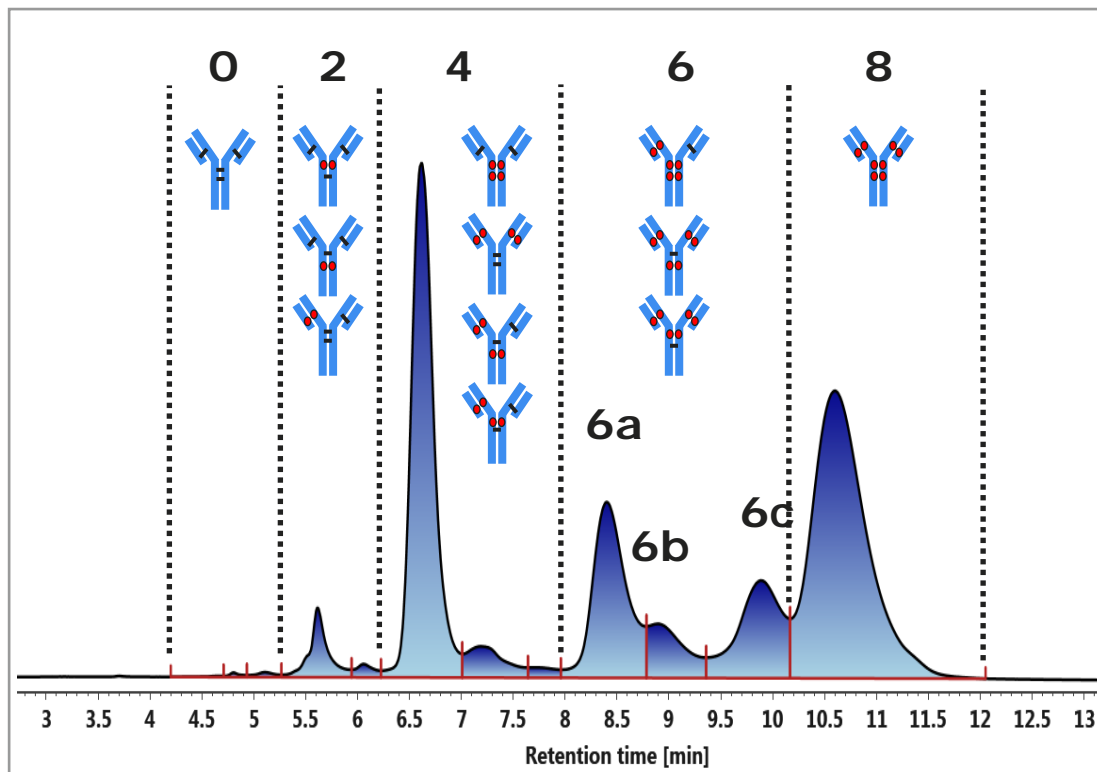
Lys-High



2D HIC/RP/MS

Multiple Isomers Resolved by HIC

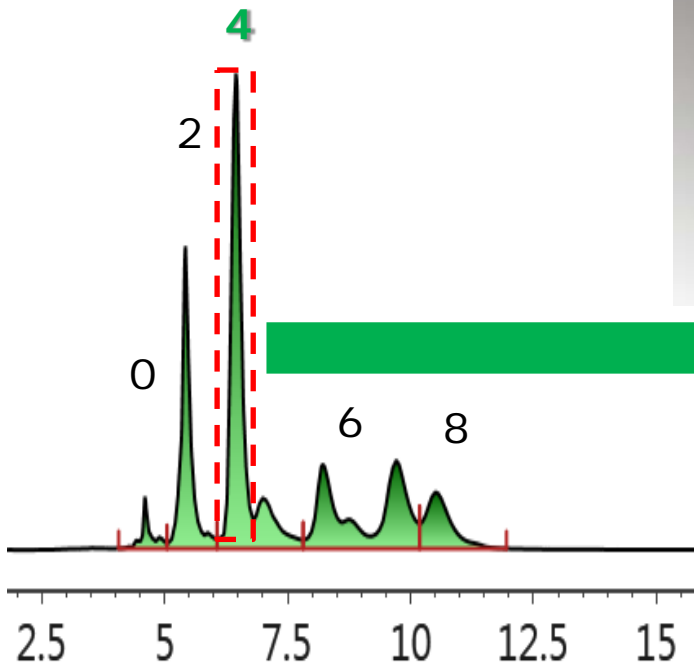
Calculated DAR from HIC Analysis



Expected DAR	Retention time (min)	Area	% Area	Individual DAR
0	4.66	3943	0.04	0.00
0	4.8	11615	0.11	0.00
0	5.11	22377	0.22	0.00
2	5.62	288610	2.84	0.06
2	6.06	58009	0.57	0.01
4	6.61	2757313	27.17	1.09
4	7.19	288157	2.84	0.11
4	7.72	63524	0.63	0.03
6	8.4	1420982	14	0.84
6	8.89	469456	4.63	0.28
6	9.89	1015204	10.01	0.60
6	10.6	3747624	36.93	2.95
Total DAR				5.97

2D-LC/MS (HIC/RPLC) for Isoform Structure Elucidation

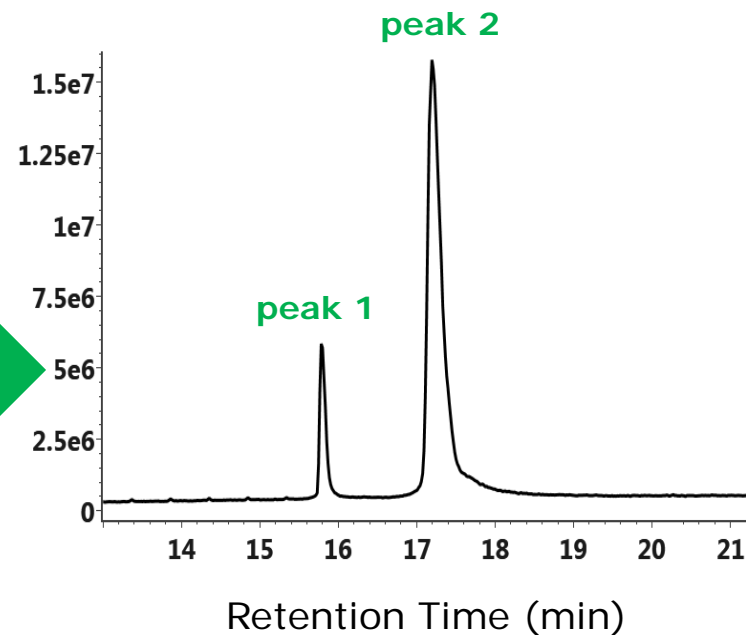
HIC (1st Dimension)



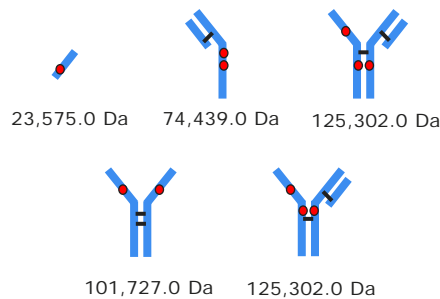
Heart Cut 2D LC-MS



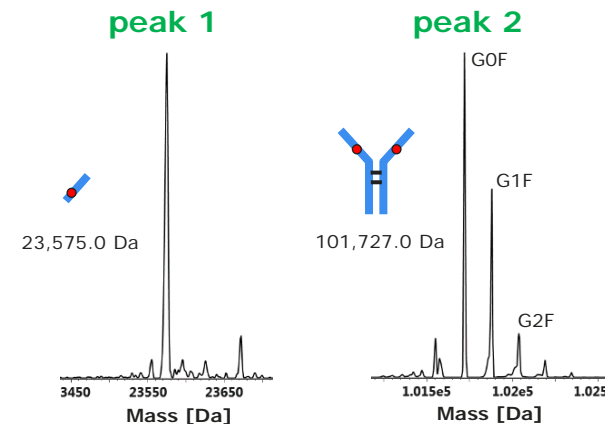
RPLC/MS (2nd Dimension)



