# **MONITORING PRODUCT AND PROCESS ATTRIBUTES** IN BIOPHARMACEUTICAL DEVELOPMENT AND QC

A)

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# INTRODUCTION

The proposal of "Multi-Attribute Method (MAM)" based LCMS peptide mapping methods for semi-targeted monitoring of biotherapeutic protein attributes has the potential to reduce manufacturing costs and bring products to market faster.

Currently, MAM-based approaches are being evaluated for their appropriateness to streamline characterization processes as industry moves towards continuous manufacturing strategies as well as their ability to transfer to QC/lot release roles.

Deploying and operating High-Resolution-MS (HRMS) methodologies in regulated environments must be weighed against more routine nominal mass detection approaches that may require more rigorous evaluation in establishing their suitability for MAM based analyses.

# **Characterizing Product Attributes using High Resolution Accurate Mass**





In this study, we have generated a common set of Trastuzumab forced degradation samples, subjected to various levels of oxidative and high pH stress to enable data-based discussions of fit-for-purpose MS for peptide map based attribute monitoring in regulated environments.

# **METHODS**

Trastuzumab samples were treated with alkaline and oxidation stress, followed by denaturation, alkylation and tryptic digestion.

LC System: ACQUTIY UPLC H-Class Bio System Column: ACQUITY UPLC CSH300 C18, 1.7 µm, 2.1 x 100 mm Column temperature: 65 °C

Control

Alkaline

Stress

Oxidation

stress

Mobile phase: A. 0.1% FA in  $H_2O$ B. 0.1% FA in MeCN Gradient: 3-33 %B over 120 min TUV Detection: 215 nm

Figure 1: A) The Vion QTof enables robust accurate mass measurement of peptides with high sensitivity over a large dynamic range with high sequence coverage. B) A MS<sup>E</sup> peptide mapping workflow in conjuction with the high MSMS fragmentation efficiency of the Vion was used to define product attributes of the trastuzumab reference sample, confirm protein identity/sequence, and determine product modification variants for confident assignment of peptides and their post-translational and chemical modifications.

#### **Building an Accurate Mass Based Scientific Library for Multi-Attribute Monitoring**

Immary Batch Overview SE pep map workflow_1 Review results Coverage Map_Spectra Fragmentation Viewer 3D viewer Trending plot	Protein name T Tmab Tmab 9 Tmab	Component name 2:T25&:Glycosylation G0F-GlcNAc N [5]+H*	Peptide	Modifiers	Observed RT (min)	AL 10 100				
Chromatogram Signature Peptides Binary Compare	10         Tmab           11         Tmab           12         Tmab           13         Tmab           14         Tmab           15         Tmab           16         Tmab           17         Tmab           18         Tmab           14         Chromatogramu           14         Tmab           15         Tmab           16         Tmab           17         Tmab           18         Tmab           10         Chromatogramu           11         Tmab           12         Tmab           13         Tmab           14         Tmab           15         Tmab           16         Tmab           17         Tmab           18         Tmab           19         5e6           9         10           10         10	2/1258:c0ycosylation GGF N [5]+H" 2/16+H" 2/174+H" 2/174+H" 2/174+H" 2/174+H" 2/174+H" 2/1728:Carbamidomethyl C [3]+H" 1/174+H" 2/1728:Carbamidomethyl C [3]+H" 1/1788:Carbamidomethyl C [3]	EEQINISTYR EEQINISTYR PYTINGYTR AEDTAVYYCSR FTISADTSK VDNALQSGNSQESVTEQD ALPAPIEK DTLMISR SLSLSPG GLEWVAR LSCAASGFNIK VYACEVTHQGLSSPVTK 44.4937.0538.5542.61 21728:Carbamidomethyl 21728:Carbamidomethyl 21728:Carbamidomethyl 21728:Carbamidomethyl 217188:Carbamidomethyl 217188:Carbamidomethyl 217188:Carbamidomethyl 2003 35 40	Giycosylation GOF-GicNAc N [5] Giycosylation GOF N [5] Carbamidomethyl C [9] Carbamidomethyl C [3] Carbamidomethyl C [3] Carbamidomethyl C [4] 5 Subtracted 147 46.80 19]+H 47.56 50 55 10] 47.56 53.50 55.79 10] 47.56 53.50 55.79 10] 47.56 53.50 55.79 10] 10] 10] 10] 10] 10] 10] 10]	334 335 1393 1727 2104 21 24 25 30 31 31 31 32 Frag Com Frag Com Frag Com Frag Com Frag Com Frag Solution Frag Com Frag Com Frag Solution Solutio	Nodified (%) 9.12 36.95 98.21 99.40 0.00 Send to Scient Comment Comment Output Output Create new Name: Description: Spectra Userray Append Item Ta Select 000 3666.12 000 3666.12 000 3666.12 000 3666.12 000 3666.12 000	Observed mass (Da)         2430.9546           2634.0314         1084.5403           1334.5650         966.4868           fic Library         966.4868           fic Library         7           Phos B HDMSE Identifi         10           ibrary         2           Critical Quality Attribu         7           gs         10           Image: State of the stat	Response         UV Response           2.9565	2243.8732 2000	) d 4.9 5.5 1.8 2.3 2.0 2.0 2.0 2.0 2.0 2.0 2.0 2.0 2.0 2.0
dministrator, UNIFI [Administrator]	976								0	8

