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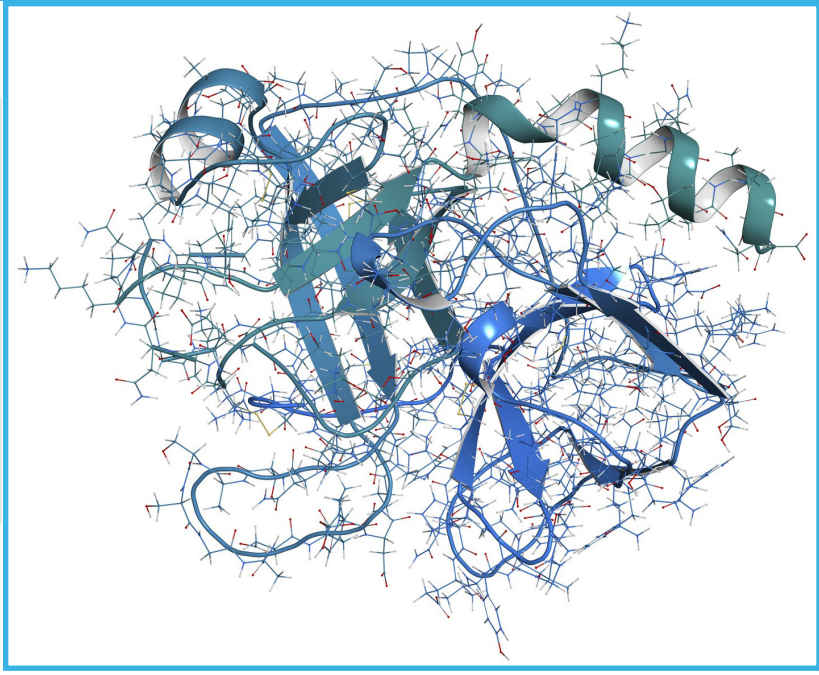
TECHNICAL REPORT: AMT_TR_BIO_21

TITLE: **CAPILLARY HILIC LC/MS SEPARATIONS OF MONOCLONAL ANTIBODY POLAR MODIFICATION DEAMIDATION PRODUCTS**

MARKET SEGMENT: BIOCLASS



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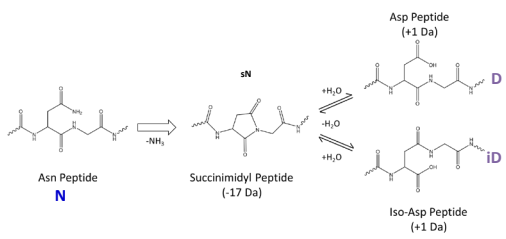
ABSTRACT

This study highlights a capillary HALO® Penta-HILIC column incorporated in an LC hyphenated high resolution MS workflow for the purpose of separating deamidation polar modification products of a monoclonal antibody, represented by a trastuzumab tryptic digest. The capillary HALO® Penta-HILIC column is an attractive alternative to the commonly used reversed-phase approaches for hyphenation with electrospray ionization MS. In addition to exploiting a low volumetric flowrate of 12 µL/min, this HILIC separation also utilizes a higher organic composition HILIC gradient.

INTRODUCTION

Deamidation is a reaction in which an amide functional group in the side chain of the amino acids asparagine or glutamine is removed or converted to another functional group. Deamidation products are of increasing importance in proteomics because they can alter a protein's structure, or possibly its function and stability, resulting in degradation. The rate of deamidation is dependent on multiple factors, including pH, the higher order structure of the protein, solution components, and temperature.

In the case of asparagine, the reaction proceeds with the loss of ammonium, then a succinimide intermediate is formed. This intermediate can then be interconverted to succinimidyl-, aspartyl- and iso-aspartyl peptides, provided it is in an aqueous solution. The analysis of protein deamidation products can be challenging for a number of reasons. The mass shifts associated with these interconversions are very well known, and can enable identification of the peptides from mass spectrometry, however, for many practical purposes, mass analysis alone is insufficient to resolve aspartate from iso-aspartate sequences, and chromatographic separation is needed. Protein deamidation has been commonly analyzed by reversed-phase liquid chromatography (RPLC) through peptide mapping, however RP separations often result in limited chromatographic selectivity for resolution of deamidated peptides from their unmodified counterparts. Recent advancements, however, have shown that hydrophilic interaction chromatography (HILIC) has advantages to RP separations [1–6]. Peptide deamidation products exhibit increased hydrophobicity, which affects peptide selectivity differences between HILIC and RP separations. Furthermore, operating in HILIC mode offers improved chromatographic separations for peptide deamidation products that are not resolved in RP [1–6]. Here we present the HALO® Penta-HILIC column for the analysis of deamidation products of trastuzumab in a high-resolution workflow and to enable identification of the polar modifications.



