

Liquid Chromatograph Mass Spectrometer LCMS™-9030

Characterization of control and stress induced samples of trastuzumab biosimilar using LCMS-9030 by bottom-up approach

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User Benefits

- ◆ In-depth peptide mapping and PTM analysis methodology is described for the analysis of control and stress induced samples of mAb which can be useful in identification of sites susceptible to oxidation and deamidation modifications
- ◆ Excellent and stable mass accuracy, comprehensive fragmentation pattern and high sensitivity offered by LCMS-9030 helped obtain good sequence coverage, identification of PTMs and sites susceptible for modifications

Introduction

Monoclonal antibodies (mAbs) are a major class of biopharmaceuticals covering a large panel of diseases, from cancer to asthma, including central nervous system disorders, infectious diseases and cardiovascular diseases¹. Throughout manufacturing, storage, transportation, and administration, mAbs are subjected to biophysical and biochemical stress from multiple sources, which may lead to their degradation via aggregation, fragmentation, and chemical modifications, such as oxidation, deamidation, or isomerization². Among these undesirable degradation products, oxidation and deamidation are the most commonly observed Post-Translational Modifications (PTMs). Early identification of these prone sites enables antibody engineering to eliminate liability of leading candidates to such modifications while maintaining binding activity.

Bottom-up approach which is essentially peptide mapping helps in determination of primary amino acid sequence and identification of site-specific PTMs. In this application note, methodology for identification of sites susceptible to oxidation and deamidation is described by analyzing the control and artificial chemically stress induced (forced degradation) samples of mAb.

Experimental

Trastuzumab biosimilar sample (2 mg/mL) was incubated in water with pH 9 (adjusted using tris base solution) for 7 days at 37 ° C to induce deamidation. Similarly, 10 µL of 30 % H₂O₂ solution was added to 2 mg/mL of trastuzumab sample and incubated for 1 hour at 37 ° C to induce oxidation. Oxidation reaction was quenched by adding pinch of methionine.

100 µg of trastuzumab control and stress induced samples were incubated with reduction buffer containing 8 M urea and 5 mM dithiothreitol at 37 ° C for 60 mins. Solutions were then alkylated with 20 mM of iodoacetamide at room temperature for 30 mins. At the end of incubation period, 50 mM Tris-HCl buffer (pH 8) was added to the solutions. Finally, trypsin was added in 1: 50 ratio and overnight digestion was carried out. Reaction was quenched with diluted formic acid and desalting was carried out using Solid Phase Extraction (SPE) cartridges. Eluent obtained at the end of SPE clean-up was evaporated using vacuum centrifugation and reconstituted in water: formic acid: acetonitrile (100:1:2 v/v) solution and analysed with LCMS-9030 (shown in Fig. 1) for peptide mapping analysis. Mobile phase consisting of 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile was used on Shim-pack™ Arata Peptide C18 column for the chromatographic separation. Analysis was performed in Data Dependent Acquisition (DDA) mode in positive polarity using Electro Spray Ionization (ESI) interface³. DDA data acquisition was

controlled by the LabSolutions™ LCMS software. Mass range of 200-2500 *m/z* was used for MS1 TOF survey scan. Base peak chromatogram intensity threshold of more than 1000 was used to trigger the MS/MS fragmentation with collision energy spread of 18-52 V. Use of collision energy spread allowed acquisition of comprehensive fragmentation pattern for any given precursor ion. Seven dependent (MS/MS) events were set to allow sufficient MS/MS data collection. Mass range of 100 to 2800 *m/z* was used to obtain MS/MS spectra. Ion exclusion and inclusion settings are available in the LabSolutions LCMS software to automatically exclude background ions and include ions of interest, respectively.

All data acquisition was performed with single external TOF calibration. No intermediate TOF calibration/lock masses were used during the data acquisition/processing. Details of analytical conditions are given in Table 1.

