# HILIC Analysis for Polar Modifications of Proteins and Peptides

## Introduction

Post-translational modifications (PTMs) occur for proteins intended for both intracellular and extracellular functions. These biochemical changes to a protein can be enzymatic or non-enzymatic and may involve changes to amino acids side-chain functional groups and their N-termini, as well as peptide hydrolysis.[1] Many PTMs are polar by their nature, including glycosylation, deamidation, and oxidation.

These types of polar modifications are more readily analyzed (as released species or as modified proteins) using hydrophilic interaction liquid chromatography (HILIC) because they are retained and resolved better compared to reversed-phase liquid chromatography (RPLC).

PTMs must be characterized and controlled for the development of effective and safe biotherapeutics such as monoclonal antibody (mAb) drugs. PTMs can form during the production, formulation, and storage stages of drug development. With the continued market growth of mAb biotherapeutics and the emergence of biosimilars (generic biological medicines similar to the innovator biological drug), the focus on PTM characterization is not diminishing, but expanding. Improved characterization leads to better understanding of the impact that PTMs have on mAb biotherapeutics for the ultimate goal of delivering safe, efficacious treatment to patients. In this white paper we investigate the characterization of glycosylation, deamidation, and oxidation as different types of PTMs.

## Glycosylation

Glycosylation is a protein modification that plays a major role in determining protein structure, function, and stability. The various carbohydrates, or glycans, are classified by how they are linked to the amino acids in the protein: either N-linked or O-linked. The N-linked glycans are attached to the protein via an N-glycosidic bond when the amino acid sequence is Asn-X-Ser/Thr, with X being any amino acid except a proline. The O-linked glycans are β-O-(N-acetylglucosamine) (O-GlcNAc) attached to the hydroxy group of either a serine or threonine residue of a protein.[2]

The importance of glycosylation cannot be underestimated. Glycosylation impacts the safety/immunogenicity, influences the efficacy and clearance, and affects the stability and solubility of all therapeutic proteins, particularly monoclonal antibodies (mAbs). As a critical quality attribute of mAbs, glycosylation must be characterized and monitored.

Regardless of the type of glycan, its presence increases the polarity of the protein or peptide, making it more suitable for HILIC analysis than reversed-phase LC analysis. A comparison of the analysis of the glycopeptides in a bovine Ribonuclease B tryptic digest using both reversed-phase and HILIC conditions is shown in **Figure 1**.

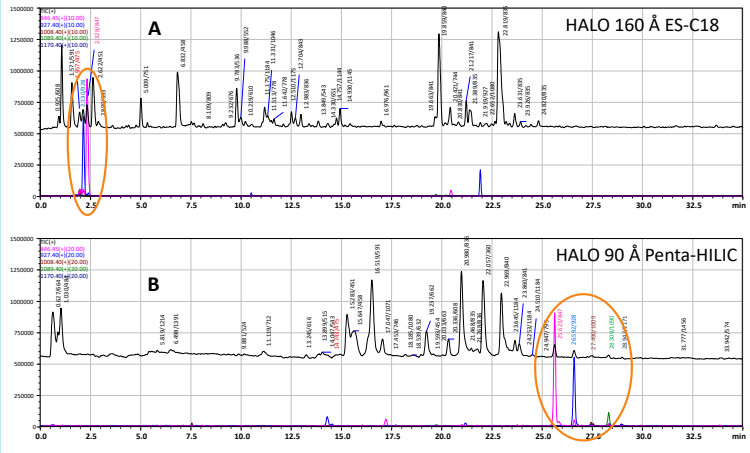


**Figure 1.** A: Reversed-phase LC separation of high mannose variant glycopeptides from bovine Ribonuclease B; B: HILIC separation of high mannose variant glycopeptides from bovine Ribonuclease B; Conditions: For A: HALO 160 Å ES-C18, 2.7 µm, 2.1 x 100 mm, For B: HALO 90 Å Penta-HILIC, 2.7 µm, 2.1 x 100 mm, 0.35 mL/min, RP Mobile Phase A: 0.1% formic acid/10 mM ammonium formate, RP Mobile Phase B: 80/20 ACN/water/0.1% formic acid/10 mM ammonium formate RP Gradient: 2–58.2% B in 45 min.

