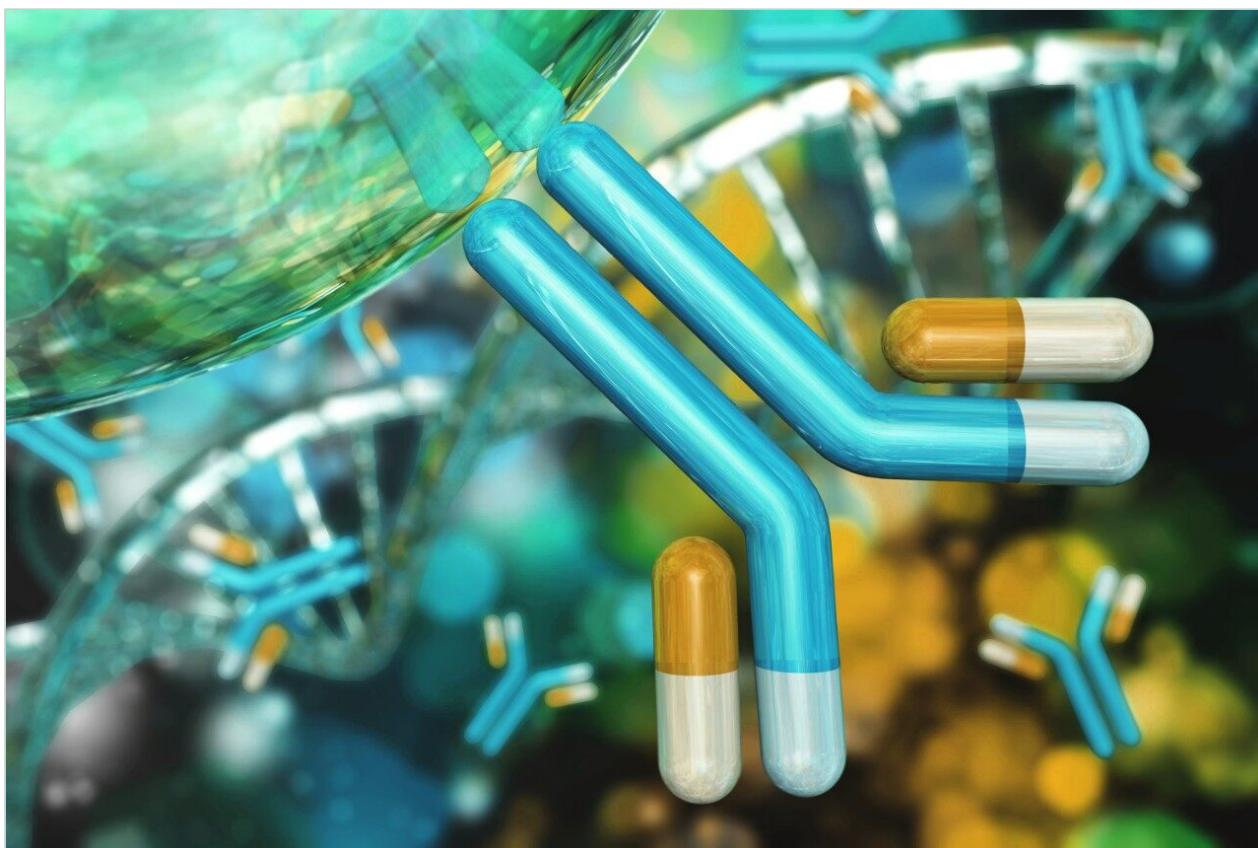


## UPLC Technology for the Analysis of Antibody Glycopeptides

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Beth L. Gillece-Castro, Thomas E. Wheat, Jeffrey R. Mazzeo, Diane M. Diehl

Waters Corporation



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### Abstract

The Waters UPLC Peptide Analysis Solution was described as a tool for peptide mapping protein characterization. The technique has proven useful for improved resolution and speed in peptide mapping. The significantly improved peptide resolution is now shown to provide separation of glycopeptides with mixed glycan structures, even the separation of glycopeptide positional isomers. Quantitation of the relative amounts of glycan structures can thus be approached within the peptide map without the cumbersome procedures required for released glycan analysis.

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## Introduction

The development of protein biopharmaceuticals requires complete structural characterization. Definition of a protein structure includes the amino acid sequence, storage-induced modifications, and post-translational modifications of the protein. Glycosylation is one of the most important classes of post-translational modifications. Within the human body many circulating proteins are glycosylated, and therefore the most promising candidate drugs for biopharmaceutical use have glycan chains. The oligosaccharide components can directly affect the efficacy and safety of these drugs by influencing binding, immunogenicity, and turnover. It is, therefore, critical to characterize oligosaccharide structure in biopharmaceutical development.

