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# HILIC Glycopeptide Mapping with a Wide-Pore Amide Stationary Phase

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## APPLICATION BENEFITS

- Orthogonal selectivity to conventional reversed phase (RP) peptide mapping for enhanced characterization of hydrophilic protein modifications, such as glycosylation
- Class-leading HILIC separations of IgG glycopeptides to interrogate sites of modification
- MS compatible HILIC to enable detailed investigations of sample constituents
- Enhanced glycan information that complements *Rapi*Fluor-MS released N-glycan analyses
- Glycoprotein BEH Amide 300Å 1.7 µm stationary phase is QC tested via a glycoprotein separation to ensure consistent batch to batch reproducibility

## WATERS SOLUTIONS

ACQUITY UPLC® Glycoprotein BEH Amide 300Å Column (patent pending)

Glycoprotein Performance Test Standard

ACQUITY UPLC H-Class Bio System

Waters SYNAPT® G2-S HDMS

## KEY WORDS

ACQUITY UPLC H-Class Bio System, BEH Amide 300Å, glycans, glycosylated proteins, glycosylation, HILIC, mab, glycopeptide, glycoprotein

## INTRODUCTION

Peptide mapping of biopharmaceuticals has longed been used as a tool for identity tests and for monitoring residue-specific modifications.[1-2] In a traditional analysis, peptides resulting from the use of high fidelity proteases, like trypsin and Lys-C, are separated with very high peak capacities by reversed phase (RP) separations with $C_{18}$ bonded stationary phases using ion-pairing reagents. Separations such as these are able to resolve peptides with single amino acid differences such as asparagine; and the two potential products of asparagine deamidation, aspartic acid and isoaspartic acid.[3-4]

Nevertheless, not all protein modifications are so easily resolved by RP separations. Glycosylated peptides, in comparison, are often separated with relatively poor selectivity, particularly if one considers that glycopeptide isoforms usually differ in their glycan mass by about 10 to 2,000 Da. So, while RP separations are advantageous for generic peptide mapping, they are limited in their ability to resolve hydrophilic modifications. Previous studies have demonstrated that hydrophilic interaction chromatography (HILIC) with an amide-bonded stationary phase can provide complementary and highly resolving separations of glycosylated peptides.[5-6] These studies have demonstrated that amide-bonded stationary phases are particularly effective for these separations, because they afford high retentivity as a consequence of their hydrophilicity and propensity for hydrogen bonding.[7]

Expanding upon this technology, we have developed an amide-bonded stationary phase with a nominally larger pore diameter, a so-called "wide-pore" material, such that amide HILIC separations can be universally applied to separating the glycoforms of both intact and digested glycoproteins. This stationary phase found in ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Columns ensures that glycopeptides, regardless of their size, will have access to the majority of the porous network and be less prone to restricted diffusion.[8-9] In previous work, we have demonstrated the use of this HILIC column to assay the glycan occupancy of an intact monoclonal antibody (mAb),[10] to map the domain-specific glycosylation of IgG subunits,[11] and to improve the resolution of tri- and tetra-antennary GlycoWorks,™ *Rapi*Fluor-MS™ labeled N-glycans.[12] Here, we explore the use of the Glycoprotein BEH Amide 300Å 1.7 µm Column to produce high resolution HILIC separations of glycopeptides from three different monoclonal antibodies: trastuzumab, cetuximab and an IgG1K candidate reference material from NIST.

## EXPERIMENTAL

### Sample description

#### Lys-C digest of trastuzumab and NIST candidate reference material

An adaptation of a previously published single reaction vial, overnight (16+ hours) procedure[4] was employed to prepare non-reduced Lys-C digests of trastuzumab and a IgG1K monoclonal antibody candidate reference material obtained from NIST (#8670, lot# 3F1b). TFA quenched digests were stored at -80 °C until analyzed. In preparation for HILIC chromatography, aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide and were then centrifuged at 16 x 1000 g for 10 minutes to remove any insoluble composition. Supernatant from the centrifuged digest was thereafter injected.

