# IMPROVING GLYCOPEPTIDE FINGERPRINTS WITH HIGH RESOLUTION CHROMATOGRAPHY

*Beth L. Gillece-Casto, Thomas E. Wheat, Ziling Lu, and Jeffrey R. Mazzeo*
*Waters Corp., Milford MA*

## INTRODUCTION

**Characterization of glycoproteins entails analysis of the primary structure of the protein and analysis of the attached oligosaccharides. Oligosaccharides are routinely characterized by glycan mapping after release from the protein. Peptide maps can provide information about the site-specific micro heterogeneity of the glycans if the glycopeptides are resolved. The enhanced chromatographic resolution associated with Ultra Performance Liquid Chromatography™ has been demonstrated for peptide mapping.[1] The technique improves resolution by a factor of three or more. The Peptide Separation Technology Columns for UPLC™ include both 130Å and 300Å pore size materials. Coupling UPLC™ to oa-Tof mass spectrometry allows high sensitivity identification of glycopeptides through exact mass measurement and deconvolution of complex mass spectra. Optimized chromatographic resolution of individual glycoforms of glycopeptides yields high quality mass spectra. This spectral information can be used to develop UPLC™ methods for quantitative glycopeptide mapping with UV detection.**

## METHODS

### UPLC Conditions

| | |
|---|---|
| Injection Volume: | 10 µL of 3-5 pmol/µL digest solution |
| Columns: | Peptide Separation Technology<br>ACQUITY UPLC™ BEH 130 $C_{18}$ 1.7 µm, 2.1 x 100mm<br>ACQUITY UPLC™ BEH 300 $C_{18}$ 1.7 µm, 2.1 x 100mm<br>X-Bridge™ BEH $C_{18}$ 3.5µm, 2.1 x 150mm<br>Vydac 238MS™ 5µm, 2.1 x 250 mm |
| Temperature: | 40°C (unless otherwise noted) |
| Flow Rate: | 100 µL/min (unless otherwise noted) |
| Solvent A: | 0.1% TFA in water |
| Solvent B: | 0.08% TFA in acetonitrile |
| Mixer: | High Sensitivity Peptide Analysis mixer (P/N 205000403) |

Gradient Table:

| Time (min) | %A | %B |
|---|---|---|
| Init | 100 | 0 |
| 2 | 100 | 0 |
| 118 | 50 | 50 |
| 120 | 25 | 75 |

### MS Conditions

| | |
|---|---|
| LCT Premier™ | oa-Tof mass spectrometer |
| Capillary: | 3000V |
| Cone: | 35V |
| Source Temperature: | 100°C |
| Source Pressure: | 1.4 x $e^0$ Torr |
| Scan Range: | 300-3000 *m/z* |
| Scan Rate: | 2 scans/sec |

## RESULTS

### Glycopeptides with Neutral N-linked Glycans

**Figure 1** **Glycopeptides of IgG**

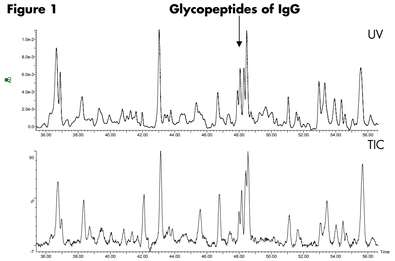


*Figure 1. Small portion of mouse IgG tryptic digest UPLC™ chromatograms. The glycoforms have been significantly resolved.*

**Figure 2**

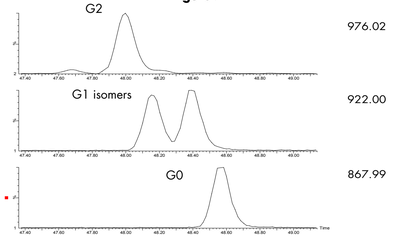


*Figure 2. Mouse IgG glycoforms were also detected with MS, and extracted ion chromatograms of the triply-charged glycopeptides are shown. Four peaks were observed for the molecular weights indicating*