The analysis of glycoproteins is a challenging enterprise because of the extreme complexity of oligosaccharides and the heterogeneity of the cellular products. The molecular weights and branched structure of glycans that have been released from a protein can be determined by applying MALDI or ESI mass spectrometry with collision-induced dissociation (CID). Proposed structures can also be based on elution times from anion-exchange chromatography or from HILIC on amino phases. These analytical techniques do not, however, yield information about the sites of modifications. To approach the modification site question, glycoproteins are subjected to proteolytic cleavage digestion without removal of the glycans. The reversed-phase HPLC peptide maps usually include glycopeptides as broad, asymmetric, poorly resolved peaks. Better chromatographic separations are required for assessing the safety and efficacy of biopharmaceuticals.

Recently, UltraPerformance LC (UPLC Technology) has been proposed as a tool for improving resolution in peptide mapping. This technique has now been tested for the analysis of glycoforms of a monoclonal IgG.



Figure 1. UPLC Peptide Analysis Solution.

## Experimental

### Sample Preparation

A monoclonal mouse IgG1 (VICAM division of Waters) was reduced, alkylated, and digested in the presence of 0.1% *Rapi*Gest SF surfactant (Waters Part Number: [186001860](https://www.waters.com/waters/partDetail.htm?cid=1000941&id=27026) < <https://www.waters.com/waters/partDetail.htm?cid=1000941&id=27026>> ) in a Certified Maximum Recovery vial (Waters, Part Number: [600000670CV](https://www.waters.com/waters/partDetail.htm?cid=10000711&id=28881) < <https://www.waters.com/waters/partDetail.htm?cid=10000711&id=28881>> ). The reducing agent, TCEP (tris[2-carboxyethyl] phosphine, Pierce) was added to a concentration of 10 mM. After 30 minutes of reduction, the alkylation reagent, iodoacetamide, was added to a concentration of 20 mM for 30 minutes. The protein was then digested overnight with Modified Trypsin (Promega) at an enzyme-to-substrate ratio of 1:25.

### LC Conditions

|             |                                                                                                                        |
|-------------|------------------------------------------------------------------------------------------------------------------------|
| Instrument: | ACQUITY UPLC Peptide Mapping System                                                                                    |
| Detector:   | TUV @ 214 nm                                                                                                           |
| Column:     | Peptide Separation Technology Column 2.1 x 100 mm, ACQUITY BEH130 C <sub>18</sub> , 1.7 $\mu$ m Part Number: 186003555 |

|                   |                           |
|-------------------|---------------------------|
| Column Temp:      | 40 °C                     |
| Injection Volume: | 10 µL                     |
| Mobile Phase A:   | 0.1% TFA in water         |
| Mobile Phase B:   | 0.08% TFA in acetonitrile |
| Flow Rate:        | 100 µL/min                |

## Gradient

| Time (min) | A    | B   | Curve |
|------------|------|-----|-------|
| 2          | 100% | 0%  | *     |
| 118        | 50%  | 50% | 6     |
| 122        | 25%  | 75% | 6     |
| 123        | 0%   | 75% | 6     |
| 125        | 100% | 0%  | 6     |
| 143        | 100% | 0%  | 6     |

## MS Conditions

|                    |                                      |
|--------------------|--------------------------------------|
| Instrument:        | Waters LCT Premier Mass Spectrometer |
| Ionization Mode:   | ESI Positive                         |
| Capillary Voltage: | 3200 V                               |
| Cone Voltage:      | 30 V                                 |