#### Lys-C/tryptic digest of cetuximab

Reduced and alkylated cetuximab was digested with a combination of Achromobacter protease I (Lys-C) and trypsin. Formulated cetuximab was concentrated to 10 mg/mL and buffer exchanged with a 10 kDa MWCO centrifugal filter (Millipore, Billerica, MA) into a solution of 6 M GuHCl, 50 mM DTT, and 0.2 M phosphate (pH 8.1), then incubated at 37 °C for 2 hours. Thereafter, the sample was diluted with a solution of iodoacetamide, bringing the antibody concentration to 8 mg/mL and the buffer composition to 4.8 M GuHCl, 40 mM DTT, 50 mM iodoacetamide, and 0.17 M phosphate (pH 8.1). Alkylation with iodoacetamide was allowed to proceed under these conditions for 10 min in the dark at 37 °C, before being quenched by the addition of cysteine, diluted with a urea-containing buffer, and mixed with Achromobacter protease I (Lys-C) at a 4:1 w/w ratio. The resulting digest solution of 0.8 mg/mL cetuximab, 0.5 M GuHCl, 3 M Urea, 40 mM $NH_2OH$, 4 mM DTT, 5 mM iodoacetamide, 6 mM cysteine, and 0.1 M phosphate (pH ~7.1) was incubated at 37 °C. After 2 hours of incubation, this digest solution was diluted two fold with water and an aliquot of trypsin (Sigma T6567), such that the protein:trypsin ratio was 4:1 (w/w). After incubation at 37 °C for another 2 hours, the digest solution was again diluted two fold with water and a fresh aliquot of trypsin. With a total protein:trypsin ratio of 2:1 (w/w), the digest was left to incubate at 37 °C for 16 hours. Following this incubation, the digest was quenched by acidification with TFA and stored at -80 °C until analyzed. In preparation for HILIC chromatography, aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide and were then centrifuged at 16 x 1000 g for 10 minutes to remove any insoluble composition. Supernatant from the centrifuged digest was thereafter injected.

### Method conditions

(unless otherwise noted):

#### Column conditioning

ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Columns (as well as other amide columns intended for glycoprotein or glycopeptide separations) should be conditioned via two sequential injections and separations of 40 µg Glycoprotein Performance Test Standard (p/n 186008010; 10 µL injections of 4 mg/mL in 0.1% TFA, 80% ACN) or with equivalent loads of a test sample for which the column has been acquired. The separation outlined by the following method can be employed for conditioning with the Glycoprotein Performance Test Standard.

#### Column conditioning gradient

2.1 x 150 mm

Mobile phase A: 0.1% (v/v) TFA, H2O

Mobile phase B: 0.1% (v/v) TFA, ACN

| Time | %A | %B | Curve |
|---|---|---|---|
| 0.0 | 15.0 | 85.0 | 6 |
| 0.5 | 15.0 | 85.0 | 6 |
| 1.0 | 33.0 | 67.0 | 6 |
| 21.0 | 40.0 | 60.0 | 6 |
| 22.0 | 100.0 | 0.0 | 6 |
| 24.0 | 100.0 | 0.0 | 6 |
| 25.0 | 15.0 | 85.0 | 6 |
| 35.0 | 15.0 | 85.0 | 6 |

#### LC conditions for LC-UV-MS of mAb glycopeptides (Figures 1–6):

| | |
|---|---|
| LC system: | ACQUITY UPLC H-Class Bio System |
| Sample temp.: | 10 °C |
| Analytical column temp.: | 30 °C (trastuzumab Lys-C digest HILIC separations) |
| | 60 °C (cetuximab Lys-C/tryptic digest HILIC separations) |
| | 60 °C (trastuzumab Lys-C reversed phase separations) |

| | |
|---|---|
| Flow Rate: | 0.2 mL/min |
| Mobile phase A: | 0.1% (v/v) TFA, $H_2O$ |
| Mobile phase B: | 0.1% (v/v) TFA, ACN |
| HILIC injection volume: | 100–250 µL (Aqueous digests were diluted with 4 parts acetonitrile and 0.1 parts dimethylsulfoxide to obtain a miscible, HILIC compatible diluent.) |
| Reversed phase injection volume: | 24.2 µL (Aqueous digest) |
| Columns: | ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard) |
| | ACQUITY UPLC Peptide BEH $C_{18}$ 300 Å 1.7 µm, 2.1 x 150 mm (p/n 186003687) |
| Vials: | Polypropylene 12 x 32 mm Screw Neck Vial, 300 µL Volume (p/n 186002640) |

**Gradient used for reversed phase separations of trastuzumab Lys-C digests (Figure 1A):**

| Time | %A | %B | Curve |
|---|---|---|---|
| 0.0 | 98.0 | 2.0 | 6 |
| 96.0 | 50.0 | 50.0 | 6 |
| 99.0 | 20.0 | 80.0 | 6 |
| 101.0 | 20.0 | 80.0 | 6 |
| 102.0 | 98.0 | 2.0 | 6 |
| 113.0 | 98.0 | 2.0 | 6 |

**Gradient used for HILIC separations of trastuzumab Lys-C digests and Lys-C/tryptic digests of cetuximab (Figures 1B-6):**

| Time | %A | %B | Curve |
|---|---|---|---|
| 0.0 | 20.0 | 80.0 | 6 |
| 60.0 | 50.0 | 50.0 | 6 |
| 61.0 | 80.0 | 20.0 | 6 |
| 63.0 | 80.0 | 20.0 | 6 |
| 64.0 | 20.0 | 80.0 | 6 |
| 75.0 | 20.0 | 80.0 | 6 |