*Figure 2:* The UNIFI Scientific Library is a repository where information from multiple characterization runs can be aggregated and managed to produce target lists for subsequent screening based analyses.

	Results 1		C. [Critical Quality Attributor	Iractuzumahl			a anist and differentiations and Destands and and	ATaols T	Purpose Tasks	Create Import Paste Results Delete Edit Fragments Edit Adducts	Add To Common Fragments Add To Neutral Lo		Mass	Fragment Charg	e Waveler
		VTACEVTHQGESSPVTK Carbanitdomethyr	C [Critical Quality Attributes -	nastuzumanj		Amino AA	acid modifications		Home	Component name	Item tags	Expected RT (min)	Expected neutral mass (Da)	Expected fragment ( Adducts	Expected wavelength (nm)
	Search results (17 items found)	Property Value		1: 1 to 17 VYACEVT	THQG LSSPVTK				Manage Components	1 1:T18:Oxidation M [4]+H*	DIOMTOSPSSLSASVGDR	37.35	1893.8738	691.3320 2x(+H). 3x(+H)	215.0
		Item type Peptide Seq	uence		<b>C</b>				Default Amounts	2 1:T1+H*	DIQMTQSPSSLSASVGDR	46.07	1877.8789	2x(+H), 3x(+H)	215.0
	Name	1:T188:Cart	amidomethyl C [4]+H* -		Seq	uence				3 1:T10+H*	TVAAPSVFIFPPSDEQLK	72.81	1945.0197	3x(+H)	215.0
ations manual updat 👻	1 AEDTAVYYCSR Carbamidomethyl C	Item description Tmab								4 1:T11&:Carbamidomethyl C [8], Deamidation N [11]+H*_1	SGTASVVCLLNNFYPR	66.74	1797.8719	811.3750 3x(+H), 2x(+H)	215.0
	2 ALPAPIEK	IUPAC name								5 1:T11&:Carbamidomethyl C [8], Deamidation N [11]+H*_2	SGTASVVCLLNNFYPR	79.22	1797.8719	811.3750 3x(+H), 2x(+H)	215.0
	2 DTI MCD	Formula CR1H120N/3								6 1:T11&:Carbamidomethyl C [8]+H*	SGTASVVCLLNNFYPR	76.11	1796.8879	2x(+H), 3x(+H)	215.0
	5 DILMISR	Formula C61H150N2	Formul	a						7 1:T13+H*	VQWK	4.41	559.3118		215.0
	4 EEQYNSTYR Glycosylation G0F-GlcNAc N	Hill formula C81H130N2	20275							8 1:T14+H*	VDNALQSGNSQESVTEQDSK	25.08	2134.9614		215.0
	5 EEQYNSTYR Glycosylation GOF N	Average molar mass 1876.0951								9 1:T15+H*	DSTYSLSSTLTLSK	52.65	1501.7511		215.0
	6 EEOVINSTVR Glycosylation G0 N	Monoisotonic mass 1874 9196	Neutra	Mass						10 1:T16+H*	ADYEK	1.36	624.2755		215.0
										11 1:1188:Carbamidomethyl C [4]+H*	VYACEVTHQGLSSPVTK	32.02	18/4.9196	2x(+H), 3x(+H)	215.0
	/ EEQYNSTYR Glycosylation G1F N	Item tag								12 1.119+H	SFINK	1.65	522.2550		215.0
	8 EEQYNSTYR Glycosylation G1 N									14 1-138: Deamidation N (6)+H*	ASODVNTAVAWYOOKPGK	34 30	1000 0748	853.4300 3v(+H)	215.0
Clear	9 EEOYNSTYR Glycosylation G2F N			Detection	1 Resultsind next	+ Find previous Ch: 1 A	A: V No: 1 Abs. No.: 1 Sel: 0			15 1:T38:Deamidation N [6]+H <sup>+</sup> 1	ASODVNTAVAWYQQKI GK	36.70	1990.9748	853.4300 3x(+H)	215.0