Table 1 Details of analytical conditions for bottom-up approach

HPLC system	Nexera™ X2
Column	Shim-pack™ Arata Peptide C18 (100 mm × 2.0 mm I.D., 2.2 µm) (P/N: 227-32806-02)
Column oven	40 ° C
Mobile phases	A-0.1 % formic acid in water B-0.1 % formic acid in acetonitrile
Flow rate	0.3 mL/min
Gradient program (B%)	0-3 min → 1 (%); 3-45 min → 1-30 (%); 45-46 min → 30 (%); 46-46.1 min → 30-90 (%); 46.1-50 min → 90 (%); 50-50.1 min → 1 (%); 60 min → stop
LCMS system	LCMS-9030
Interface	Heated ESI
Polarity	Positive
Acquisition mode	DDA
Mass range for TOF survey scan	200-2500 <i>m/z</i>
Mass range for precursor ion	220-2000 <i>m/z</i>
Mass range for MS/MS scan	100-2800 <i>m/z</i>
Collision energy spread	18-52 V
Temperatures	Interface: 300 ° C Desolvation line: 200 ° C Heater block: 400 ° C
Gas flow rates	Heating gas: 15 L/min Nebulizing gas: 3 L/min Drying gas: 15 L/min

The LCMS-9030 quadrupole time-of-flight (Q-TOF) mass spectrometer is a powerful instrument that integrates the world's fastest and most sensitive quadrupole technology with TOF capabilities for accurate mass measurement. Patented technologies of LCMS-9030, UF-FlightTube™ and iRefTOF™, ensure excellent Mass Measurement Accuracy (MMA) with stability which helps in identification of different peptides and PTMs present in the sample. UFaccumulation™ and UFgrating™ offer superior sensitivity which helps in detecting low abundant PTMs present in the samples.



- ❖ **UFaccumulation™**
- ❖ **UFgrating™**
- ❖ **UF-FlightTube™**
- ❖ **iRefTOF™**

Fig. 1 LCMS™-9030 Quadrupole Time of Flight Mass Spectrometer

DDA data acquired by LCMS-9030 was processed using 'Protein Metrics' software suite⁴. Settings of precursor mass tolerance of '6 ppm' and fragment mass tolerance of '20 ppm'; maximum 2 missed cleavages; and fully specific trypsin digestion efficiency were used for peptide/PTMs identification. Carbamidomethyl modification was considered as fixed. Other PTMs like oxidation, deamidation, Gln->pyro-Glu, Glu->pyro-Glu, ammonia-loss/ succinamide formation, dioxidation, dethiomethylation were considered as variable. N-glycan 52 common biantennary database present in the software was used to obtain information about glycosylation.

■ Results and Discussion

Therapeutic proteins may undergo a series of modifications throughout their cellular production, upstream/downstream processing, and storage. These modifications can include the addition or replacement of functional groups, or structural changes such as folding/unfolding, cleavage, and racemization. Presence of these modifications can affect biological activity, half-life and immunogenicity⁵. Hence, it is of utmost importance to identify these modifications accurately. Moreover, it is also important to find out the sites which are prone to undergo such modifications.

Analysis of the control and artificially stress induced mAb samples can provide understanding of such susceptible sites. The bottom-up approach for mAb characterization is typically referred to as "peptide mapping,". Peptide mapping analysis not only provides information about primary sequence of mAbs but also useful in identifying sites that are susceptible to oxidation, deamidation etc.

Hence, control and stress induced samples of trastuzumab biosimilar were subjected to peptide mapping and PTMs analysis. Overlay of extracted ion chromatograms from TOF survey scan (MS1) for trastuzumab control sample is shown in Fig. 2. More than 92 % of peptide sequence coverage was obtained for both heavy and light chains of trastuzumab control sample even with single enzyme digestion (shown in Fig. 3). Some of the short peptide chains (around 3 to 4 amino acid) are found to be not covered; however, use of multiple enzymes for the digestion can improve the sequence coverage.

LCMS-9030 offered excellent mass accuracies for the peptides with different chain length. Representative data demonstrating the mass accuracy (less than 2 ppm) obtained for peptides with chain length as short as 4 amino acid and as long as 63 amino acid is shown in Table 2. Obtaining such mass accuracies and stability for longer duration is of utmost important to accurately identify the PTMs.