Possible sub-units using RPLC

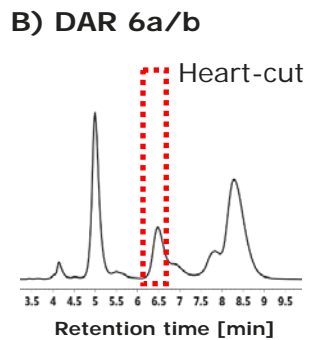


Retention Time (min)

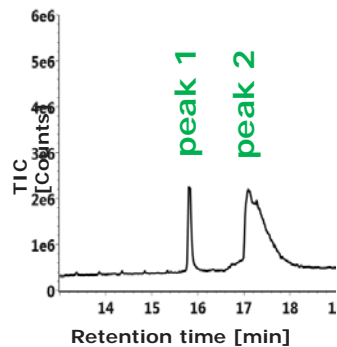


Positional Isomers Determination Using 2D-LC/MS

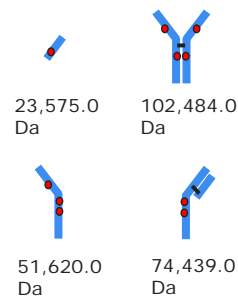
1st Dimension



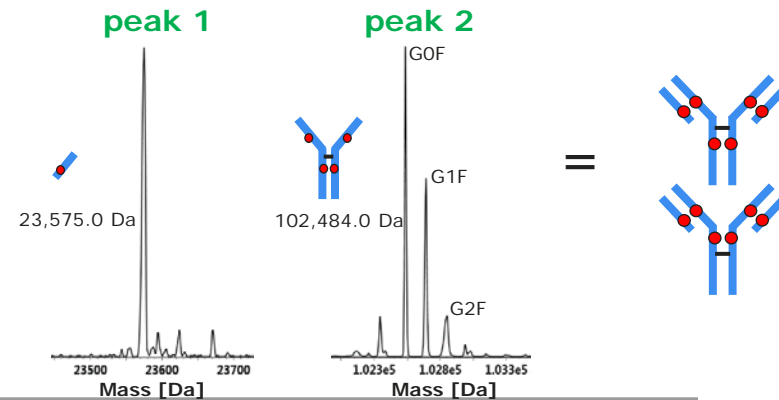
2nd Dimension



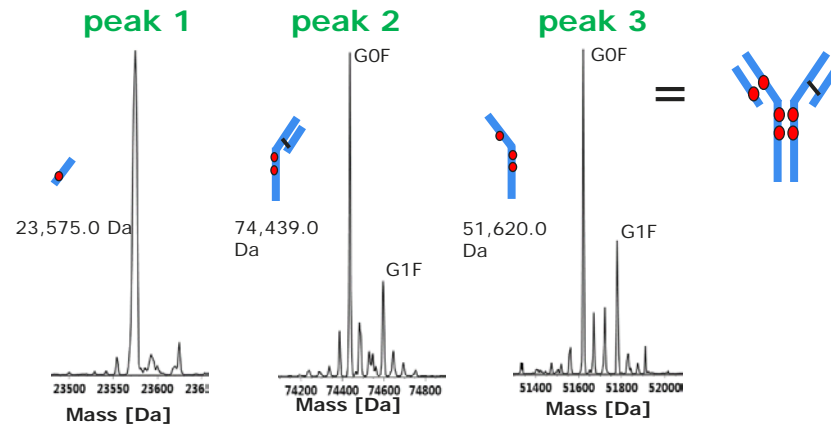
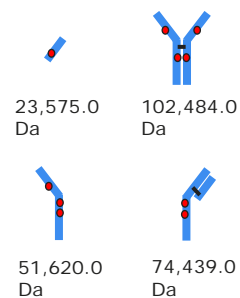
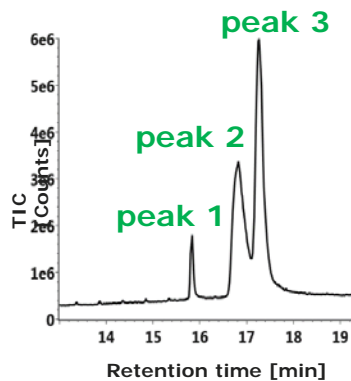
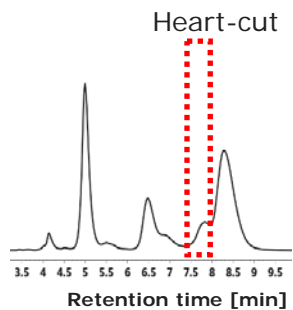
Possible Structures