**MS conditions for IgG subunit separations**

| | |
|---|---|
| MS system: | SYNAPT G2-S HDMS |
| Ionization mode: | ESI+ |
| Analyzer mode: | Resolution (~20 K) |
| Capillary voltage: | 3.0 kV |
| Cone voltage: | 25 V |
| Source temp.: | 120 °C |
| Desolvation temp.: | 350 °C |
| Desolvation gas flow: | 800 L/Hr |
| Calibration: | NaI, 1 µg/µL from 100–2000 *m/z* |
| Lockspray: | 300 fmol/µL Human Glufibrinopeptide B in 0.1% (v/v) formic acid, 70:30 water/acetonitrile every 90 seconds |
| Acquisition: | 50–2500 *m/z*, 0.1 sec scan rate |
| Data management: | MassLynx Software (V4.1) / UNIFI V1.7 |

**LC Conditions for a Glycopeptide Mapping of an IgG1K with Fluorescence Detection (Figure 7):**

| | |
|---|---|
| LC system: | ACQUITY UPLC H-Class Bio System |
| Sampletemp.: | 10 °C |
| Analytical column temp.: | 45 °C |
| Fluorescence detection: | Ex 280/Em 320 nm (10 Hz scan rate, Gain =1) |
| Injection volume: | 100 µL (DMF/ACN diluted sample) |
| Mobile phase A: | 0.1% TFA in water |
| Mobile phase B: | 0.1% TFA in ACN |
| Columns: | ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm, 2.1 x 150 mm (p/n 176003702, with Glycoprotein Performance Test Standard) |
| Other columns: | Column A: 2.6 µm, 2.1 x 150 mm |
| | Column B: 1.8 µm, 2.1 x 150 mm |
| Vials: | Polypropylene 12 x 32mm Screw Neck Vial, 300 µL Volume (p/n 186002640) |

**Gradient (Figure 7):**

| Time (min) | %A | %B | Curve |
|---|---|---|---|
| 0.0 | 15.0 | 85.0 | 6 |
| 0.5 | 15.0 | 85.0 | 6 |
| 1.0 | 30.0 | 70.0 | 6 |
| 21.0 | 37.0 | 63.0 | 6 |
| 22.0 | 100.0 | 0.0 | 6 |
| 24.0 | 100.0 | 0.0 | 6 |
| 25.0 | 15.0 | 85.0 | 6 |
| 35.0 | 15.0 | 85.0 | 6 |

Data management: UNIFI v1.7

## RESULTS AND DISCUSSION

### Orthogonal and complementary glycopeptide mapping separations

To demonstrate a conventional approach to peptide mapping, we first performed LC-UV-MS analysis on a Lys-C digest of a mAb using a RP chromatographic separation with a wide-pore $C_{18}$ bonded stationary phase (Peptide BEH $C_{18}$ 300Å 1.7 µm). Trastuzumab was selected for this study, given its prominence as a first generation mAb drug product and a potential target for biosimilar development.[13] Figure 1A shows a UPLC chromatogram that is typical for a Lys-C digest of trastuzumab, wherein peptides are broadly resolved across a separation with a gradient corresponding to a change of 0.5% acetonitrile per minute. The non-glycosylated peptides of the digest spread across the extremes of the chromatogram while the glycopeptides elute in an approximately one minute wide window at a retention time of about 60 minutes. The conditions to produce this high resolution separation involve the use of mobile phases modified with trifluoroacetic acid (TFA); the same mobile phases that have proven to be optimal for HILIC of proteinaceous analytes.[10-11]

Accordingly, an orthogonal method to the RP separation can be achieved via HILIC by simply reversing the gradient and using the newly developed wide-pore amide bonded stationary phase (Glycoprotein BEH Amide 300Å 1.7 µm). An example of a chromatogram obtained from a column packed with this wide-pore amide material and a gradient ramp of 0.5% acetonitrile per minute is shown in Figure 1B. Here, the peptides from the Lys-C digested trastuzumab are very clearly segregated into early and late eluting species, corresponding to the non-glycosylated and glycosylated species, respectively. The use of TFA ion pairing facilitates obtaining this separation, as it masks the hydrophilicity of the peptide residues and provides improved selectivity for the hydrophilic modifications. Also note that the glycopeptides have not only been class separated with the amide column, but the selectivity of the peptide glycoforms is remarkably improved over the analogous RP separation.