HILIC Mobile Phase A: 40/60 ACN/water/0.1% formic acid/10 mM ammonium formate, HILIC Mobile Phase B: 90/10 ACN/water/0.1% formic acid/10 mM ammonium formate, HILIC Gradient: 100–10% B in 45 min, For both A and B: Flow rate, 0.35 mL/min; Column Temperature, 40 °C; MS: SQ TIC (+ 300-2000 m/z) @ 0.35/s; Sample, 20 µg Bovine Ribonuclease B tryptic digest with carbamidomethylation.

This experiment was purposely designed to use the same gradient slope (± 1%) and column dimension to refrain from biasing the results of one condition over the other. Using reversed-phase conditions (**Figure 1A**), the high mannose variant glycopeptides are not well retained (circled), nor are they well resolved from one another. HILIC, in contrast (**Figure 1B**), shows much more retention and excellent resolution of the high mannose variant glycopeptides (circled).

Now that the advantages of using HILIC to analyze glycosylation have been established, a few specific examples highlighting the excellent resolution that can be achieved will be discussed. The first example is the analysis of the glycans from the mAb, trastuzumab. The N-linked glycans were enzymatically released by the addition of the enzyme PNGase F. Then, the glycans were reductively aminated with procainamide to label them for either UV or fluorescence detection. For identification purposes, a mass spectrometer is used for detection. **Figure 2** shows the HILIC separation of the glycosylation variants of trastuzumab. Each of the glycan peaks is labeled with their code and structure diagram that describes its structure.

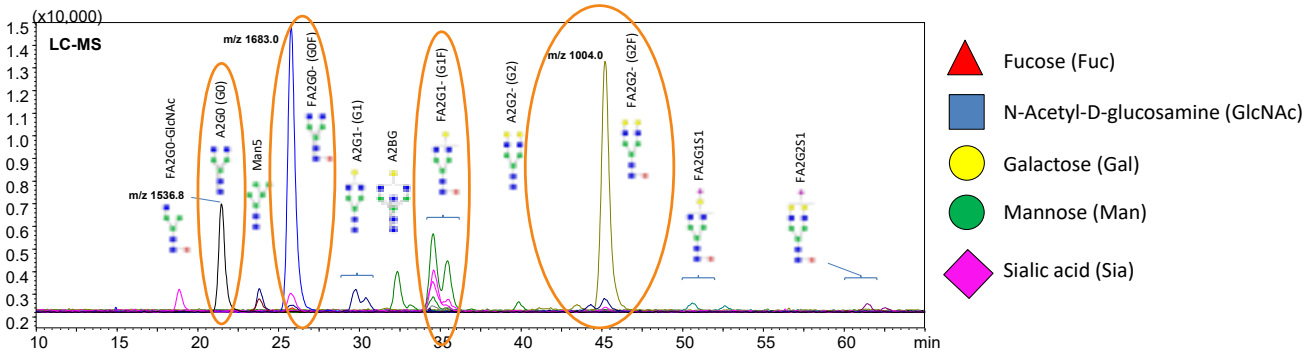


**Figure 2.** Glycosylation variants of trastuzumab with MS detection. Conditions: HALO 90 Å Glycan, 2.7 µm, 2.1 x 150 mm; 0.35 mL/min; 40 °C; Gradient Eluents: A: 50 mM ammonium formate (pH 4.5); B: ACN; Gradient: 75% to 68% ACN in 75 min.

