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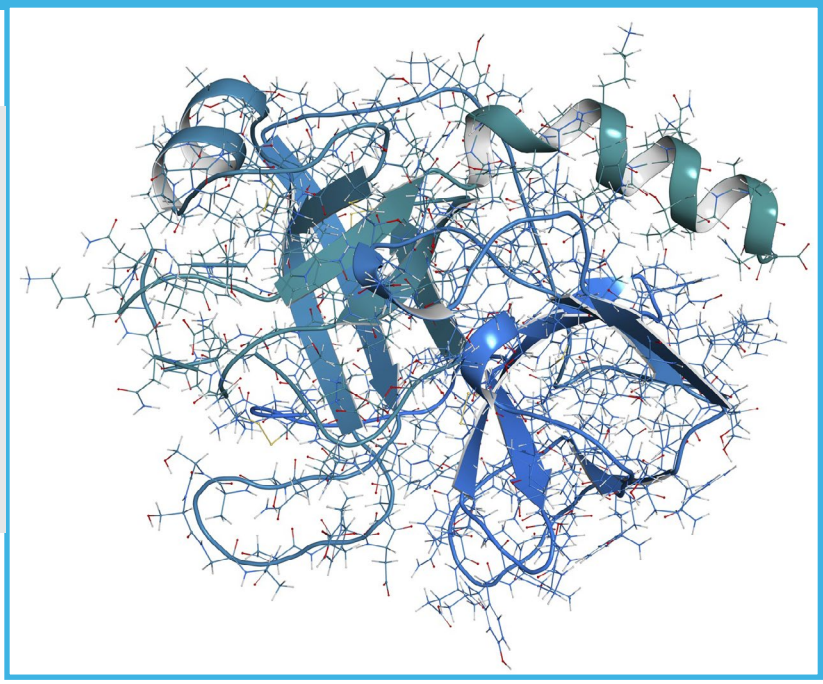
TECHNICAL REPORT: AMT_TR_BIO_21_2

TITLE: **ANALYSIS OF THE OXIDATION OF NIST MAB FRAGMENT USING THE HALO® PENTA-HILIC CAPILLARY COLUMN AND HRAM MS**

MARKET SEGMENT: BIOPHARMACEUTICAL



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ABSTRACT

Post-translational modifications (PTMs), such as oxidation, are a critical variable that must be accounted for during protein analysis. Often times the minor mass shifts associated with these modifications are too small to be resolved during intact protein analysis, due to the charge envelope produced by large proteins, such as monoclonal antibodies (mAbs). However, chromatographically, these compounds will have a difference in retention time relative to the native, and can be separated before getting to the detector. Peptide analysis is an important method of characterization for mAbs because, in addition to revealing modifications such as oxidation, it can provide valuable insight into additional post-translational modifications, which may not be evident during intact mass analysis. In this experiment the digested NIST mAb was exposed to high temperature in order to induce oxidation, and then analyzed by the HALO® Penta-HILIC capillary column, demonstrating it is an ideal choice for peptide oxidation analysis of mAbs.

INTRODUCTION

Monoclonal antibody (mAb) based drugs, are biotherapeutic proteins, which have shown bioactivity against many types of diseases, including cardiovascular and cancer.[1-2] These drugs, like most proteins, can be subject to a multitude of biochemical modifications during their lifetime, which can change the efficacy of the biotherapeutic, and lead to variant forms of the drug.[2] These variants, often do not have the intended effect on the targeted disease, and therefore need to be studied and evaluated. One such variant causing modification, oxidation, is of major concern because it has a negative effect on the shelf life and bioactivity of the drug.[1-4]

The amino acids methionine (Met), cysteine (Cys), tryptophan (Trp), and histidine (His) are susceptible to oxidation, and if this occurs, the hydrophobic nature of the protein can change. In particular, oxidation of Met is of major concern as it can result in a product with altered binding.[4] Similar to other biotherapeutic proteins, mAbs can undergo oxidation at various stages such as during production, the formulation process, or during post formulation storage.[3] Therefore, it is critical to analyze potential oxidation changes during these processes.