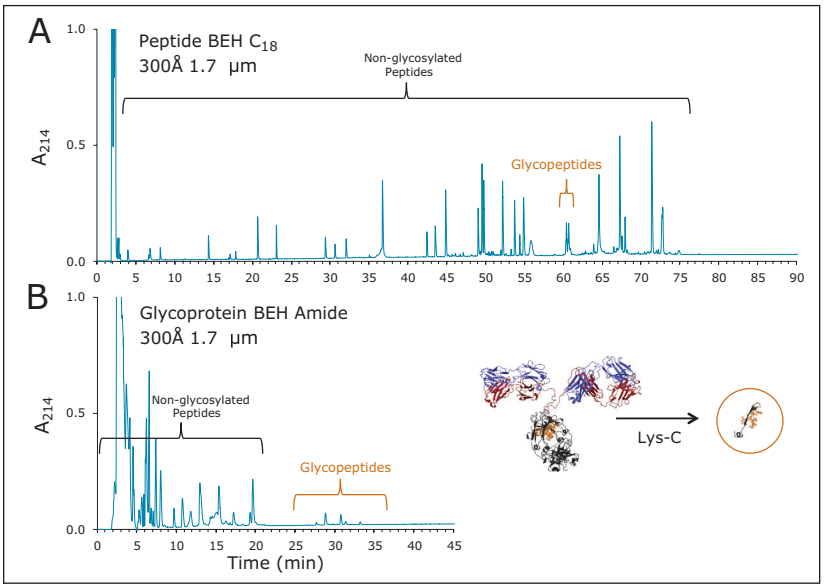


*Figure 1. Lys-C glycopeptide mapping of trastuzumab. (A) A traditional reversed phase separation of the Lys-C digest using a 2.1 x 150 mm ACQUITY UPLC Peptide BEH $C_{18}$ 300Å 1.7 µm Column. (B) A HILIC separation of the Lys-C digest using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column. In each analysis, 9.2 µg of the Lys-C digest was separated using the same gradient slope and injecting sample from a diluent comprised of either approximately 0.2% TFA in 80:20 ACN/water (HILIC) or 100% water (reversed phase).*

By focusing on the strongly retained peaks, one can begin to interrogate the glycosylation of the trastuzumab molecule (Figure 2A). In particular, MS data acquired from online mass detection and a total ion chromatogram (TIC) can be applied to identify the peptide species and its corresponding glycoforms, as shown in Figure 2B. This Lys-C glycopeptide map presents a 29 amino acid residue peptide (K16) from the Fc domain of trastuzumab. From an analysis of the MS data, many biantennary structures typical found on mAbs in relatively high abundance can be readily identified (Figure 3A). Further interrogation of the MS data, also shows that low abundance N-glycan species can likewise be detected. Figure 3B, for instance, provides MS data supporting the identification of monosialylated and disialylated glycoforms at retention times of approximately 34.7 minutes and 36.4 minutes, respectively. These identifications correlate extremely well with the released N-glycan profiles of trastuzumab that have been previously reported.[14-15]

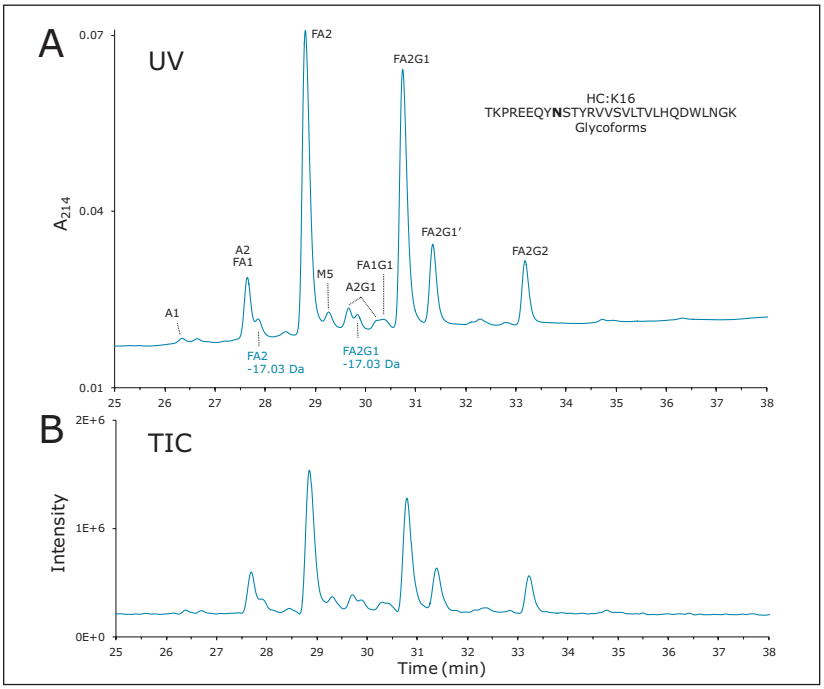


*Figure 2. Lys-C glycopeptide mapping of trastuzumab with HILIC and an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column. (A) UV chromatogram for the Lys-C glycopeptide retention window. (B) Total ion chromatogram (TIC) for the same retention window.*

