Identification of Oxidation Sites on a Monoclonal Antibody Using an Agilent 1260 Infinity HPLC-Chip/MS System Coupled to an Accurate-Mass 6520 Q-TOF LC/MS

Application Note

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Abstract

The identification of oxidation sites on a monoclonal antibody (mAb) using an Agilent 1260 Infinity HPLC-Chip/MS system coupled to an Agilent Accurate-Mass 6520 Quadrupole Time-of-Flight (Q-TOF) LC/MS is described. A monoclonal antibody was oxidized by treatment with hydrogen peroxide to stimulate potential oxidative modifications that may occur during the manufacturing process. The accurate mass measurement of the 6520 Q-TOF LC/MS combined with Agilent MassHunter BioConfirm software facilitated easy identification of the oxidation sites. Peptides containing amino acid residues such as methionine and tryptophan, which are sensitive to oxidation, were easily identified using this approach.

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Introduction

Monoclonal antibodies, like any other protein drug, can undergo oxidation during production, purification, formulation, and storage, leading to altered activity. Therefore, it is very important to analyze potential oxidation changes during such processes.

LC/MS technology is a powerful and sensitive technique for the identification of oxidation sites on proteins such as monoclonal antibodies. Nanoflow LC/MS enables faster and more sensitive characterization of proteins while minimizing sample and solvent consumption. Agilent's microfluidic-based HPLC-Chip integrates sample preparation and chromatographic separation for efficient, high-sensitivity nanospray LC/MS. Agilent Accurate-Mass 6520 Q-TOF LC/MS systems deliver exceptional mass resolution, mass accuracy, sensitivity, and data analysis capabilities for optimal MS characterization of proteins.

In this note, we describe how the combination of an Agilent 1260 Infinity HPLC-Chip/MS system, a 6520 Accurate-Mass Q-TOF LC/MS, and MassHunter BioConfirm software was used to analyze the oxidation sites of a monoclonal antibody (mAb) treated with hydrogen peroxide to simulate potential oxidative modifications that may occur during the manufacturing process. The oxidized protein was analyzed in order to find changes in the mAb's mass and to identify the sites of modification. The accurate mass measurement of the 6520 Q-TOF LC/MS enabled easy assignment of peptides carrying the oxidative modification of amino acids such as methionine or tryptophan in the peptide sequence. Furthermore, MS/MS results confirmed the site of modification in the peptide sequence. This application note continues our studies toward complete characterization of monoclonal antibodies using advanced Agilent platforms ideal for the biopharmaceutical market [1, 2].

Experimental

Sample Preparation

Materials

Immunoglobulin G (IgG) was obtained from ProMab Biotechnologies (USA), Inc. DL-Dithiothreitol (DTT), iodoacetamide, and Tris (hydroxymethyl)-aminomethane (Tris Base) were purchased from Sigma Aldrich (I ndia). High quality, sequence grade trypsin was obtained from Agilent Technologies (USA). Hydrogen peroxide was purchased from Qualigens Fine Chemicals (India).

Oxidation of mAb using hydrogen peroxide

A 2 µL sample of antibody (1 mg/mL) was diluted with 20 µL of Milli-Q deionized water, and 2 µL of H_2O_2 (50 % concentration) was added to this solution to cause oxidation. The oxidation reaction was kept for 20 min at room temperature before further analysis.

Reduction and alkylation of an antibody under denaturation conditions
Before the digestion of the mAb with various proteases, the disulfides were reduced and alkylated under denaturation conditions [3, 4].

The mAb was lyophilized, reconstituted in 2 µL of 8 M urea in 0.25 M Tris buffer, pH 7.6, containing DTT and then incubated at 37 °C for 30 min. To this solution, 2 µL of a solution containing iodoacetamide in 8 M urea in 0.25 M Tris buffer, pH 7.6, was added, and the sample was incubated at ambient temperature in the dark for 15 min. The solution was diluted with 160 µL of 0.25 M Tris buffer, pH 7.6, prior to digestion with trypsin.

Trypsin digestion
Trypsin was added to the above pretreated mAb solution at a ratio of 20:1 (protein to protease w/w). The reaction was incubated overnight at 37 °C. The enzymatic activity was quenched by adding 1 µL of 10 % formic acid solution. The samples were either immediately analyzed by LC/MS/MS or stored at -80 °C prior to LC/MS/MS analysis.