Generally, mass shift in precursor ion m/z for the modified peptide and change in the retention time are considered to identify the presence of PTMs. Furthermore, acquiring good quality MS/MS spectra is equally important to confidently assign the location of the modification on a given peptide. Collision energy spread function of LCMS-9030 helps acquire MS/MS fragmentation pattern over a range of collision energy (18-52 V, in this case) instead of obtaining MS/MS spectra at single or few selected collision energies. Thus, comprehensive MS/MS fragmentation pattern can be obtained which in turn helps in confident site-specific PTM assignment. Examples of identified modifications like oxidation, deamidation etc. are discussed below.

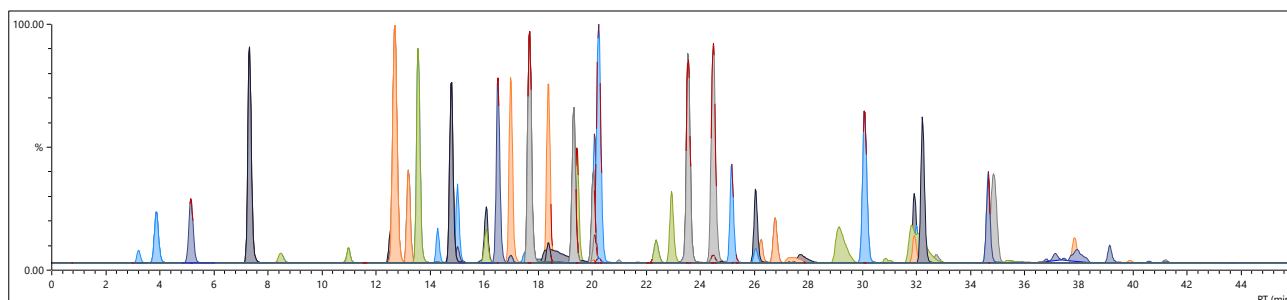


Fig. 2 Overlay of extracted ion chromatograms of TOF survey scan (MS1) for trastuzumab biosimilar control sample

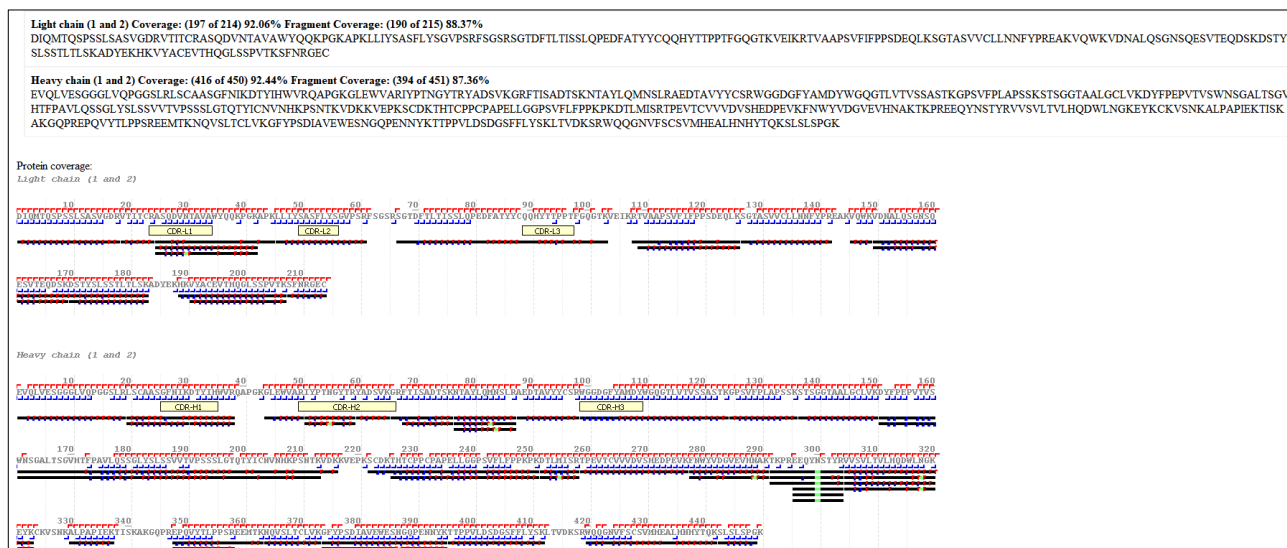


Fig. 3 Summary of peptide coverage, fragmentation coverage and PTMs for trastuzumab biosimilar control sample