A

| Species | Modification | Calculated Mass (Da) | RT (Min) | Intensity | Error (ppm) |
|---|---|---|---|---|---|
| HC:K16 | A1 | 4555.1865 | 26.4 | 15461 | -1.0 |
| HC:K16 | A2 | 4758.2656 | 27.7 | 219414 | -1.1 |
| HC:K16 | FA1 | 4701.2441 | 27.7 | 17931 | -3.3 |
| HC:K16 | FA2 | 4904.3237 | 28.8 | 1502968 | -1.7 |
| HC:K16 | M5 | 4676.2129 | 29.3 | 51057 | -0.7 |
| HC:K16 | A2G1 | 4920.3188 | 29.7 | 89714 | -0.9 |
| HC:K16 | A2G1 | 4920.3188 | 30.3 | 33516 | -1.9 |
| HC:K16 | FA1G1 | 4863.2974 | 30.4 | 17005 | -1.1 |
| HC:K16 | FA2G1 | 5066.3765 | 30.8 | 1156785 | -0.7 |
| HC:K16 | FA2G1' | 5066.3765 | 31.4 | 312270 | -1.0 |
| HC:K16 | FA2G2 | 5228.4292 | 33.2 | 226743 | -2.0 |

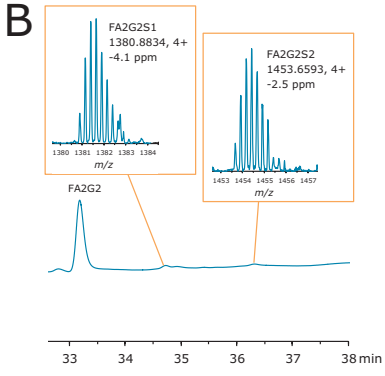


*Figure 3. Mass spectrometric data supporting trastuzumab Lys-C glycopeptide identifications. (A) Retention times, MS intensities and mass errors for assignments labeled in Figure 2. (B) MS spectra supporting the identification of low abundance Lys-C glycopeptides modified with mono and di-sialylated N-glycans.*

### Lot-to-lot analysis of trastuzumab glycosylation via HILIC-UV glycopeptide mapping

HILIC-MS based glycopeptide mapping clearly yields information-rich data. However, these HILIC glycopeptide mapping separations also lend themselves to methods based only on optical detection. We have, for example, applied a HILIC-UV method to perform lot-to-lot analysis of trastuzumab glycosylation for two drug product samples. Representative HILIC chromatograms for glycopeptide K16 obtained from two different lots of trastuzumab are shown in Figure 4A. Previous released glycan analyses on these lots have shown there to be differences in glycosylation.[14] Through comparison of peak areas across the glycopeptide profile, we have found that these two lots of trastuzumab indeed differ with respect to their glycosylation. Specifically, these lots of trastuzumab appear to have different extents of terminal galactosylation, as can be seen in the differing abundances of FA2, FA2G1 and FA2G2 glycoforms (Figure 4B). This observation was consistent with data obtained from previous released glycan analyses and previous HILIC based profiling of trastuzumab subunits.[11]