Deconvoluted Mass Spectrum

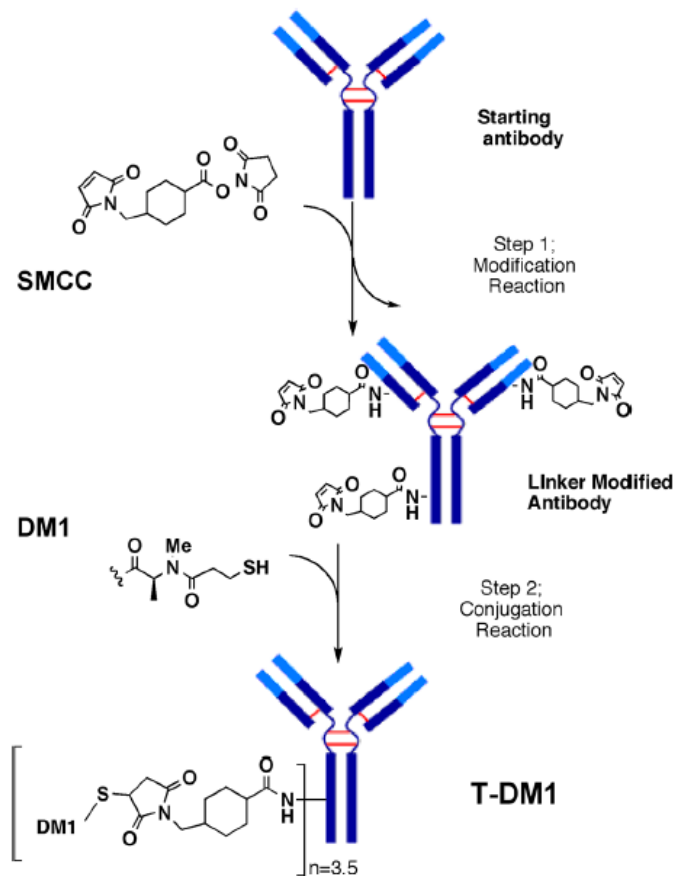


C) DAR 6c

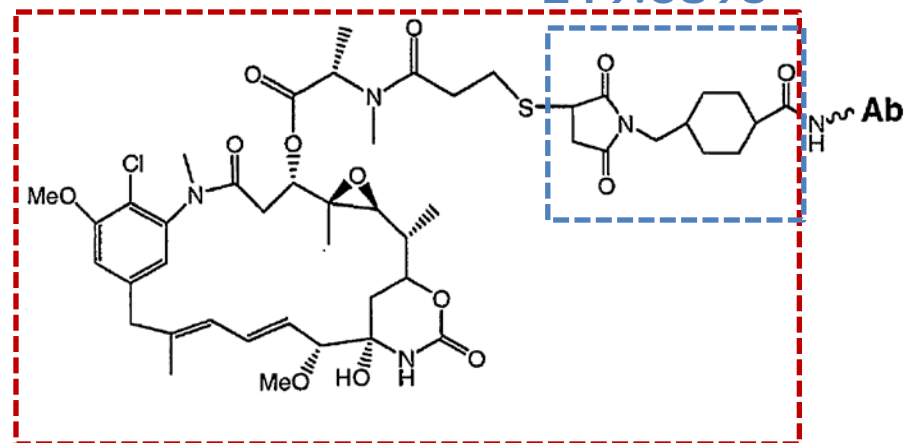


Kadcyla - Trastuzumab Emtansine Peptide Mapping Updates

Kadcyla - Trastuzumab Emtansine



MCC = C₁₂H₁₃O₃N
219.0895



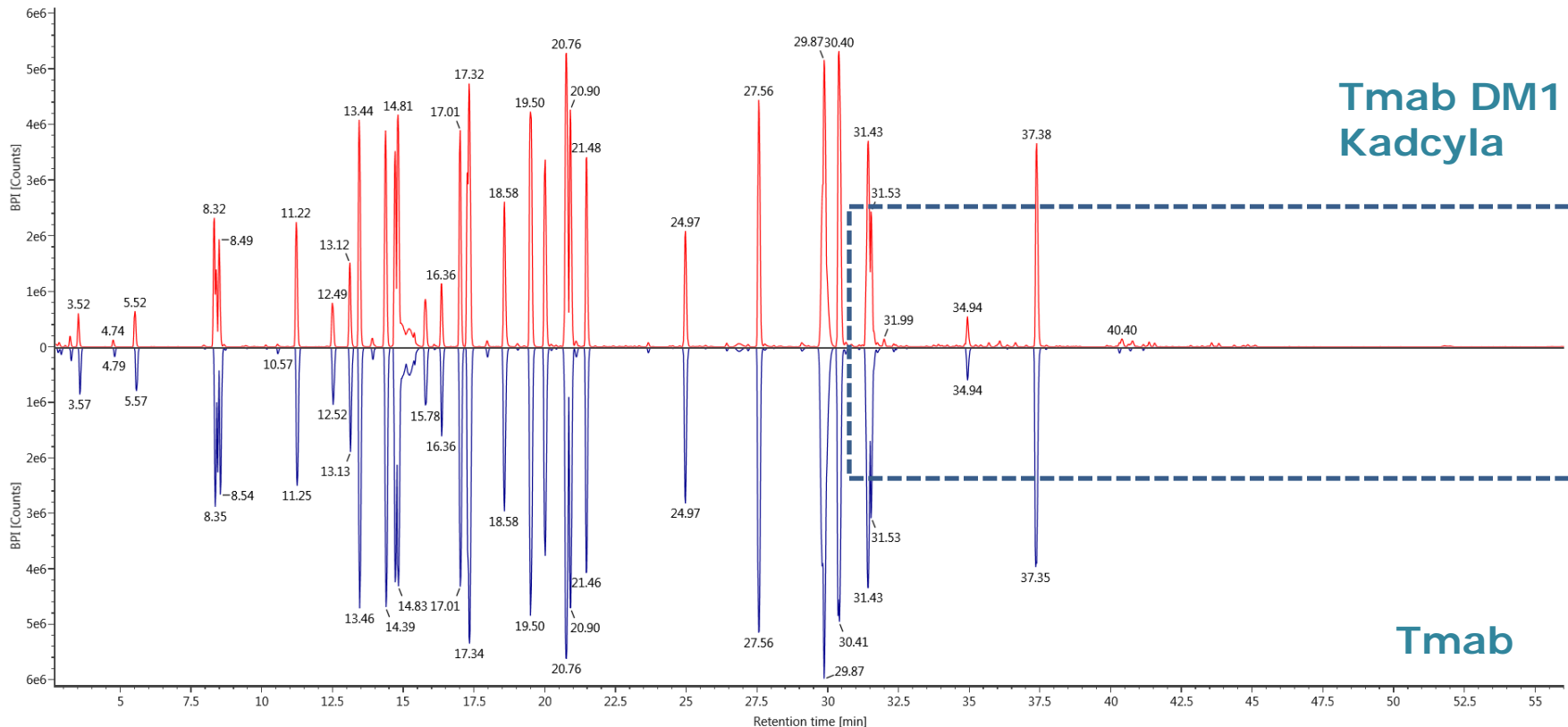
DM1 + MCC = C₄₇H₆₁N₄O₁₃SCl
956.3644

Figure 1. Schematic of T-DM1 conjugation: modification of lysines on trastuzumab with SMCC linker and subsequent reaction with the sulfhydryl of the DM1 drug. Figure adapted from Wakankar et al.¹⁷

MS^E Chromatograms (LC/MS): Trypsin

Trypsin Peptide Mapping

Item name: 20150423-YEA615-LXC-MSE-70min-Roche1335-01
Channel name: 1: TOF MS^E BPI (100-2000) 6eV ESI+



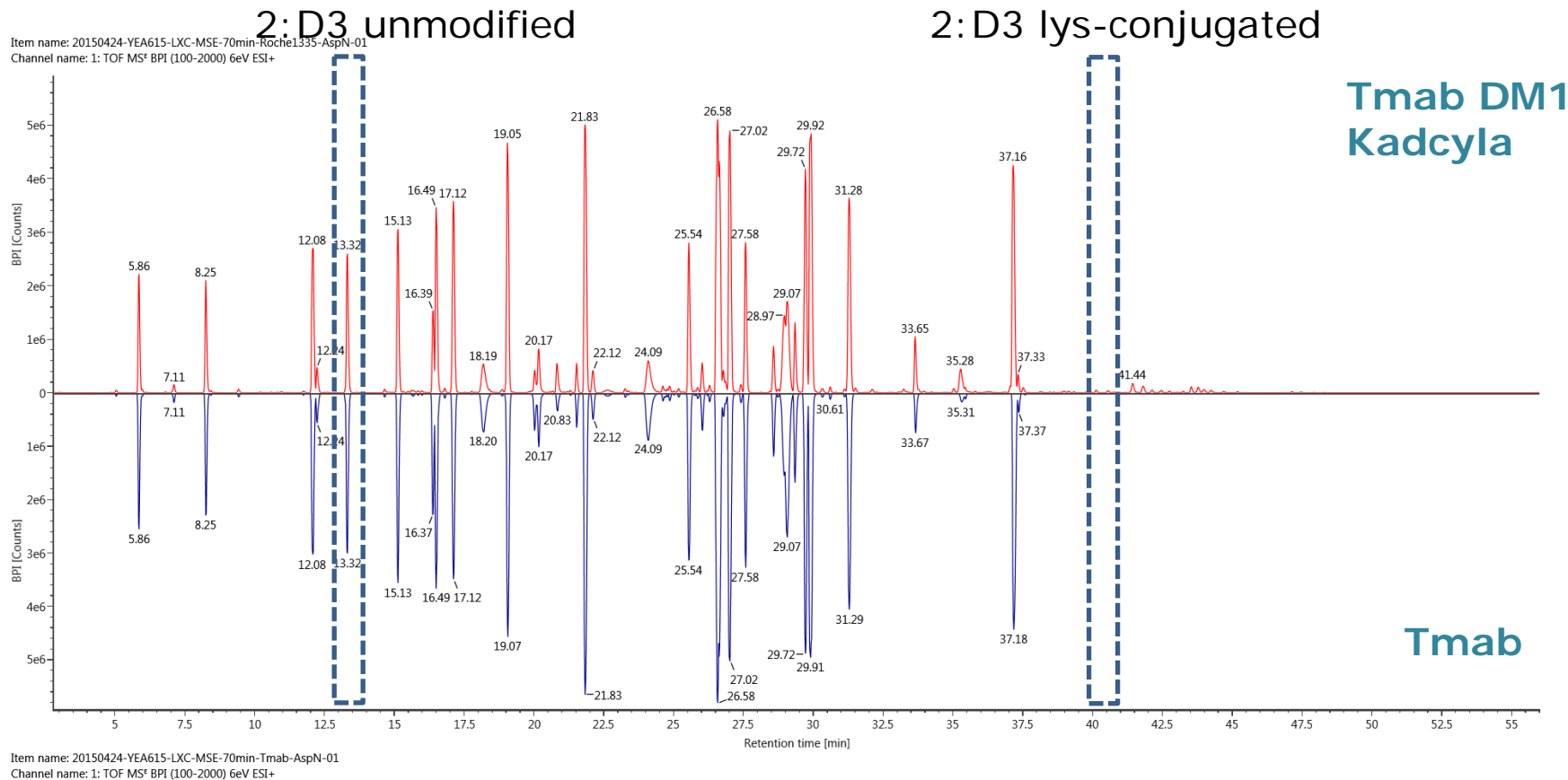
Item name: 20150423-YEA615-LXC-MSE-70min-Tmab-01
Channel name: 1: TOF MS^E BPI (100-2000) 6eV ESI+