The analysis of peptide oxidation is relatively straightforward. The mass shifts associated with these interconversions are very well known, and can enable identification of the oxidized peptides from mass spectrometry. In general, peptide-based mass spectrometry methods are used for analysis of oxidation of biotherapeutics. Although sample prep can be labor intensive and the methods can have longer run times, they can provide better chromatographic resolution of modified and unmodified features for accurate analysis.

KEY WORDS:

NIST mAb, Oxidation, HRAM, HALO® Penta-HILIC capillary

Traditionally, reversed-phase liquid chromatography (RPLC) methods are often used, as it is a hydrophobic based method and well suited to the analysis. However, Hydrophilic interaction chromatography (HILIC), a complementary technique to traditional RPLC, is also an ideal choice for this analysis because polar compounds can be retained while using MS friendly mobile phases and buffers. HILIC can have an advantage over RPLC due to the higher organic content of the mobile phase.[5-7] Whereas RPLC elutes from low to high organic, HILIC elutes from high to low organic. This will allow a higher percentage of organic into the ion source during the elution than RPLC, and will enhance the desolvation of the analyte and contribute to better spray stability, which will therefore lead to an increase in sensitivity.[5] There are also selectivity differences between HILIC and RPLC, which can have definitive advantages depending on the analyte of interest.[6,7]

Here we present the HALO® Penta-HILIC capillary column as an alternative to RPLC for analysis of oxidative stress of peptides.

EXPERIMENTAL DATA

All solvents used were MS grade. Methanol, acetonitrile, mobile phase additives, and individual standards were obtained from MilliporeSigma (St. Louis, MO), unless specified otherwise.

Trypsin digestion of the NIST mAb

The NIST mAb sample was denatured and alkylated using 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, and 2-iodoacetamide (Sigma Aldrich). Trypsin (Promega) was added in a ratio of 1:30 (w:w; Trypsin:mAb) followed by an incubation at 37 °C overnight. The reaction was quenched by 0.5% formic acid and analyzed by LCMS.

Samples were analyzed on a Shimadzu Nexera X2 (Shimadzu Scientific Instruments, USA). Mass spectra were acquired using a Thermo Scientific Velos Pro LTQ Orbitrap mass spectrometer (Bremen, Germany) using a heated electrospray (HESI-II) probe on the Ion Max source.
A 90 Å 2.7 µm, (0.5 mm ID x 150mm)
HALO® Penta-HILIC column was used (Advanced Materials Technology, Inc. Wilmington, DE). Flow rate was 50 µL/min, and 60 °C for initial analysis. In the oxidation experiments the temperature was raised to 80 °C.

LC Gradient

Time	%B
0	80
4	80
55	48
59	48
63	80
70	end

Column: HALO® 90 Å Penta-HILIC, 2.7 µm, 0.5 x 150 mm
Part Number: 98215-705
Flow Rate: 50 µL/min
Pressure: 158 bar
Temperature: 60 °C characterization run, 80°C for oxidation run.
Detection: +ESI
Injection Volume: 5.0 µL
Sample Solvent: 70% ACN 30% water
LC System: Shimadzu Nexera X2
MS System: Thermo Scientific Velos Pro LTQ Orbitrap
Mobile phase A: 50 mM ammonium formate pH 4.4 and
Mobile phase B: 0.1% formic acid in acetonitrile.

MS Conditions

Voltage	3.5 kV
Aux gas	4 arbitrary units
Sheath gas	2 arbitrary units
Sweep gas	0 arbitrary units
Rf lens	45 v
Heater temp	225 °C

Oxidation experiment:

After digestion, the oxidation was carried out by injecting the sample onto the column at 80 °C, then the flow was stopped for 30 minutes. After the 30-minute hold had been completed, the mobile phase gradient was started and the analysis was begun.

RESULTS AND DISCUSSION

In order to determine the validity of the HALO® Penta-HILIC column for oxidative analysis, a rudimentary oxidation experiment was performed. The digested peptide was injected onto the column at an elevated temperature of 80 °C, and left on the column for 30 minutes. After 30 minutes the mobile phase gradient was started and the digest was eluted from the column. Figure 1 shows the extracted ion chromatogram of (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A), m/z=1305.60167, before the oxidation experiment.

