Quantitation 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 various concentrations of t-butyl hydroperoxide (t-BHP) to stimulate potential oxidative modifications that may occur during a 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 are sensitive to oxidation, but were easily identified using this approach compared to a non-oxidized control sample. Statistical software was used to demonstrate the degree of grouping and clear differentiation between levels of oxidation. Difference mapping was also used to illustrate differences between oxidation levels.

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Introduction

Protein oxidation is one of the major concerns in the biopharmaceutical industry. Proteins are sensitive to oxidation during production, formulation, storage, and other processes. Amino acids such as methionine (Met) cysteine (Cys), tryptophan (Trp), and histidine (His) are known to be sensitive to oxidation, which can lead to a loss of desired activity of protein biotherapeutics [1]. Oxidation of a Met residue in a monoclonal antibody is of major concern because it can lead to a product with altered binding properties [2]. Forced oxidation studies using either hydrogen peroxide or t-BHP have been performed in order to investigate the possible sites that are susceptible to oxidation and the effect of oxidation on activity [3].

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, a 6520 Accurate-Mass Q-TOF LC/MS, and MassHunter BioConfirm software was used to analyze the oxidation sites of a mAb treated with t-BHP to simulate potential oxidative modifications. The oxidized and non-oxidized control samples were digested with trypsin to generate peptide maps, and analyzed with LC/MS to identify modification sites and quantify the degree of oxidation.

The commonly used DLTMISR peptide sequence from the mAb was used to quantify the degree of oxidation in the monoclonal antibody. MassHunter Comparative Analysis software was used to display differential results and to highlight oxidation levels, and MassProfiler Professional software was used to confirm the statistical significance between differing levels of oxidation. Studies such as these are particularly important at the early stages of biopharmaceutical product development. It is in these early stages that limited amounts of antibody are available, so the use of the high-sensitivity HPLC-Chip/MS technique achieves high-quality results with low (50 ng) sample levels.

Experimental

Sample Preparation

Materials

Immunoglobulin G (IgG) was obtained from ProMab Biotechnologies, Inc. DL-Dithiothreitol (DTT), iodoacetamide, Tris (hydroxymethyl)-aminomethane (Tris Base), t-butyl hydroperoxide (t-BHP) were purchased from Sigma Aldrich (India). High quality sequence grade trypsin was obtained from Agilent Technologies (USA).

Oxidation of mAb using t-BHP

The monoclonal antibody samples were incubated with different concentrations of t-BHP in Tris-HCl, pH 7.5, overnight at ambient temperature. Samples of mAb were incubated with 0, 0.1, 0.3, 0.6, 1.0, 1.5, and 3 % t-BHP. After overnight reaction, the treated samples were vacuum dried and subjected to trypsin digestion.

Trypsin digestion

Trypsin was added to the mAb samples at a ratio of 20:1 (protein to protease w/w, after reduction and alkylation treatment). 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 until further 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	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 oxidation 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

Samples were analyzed by HPLC-Chip/MS and Met oxidation was determined. As initial screening, a Principle Component Analysis (PCA) plot was generated to compare and contrast the different levels of oxidation of the mAb. PCA is a clustering tool often applied to reduce the dimensionality of complex data sets. In the context of this experiment, PCA was applied as a screening tool to check the grouping and differentiation prior to determining the extent of oxidation, and producing the relative percentage oxidation graph. The result for the oxidation samples demonstrates correct grouping of the technical replicates (same color), confirming the reproducibility of the data. It is clearly observed that the control (cyan) sample and 0.1 % (red) t-BHP treated mAb were well separated from the other samples treated with increasing concentration of the oxidizing reagent (Figure 1). This suggested the control and mildly oxidized mAb were very different from the mAb that had been treated with a higher concentration of t-BHP.