Tmab DM1
Kadcyła

Tmab

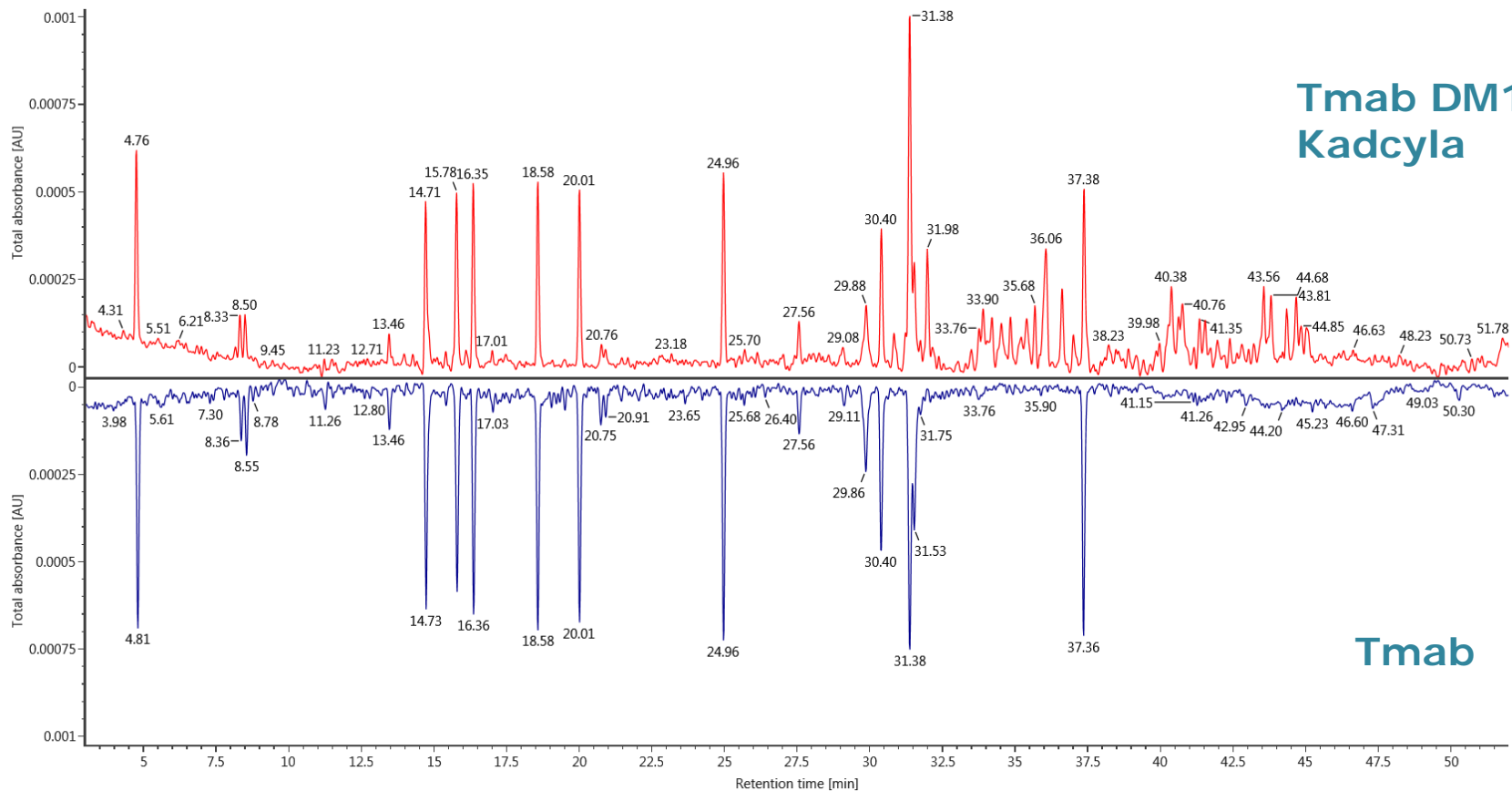
MSE Chromatograms (LC/MS): AspN

AspN Peptide Mapping



UV 254 Chromatograms: Trypsin Digest

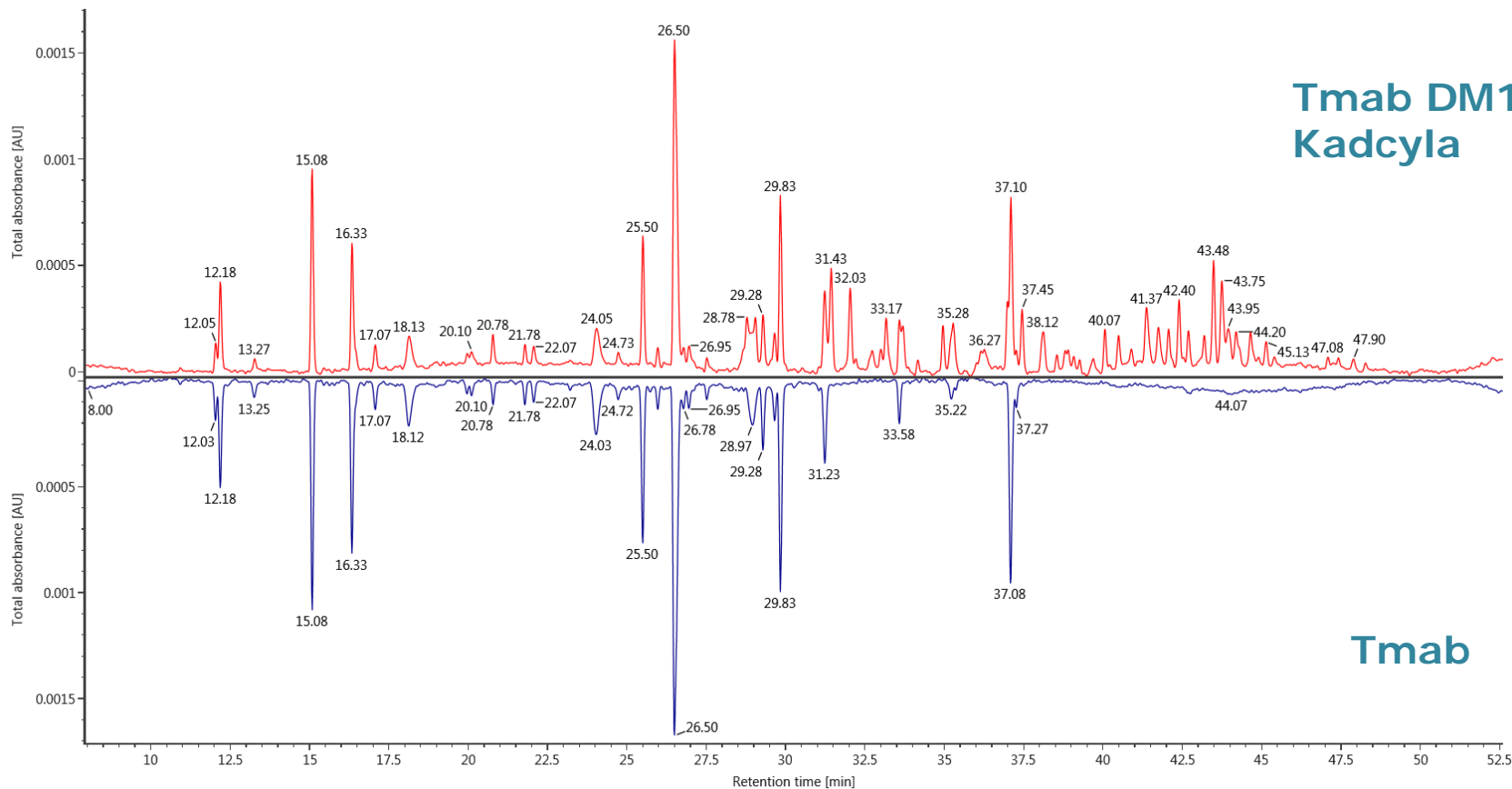
Item name: 20150423-YEA615-LXC-MSE-70min-Roche1335-01
Channel name: TUV 254 : Background Subtracted