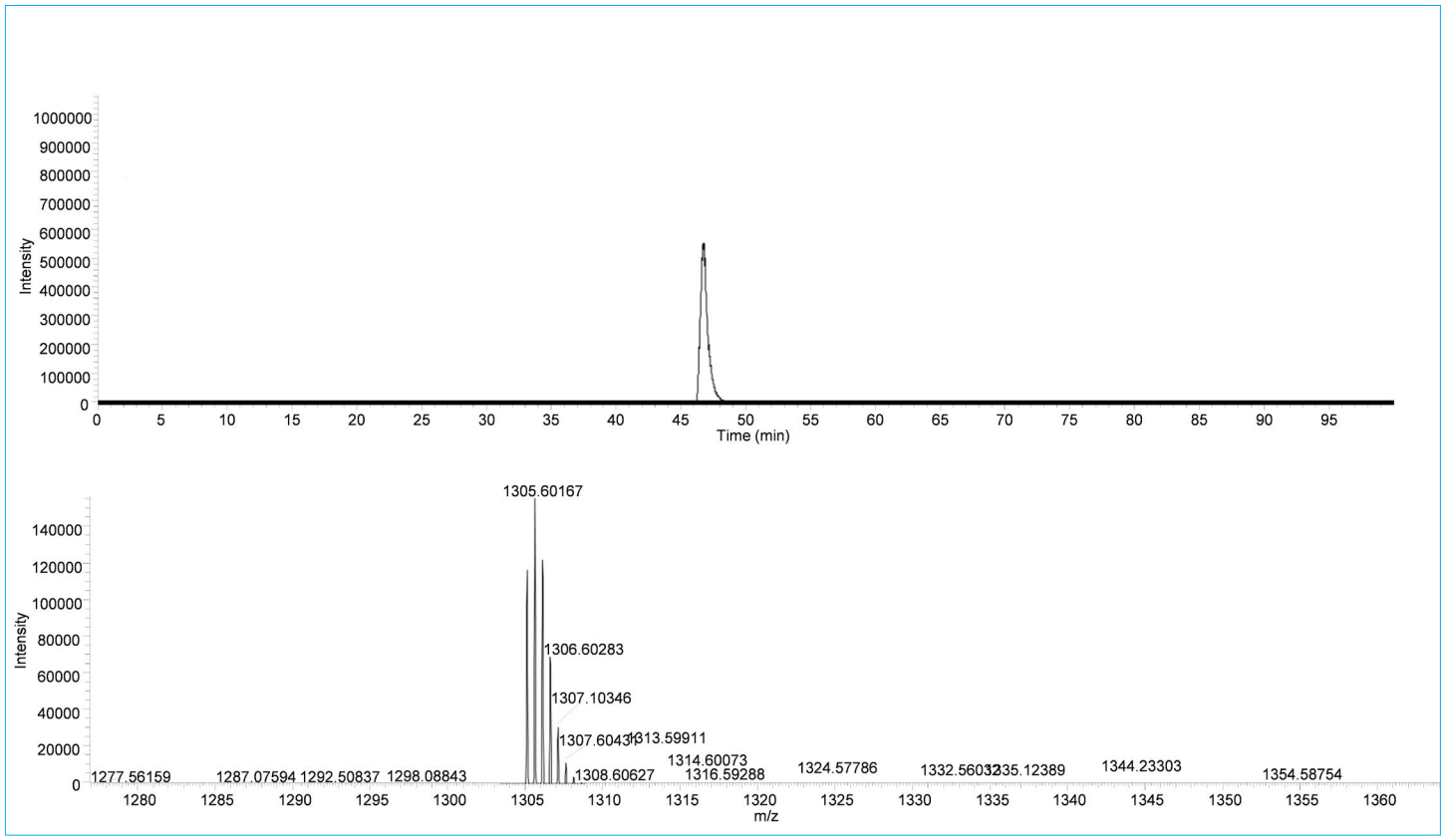

Figure 1. extracted ion chromatogram of (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A)

Figure 2 shows the same peptide after the oxidation and shows the characteristic mass shift of 8 Da associated with oxidation for a doubly charged peptide species.