Table 2 Representative results of mass accuracies for peptide sequences of different chain length for control and stress induced samples of Trastuzumab biosimilar

#	Name	m/z	TmAb_Control		TmAb_Oxidation_1		TmAb_Deamidation_pH 9	
			Found RT	Mass Error (ppm)	Found RT	Mass Error (ppm)	Found RT	Mass Error (ppm)
1	VQWK	280.6632	5.140	-1.033	5.092	-0.713	5.144	-1.710
2	YADSVK	341.6740	3.206	-0.819	3.318	-0.966	3.302	-1.727
3	DTLMISR	418.2207	14.779	0.096	14.833	-0.550	14.810	-1.124
4	DTLm (+16)ISR	426.2182	12.530	-0.493	12.572	-0.094	12.547	-1.103
5	ASQDVNTAVAWYQQKPGK	664.3376	19.423	-0.045	19.460	0.241	19.464	-0.557
6	ASQDVhTAVAWYQQKPGK	664.6656	20.432	0.135	20.454	-0.361	20.446	-0.451
7	EEQYnSTYR(Nglycan/1606.5867)	932.7044	10.889	-0.279	10.923	0.118	10.902	0.450
8	DYFPEPTVSWNSGALTSVHTFPAVLQSSGLYSLSVTVPSSSLGTQTYICNVNHKPSNTK	1343.4687	41.202	0.298	41.173	1.243	41.150	0.789

Oxidation of biotherapeutic proteins can alter their physical and biological properties, affecting their potency and stability characteristics. The most commonly oxidized amino acid is methionine (Met). Oxidized peptides are easily identified using MS since the addition of one oxygen atom to the Met side-chain upon conversion to Met sulfoxide increases the mass of the affected residue by +16 Da⁵. Location of oxidation modification can be easily identified from MS/MS fragmentation pattern. Identification of oxidation modification is illustrated with 'DTLMISR' peptide and shown in Fig. 4. Oxidized peptide 'DTLMISR' elutes earlier as compared to its unmodified counterpart as 'oxidation' imparts hydrophilicity to the peptide. Mass shift of +8 Da (for '+2' charge state) for oxidized peptide precursor ion *m/z* can be seen from MS1 spectra. MS/MS fragmentation spectra revealed that y3 fragment is

same for both the peptide versions, however mass shift in fragment ions can be seen from y4 fragment onwards indicating the location of modification as y4 methionine. Met sulfoxide-containing peptides readily lose methane sulfenic acid (CH₃SOH) upon CID fragmentation and are thus easily identified by a characteristic loss of 64 Da from the fragment mass which can be seen in MS/MS spectra of modified peptide shown in Fig. 4.

Deamidation of asparagine (Asn) and glutamine (Gln) has an important role in regulating the heterogeneity and stability of recombinant mAbs. Deamidation is one of the most challenging PTMs to characterize using MS-based techniques. Deamidation results in conversion of –NH₂ to –OH (+0.984 Da), which has a very similar mass shift as the first ¹³C isotope peak of the native peptide (+1.0034 Da)⁵.