	10 SSOVAUSTVD Stress Inters Mars N									16 1:T3+H*	ASODVNTAVAWYOOKPGK	34.54	1989.9908	3x(+H)	215.0
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UK Cancel	11 FTISADTSK	Add Edit Delete Intensity	Charo	e		RT				18 1:T78:Carbamidomethyl C [22]+H*	SGTDFTLTISSLQPEDFATYYCQQHYTTP PTFGQGTK	82.62	4186.9106		215.0
2242 0722		Distantes des Interaction des Franceires 1	Handard Marce (Dat)	······································	Evented in the Observed in the Ob	annual DT (min) Instantion to	Detail to a			19 1:T8+H*	VEIK	3.06	487.3006		215.0
2243.8732	13 IYPTNGYTR	Priority 1 Intensity 2 Pormula	veutrai Mass (Da) Adduct 3 C	ragmentation type	Expected m/2 Observed m/2 Ob	served KT (min) Jonization te	echnique Detail type			20 2:T1&:Pyroglutamic Acid E N-TERM [1]+H*	EVQLVESGGGLVQPGGSLR	69.90	1862.9850		215.0
	14 LSCAASGFNIK Carbamidomethyl C	Detection result: Instrument model: ACQUI	TY Sample Manager FTN, ACQUIT	Column Manager, Osprey, A	ACQUITY TUV Detector, ACQUITY Qua	ternary Solvent Manager, , Ir	instrument serial no: , Analysis, Created by ad	ninistrator on Sep		21 2:T1+H*	EVQLVESGGGLVQPGGSLR	53.92	1880.9956		215.0
	15 SI SI SPG	1 10466437	1874.9196 3x(+H) 3	None	625.9805 625.9787	32.030 ESI+	MSe			22 2:T10&:Deamidation N [8]+H*_1	NTAYLQMNSLR	41.72	1310.6289	2x(+H)	215.0
	16	2 1069877	1874.9196 2x(+H) 2	None	938.4671 938.4649	32.028 ESI+	MSe			23 2:T10&:Deamidation N [8]+H <sup>+</sup> _2	NTAYLQMNSLR	40.12	1310.6289	2x(+H)	215.0
2000	16 VDNALQSGNSQESVTEQDSK		1074.0400	Nees	4075 0060 4075 0040	20.024 557	115-			24 2:T10&:Oxidation M [7]+H*_1	NTAYLQMNSLR	27.30	1325.6398	2x(+H)	215.0
*	17 VYACEVTHQGLSSPVTK Carbamidomethyl	5 8045	18/4/3130 +H 1	None	18/5/9269 18/5/9218	32.051 ESI+	Mise			25 2:10+H*	NTAYLQMNSLR	38.43	1309.6448	2x(+H)	215.0
		4 3466	1874.9196 4x(+H) 4	None	469.7372 469.7356	32.027 ESI+	MSe			20 2:1118:Carbamidomethyl C [9]+H	AEDTAVIYCSR	22.46	1333.5608	2.(14) 4.(14)	215.0
										27 2.112ccOxtdation W [5]Th	WGGDGFTAMDTWGQGTEVTV35A5TK	/3.3/	2755.2400	5X(TTI), 4X(TTI)	215.0

Import into Accurate mass Screening Method

# **RESULTS AND DISCUSSION**

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## Monitoring Product Attributes with a HRMS Accurate Mass Workflow

No treatment

pH 9.0, 37 °C, Time

4d

H<sub>2</sub>O<sub>2</sub>% (v/v), 24 hr @ RT

0.003% 0.01% 0.015%

7d

2d



Accurate Mass System: Vion & Xevo QTof MS Data Acquisition: MS<sup>E</sup> Capillary Voltage: 3.0 kV Cone Voltage: 30 V Source Temperature: 100 °C Desolvation Temperature: 250 °C Mass Range (*m/z*): 100-2000