Item name: 20150423-YEA615-LXC-MSE-70min-Tmab-01
Channel name: TUV 254 : Background Subtracted

Control mAb & Lysine-Conjugated Sample UV 254 Chromatograms: AspN Digest

Item name: 20150424-YEA615-LXC-DDA-70min-Roche1335-AspN-01
Channel name: TUV 254 : Background Subtracted : Background Subtracted

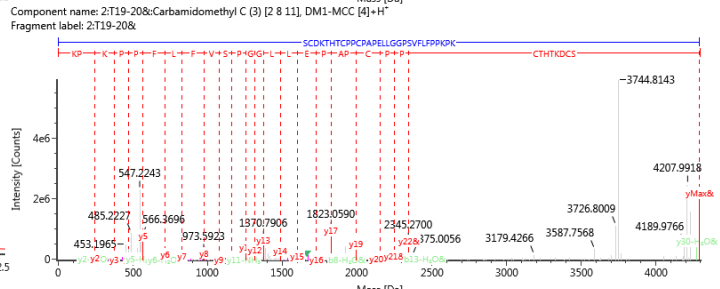
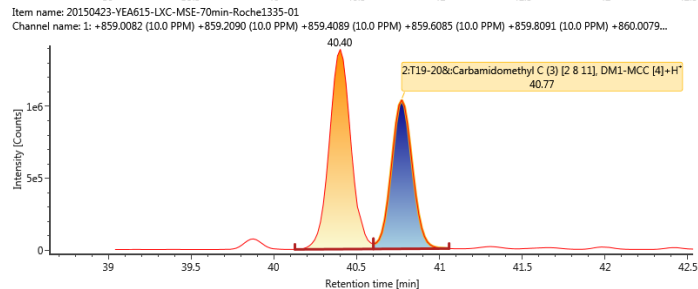
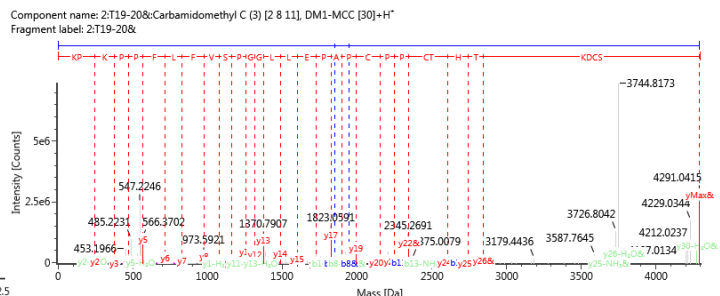
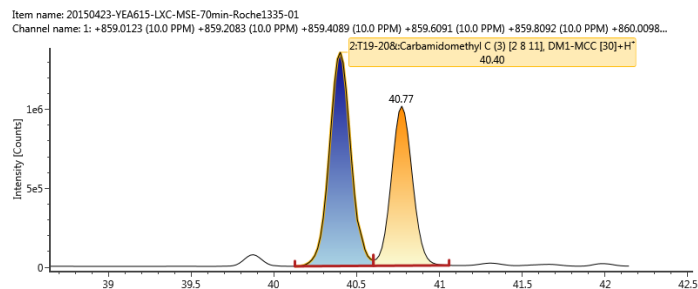


Item name: 20150424-YEA615-LXC-DDA-70min-Tmab-AspN-01
Channel name: TUV 254 : Background Subtracted : Background Subtracted