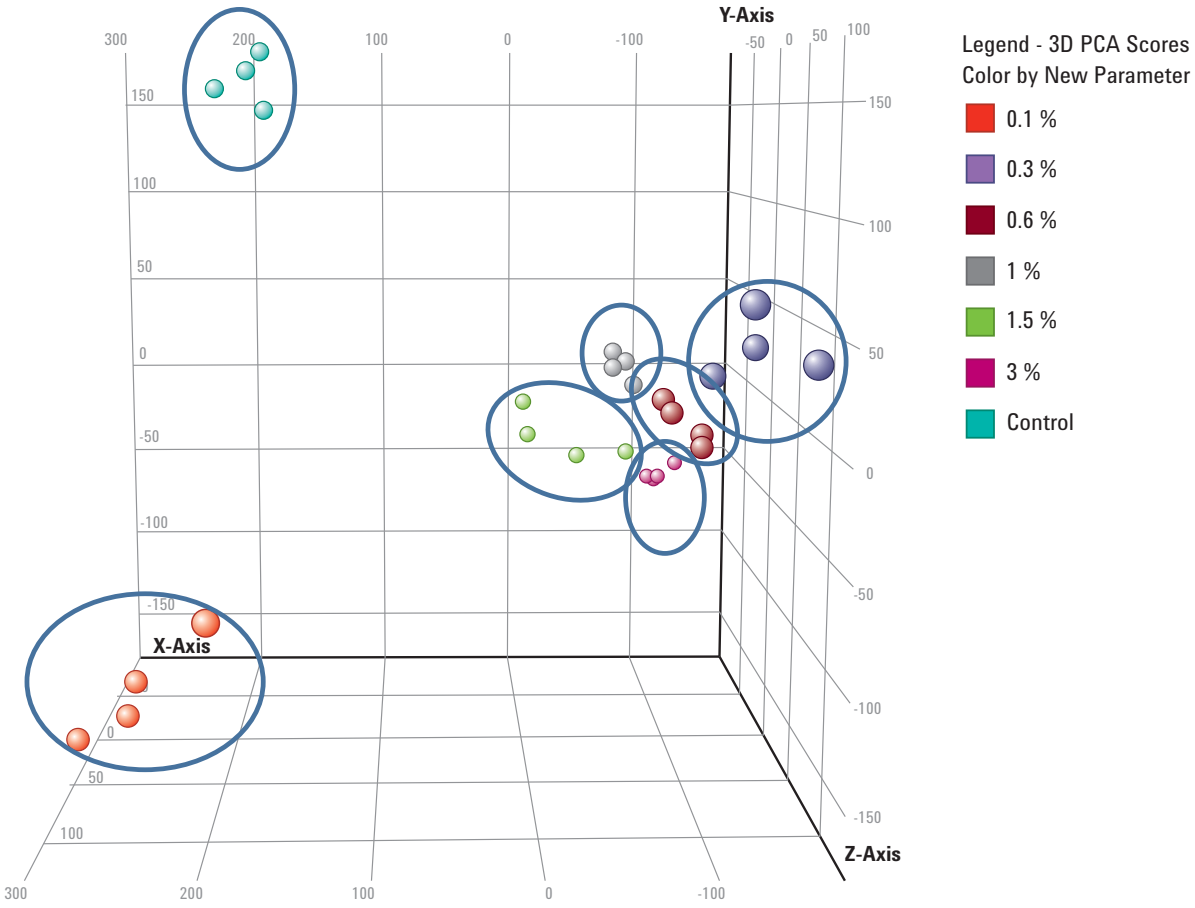

Figure 1. PCA analysis (3D scatter plot) of the different oxidation conditions of the mAb.

The peptide masses obtained for the oxidized mAb from the MFE analysis were matched with the theoretical digest, with a preferred modification of oxidation included for the theoretical peptide digest generated using the BioConfirm sequence editor (define and match sequences). Using Agilent MassHunter Comparative Analysis software, it was clear that the oxidized peptide was much more abundant in the sample treated with 3 % t-BHP. From the differential display and table of results (Figure 2A and 2C), we can easily see the peptide ratio changed when oxidation occurred.