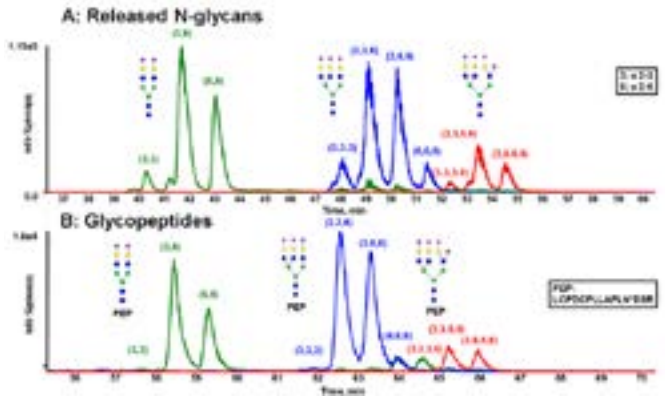


**Figure 3.** Adapted from J. Biomol. Technol., (2016) 27, 98-104. A: Released N-glycans from fetuin. B: Glycopeptides from fetuin. Conditions: HALO 90 Å Penta-HILIC 2.7 µm, 2.1 x 150 mm, 0.4 mL/min; 60 °C; Mobile Phase A: 95% 50 mM ammonium formate (pH 4.4)/5% ACN; Mobile Phase B: ACN; Gradient – Released N-Glycans: 80% to 62% ACN in 60 min (-1%/min); Glycopeptides: 85% to 48% ACN in 75 min.

This result encompasses the entire range of N-linked glycovariants that are present in mAbs. The main glycans found in trastuzumab are G0, G0F, G1F, and G2F. Great peak shape and great resolution are observed for the compositional isomers. For a more in-depth investigation of method development for glycans from mAbs, please refer to the white paper A Novel Approach to Glycan Method Development for Biotherapeutics using Superficially Porous Particle Technology.

One of the challenging aspects of glycan characterization is that many glycans are isomers and can be difficult to resolve, but with the HALO® Penta-HILIC column, resolution is accomplished. For example, **Figure 3** shows the isomeric glycans and glycopeptides from fetuin resolved by analysis using a HALO® Penta-HILIC column.[3]

With the use of a 4000 QTRAP mass spectrometer, another experiment can be conducted to show the occupancy of the glycans.

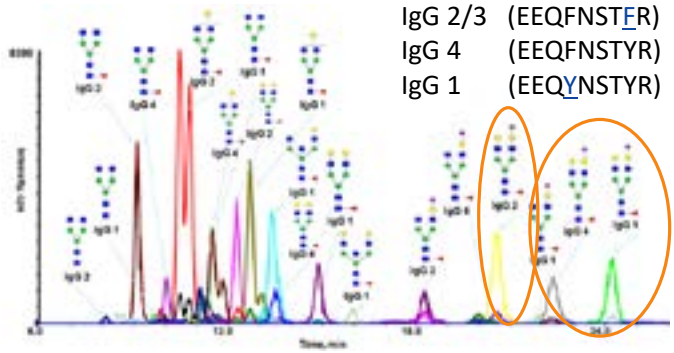


Figure 4. Adapted from J. Biomol. Technol., (2016) 27, 98-104. Isomeric glycopeptide glycoforms from human serum IgGs. Conditions: same as Figure 3 except using this segmented gradient: 1) 62% mobile phase B to 61.2% mobile phase B in 9 min, 2) 61.2%mobile phase B to 60.2% mobile phase B in 10 min, and 3) 60.2%mobile phase B to 58% mobile phase B in 11 min.

The circled glycans (**Figure 4**) all have the same mass but are attached to different peptide sequences (different masses) that can be resolved both chromatographically and by using the mass spectrometer. The mass of the peptide fragment and the glycan is known, so this is selected in the first quadrupole. Then, the selected ion is fragmented in the second quadrupole and an identifying ion is detected in the third quadrupole.

A comparison of the glycopeptides from hemopexin, a protein found in plasma and responsible for binding heme, was conducted using three different HILIC columns.[4] See **Figure 5** for the comparative results.

The same gradient conditions were used for both the (A) HALO® Penta-HILIC column and the (B) Glycan BEH Amide column, while a shallower gradient was used for the (C) ZIC-HILIC column. Increased retention was observed with the Penta-HILIC column for the di-sialylated glycopeptides (circled region in 5A) along with a much larger range of retention (17–34 minutes). A much narrower elution range was observed on the Glycan BEH Amide column (circled region in 5B) (< 2 min) with all the glycans eluting between 30 and 32 minutes. Reduced retention of the sialylated glycans (circled region in 5C) using the ZIC-HILIC column may be due to ion repulsion between the negatively charged sulfobetaine functional group and the negatively charged sialic groups of the glycans. The ZIC-HILIC column also has a narrower elution range than the HALO® Penta-HILIC column with the glycans eluting between 39 and 44 minutes using the former.