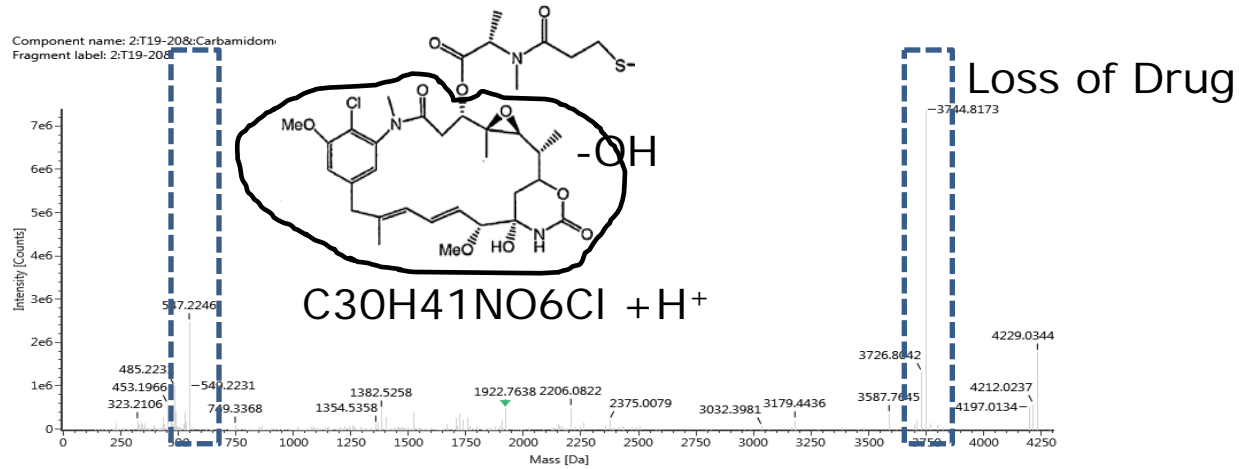
Tmab DM1
Kadcyla

Tmab

2:T19-20 Conjugated Peptides Elute in Pairs

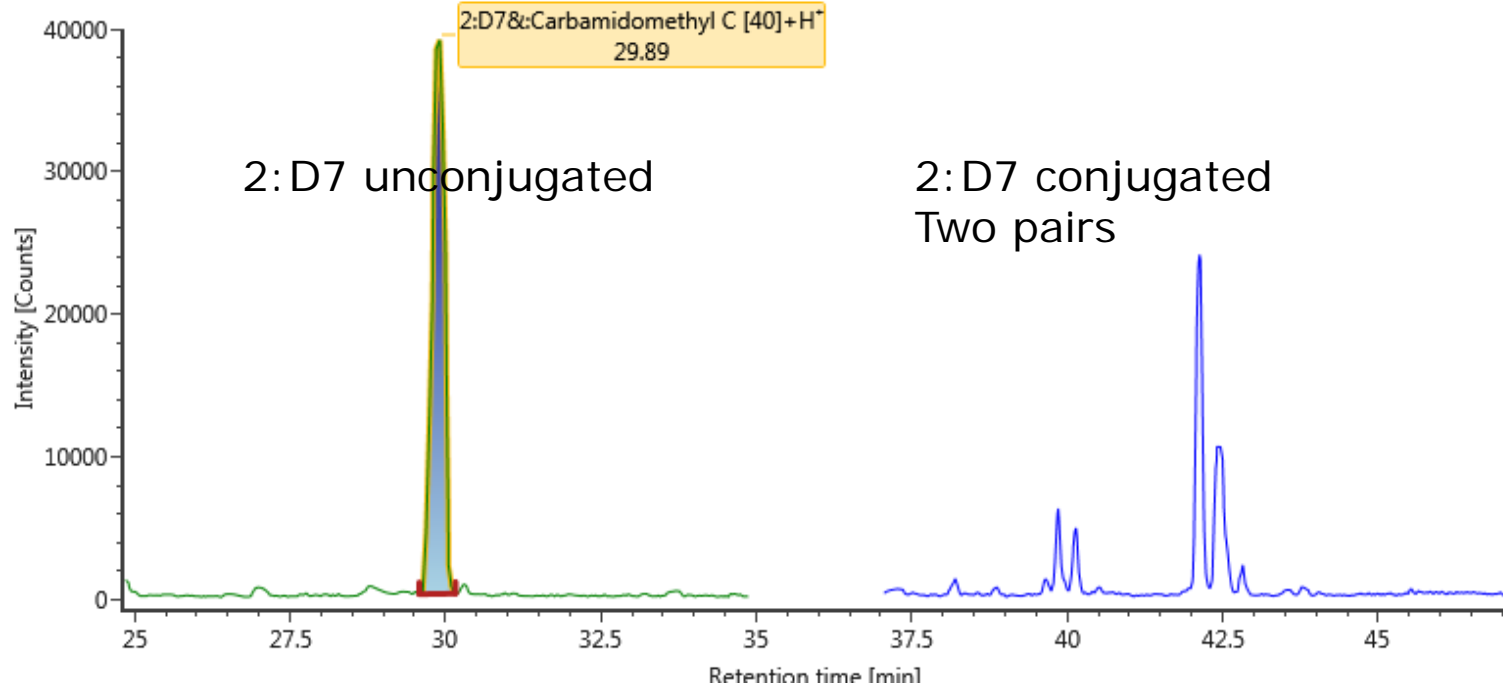


Unassigned Ions



2:D7 Unconjugated vs. Conjugated

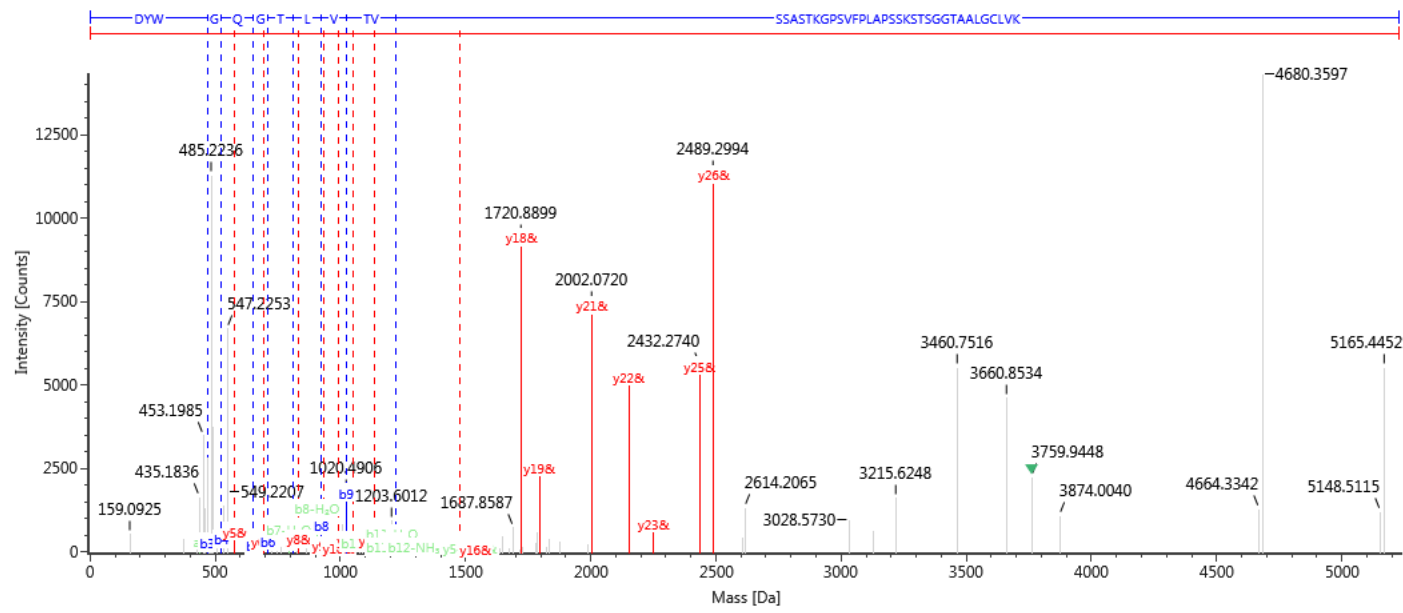
Item name: 20150424-YEA615-LXC-DDA-70min-Roche1335-AspN-01
Channel name: 1: +854.8331 (45.5 PPM) : TOF MS (100-2000) ESI+ : Integrated : Smoothed