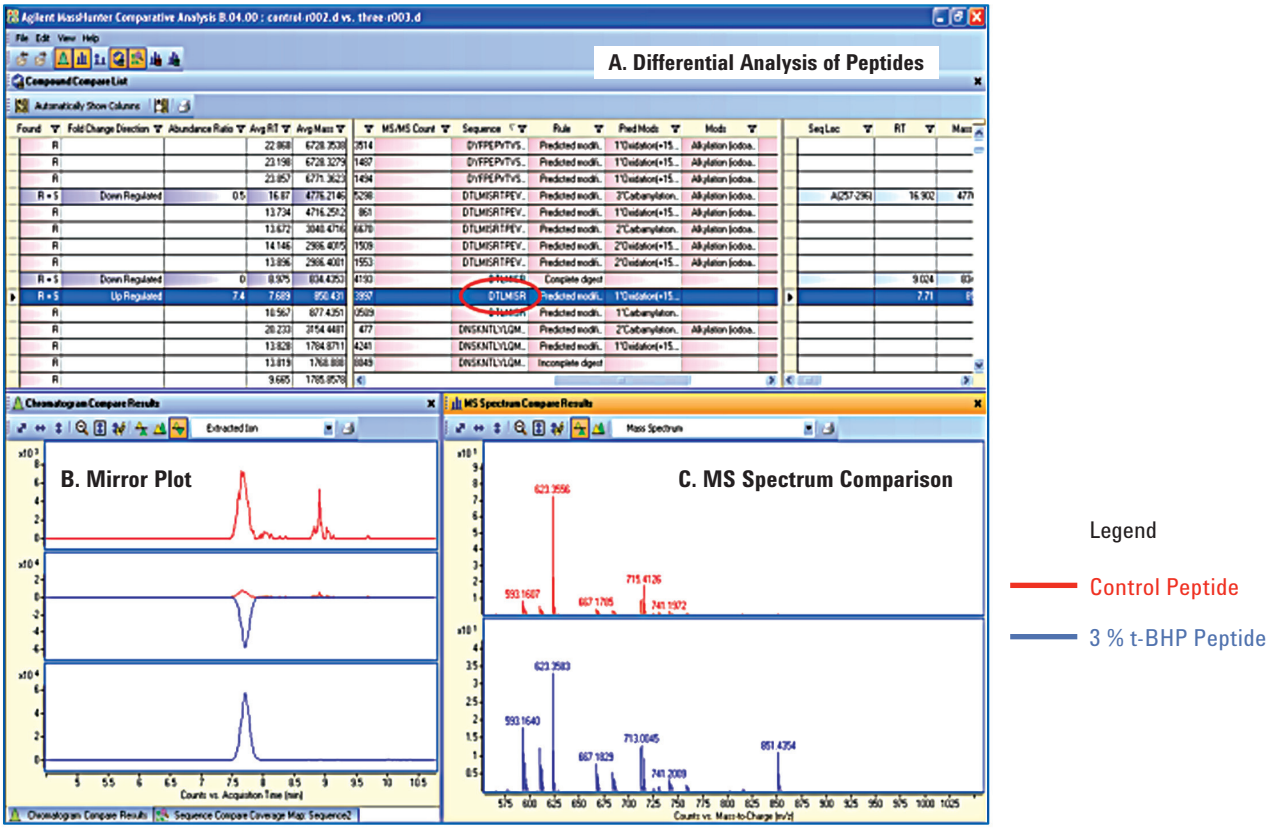

Figure 2. (A) Differential analysis of oxidized DLTMISR peptide in control (red) and 3 % t-BHP treated peptide (blue) of mAb. (B) The mirror plot of the extracted ion chromatograms clearly shows the oxidized DLTMISR peptide is more prominent in 3 % t-BHP treated sample. (C) Oxidation of Met results in sulfoxide with a molecular weight (MW) increase of ~ 16 Da. LC-MS analysis of the peptides containing Met from the samples treated with t-BHP revealed an additional peak with a MW increase of ~ 16 Da.

Quantitative analysis and relative percent oxidation for each Met-containing peptide was calculated using the formula displayed to the right:

Relative Percentage Oxidation

$$= \frac{\sum \text{ Height of Met-oxidized peptide ions}}{(\sum \text{ Height of Met-oxidized peptide ions}) + (\sum \text{Height of Met-(non-oxidized peptide ions)})} \times 100$$

An increase in oxidation for the DLTMISR peptide was demonstrated in the extracted compound chromatograms of oxidized and non-oxidized mAb (Figure 3). The oxidized Met containing peptide eluted earlier (~ 7.7 min) than the non-oxidized (~ 9.0 min) due to the more polar nature of the methionine sulfoxide compared to methionine. It should be noted that the control sample (0 % t-BHP) also contained some degree of oxidation of the Met residue (~ 1 %). A comparable ionization efficiency of non-oxidized/oxidized peptide pairs of interest was assumed in this study.