## Deamidation

Deamidation (loss or conversion of an amide functional group from a side chain) occurs for asparagine (N) and glutamine (Q) residues in both peptides and intact proteins. The rate of the deamidation reaction is strongly pH- and temperature-dependent as well as sequence-dependent. **Figure 6** shows the mechanism of the

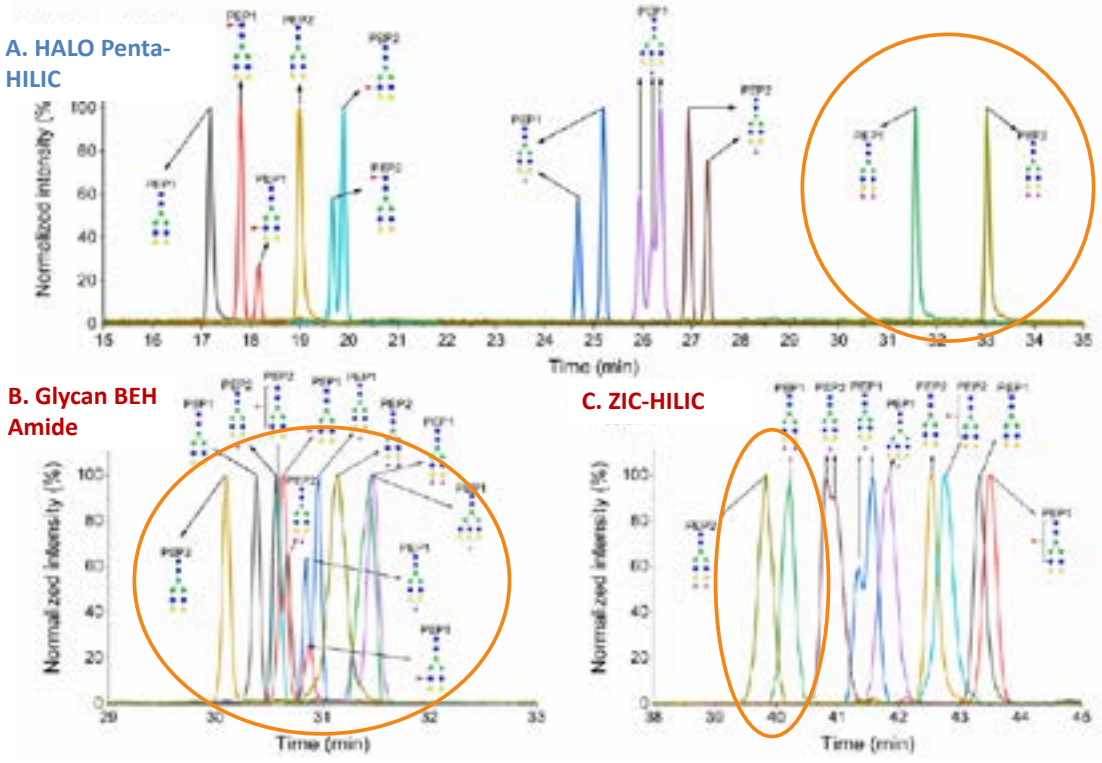


**Figure 5.** Adapted from Molecules 2020, 25, 4655. Glycopeptides from hemopexin analyzed on three different stationary phases: A: HALO® Penta-HILIC; B: Glycan BEH Amide; C: ZIC-HILIC. Conditions in reference.