2:D7 Lys site 124

10 20 30 40
 DYWGQGLVTVSSAST**K**GPSVFPLAPSS**K**STSGGTAALGCLV**K**

Component name: 2:D7&:Carbamidomethyl C [40], DM1-MCC [43]+H⁺
 Label: 2:D7&



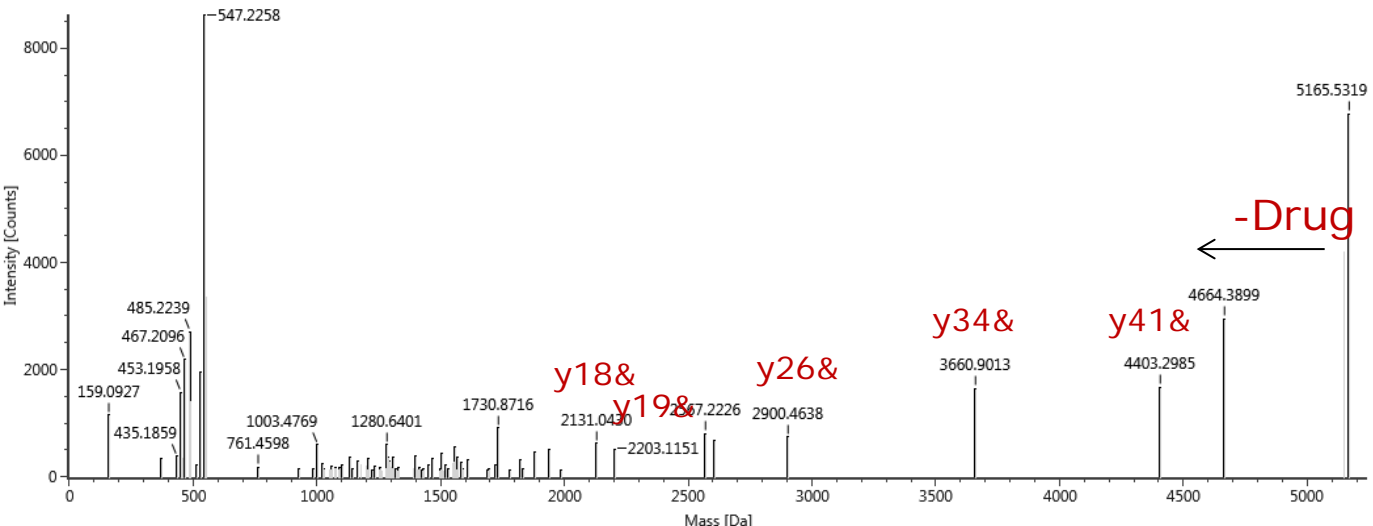
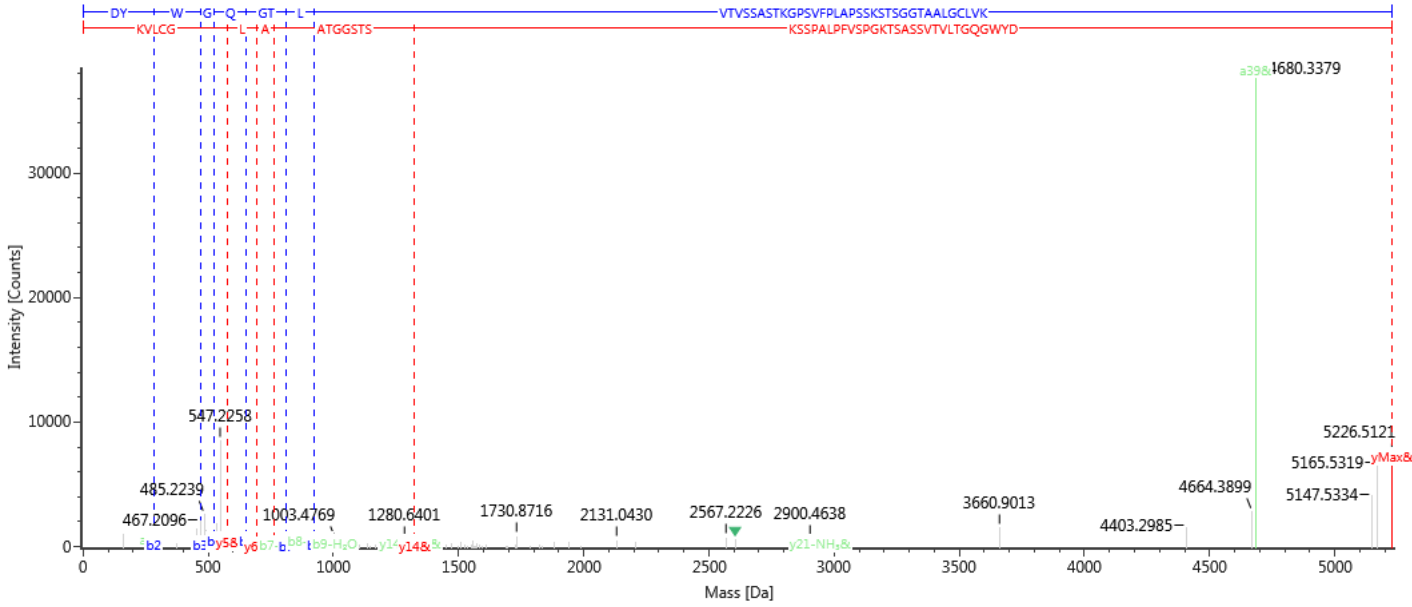
2:D7 Lys site 136

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10 20 30 40
DYWGOGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVK

Component name: 2:D7&:Carbamidomethyl C [40], DMI-MCC [17]+H
 Label: 2:D7&

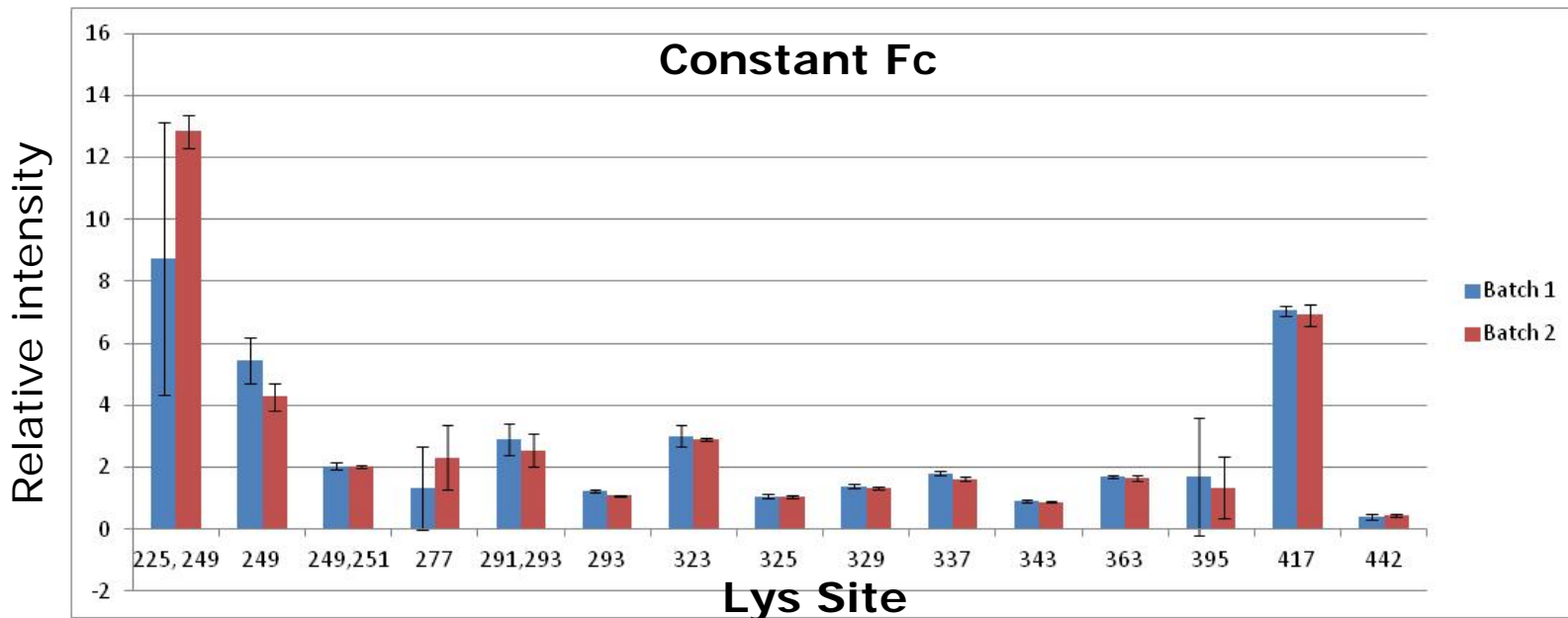
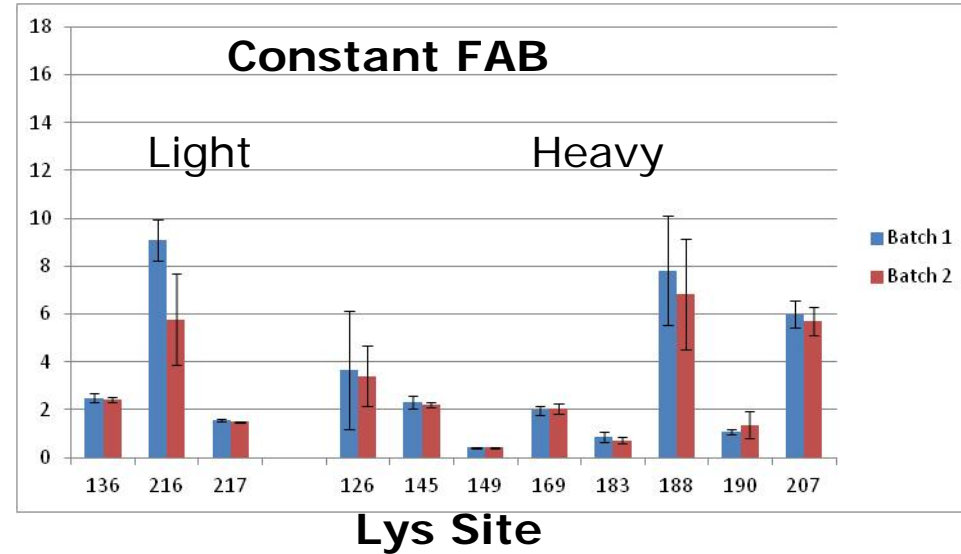
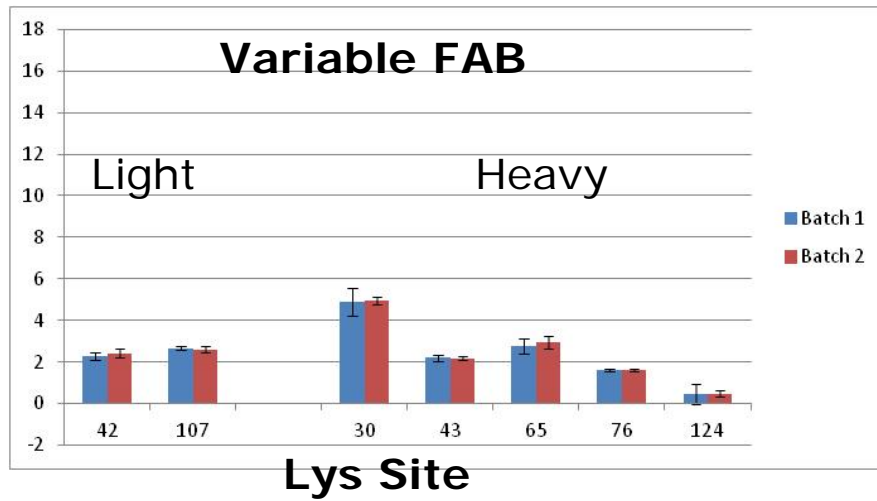