LC/MS Analysis

Instrumentation
The Agilent 1260 Infinity HPLC-Chip/MS was coupled to the Agilent 6520 Accurate-Mass Q-TOF LC/MS platform for LC/MS analyses.

LC Parameters
HPLC-Chip: 5 µm, ZORBAX 300SB-C18 (300 Å), 40 nL enrichment column and a 75 mm x 43 mm analytical column.

Flow rate: 3 µL/min from capillary pump to the enrichment column and 600 nL/min from nanoflow LC pump to the analytical column.

Solvents: 0.1 % formic acid in water (A); 90 % acetonitrile in water with 0.1 % formic acid (B). Flush volume was set at 4 µL.

Sample Loading: With capillary pump at 3 % B.

Sample Load: 50 ng of the protein digest.

Sample analysis: Gradient with nanoflow LC pump as shown below.

Time (min)	B (%)
Initial	3
30	50
32	95
34	95
34.10	3

Stop time: 36 min

MS Parameters
Spectra were recorded in positive ion and in centroid mode.

Vcap: 1,900 V and drying gas flow of 5 L/min at 325 °C was used.

Fragmentor voltage: 175 V.

Data were acquired on high resolution (3,200 *m/z*), 4 GHz, MS only mode, range 300-3,200 *m/z*; for MS/MS mode, range 50-3,000 *m/z*. An internal mass calibration sample was infused continuously during the LC/MS runs. This internal reference mass system allowed accurate, precise, and automated mass accuracy measurements during the LC/MS runs.

Data Analysis
The data obtained were analyzed using Agilent MassHunter Qualitative Analysis software and Agilent MassHunter BioConfirm software. All peptide components were found using the Molecular Feature Extractor (MFE) program within Agilent MassHunter Qualitative Analysis software.

Define and match sequence: Both the light and heavy chain sequences were digested using trypsin containing 2 missed cleavages with preferred modification of mono-oxidation and dioxidation to generate a theoretical peptide digest list. The compounds extracted using MFE were matched against this list.

MS/MS spectrum assignments:
MassHunter BioConfirm software was used to assign and match the MS/MS data obtained for the mAb trypsin digest against the theoretical peptide fragments.

Results and Discussion

The oxidized protein was analyzed using LC/MS to assess the number of oxidative modifications. Figure 1 shows the deconvoluted mass spectrum of the intact mAb, with and without oxidative modification. Analysis of the deconvoluted spectrum clearly shows the broad peak for the oxidatively modified protein implying a different degree of oxidation. The observed molecular weight of 148811.90 Da for the unmodified protein is in excellent agreement with the calculated molecular weight of 148811.95 Da, which corresponds to the glycosylated form of the protein[1].

The observed molecular weight for the oxidized mAb of 148950.45 Da corresponds to the addition of an average of 9 oxygen moieties (each oxidation adds 15.9949 Da). These 9 oxidations can happen randomly at any of the oxidatively sensitive amino acid residues in the mAb. In order to identify the modification sites, the mAb was subjected to trypsin digestion followed by LC/MS.

Figure 2 shows the total ion chromatogram (TIC) of the trypsin-digested oxidized mAb obtained using LC/MS, revealing strong chromatographic peaks with narrow peak widths. The peptide masses obtained for oxidized mAb were then matched with the theoretical digest using a mass tolerance of +/- 5 ppm with preferred modification of mono-oxidation and dioxidation included for the theoretical peptide digest.