Lock mass used:

## Monitoring Product Attributes with a Nominal Mass Workflow

39.8 39.2 40.4

60.0 60.7 59.4



Fragment	Average Mass	[CH+1H] <sup>+1</sup>	[CH+2H] <sup>+2</sup>	[CH+3H] <sup>+3</sup>	[CH+4H] <sup>+4</sup>	[CH+5H] <sup>+5</sup>	[CH+6H] <sup>+6</sup>	[CH+7H] <sup>+7</sup>	[CH+8H] <sup>+8</sup>	[CH+9H] <sup>+9</sup>	[CH+10H] <sup>+10</sup>
Т39	574.3	575.3	288.2	192.4	144.6	115.9	96.7	83.0	72.8	64.8	58.4
T7	681.3	682.3	341.7	228.1	171.3	137.3	114.6	98.3	86.2	76.7	69.1
Т5	830.0	831.0	416.0	277 7	208 5	167.0	139.3	119.6	104 7	93.2	84.0

LeuEnk ([M+2H]<sup>2+</sup>, 556.2763)

MS<sup>E</sup> settings:

Low/high Energy Scan rate: 0.5 sec Low energy: 6 V High energy ramp: 20-45 V

#### **Deamidated Peptide Monitoring**



Figure 3: Monitoring variation in asparagine deamidation. A) Reviewing processed and integrated results in UNIFI can be obtained for optical and MS data channels, including XICs for individual components. B) Summary plots demonstrate relative % abundance of UV and MS response. Robust quantification was also obtained for peptides less susceptible to alkaline stress.

#### Glycopeptide Profiles Monitoring (HC T25: EEQYN<sub>300</sub>STYR)



Table 1: Peptide map charge states table. Multiple charge states observed for heavy chain tryptic peptides of trastuzumab across a broad molecular weight range (inset) using TFA and FA based methods, affords significant flexibility in method development of monitoring assays using the ACQUITY QDa.

T21	835.0	836.0	418.5	279.3	209.7		N	Aass Error Vs. Mole	cular Weight		
Т30	838.0	839.0	420.0	280.3	210.5	0.30					
Т9	969.1	970.1	485.5	324.0	243.3	0.10		8 0			) Inst
Т6	1084.2	1085.2	543.1	362.4	272.1			80 ° (			rum
Т3	1089.2	1090.2	545.6	364.1	273.3	-0.20					J tion
T36*	1161.4	1162.4	581.7	388.1	291.3	-0.30					
T2*	1167.4	1168.4	584.7	390.1	292.8	0	1,000 2,000	3,000 4,00	0 5,000 e	5,000 7,000	8,000
T8-9	1182.3	1183.3	592.2	395.1	296.6	T	Theore	tical Average Iviole	cular weight (Da	)	
T13	1186.4	1187.4	594.2	396.5	297.6	238.3	198.7	170.5	149.3	132.8	119.6
T10	1310.5	1311.5	656.3	437.8	328.6	263.1	219.4	188.2	164.8	146.6	132.1
T4-5	1311.5	1312.5	656.8	438.2	328.9	263.3	219.6	188.4	164.9	146.7	132.2
T14*	1321.5	1322.5	661.8	441.5	331.4	265.3	221.3	189.8	166.2	147.8	133.2
T11*	1334.4	1335.4	668.2	445.8	334.6	267.9	223.4	191.6	167.8	149.3	134.4
T23	1677.8	1678.8	839.9	560.3	420.5	336.6	280.6	240.7	210.7	187.4	168.8
T33-34	1724.9	1725.9	863.5	576.0	432.2	346.0	288.5	247.4	216.6	192.7	173.5
T26	1808.1	1809.1	905.1	603.7	453.0	362.6	302.4	259.3	227.0	201.9	181.8
T38	1874.1	1875.1	938.0	625.7	469.5	375.8	313.3	268.7	235.3	209.2	188.4
T1	1882.1	1883.1	942.1	628.4	471.5	377.4	314.7	269.9	236.3	210.1	189.2
T22*	2139.4	2140.4	1070.7	714.1	535.8	428.9	357.6	306.6	268.4	238.7	214.9
T26-27	2228.6	2229.6	1115.3	743.9	558.1	446.7	372.4	319.4	279.6	248.6	223.9
T2-3*	2238.6	2239.6	1120.3	747.2	560.6	448.7	374.1	320.8	280.8	249.7	224.9
T37	2544.7	2545.7	1273.3	849.2	637.2	509.9	425.1	364.5	319.1	283.7	255.5
T12	2785.0	2786.0	1393.5	929.3	697.3	558.0	465.2	398.9	349.1	310.4	279.5
T41*	2802.1	2803.1	1402.1	935.0	701.5	561.4	468.0	401.3	351.3	312.3	281.2
T19-20*	3335.9	3336.9	1669.0	1113.0	835.0	668.2	557.0	477.6	418.0	371.7	334.6
T15*	6716.5	6717.5	3359.2	2239.8	1680.1	1344.3	1120.4	960.5	840.6	747.3	672.6
T15-16*	7058.9	7059.9	3530.4	2354.0	1765.7	1412.8	1177.5	1009.4	883.4	785.3	706.9
T1E 17*	7197 0	7100 0	2E04 E	2206 7	1707 0	1/20 /	1100 0	1027.7	000 /	700 6	710 7