Desolvation Temp: 150 °C

Desolvation Gas: 500 L/Hr

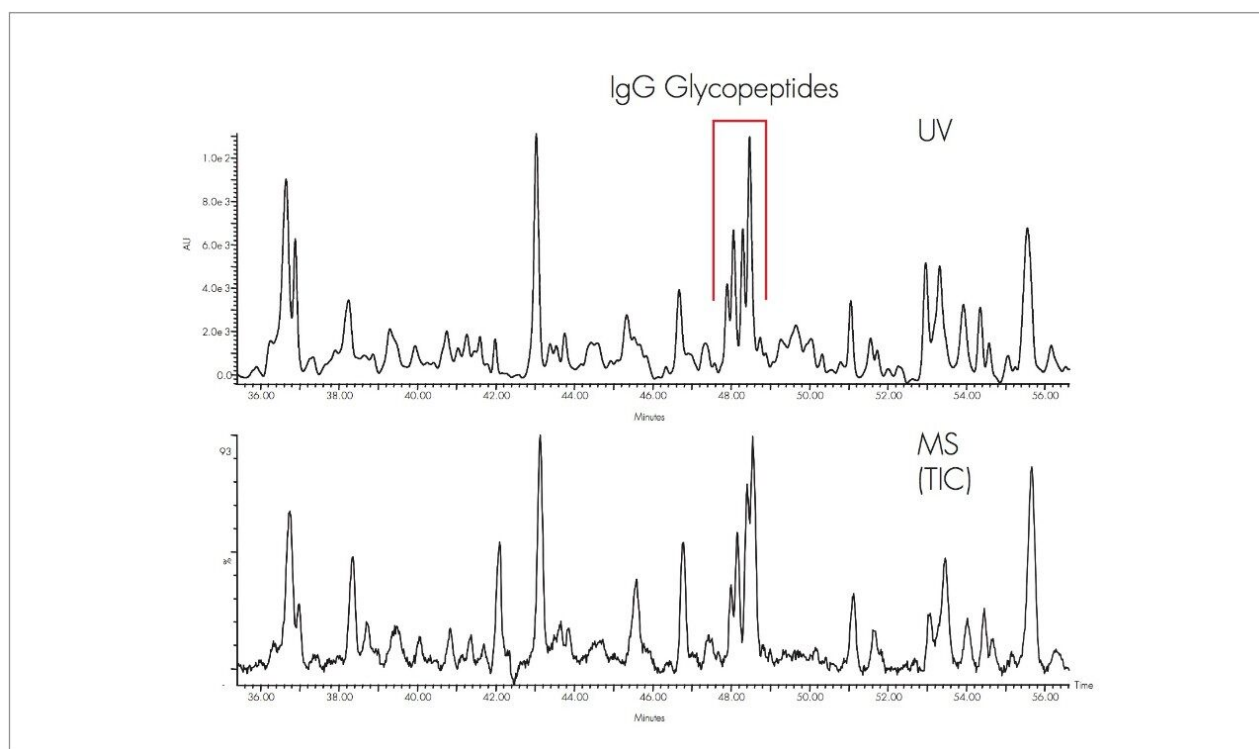
Source Temp: 100 °C

Acquisition Range: 50-2000  $m/z$

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## Results and Discussion

The tryptic peptides of the reduced and alkylated monoclonal IgG were separated using UPLC Peptide Mapping System, as shown in Figure 2. The same chromatogram could be observed with either UV or MS detection, and the glycopeptides were recognized by their masses as described in Figures 3 and 4.



*Figure 2. UV and MS UPLC chromatograms of a mouse antibody tryptic digest. The selected time range is focused on the glycopeptides.*

The cluster of four marked peaks represent the elution of the major glycoforms of a monoclonal IgG. These antibodies have only one conserved glycosylation site. There are two N-linked glycans tucked between the two heavy chains to which they are attached, one to each heavy chain, at the same conserved sequence. They are heterogeneous with respect to the number of terminal galactose residues, so that a given peptide may exist with zero, one, or two galactose residues. The structures of the "G0F", "G1F", and "G2F" glycans are shown in Figure 3.