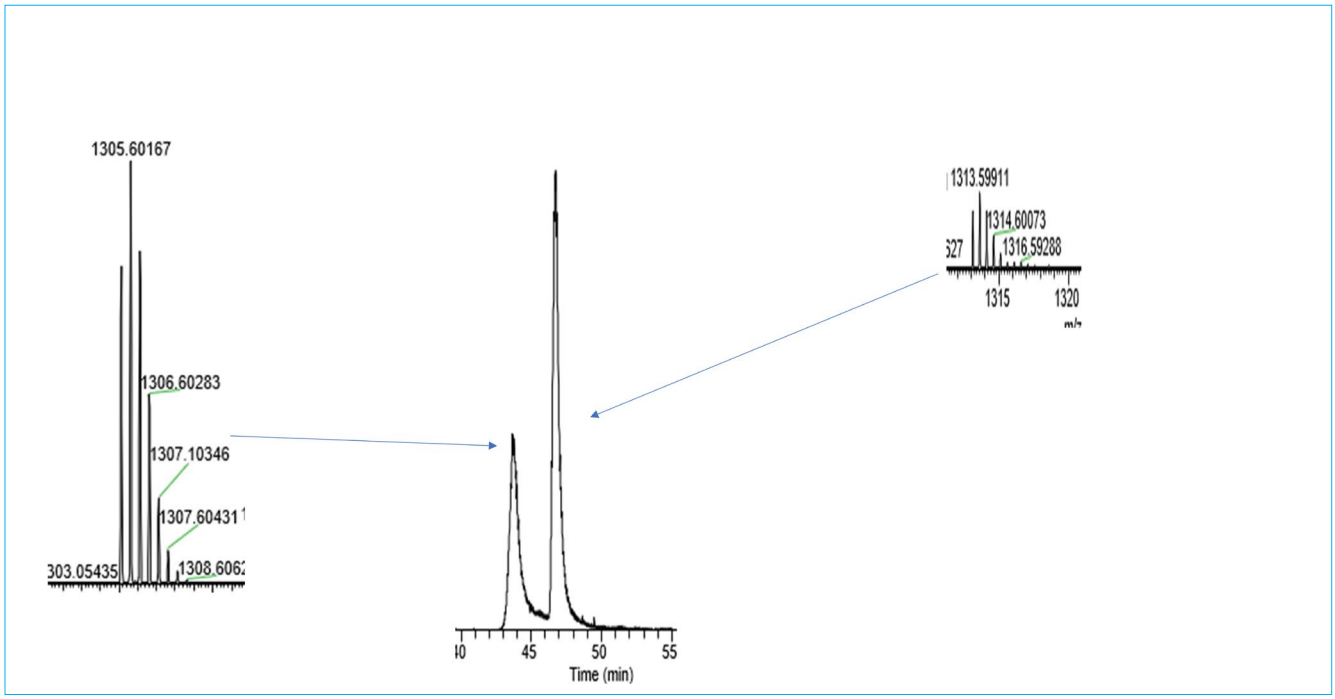

Figure 2. Extracted ion chromatogram oxidized (-) DIQMTQSPSSLSASVGDRVTITC(Carbamidomethyl)R(A) showing a mass shift of 8.

The oxidized species is detected at m/z= 1313.59911, and is 8 Da away from the native as would be expected for a doubly charged peptide. The shift in retention time relative to the native is evident and indicative of a modification having occurred on this peptide.

This pattern was also observed in Figure 3, which shows m/z=959.96868, the extracted ion chromatogram of the doubly charged peptide fragment (R)EPQVYTLPPSREEMTK(N). The oxidized species is detected at m/z=967.46419, and is 8 Da away from the native as would be expected for a doubly charged peptide.