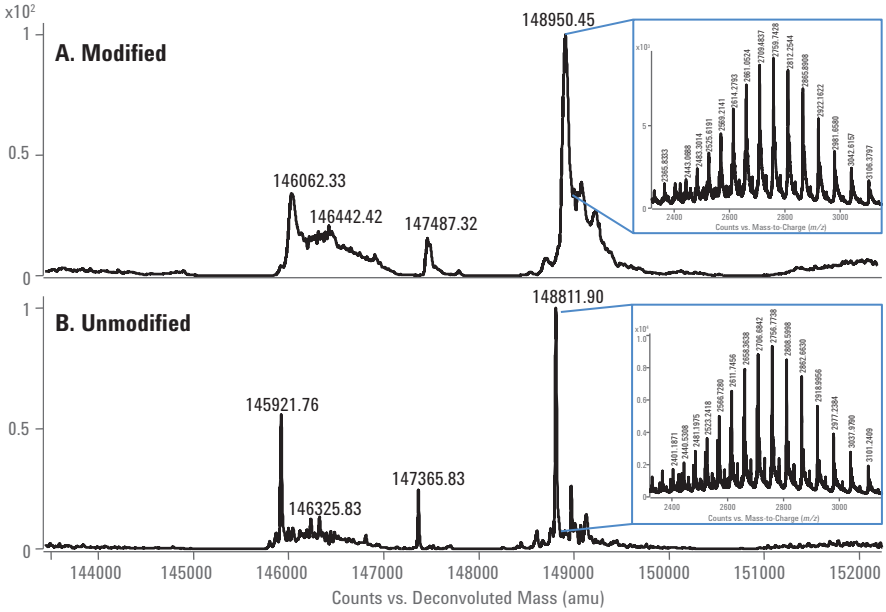

Figure 1. Deconvoluted spectrum of the intact antibody, oxidatively modified (A) and unmodified (B), with insets showing the mass spectrum of intact antibody.

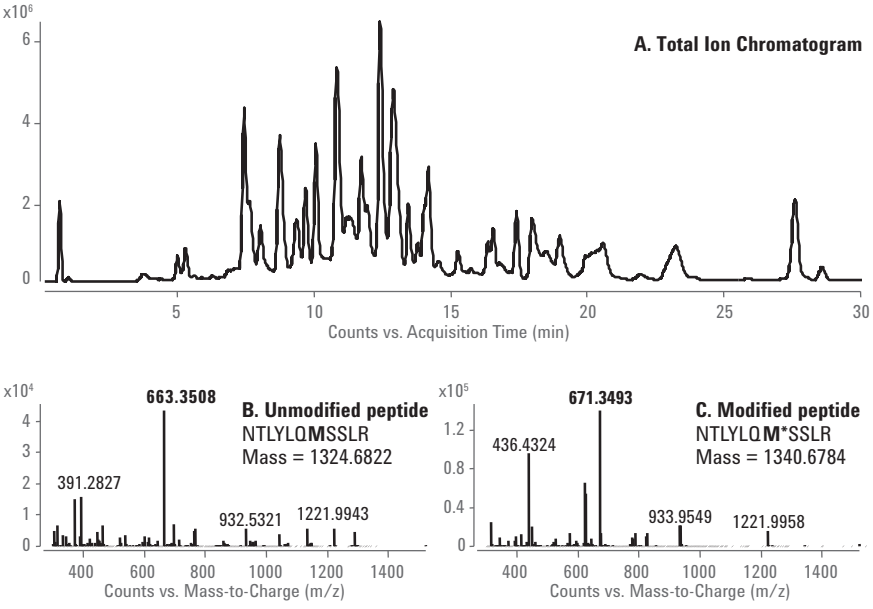

Figure 2. (A) Total ion chromatogram of trypsin digested mAb after oxidative modification with H_2O_2 on a C_{18} HPLC-Chip. Representative mass spectrum showing both the (B) unmodified and (C) modified peptide.

The theoretical digestion list of peptides for the trypsin-digested oxidized mAb was generated using the MassHunter BioConfirm sequence editor (define and match sequences). A partial list of peptides following with a match for the target mass having the modification is shown in Table 1.

From Table 1, it can be observed that peptides containing methionine (Met) and tryptophan (Trp) are readily oxidized. Oxidation of these residues results in increased molecular weights of ~16 or ~32 Da, depending on the number of oxygens added. The fifteen oxidation sites in Table 1 show the random nature of oxidation and explain the discrepancy from the intact protein analysis, highlighting the value of closely examining the tryptic digest of the antibody.