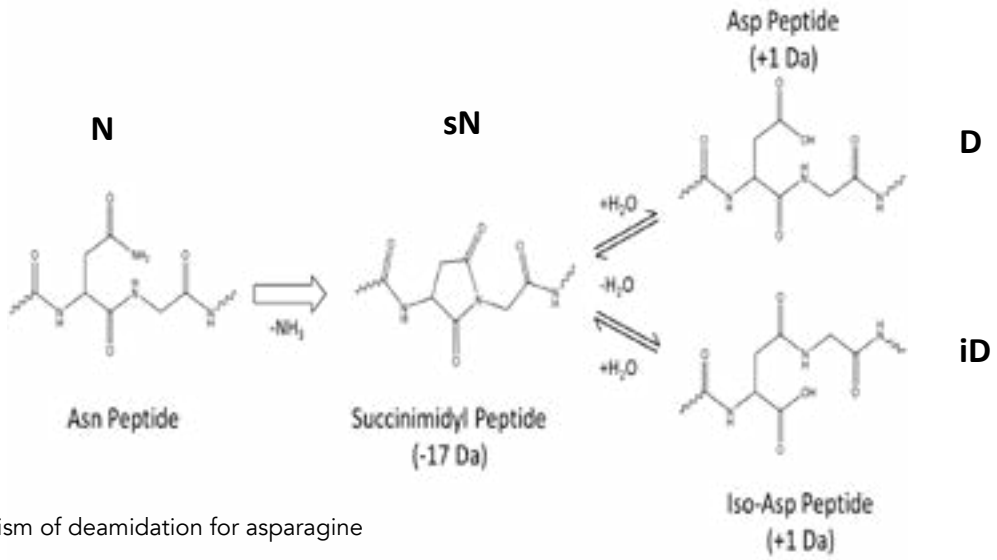


**Figure 6.** Mechanism of deamidation for asparagine

deamidation reaction for Asn. With the loss of ammonia, a cyclic intermediate called a succinimidyl peptide is formed, which with the addition of water, can form either aspartate (D) or isoaspartate (iD). The formation of both aspartate and isoaspartate results in a +1 Dalton mass shift.

Much like glycosylation, deamidation is important because it changes the structure and function of the protein or mAb. Deamidation could lead to reduced bioactivity, a change to the pharmacokinetics, or a change to the protein's antigenicity. Deamidation could change the stability of the protein, which could then lead to degradation. Deamidation results in a more polar amino acid being created which aligns well for analysis via HILIC. **Figure 7** shows the comparison of three synthetic peptides that have either N, D, or iD at position 14 in the sequence GFYPSDIAVEWESNGQPENNYK analyzed using either HILIC conditions or reversed-phase conditions. This particular peptide fragment was selected for investigation because it contains three Asn residues, so it could have multiple deamidations.

An increased selectivity difference is observed for resolving these peptides using HILIC conditions compared to reversed-phase conditions. Unlike reversed phase, HILIC reliably resolves Asn/Asp/iAsp, with the retention order shown in **Figure 7**.

Furthermore, this specific synthetic peptide GFYPSDIA VEWESNGQPENNYK was also subjected to 18 hours of pH 9 and 37 °C to determine which deamidations could be observed, and was analyzed using a 0.5 x 150 mm, 2.7 µm HALO® Penta-HILIC column. This treatment was