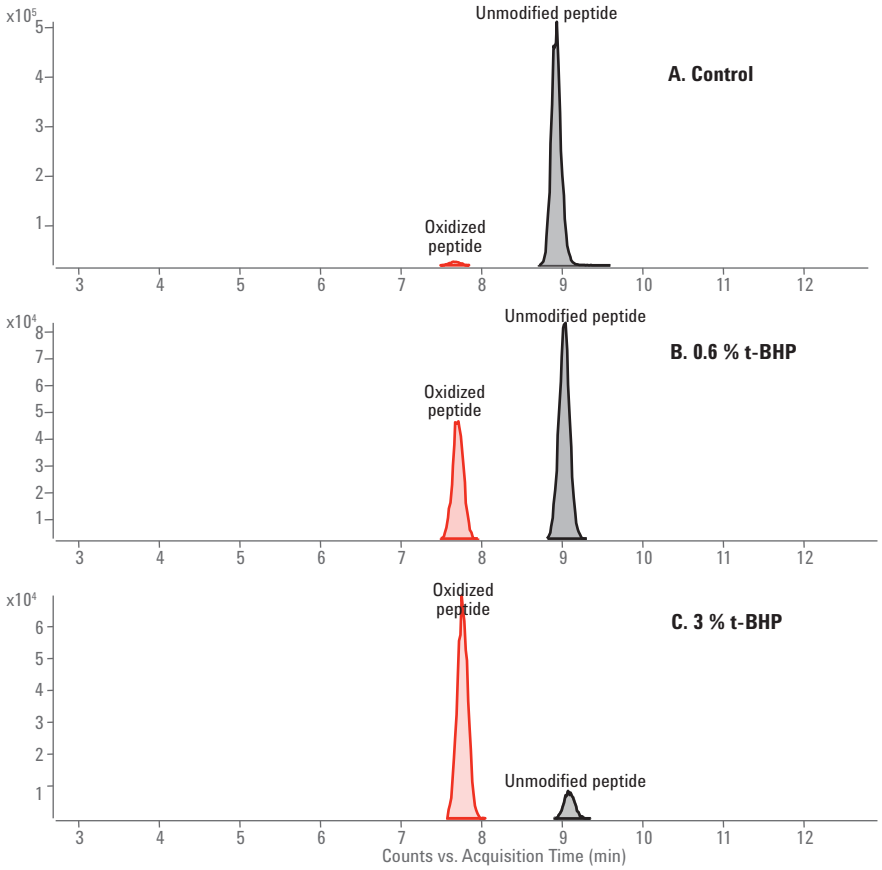

Figure 3. Extracted compound chromatograms (ECC) of the DLTMISR peptide of a mAb oxidized with 0.6 % (B) and 3 % t-BHP (C), and a non-oxidized mAb control (A). The oxidized Met peptide (red) eluted earlier compared to non-oxidized Met peptide (grey).

The study also inferred that there were differences in susceptibility to oxidation for different peptides containing Met residues (Figure 4). Two Met residues in peptides DLT**M**ISR (Met 256) and SRWQQGNVFSCSV**M**HEALHNHYTQK (Met 432) were found to be susceptible to oxidation, while the Met residue from peptide NTLYLQ**M**SSLR (Met 84) was found to be more resistant to oxidation. The mild oxidizing reagent t-BHP has been previously used to probe the susceptibility of Met residues of recombinant monoclonal antibodies because it specifically oxidizes surface-exposed Met residues to form the sulfoxide. Therefore, Met residues 256 and 432 are likely to be surface exposed in the mAb. The results obtained from this study are in agreement with previous published studies on mAbs [4].

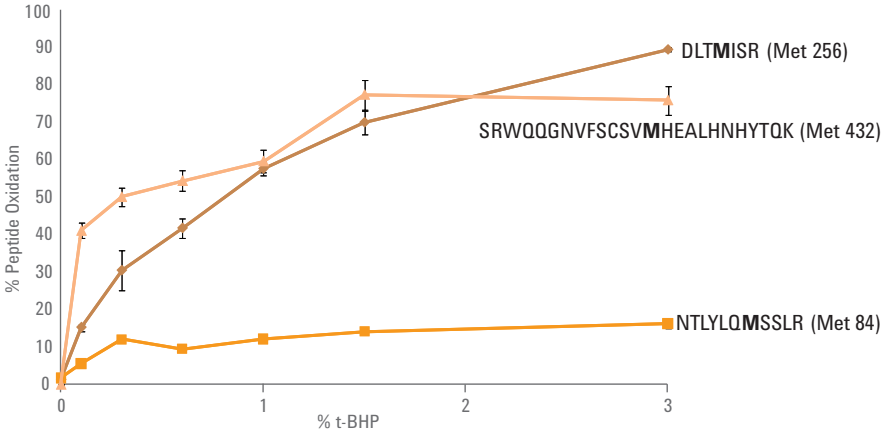

Figure 4. Monoclonal antibody oxidation levels of Met containing peptides incubated with 0, 0.1, 0.3, 0.6, 1.0, 1.5, and 3 % t-BHP. The relative percentage oxidation was calculated using the previously shown equation.

Conclusions

The relative quantitation of Met oxidation in a mAb was demonstrated. Met 256 and Met 432, which are in the CH2-CH3 interface of the mAb, are more prone to oxidation than Met 84 which is not exposed on the surface, as described in previous studies.

Valuable early stage antibody material can be examined for oxidation susceptibility at low sample levels by using an 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 study. Agilent MassHunter BioConfirm and Comparative Analysis software enabled the quick assignment and quantitation of the methionine-containing peptide peaks based upon their oxidation states. Prescreening of the results with statistical analysis software (Agilent MassProfiler Professional) allowed rapid analysis of a large batch of samples prior to developing a full relative oxidation curve.

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