Peptide Mapping Results

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		Lys Position	Trypsin Digest				AspN Digest				AspN Peptides	
			Trypsin Peptides	MSE		DDA		MSE		DDA		
				Drug	y/b	Drug	y/b	Drug	y/b	Drug		y/b
Light Chain	Variable Fab	39	1:T3*	n/a		n/a						
		42	1:T3-4*	yes	>5	yes	>5	yes	>5	n/a	1:D3	
		45	1:T4-5*	yes	>5		n/a					
		103	1:T7-8*	n/a		n/a						
		107	1:T8-9*	yes	3	yes	1		n/a	n/a	1:D5	
	Constant Fab	126	1:T10-11*	yes	>5	yes	>5					
		145	1:T12-13*	yes	3	yes	3	yes	>5	yes	>5	1:D6
		149	1:T13-14*	yes	3	yes	>5					
		169	1:T14-15*	yes	>5	yes	>5	yes	0	yes	1	1:D8
		183	1:T15-16*	yes	>5	yes	>5	yes	>5	yes	3	1:D9
188		1:T16-17*	yes	>5	yes	>5						
190		1:T17-18*	yes	3	yes	>5	yes	>5	yes	>5	1:D10	
207	1:T18-19*	yes	>5	yes	>5							
Heavy Chain	Variable Fab	30	2:T2-3*	yes	>5	yes	>5	yes	>5	yes	>5	2:D1
		43	2:T4-5*	yes	>5	yes	>5	yes	>5	yes	>5	2:D2
		65	2:T7-8*	yes	2	yes	3	yes	>5	yes	3	2:D3
		76	2:T9-10*	yes	>5	yes	>5	yes	>5	yes	>5	2:D4
		124	2:T12-13*	yes	>5	yes	>5					
	Constant Fab	136	2:T13-14*	yes	>5	yes	>5	yes	1	yes	>5	2:D7
		150, 208	2:T14-15*	n/a		n/a						
		208	2:T15*	yes	>5	yes	>5	yes	>5	yes	>5	2:D8
		213	2:T15-16*	yes	>5	yes	>5					
		216	2:T16-17*	yes	0	yes	0					
		217	2:T17-18*	yes	3	yes	0	yes	>5	yes	4	2:D9
		221	2:T18-19*	n/a		n/a						
	Hinge	225, 249	2:T19-20*	yes	>5	yes	4					
		249	2:T20*	yes	>5	yes	>5	yes	>5	yes	>5	2:D10
	Constant Fc	249, 251	2:T20-21*	yes	5	yes	>5					
		277	2:T22-23*	yes	>5	yes	>5	yes	4	yes	2	2:D13
		291, 293	2:T23-24*	yes	>5	yes	>5					
		293	2:T24*	yes	1	yes	1		n/a		n/a	2:D14
		320	2:T26-27*	yes	>5	yes	>5					
		323	2:T27-28*	yes	1	yes	1					
		325	2:T28-29*	yes	3	yes	3					
		329	2:T29-30*	yes	>5	yes	>5					
		337	2:T30-31*	yes	>5	yes	>5	yes	3	yes	>5	2:D15
		341	2:T31-32*	yes	0		n/a					
		343	2:T32-33*	yes	2	yes	2					
		363	2:T35-36*	yes	>5	yes	>5					
		373	2:T36-37*	n/a		n/a						
395		2:T37-38*	yes	>5	yes	>5	yes	>5	yes	>5	2:D16	
412		2:T38-39*	n/a		n/a			n/a		n/a	2:D18	
417	2:T39-40*	yes	3	yes	4							
442	2:T41-42*	yes	2	yes	>5	yes	>5	yes	2	2:D19		



Key Technologies for ADCs

■ LC-UV

- [ACQUITY UPLC H-Class System](#) with TUV Detector (or, ACQUITY UPLC H-Class Bio System)
- [Empower 3 Software](#)

■ LC-MS

- [Waters Biopharmaceutical Platform Solution with UNIFI:](#)
 - ACQUITY UPLC H-Class System with TUV Detector
 - [Xevo G2-XS QToF Mass Spectrometer](#)
 - [UNIFI Scientific Information System](#)

■ 2D LC-MS

- [ACQUITY UPLC H-Class Bio System with 2D Technology](#)
- Xevo G2-XS QToF Mass Spectrometer
- UNIFI Scientific Information System

■ Columns

- Hydrophobic interaction (HIC): Protein-Pak HiRes HIC
- Intact & subunit analysis: ACQUITY UPLC Protein BEH C4
- Peptide mapping: ACQUITY UPLC Peptide CSH C18
- Released glycan analysis: ACQUITY UPLC Glycan BEH
- Size exclusion chromatography: ACQUITY UPLC Protein BEH SEC
- [Bioseparations Columns Advisor](#)



Contributors & More Details

Acknowledgements

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- Henry Shion
- Robert Birdsall
- Ying Qing Yu
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ADC Resources

- Waters.com/ADC
 - Molecular insights video
 - Presentations by collaborative industry thought leaders on ADCs (Slideshare)
 - Case studies & applications work

Key application notes

- [Automating the Determination of Drug-to-Antibody Ratio \(DAR\) of Antibody Drug Conjugates \(ADCs\) Based on Separation by Hydrophobic Interaction Chromatography \(HIC\)](#)
- [Development of Integrated Informatics Workflows for the Automated Assessment of Comparability for Antibody Drug Conjugates \(ADCs\) Using LC-UV and LC-UV/MS](#)

[COLLABORATE. INNOVATE. SUCCEED.]

Thank you

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