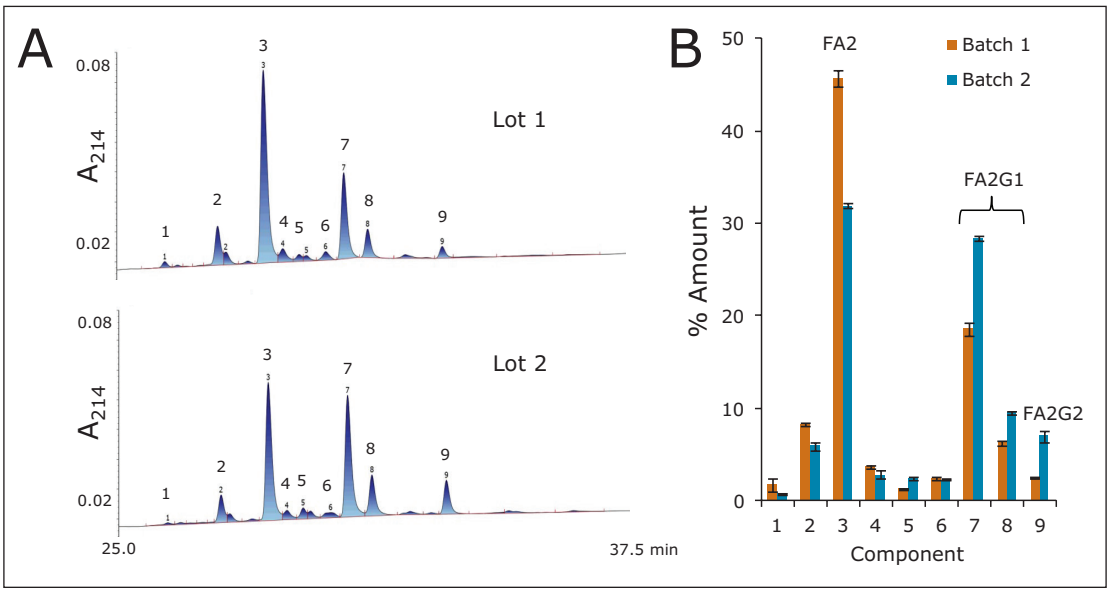


*Figure 4. Lot-to-lot profiling of trastuzumab Lys-C peptide glycoforms. (A) HILIC chromatograms of trastuzumab Lys-C glycopeptides from two different lots of drug product. (B) Relative abundances of the major sample components. Analyses were performed in triplicate using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column.*

### Complementing GlycoWorks *Rapi*Fluor-MS N-glycan analyses with domain and peptide specific information about mAb glycosylation

An appealing aspect of glycopeptide mapping is that it can be applied to the elucidation of domain and peptide specific information. By inference or ETD fragmentation analyses, or both, glycopeptide mapping can also be used to detail the exact sites of glycosylation.[16] As we have noted before,[11] IgGs contain one conserved N-glycosylation site at Asn297 of the heavy chain, meaning they will be modified with two glycans in their Fc subunit. In addition, some IgGs and even some mAb IgG therapeutics exhibit multi-domain glycosylation. Cetuximab, for instance, is glycosylated in both its Fc and Fab domains,[17] making it a very interesting case study for this work.

A HILIC glycopeptide map of a Lys-C/tryptic digest of cetuximab provides a clear indication of the complicated glycan profile of this molecule (Figure 5). In the presented chromatogram, approximately thirty chromatographic peaks are observed. Furthermore, a cursory analysis of the MS data has shown there to be at a minimum twenty five different glycoform species with rather high relative abundances of greater than 1–2%. Figure 6 provides the MS data supporting these assignments. As can be seen, 9 unique glycoforms could be assigned to tryptic peptide T22 from the Fc domain, while the other 16 glycoforms could be assigned to tryptic peptide T8 from the Fab domain of cetuximab. It is interesting to note that the majority of the Fab domain (T8) glycans contain immunogenic epitopes, such as non-human alpa-1,3-galactose or non-human N-glycolyl-neuraminic acid moieties.[18] In previous work, these glycan species were identified through complementary subunit mapping and *Rapi*Fluor-MS released N-glycan analyses.[11] With these results on glycopeptide mapping, we show yet another complementary technique for assessing protein glycosylation.

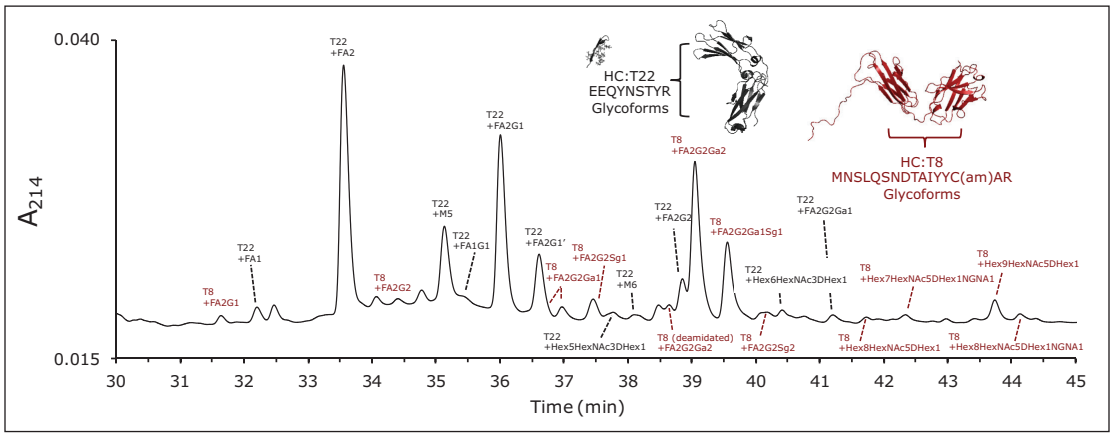


*Figure 5. Assaying the N-linked glycan sites of cetuximab and their microheterogeneity using a combined Lys-C/tryptic digest. Assignments for glycoforms of peptide T22 from the Fc domain of cetuximab are shown in dark gray, while assignments to the glycoforms of peptide T8 from the Fab domain are shown in red. Analyses were performed on 9.2 µg of Lys-C/tryptic digest using a 2.1 x 150 mm ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column and a sample diluent of approximately 0.2% TFA in 80:20 ACN/water.*