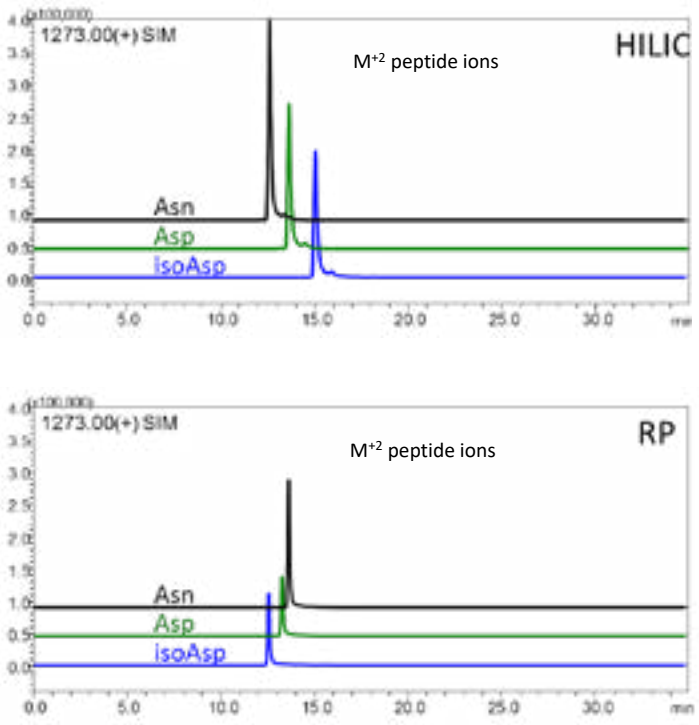


Figure 7. Comparison of HILIC and RP modes for resolving deamidated and isomerized Asn-containing peptides. Conditions: HALO 90 Å Penta-HILIC, 2.7 µm, 2.1 x 150 mm; HALO 160 Å ES-C18, 2.7 µm, 2.1 x 150 mm; 0.4 mL/min; 60 °C; Detection: Shimadzu MS-2020 Single Quad MS; Mobile Phase A: water/50 mM ammonium formate, pH 4.4; Mobile Phase B: acetonitrile/0.1% formic acid; Gradients: HILIC – 80%–46.2% B in 60 min.; RP - 10–70% B in 60 min; Injection volume: 4 µL (0.1 µg).

conducted before analysis on the HALO® Penta-HILIC column. **Figure 8** shows both the UV and MS results of the deamidation experiment. In **Figure 8A** the N/D/iD pattern for deamidation at 388 is observed. Using the XIC for $N^{388}G$, it is confirmed that some of the unmodified peptide is still present (**Figure 8B**). The mass increase of + 1 Da, but with 2+ charge (net gain of 0.5) is shown in **Figure 8C**. Here the $D^{388}G$ and $iD^{388}G$ are confirmed to be present. The $N^{388}G$ that is also observed is due to the carbon isotope. The presence of $N^{388}G$-D/$iD^{393}N$, which is another deamidation at the second N in GFYPSDIAVEWESNGQPENNYK is circled. Finally, **Figure 8D** shows the presence of two deamidations: $iD^{388}G$-$iD^{393}N$. These results were supported by mass analysis, using CID fragmentation. No evidence of the formation of a triple deamidation was obtained ($N^{394}$), which shows that

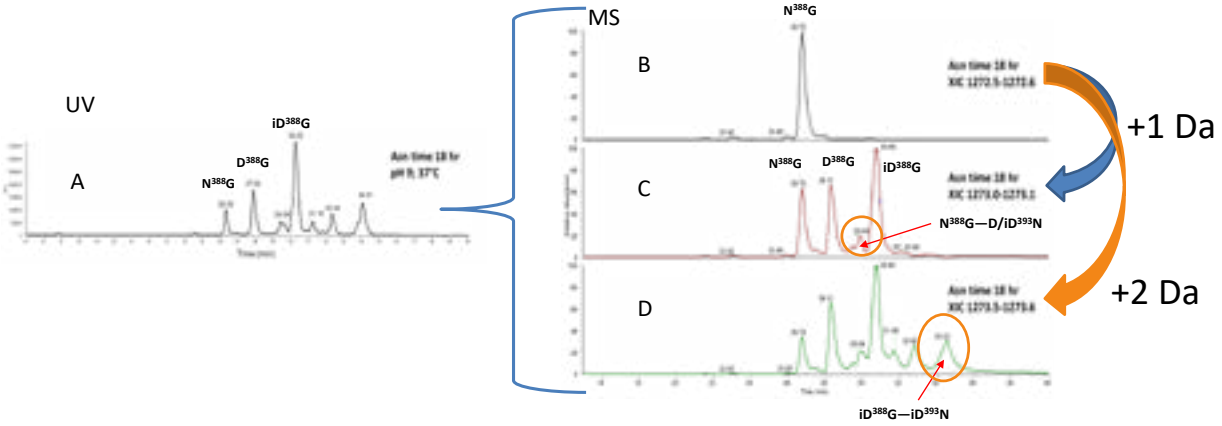


**Figure 8.** UV and MS results for HILIC analysis of GFYPSDIAVEWESNGQPENNYK after treatment for 18 hours of pH 9 and 37 °C. Conditions: HALO 90 Å Penta-HILIC, 2.7 µm, 0.5 x 150 mm; 12 µL/min; 60 °C; UV (220 nm) or Orbitrap Velos Pro MS; Mobile Phase A: water/50 mM ammonium formate, pH 4.4; Mobile Phase B: acetonitrile/0.1% formic acid; Gradient: Hold at 80% B for 4 min.; 80%–48% B in 64 min.