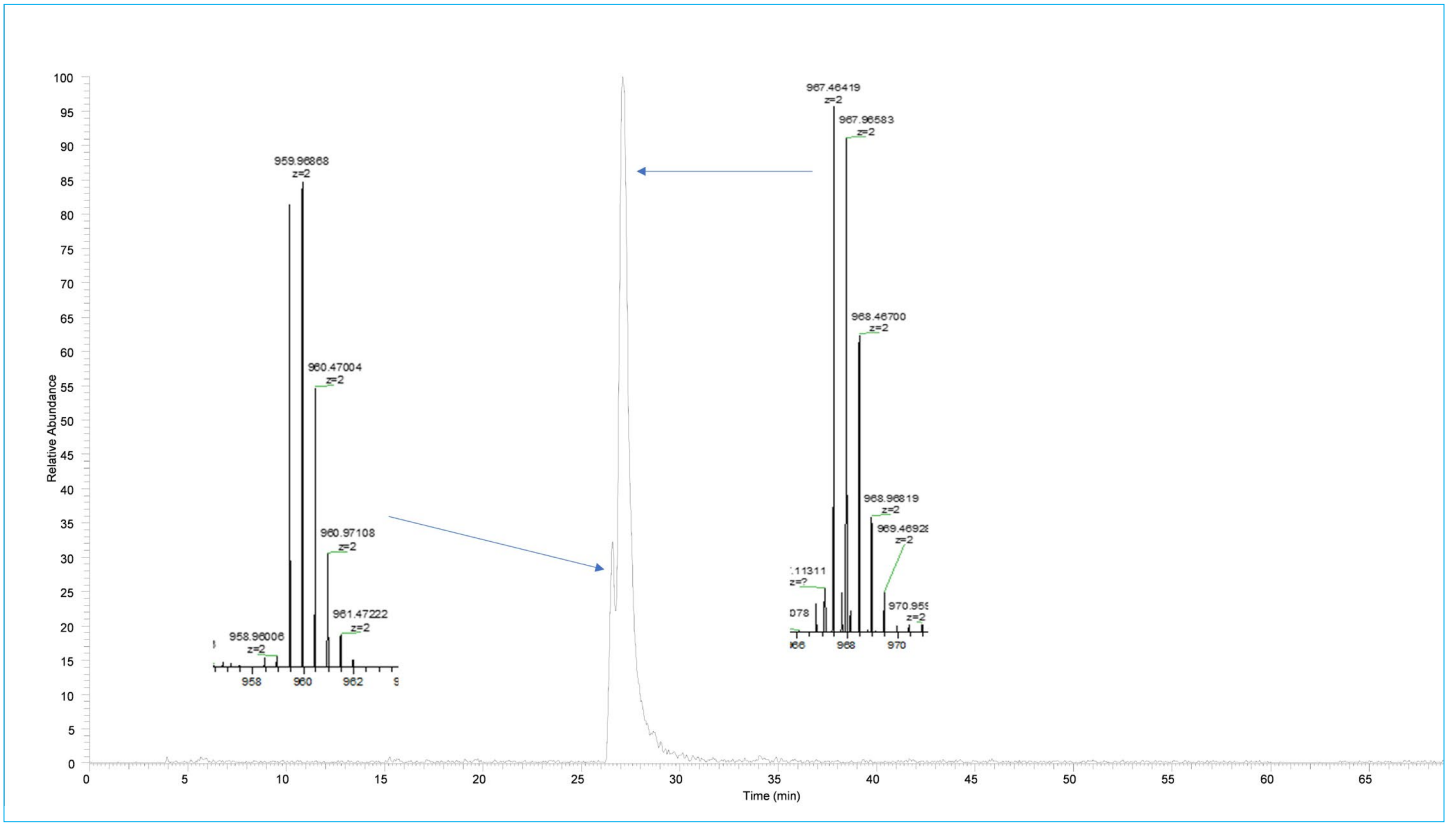

Figure 3. The extracted ion chromatogram of the doubly charged peptide fragment (R)EPQVYTLPPSREEMTK(N) showing a mass shift of 8.

The mass shift that was observed with both doubly charged peptide fragments was indicative of a successful oxidation. Despite the rudimentary aspects of the experiment, the HALO® Penta-HILIC capillary column displayed the selectivity and sensitivity to resolve the structural modification that occurred with oxidation.

CONCLUSION:

Polar modifications, such as oxidation, can have a deleterious effect on the bioactivity of a mAb biotherapeutic, and must be monitored. Peptide analysis is critical to test for oxidization, due to the negative effect that can result from the process, and a loss of integrity of the mAb. The HALO® Penta-HILIC capillary column coupled with HRMS delivers the sensitivity and resolution needed to analyze oxidation of mAbs.

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