| Species | Modification | Calculated Mass (Da) | RT (Min) | Intensity | Error (ppm) |
|---|---|---|---|---|---|
| HC:T22 | FA1 | 2429.9592 | 32.2 | 9047 | 1.5 |
| HC:T22 | FA2 | 2633.0386 | 33.6 | 263523 | 1.4 |
| HC:T22 | M5 | 2404.9275 | 35.2 | 78988 | 0.0 |
| HC:T22 | FA2G1 | 2795.0913 | 36.1 | 186156 | 1.2 |
| HC:T22 | FA2G1' | 2795.0913 | 36.7 | 57185 | 0.7 |
| HC:T22 | [Hex5HexNAc3DHex1] | 2754.0647 | 37.8 | 7783 | 1.0 |
| HC:T22 | M6 | 2566.9805 | 38.1 | 5432 | -1.8 |
| HC:T22 | FA2G2 | 2957.1443 | 38.9 | 38257 | 1.1 |
| HC:T22 | [Hex6HexNAc3DHex1] | 2916.1177 | 40.5 | 12246 | -1.3 |
| HC:T8 | FA2 | 3350.3689 | 29.4 | 5688 | 4.9 |
| HC:T8 | M5 | 3122.2578 | 30.4 | 6437 | 2.7 |
| HC:T8 | FA2G1 | 3512.4216 | 31.7 | 8925 | 3.2 |
| HC:T8 | FA2G2 | 3674.4746 | 34.1 | 11695 | 8.2 |
| HC:T8 | FA2G2Ga1 | 3836.5273 | 36.7 | 9657 | -0.6 |
| HC:T8 | FA2G2Ga1' | 3836.5273 | 37.0 | 15605 | 0.3 |
| HC:T8 | FA2G2Sg1 | 3981.5649 | 37.5 | 25532 | 1.9 |
| HC:T8 | FA2G2Ga2 | 3998.5801 | 39.1 | 212644 | -0.7 |
| HC:T8 | A2G2 | 3471.3950 | 39.1 | 21542 | 2.0 |
| HC:T8 | [Hex7HexNAc5DHex1] | 4144.6377 | 39.2 | 6944 | -10.8 |
| HC:T8 | FA2G2Ga1Sg1 | 4143.6177 | 39.6 | 90905 | -0.7 |
| HC:T8 | FA2G2Sg2 | 4288.6553 | 40.2 | 7428 | -2.5 |
| HC:T8 | [Hex8HexNAc5DHex1] | 4363.7124 | 41.8 | 5280 | 1.7 |
| HC:T8 | [Hex7HexNAc5DHex1NGNA1] | 4508.7498 | 42.4 | 7114 | -3.6 |
| HC:T8 | [Hex9HexNAc5DHex1] | 4525.7651 | 43.8 | 20104 | 4.3 |
| HC:T8 | [Hex8HexNAc5DHex1NGNA1] | 4670.8027 | 44.2 | 7640 | 1.2 |

*Hex: hexose, HexNAc: N-acetylate hexosamine, DHex: deoxyhexose, NGNA: N-glycoyl neuraminic acid / (`): structural isomer

*Figure 6. Mass spectrometic data supporting glycopeptide assignments from the HILIC-UV-MS analysis of Lys-C/trypsin digested cetuximab. From a cursory analysis of MS data, 9 unique glycoforms could be assigned to the Fc domain, and 16 unique glycoforms could be assigned to the Fab domain.*

## Benchmarking the Capabilities of the Glycoprotein BEH Amide 300Å 1.7 µm Column

The peak capacities obtained in these example glycopeptide separations are particularly noteworthy when a comparison is made to otherwise available column technologies. To benchmark the performance of the Glycoprotein BEH Amide 300Å 1.7 µm Column, we have analyzed a Lys-C digest of a NIST candidate reference material, an IgG1K mAb. In this testing, a focused gradient was used along with intrinsic peptide fluorescence instead of low wavelength UV detection so that higher signal-to-noise could be achieved in the obtained chromatograms. The glycopeptide that originates from the Fc domain of a mAb will contain a tryptophan residue upon Lys-C cleavage, which in large part makes this detection mechanism feasible. Three fluorescence chromatograms obtained for the Lys-C glycopeptides from the NIST IgG1K are presented in Figure 7. These three chromatograms were obtained from the use of the ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm as well as two commercially available alternatives (Columns A and B). Peak capacities have been measured for each specific column using the retention windows demarcated by the most extreme glycopeptide retention times (*) and the half-height peak widths of the K16+FA2, K16+FA2G1, K16+FA2G1', K16+FA2G2, and K16+FA2G2Ga1 peaks. This analysis shows that these columns exhibit strikingly different resolving power. With an effective peak capacity of 72.8, the Glycoprotein BEH Amide column shows a superior peak capacity and performance increases over the alternative amide column technologies of 40 and 96%.