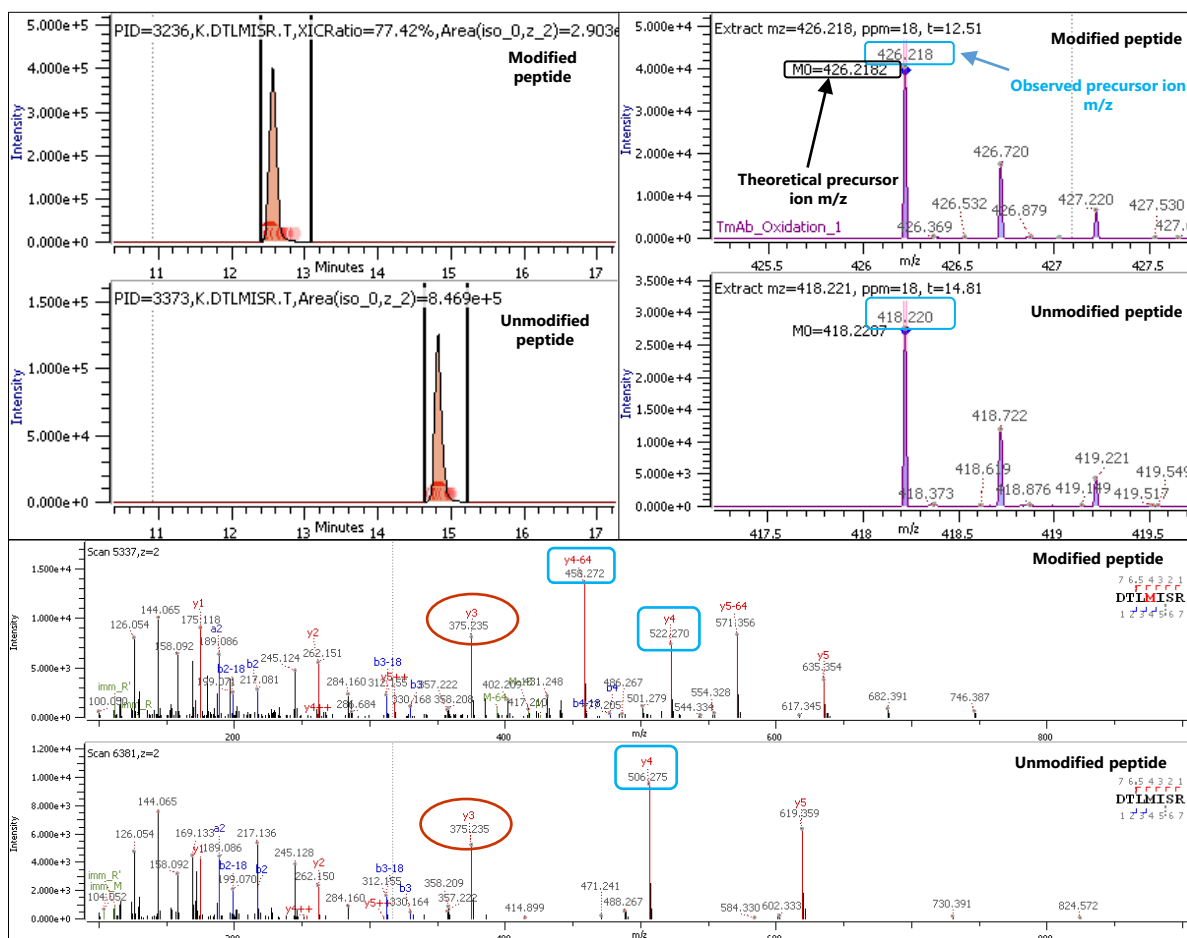


Fig. 4 Top left: Chromatograms of oxidized and unmodified 'DTLMISR'; Top right: MS1 spectra of oxidized and unmodified 'DTLMISR'; Bottom: MS/MS fragmentation spectra for oxidized and unmodified 'DTLMISR'