Sequence	Theoretical Mass (Da)	Measured Mass (Da)	Mass Difference (ppm)	Preferred Modifications
RFVSGAFDIWGQGTMVTVSSASTKGPSVFPLAPSSK	3762.8563	3762.8591	0.74	2*Dioxidation (+31.9898)
RFVSGAFDIWGQGTMVTVSSASTKGPSVFPLAPSSK	3698.8767	3698.8913	3.95	
SRWQQGNVFSCSVMHEALHNHYTQK	3091.3777	3091.3837	1.93	3*Oxidation (+15.9949)
SRWQQGNVFSCSVMHEALHNHYTQK	3043.393	3043.4066	4.46	
VQWKVDNALQSGNSQESVTEQDSK	2692.2576	2692.2675	3.68	1*Oxidation (+15.9949)
VQWKVDNALQSGNSQESVTEQDSK	2676.2627	2676.2531	-3.61	
GFYPSDIAVEWESNGQPENNYK	2559.119	2559.1255	2.52	1*Oxidation (+15.9949)
GFYPSDIAVEWESNGQPENNYK	2543.1241	2543.1347	4.18	
ASQSVSSSYLAWYQQKPGQAPR	2454.1928	2454.2025	3.96	1*Oxidation (+15.9949)
VYACEVTHQGLSSPVTKSFNR	2395.1591	2395.153	-2.55	1*Oxidation (+15.9949)
VYACEVTHQGLSSPVTKSFNR	2379.1641	2379.1563	-3.28	
GLEWVSAISGSGGSTYYADSVK	2249.0488	2249.0543	2.45	1*Oxidation (+15.9949)
GLEWVSAISGSGGSTYYADSVK	2233.0539	2233.0609	3.14	
LSCAASGFTFSSYAMSWVR	2190.935	2190.9383	1.49	2*Dioxidation (+31.9898)
HKVYACEVTHQGLSSPVTK	2156.0684	2156.075	3.03	1*Oxidation (+15.9949)
HKVYACEVTHQGLSSPVTK	2140.0735	2140.0821	3.99	
DNSKNTLYLQMSSLR	1784.8727	1784.8765	2.11	1*Oxidation (+15.9949)
DNSKNTLYLQMSSLR	1768.8778	1768.8821	2.44	
NTLYLQMSSLR	1340.6758	1340.6784	1.89	1*Oxidation (+15.9949)
NTLYLQMSSLR	1324.6809	1324.6822	0.98	

Table 1. Partial peptide mass list after digestion of the oxidized mAb with trypsin.

MS/MS data was used to map the modification sites on the peptides. The data was searched against the mAb sequence with preferred modification of oxidation using Agilent MassHunter BioConfirm software. An example of the analysis is shown in Figure 3, with the representative MS/MS spectrum for one of the peptides (NTLYLQMSSLR) in both modified and unmodified form.

If the y5 to y9 ions from the MS/MS spectrum from the modified peptide (Figure 3C) are compared to those from the unmodified peptide (Figure 3B), there is clearly an increase of ~16 Da over those ions. As both spectra show y3 and y4 ions at the same mass the oxidation must occur on the Met position. The fragment masses were within 10 ppm mass error, showing the superb mass accuracy of Agilent Q-TOF LC/MS, which enhances the reliability and validity of the peptide fragment assignments. Table 2 shows the peptide fragment ions with ppm error for the non-oxidized and oxidized peptide.