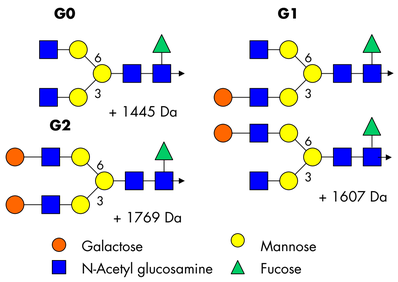


**Figure 3**

**EEQFNSTFR**

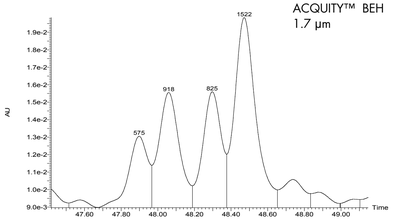


*Figure 3. Mouse IgG1glycoforms were detected with UV at 214 nm. The peptide sequence is shown above. Area was integrated for the four glycoforms shown.*

**Figure 4**

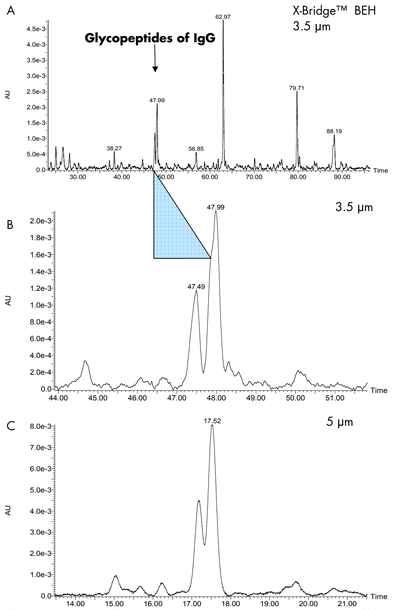


*Figure 4. Mouse IgG glycoforms were detected by UV (4a) Using HPLC. Resolution of glycopeptides on the X-Bridge*

### Glycopeptides with Sialylated N– and O-linked Glycans

**Figure 5**

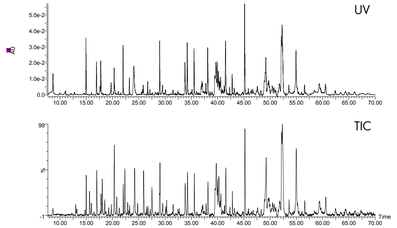


*Figure 5. Tryptic digest of bovine fetuin analyzed with UPLC on a 300Å pore size $C_{18}$ UPLC™ column with TFA mobile-phase modifier. Glycopeptide peak shapes are narrow, symmetrical and yield useful spectra.*

**Figure 6**

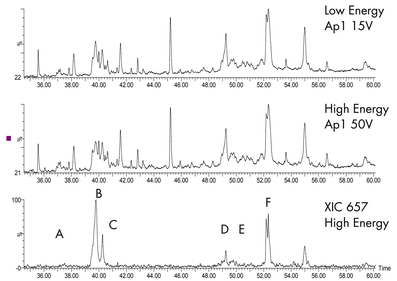


*Figure 6. The intensity of the total ion chromatograms of the fetuin trypsin digest is comparable whether the source is tuned for molecular ions or fragments. Numerous sialylated glycopeptides were observed by fragment analysis. The flow rate in this case was 200 µL/min. Labeled peptides are described in Table I*

**Table I**

| Peak | Peptide | Major Glycoforms | Resolution |
|---|---|---|---|
| A | T10 | O- 1 sialo-hex-hexNAc<br>" +1 sialic acid | Overlapped |
| B | T11-12 | N- Trisialo-Triantennary<br>" +1 sialic acid | Resolved |
| C | T12 | N- Trisialo-Triantennary<br>" +1 sialic acid | Partially Resolved |
| D | T7 | N- Trisialo-Triantennary<br>" +1 sialic acid | Overlapped |
| E | T20 | O- 3 sialo-hex-hexNAc<br>3 " +1 sialic acid<br>2 sialo-hex-hexNAc | Partially Resolved<br>Resolved |
| F | T13 | N- Trisialo-Triantennary<br>" +1 sialic acid | Partially Resolved |