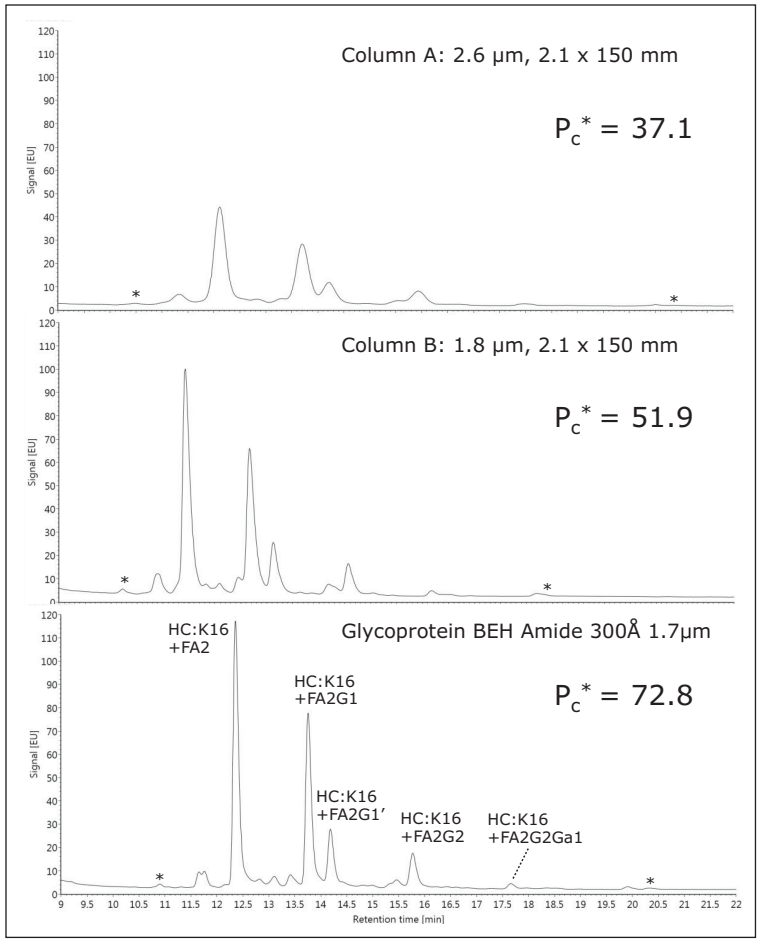


*Figure 7. Lys-C glycopeptide mapping of an IgG1K using fluorescence detection and various 2.1 x 150 mm columns packed with amide bonded stationary phase: a Competitor Column A: 150Å 2.6 µm, 2.1 x 150 mm (Top), a Competitor Column B: 1.8 µm, 2.1 x 150 mm (Middle), and on an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm column (Bottom). Peak capacities were calculated based on the half-height peak widths of the labeled glycopeptides and the retention window established by the earliest and latest eluting glycopeptide species, marked with asterisks(*). Comparative separations may not be representative in all applications.*

## CONCLUSIONS

Glycopeptide mapping of glycoproteins presents a highly effective technique that can be used to elucidate both domain and peptide-specific glycosylation. In this work, we have demonstrated the use of an ACQUITY UPLC Glycoprotein BEH Amide 300Å 1.7 µm Column to obtain HILIC separations of glycopeptides that complement the chromatographic information afforded by a reversed phase separation. In addition, our results indicate that these HILIC separations provide exemplary peak capacity in comparison to other commercially available amide column technologies. That the HILIC separation is MS-compatible means that information-rich data can be readily acquired to characterize a glycopeptide map. For instance, this work shows that it can be a relatively straightforward exercise to characterize multidomain protein glycosylation, such as the Fc and Fab domain glycosylation of cetuximab. Combined with other recently developed strategies, such as HILIC subunit mapping and GlycoWorks *Rapi*Fluor-MS released N-glycan analyses, glycopeptide mapping with the ACQUITY UPLC Glycoprotein BEH Amide Column shows significant promise for facilitating the characterization of protein glycosylation to unprecedented levels of detail.

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