KEY WORDS:

HILIC, capillary, tryptic digest, peptide mapping, polar modifications, deamidation monoclonal antibody, trastuzumab

MATERIALS AND METHODS:

All solvents used were HPLC grade. Methanol, acetonitrile, mobile phase additives, and individual standards were obtained from MilliporeSigma (St. Louis, MO), unless specified otherwise. Trypsin digestion of the mAb - trastuzumab (as obtained for pharmaceutical purposes). Reduced and alkylated proteins were digested at 1:20 enzyme to protein overnight in 50 mM Tris-HCl (pH 7.8)/1.5M Guanidine-HCl, followed by 0.5% formic acid acidification and direct injection on to capillary LC/MS using the Orbitrap/IT.

Columns of HALO® Penta-HILIC were produced at Advanced Materials Technology Inc. (Wilmington, DE). These materials employ superficially porous Fused-Core® silica particles of 2.7 µm diameter, shell thicknesses of 0.5 µm, and pore sizes of 90 Å. Analyses of trastuzumab tryptic fragments employed a 0.5 mm ID x 150mm Penta-HILIC capillary column, operated at 12.0 µL/min, and 60°C. A trap column of the same material, of 2.6 µL size (0.5 mm x 12.5 mm) was obtained from Optimize, Inc. Analytical gradient conditions were from 80%B for 4min, then 80-48%B in 64 min using a flow rate of 12 µL/min. Mobile phase modifiers were obtained from Pierce (TFA, FA), Sigma/Millipore (TFA, formic acid, ammonium formate). Acetonitrile was MS grade from JT Baker. Mobile phase A: 50 mM ammonium formate pH 4.4 and mobile phase B: 0.1% formic acid in acetonitrile.

Capillary column separations used the Dionex RSLC 3000 connected to the Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI capillary needle operated at 3.8 kV potential. MS spectra were obtained, using 30,000 resolution, with CID MS/MS obtained using a Top N=4 data dependent regime. MS data were analyzed using Chromeleon v. 7.2 for integration. Chromatographic peak widths are reported as half height (PW1/2).

Results and Discussion

We highlight the HALO® Penta-HILIC capillary column to facilitate online LC/MS to determine deamidation and isomerization of protein asparagine and aspartate residues. The HALO® Penta-HILIC separation is an attractive front-end LC separation technique for use with LC/MS, due to a low volumetric flow-rate of 12 µL/min and a HILIC gradient employing a highly organic mobile phase environment. Clearly, the ESI is amenable to these conditions, as evidenced by the large number of charged species shown in Figure 1, and no evidence of ion suppression. The sufficient ion current dictates that the HILIC phase can be used for these types of analysis.

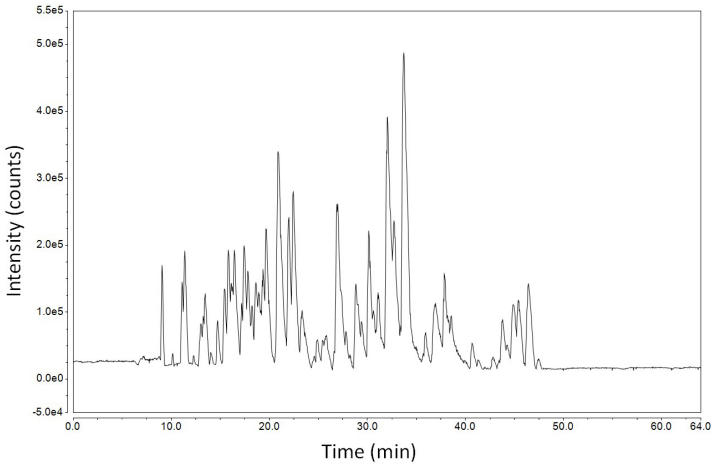


Figure 1. Total ion chromatogram of a tryptic digest between 0-64 min.

The deamidation products from the tryptic digest of the monoclonal antibody are shown in the peptide fragment GFYPSDIAVEWESNGQPENNYK and is one of many in a long list of peptide fragments identified by their mass to charge ratios (m/z) with the use of Biopharma Finder. Figure 2 illustrated the presence of the asparagine peptide (N) and the aspartyl- (D) and iso-aspartyl (iD) peptides in the peptide fragment. Both D and iD have equivalent m/z values that were separated by the capillary HALO® Penta-HILIC column. The retention pattern was supported by the analysis of standard synthetic peptides containing N, D and iD outlined previously [7].