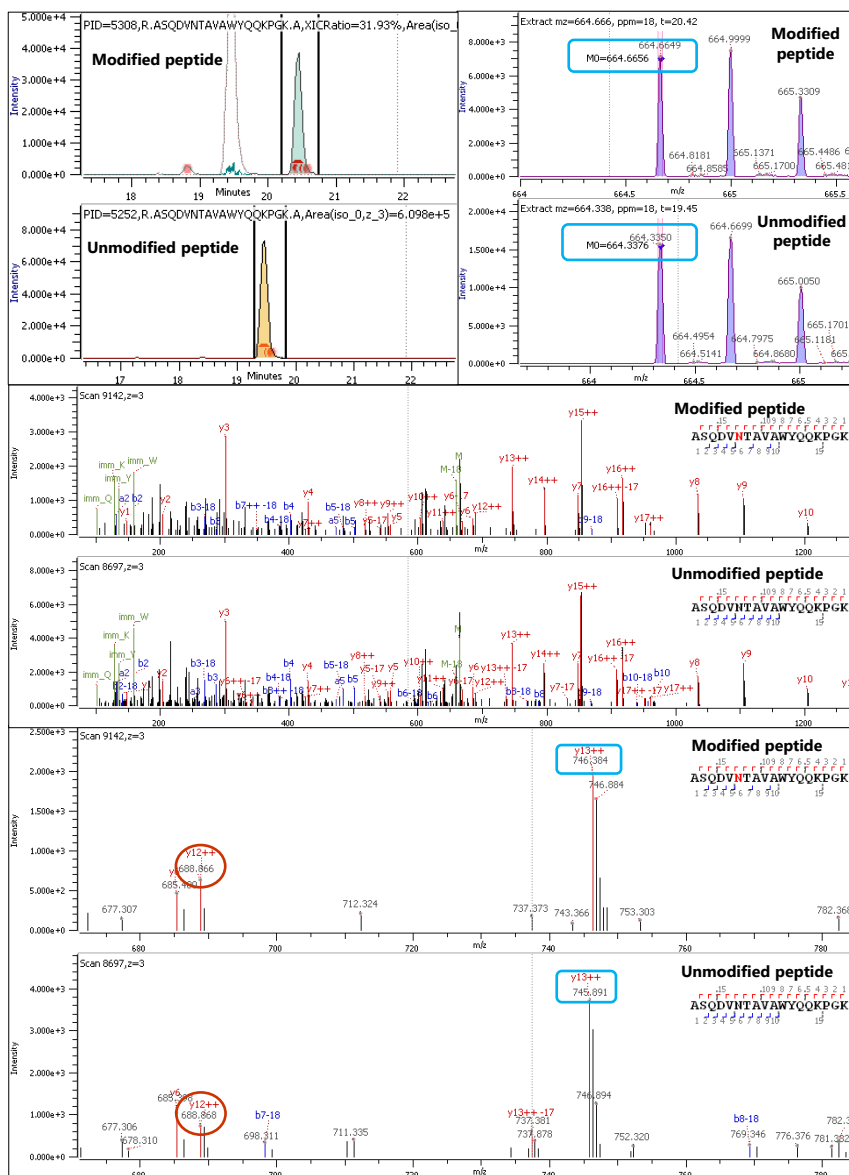


Fig. 5 Top left: Chromatograms of deamidated and unmodified 'ASQDVNTAVAWYQQKPGK'; Top right: MS1 spectra of deamidated and unmodified 'ASQDVNTAVAWYQQKPGK'; Middle: MS/MS fragmentation pattern for deamidated and unmodified 'ASQDVNTAVAWYQQKPGK' Bottom: Zoomed MS/MS fragmentation spectra for deamidated and unmodified 'ASQDVNTAVAWYQQKPGK'

Deamidation modification example is illustrated with 'ASQDVNTAVAWYQQKPGK' peptide (refer to Fig. 5). Mass shift of '+ 0.329 Da' (for +3 charge state) is observed in MS1 scan spectra of modified peptide which is very close to first ¹³C isotope m/z of unmodified precursor ion. Hence, careful evaluation of precursor ion mass shift, MS/MS pattern and change in the retention time is essential before assigning deamidation modification.

It is observed that deamidated peptide is eluting later than its unmodified counterpart. MS/MS fragmentation pattern revealed that product ion m/z up to y12++ are same for modified as well as unmodified peptide. However, y13++ fragment ion showed difference of 0.49 Da (for '+2' charge state) as seen in Fig. 5 confirming the presence of deamidation modification and its location on given peptide. Relative abundance summary of PTMs observed for control and stress induced samples of trastuzumab biosimilar is given in Table 3. It can be observed that three sites, viz., HC-361, HC-107 and HC-431 have shown methionine oxidation only in oxidative stress induced sample and are absent in control as well as deamidation stress induced

samples which shows susceptibility of these sites to oxidative stress. Site HC-255 has shown methionine-oxidation in all three samples, however, relative abundance of modified peptide is higher (77.5%) in oxidative stress induced sample as compared to control and deamidation stress induced samples (6.35 and 7.29%). This site appears to be most susceptible to oxidative stress. Site HC-83 has shown minor level of methionine oxidation in all three samples with almost comparable relative abundance which indicates that this site may not be susceptible to oxidative stress.