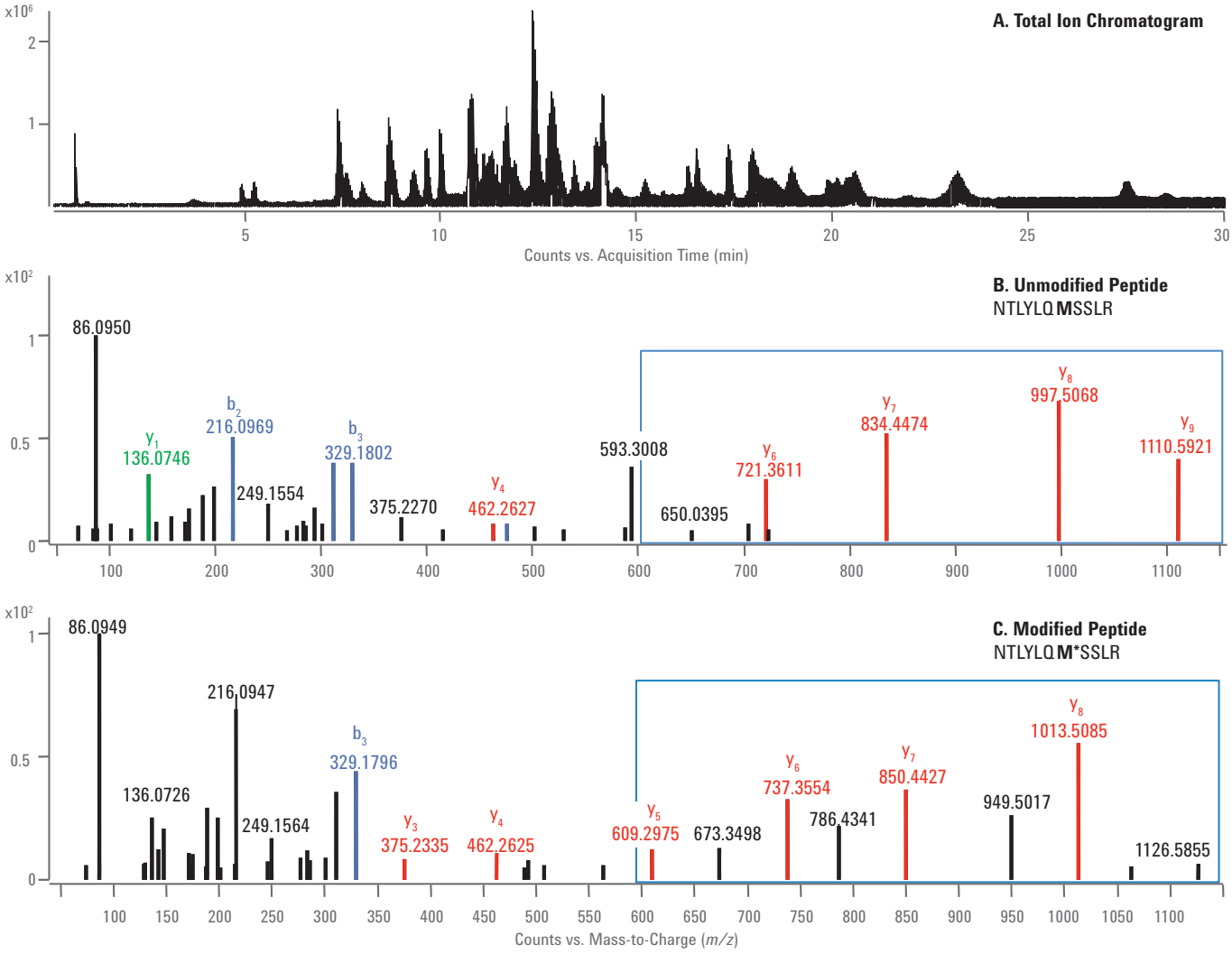

Figure 3. (A) Total ion chromatogram MS (all) of the trypsin-digested mAb after oxidative modification with H_2O_2 on a C_{18} HPLC-Chip. (B) Unmodified and (C) modified representative MS/MS spectra of peptides and their assignments. The blue box shows the difference of ~16 Da for y5 to y9 ions between the unmodified and modified peptide.

Non–oxidized peptide

Mass Difference (ppm)	Measured Mass	Theoretical Mass	Ion	Sequence
-4.7	216.0969	216.0979	b2	NT
-5.4	329.1802	329.1819	b3	NTL
-9.5	462.2627	462.2671	y4	SSLR
-7	721.3611	721.3661	y6	QMSSLR
-3.4	834.4474	834.4502	y7	LQMSSLR
-6.8	997.5068	997.5135	y8	YLQMSSLR
-5	1110.592	1110.5976	y9	LYLQMSSLR

Oxidized peptide

Mass Difference (ppm)	Measured Mass	Theoretical Mass	Ion	Sequence
-7.1	329.1796	329.1819	b3	NTL
-4.1	375.2335	375.235	y3	SLR
-9.8	462.2625	462.2671	y4	SSLR
-8.2	609.2975	609.3025	y5	MSSLR
-7.6	737.3554	737.3611	y6	QMSSLR
-2.8	850.4427	850.4451	y7	LQMSSLR
0.1	1013.509	1013.5084	y8	YLQMSSLR

Table 2. Fragment ion mass with ppm error shown for non-oxidized and oxidized peptide.

Conclusions

Rapid and sensitive characterization of an intact mAb has been demonstrated using the Agilent 1260 Infinity HPLC-Chip/MS system coupled to the Agilent 6520 Accurate-Mass Q-TOF LC/MS. The excellent chromatographic resolution and highly accurate peptide mass determination enabled accurate assignment of oxidized peptide peaks to the mAb sequence under investigation. The MS/MS analyses helped increase confidence of peptide sequence assignments by mapping the exact location of the oxidative modification in peptides.

Agilent MassHunter BioConfirm software enabled the generation of a theoretical digest with a variable modification and is a valuable tool for assessing the oxidative changes that occur in a mAb during manufacturing or other process. The b and y ions workflow feature provided another valuable benefit in assigning the MS/MS spectrum of peptides.

References

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