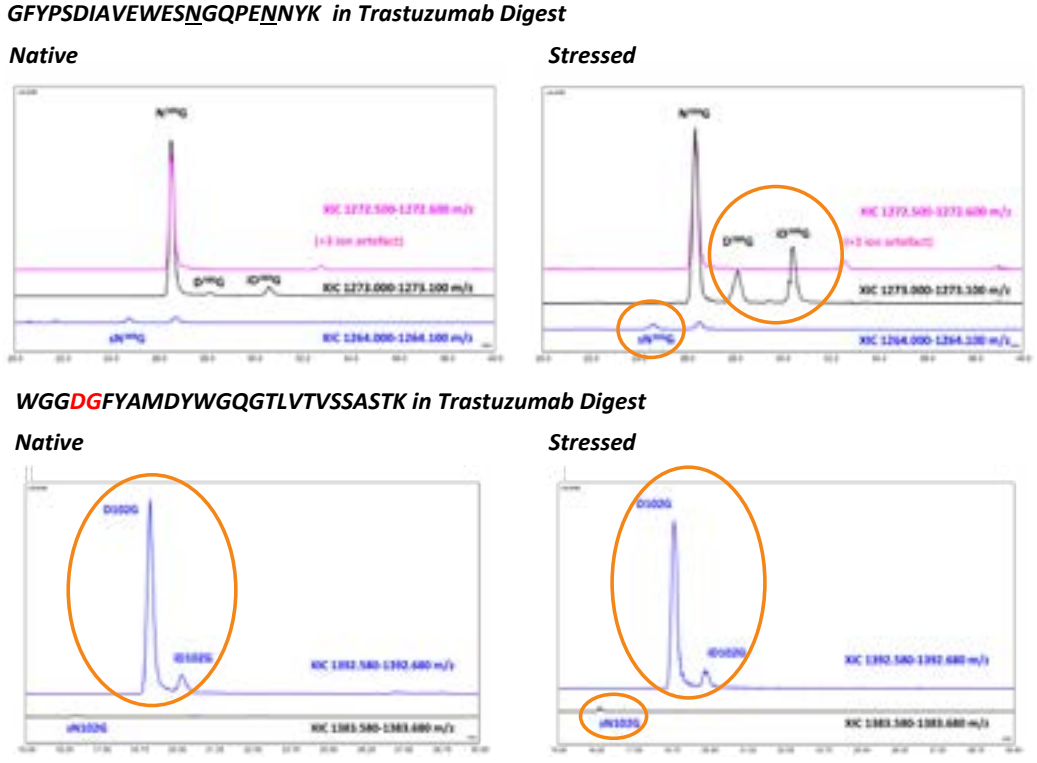


**Figure 9**. Deamidation and isomerization in trastuzumab tryptic digests. Conditions: same as Figure 8

deamidation at that position is not likely to occur. Trastuzumab tryptic digests were prepared using the native mAb and the mAb that had been subjected to stressed conditions (Tris-HCl pH 9.0 for 7 days). The goal was to find evidence of increased levels of deamidations in the digest prepared from the stressed trastuzumab. **Figure 9** shows the results from the HALO® Penta-HILIC analysis of two different tryptic digest fragments (both native and stressed).

**Figure 9A** (black trace) shows the presence of some deamidation at $N^{388}G$ from the native mAb digest. In **Figure 9B** (black trace), the deamidations at $N^{388}G$ are increased for the stressed mAb digest. The presence of the succinimidyl peptide is also observed (blue traces in both **9A and 9B**). Two sites showed presence of D/iD pairs (**Figure 9C and 9D**; blue traces), but neither were strongly affected by the stressed conditions. Evidence of the succinimidyl peptide is also observed (black traces in both **9C and 9D**). A survey of 37 additional tryptic fragments with N, D, iD, in some cases with multiple deamidations and isomers, are accurately predicted for HILIC retention using the model developed for this purpose.[5]

## Oxidation

Methionine, cysteine, tryptophan, and histidine residues are susceptible to oxidation. For the mechanism of methionine oxidation, see **Figure 10**.

Methionine (Met-S) oxidizes to form methionine sulfoxide (Met-SO). Methionine sulfoxide (Met-SO) can further oxidize to form methionine sulfone (Met-$SO_2$). Met-SO can be reduced back to methionine using methionine sulfoxide reductase A (MsrA). Met oxidation in mAbs has been linked to function loss, folding stability decrease, and the increase in the propensity for the mAb to aggregate. Met oxidation is possible at various development stages, including production, formulation, and storage. For this reason, the presence of oxidation must be monitored at each development stage to determine the condition of the mAb.