Similarly, sites LC-30, HC-289, HC-387, HC-392, HC-55, HC-84 and HC-318 have shown deamidation. Some of these modifications could be sample preparation artefacts introduced due to elevated pH used during sample preparation. Nevertheless, sites LC-30 and HC-318 have shown elevated levels of deamidation modification in deamidation stress induced sample as compared to control and oxidative stress induced samples indicating potential susceptibility of these sites.

Table 3 Relative abundance summary of PTMs for control and stress induced samples of trastuzumab biosimilar

Sequence (unformatted) †	Mod. Names †	Protein name †	Mod. AAs †	Glycans †	Var. Pos. Protein †	MS Id --	1	3	4
						MS Alias name --	TmAb_Control (%)	TmAb_Oxidation_1 (%)	TmAb_Deamidation_pH 9 (%)
ASQDVTAVAWYQQKPGK	Deamidated/0.9840	Light chain (1 and 2)	N		30		7.63	7.12	31.1
DILMSR	Oxidation/15.9949	Heavy chain (1 and 2)	M		255		6.35	77.5	7.29
EEQYNSTYR	NGlycan/1241.4545	Heavy chain (1 and 2)	N	HexNAc(3)Hex(3)Fuc(1)	300		17.6	16.5	16.2
	NGlycan/1444.5339	Heavy chain (1 and 2)	N	HexNAc(4)Hex(3)Fuc(1)	300		43	43.9	44
	NGlycan/1606.5867	Heavy chain (1 and 2)	N	HexNAc(4)Hex(4)Fuc(1)	300		39.4	39.6	39.7
EPQVYTLPPSREEMTK	Oxidation/15.9949	Heavy chain (1 and 2)	M		361			39.8	
FNWYVDGVEVHNAK	Deamidated/0.9840	Heavy chain (1 and 2)	N		289		1.4	1.42	1.43
GFYPSDIAVEWESNGQFENNYK	Deamidated/0.9840	Heavy chain (1 and 2)	N		387		17.4	18.6	22.1
					392		17.4	18.6	22.1
IYPINGYTR	Deamidated/0.9840	Heavy chain (1 and 2)	N		55		37	38.7	37.8
NTAYLQMNSLR	Deamidated/0.9840	Heavy chain (1 and 2)	N		84		1.83	2.21	1.69
	Oxidation/15.9949	Heavy chain (1 and 2)	M		83		1.96	2.08	1.43
TKPREEQYNSTYR	NGlycan/1241.4545	Heavy chain (1 and 2)	N	HexNAc(3)Hex(3)Fuc(1)	300		9.09	11.8	9.02
	NGlycan/1444.5339	Heavy chain (1 and 2)	N	HexNAc(4)Hex(3)Fuc(1)	300		33.8	46.8	33.9
	NGlycan/1606.5867	Heavy chain (1 and 2)	N	HexNAc(4)Hex(4)Fuc(1)	300		57.1	41.4	57.1
VVSVLTVLHQDWLNGK	Deamidated/0.9840	Heavy chain (1 and 2)	N		318		5.83	5.85	12.4
VVSVLTVLHQDWLNGKEYK	Deamidated/0.9840	Heavy chain (1 and 2)	N		318		11.1	12.3	49.4
WGDDGFYAMDYWGQGLTVVSSASTK	Oxidation/15.9949	Heavy chain (1 and 2)	M		107			32.3	
WQQGNVFSQVMHEALHNHYTK	Oxidation/15.9949	Heavy chain (1 and 2)	M		431			35.5	

Conclusion

- Complete peptide mapping and PTMs analysis workflow for control and stress induced mAb samples is described.
- Sequence coverage of more than 92 % was obtained for both heavy and light chain of the mAb even with single enzyme digestion.
- Excellent mass accuracy with good stability, comprehensive fragmentation pattern and sensitivity offered by LCMS-9030 helps in reliable and in-depth characterization of mAb and also has helped in identification of sites susceptible to oxidation and deamidation modifications. Such studies can help in deep understanding of Critical Quality Attributes (CQA) of the product and to further develop Multiple-Attribute Method (MAM).

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