#### **Glycopeptide Monitoring**



*Figure 7: Glycopeptide profiles are* obtained using the MS SIR scan for enhanced sensitivity to monitor co-eluted peptides. Relative modification results using QDa were comparable to UNIFI/HRMS results.

Figure 4: A) The UNIFI accurate mass screening workflow enables robust and efficient targeted MAM for qualitative assessment with an option to flag signature fragment ions for increased confidence in assignments B) across large multi-batch data sets.

#### **Setting Limits and System Suitability**

to be r	performed duri	ng analysis then fill in the desire	ed limits check parameters						Compone	ry Plot + ent: 1:T38:Deamidation N [6]+H*		Sum
a conte	ext menu on the	e Component column header.	<sup>6</sup> % Peptide Modification (MS									
Selec	ted fields and li	imits checking		,	Warnin	g and Error Lev	vel Settir	ngs	%] SM-40- 40- 40-	<b>√</b> UEL		40.2 40.4 42.0
1	Node	Field name	Component	Sample type	Error minimum	Warning mini 1 A Target value	Warning maximum	Error maximum	20-		24.8 24.8 24.8	
1	Component	%Peptide Mod-MS (%)	1:T3&:Deamidation N [6]+H*	All	-9	0	10	30		83 85 84 84 84 <b>82</b> 81	83 84 86 84	
2	Component	% Peptide Mod-UV (%)	1:T3&:Deamidation N [6]+H*	All	-11	0	10	35	0	2		, , , , , , , , , , , , , , , , , , ,
3	Component	Chromatographic width (min)	All	All	0	0.05	0.3	0.5		b_Contr, b_Oxi3, b_Oxi3, b_Oxi2, b_Oxi2,	b_Oxi_1. b_Oxi_1. b_Oxi_1. b_oxi_1. Deam_1. Deam_1.	Deam_2 Deam_2 Deam_2
/4	Component	Mass error (ppm)	·	All	-20.1	-10.1	10.1	20.1		Trink Trink Trink Trink Trink	یس موجود عسل موجود Sample Injection	Tmab_ Tmab_

Figure 5: Setting limits and system suitability criteria enables color coded highlights for samples or batches that exceed data quality criteria or breach expected limits for component ranges.

Using the UNIFI/HRMS platform, characterization and monitoring data can be acquired using a single acquisition methodology (UPLC/UV/MSE), with different informatics but processing workflows optimized for each analysis.

✓ A compliant-ready UNIFI platform ✓ Robust Quantification (UV and MS) ✓ New/differential peak detection

✓ Ability to update the scientific library



Figure 8: Complementary determining region (CDR) peptide profiles are effectively extracted from the MS full scan TIC for the rapid determination of product identity.

The AQUITY QDa mass detector can be readily added to existing Empower/ACQUITY UPLC/UV systems for expanded dynamic range in attribute monitoring over optical only assays



Figure 9: Reports can be automatically generated when linked to the method, automating the monitoring process in a regulated environment.

✓ Compliant-ready Empower platform  $\checkmark$  Accessible technology

✓ Small footprint, easier deployment

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