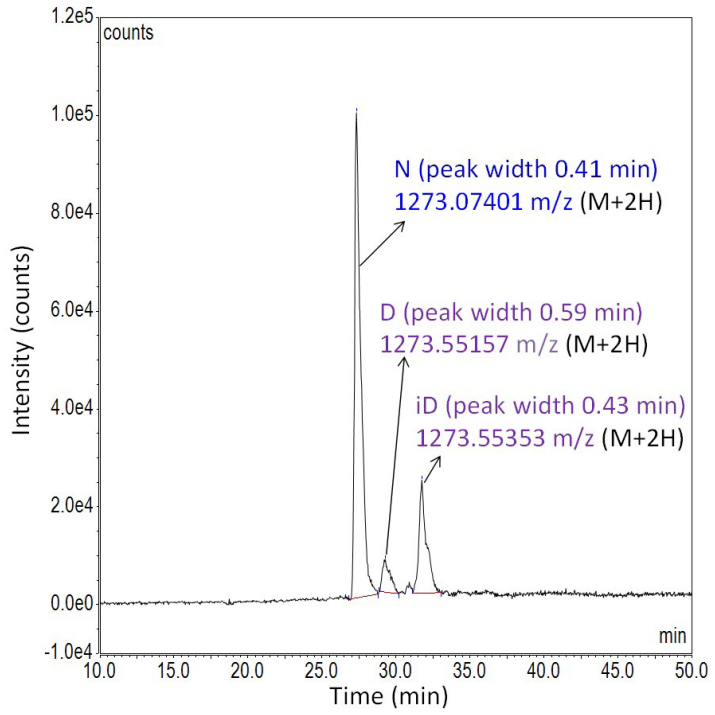


FIGURE 2. Extracted ion chromatogram 1272.5-1273.7 m/z of the peptide fragment GFYPSDIAVEWESNGQPENNYK.

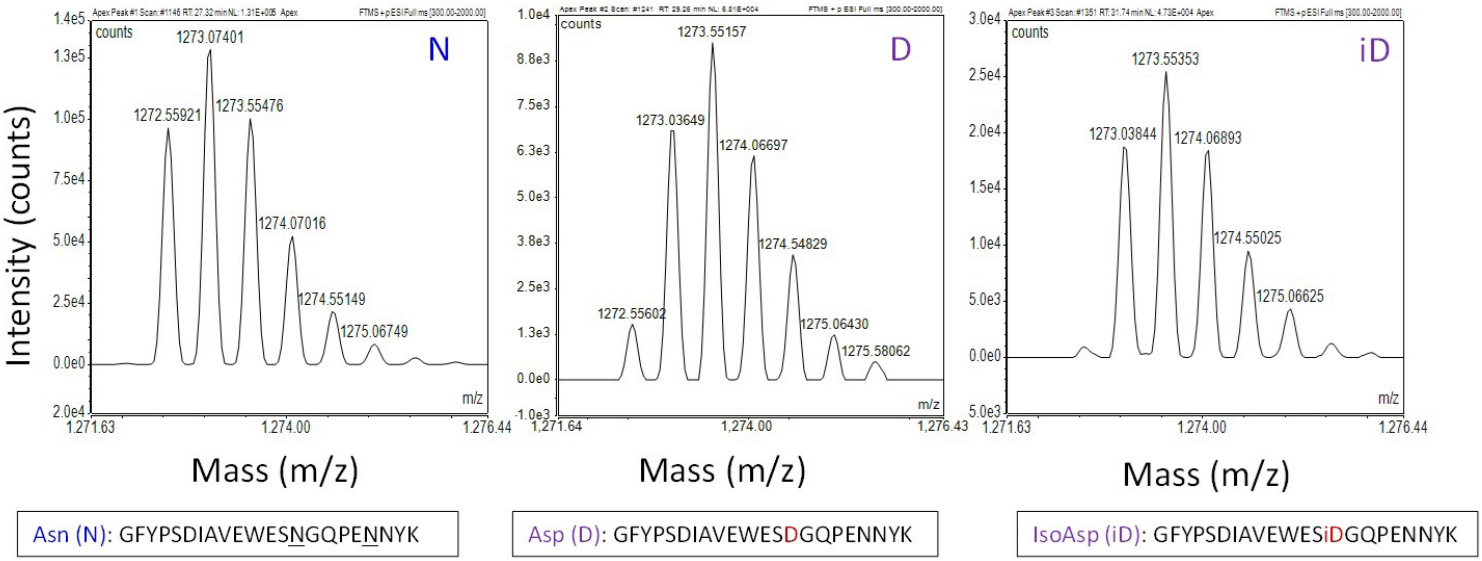


FIGURE 3. Mass spectra of the three peaks shown in Fig 2 in the order of elution.

Furthermore, the spectra for each of the asparagine peptide (N) and the aspartyl- (D) and iso-aspartyl (iD) peptide peaks represented in Figure 2 are shown in the order of elution in Figure 3. The spectra and EIC of the peptide fragment of GFYPSDIAVEWESNGQPENNYK evidenced the deamidation polar modification products of the trastuzumab tryptic digest experiments. This exercise may be repeated by searching other peptide fragments and their respective m/z that have a similar EIC pattern and spectra associated with the deamidation and isomerization of asparagine peptide and their retention pattern using standard peptides for N, D and iD [7].

CONCLUSION:

The capillary HALO® Penta-HILIC column facilitated coupling of microflow LC conditions that are ideal for use with ESI, with a low volumetric flowrate of 12 µL/min and a higher organic composition HILIC gradient. This report highlighted an LC/MS workflow for examining peptide deamidation and isomerization products of Asn, Asp, and isoAsp forms via HILIC [7].

ACKNOWLEDGEMENTS:

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AUTHOR CONTRIBUTIONS:

AS figures and writing; BP experimental, editing; AH writing.

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