In **Figure 11**, a methionine oxidation in NIST mAb was identified for the peptide fragment listed (position 4). The increase of mass of +16 Da with a doubly charged peptide fragment corresponds to a net change of 8 Daltons.

The NIST mAb digest analyzed at a column temperature of 60 °C did not show the presence of the oxidized Met. When the sample was allowed to remain on column at 80 °C for 30 min, then the oxidized Met was observed.

Another example of Met oxidation run under HILIC conditions is shown in **Figure 12**.[6]

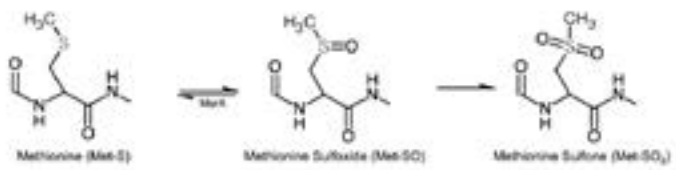


Figure 10. Oxidation of Methionine Mechanism

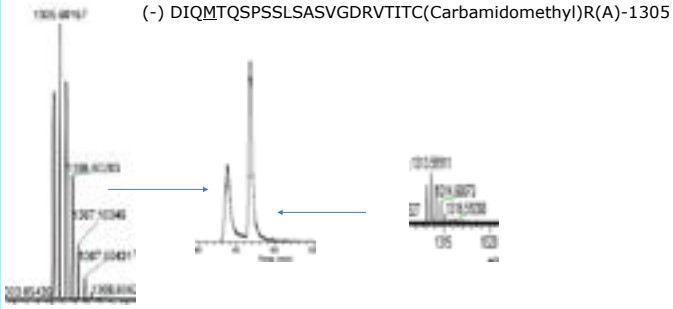


Figure 11. Evidence of Methionine oxidation in NIST mAb tryptic digest. Conditions: HALO® 90 Å Penta-HILIC, 2.7 µm, 0.5 x 150 mm; Mobile Phase A: 50 mM ammonium formate, pH 4.4; Mobile Phase B: 0.1% formic acid in acetonitrile; 50 µL/min; 80 °C; MS detection.

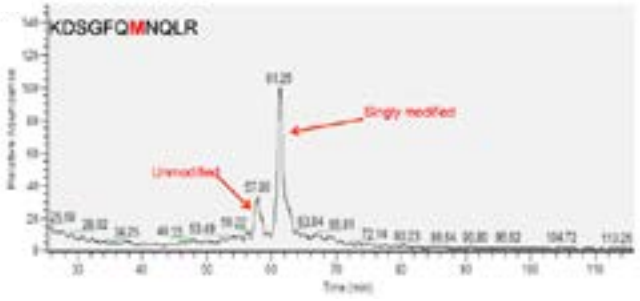


Figure 12. Adapted from J. Am. Soc. Mass Spectrom. (2017) 28:818-826. IgG oxidized peptide analyzed using HILIC conditions. Conditions: 0.2 x 150 mm HALO 90 Å Penta-HILIC; 2 µL/min; room temperature; Mobile Phase A: 50 mM ammonium formate/0.1% formic acid; Mobile Phase B: acetonitrile/0.1% formic acid; Gradient: 95%–30% B in 90 min.

The IgG oxidized peptide is well resolved from the unmodified form and can easily be quantified using the HALO® Penta-HILIC column. The benefit of using a superficially porous particle column in terms of enhanced efficiency is also demonstrated.

## Conclusion

Measuring and monitoring post-translational modifications, including glycosylation, deamidation, and oxidation are critical to assessing and maintaining the function and form of proteins and peptides, especially biotherapeutics. HILIC separations show increased resolution of polar PTMs of proteins and peptides compared to reversed-phase separations. HALO® Penta-HILIC is well suited for separations of glycovariants and analysis of site occupancy. HALO® Penta-HILIC can yield quantitative details on protein deamidations, oxidations, and other chemical modifications that are essential to correct protein function.

## References


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