**Figure 7**

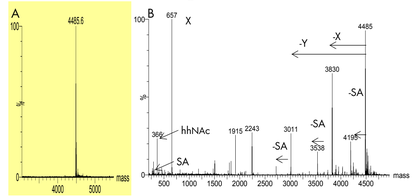


*Figure 7. Deconvoluted mass spectra of the 3 sialic acid-containing region of Peak B. The spectra were collected in the same run at low (4a) or high (4b) aperture1 potentials. Under high energy conditions fragments are observed. With good chromatographic resolution useful glycan structural information is obtained in MS only mode.*

**Figure 8**

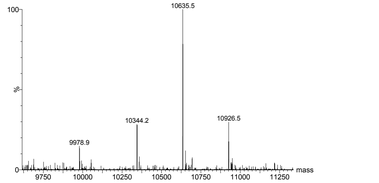


Figure 8. The largest glycopeptides observed in these experiments represents missed cleavages in the fetuin digest. The glycoforms have molecular weights approximately 10 kDa, and were separated on the 130Å.pore size, 1.7 µm particle packing material.

**Figure 9**

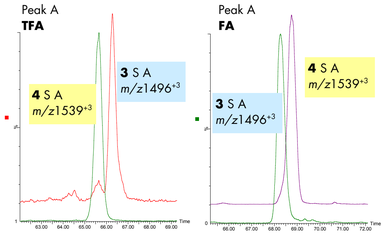


*Figure 9. The fetuin peptide T11-12 has two major glycoforms. Both are complex triantennary structures with either 3 or 4 sialic acid termini. Elution order is reversed when formic acid is replaced with TFA.[3]*

### MS Conditions for Glycan Detection

Glycopeptides were detected using the classic method of source fragmentation to create diagnostic glycan ions.[2] MS scans were alternated between normal source conditions (Aperture 1 = 15V) and fragmentation conditions (Aperture 1 = 50V). The fragment ion, *m/z* 657, identifies the presence of N- and O-linked sialylated glycans. Sialylated glycan termini, both N– and O-linked, produce *m/z* 292 (NeuAc) and *m/z* 657 (NeuAc-Gal-GlcNAc).

**Figure 10**

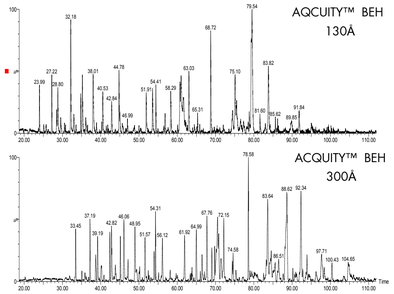


*Figure 10. Comparison of the separation of tryptic peptides of bovine fetuin. Both the 300Å and 130Å pore size Peptide Separation Technology™ 1.7µm columns provide excellent resolving power for peptides.*

## CONCLUSION

- **Chromatographic resolution provides excellence in mass spectra of glycopeptides.**
- **High resolution separation of glycopeptides has been achieved on 300Å and 130Å pore size 1.7 µm particles.**
- **Good peak shape is obtained for sialylated and non-sialylated glycopeptides including positional isomers.**
- **Changing the gradient slope, temperature or mobile phase modifier can optimize separations.**
- **Data obtained in MS only mode produced spectra characterizing complex sialylated glycan structures.**

### References

1. Mazzeo, J.R.; Wheat, T.A., Gillece-Castro, B.L. and Lu, Ziling *BioPharm International*, **2006**, *19-1*, 1-9
2. Huddleston; M.J., Bean; M.F. and Carr, S.A. *Anal. Chem.* **1993**, *65* 877-884.
3. Medzihradszky, K.F.; Besman, M.J. and Burlingame, A.L. *Anal Chem.* **1997,** *69*, 3986-3994.

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