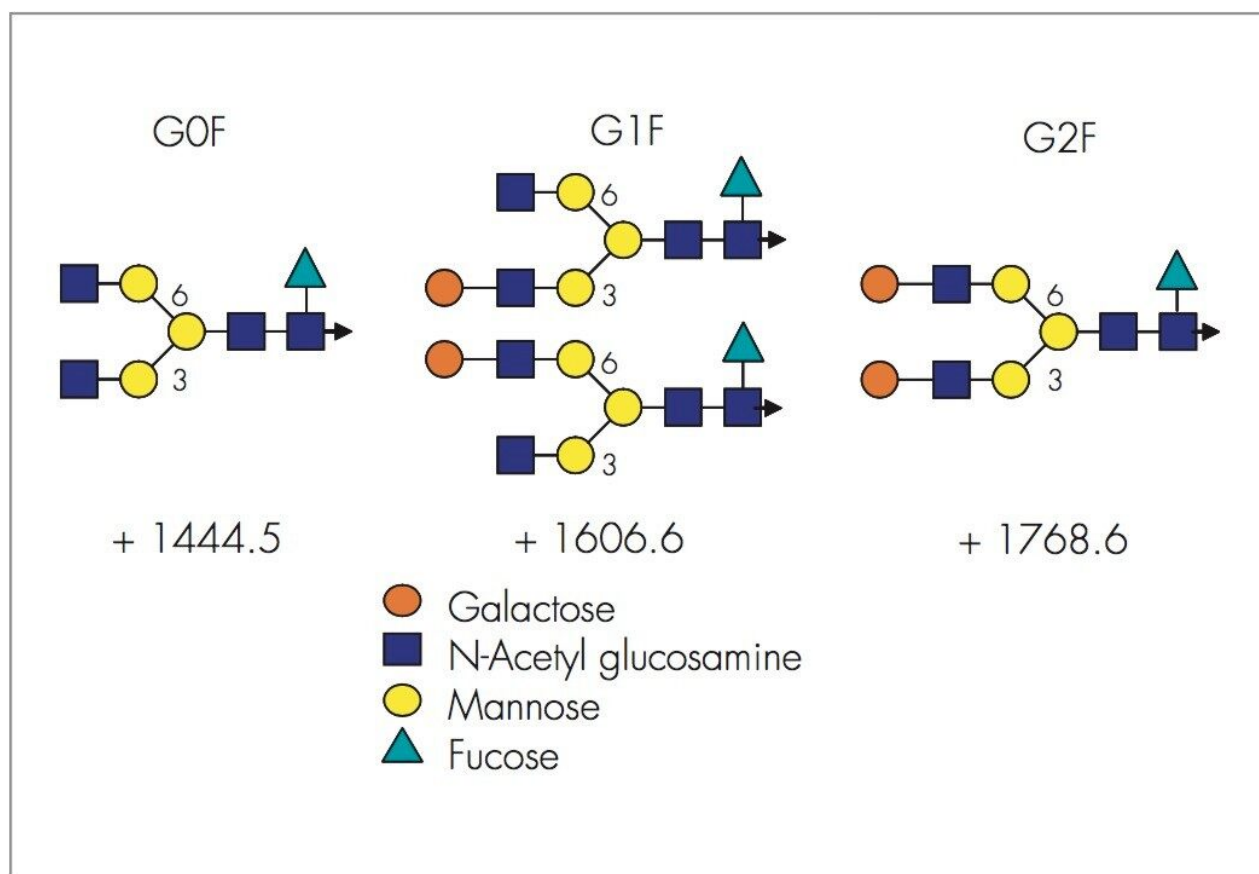


Figure 3. N-linked Glycans of Monoclonal IgG.

Extracted ion chromatograms for the three glycopeptide molecular weights, in each case the triply-charged ion, are shown in Figure 4. The G2F glycoform elutes first in the TFA-containing gradient, followed by baseline-separated glycopeptides displaying the two isomers of G1F. Chromatographic peak widths at half height were 0.11 minutes or 6–7 seconds. The improved resolving power allowed the resolution of individual glycopeptide structures.

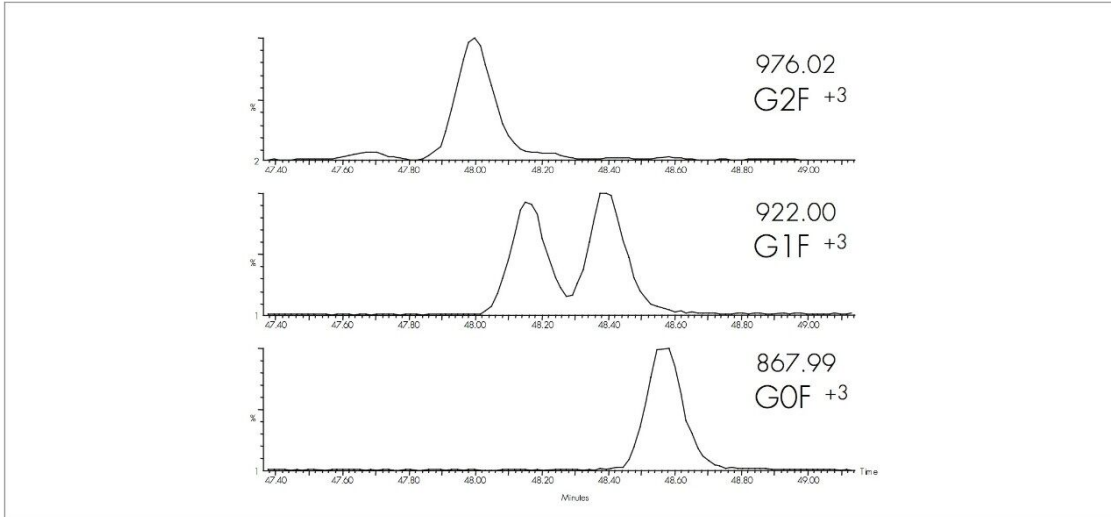


Figure 4. Extracted Ion Chromatograms for the Triply-charged State for Each of the Three Expected Glycopeptides.

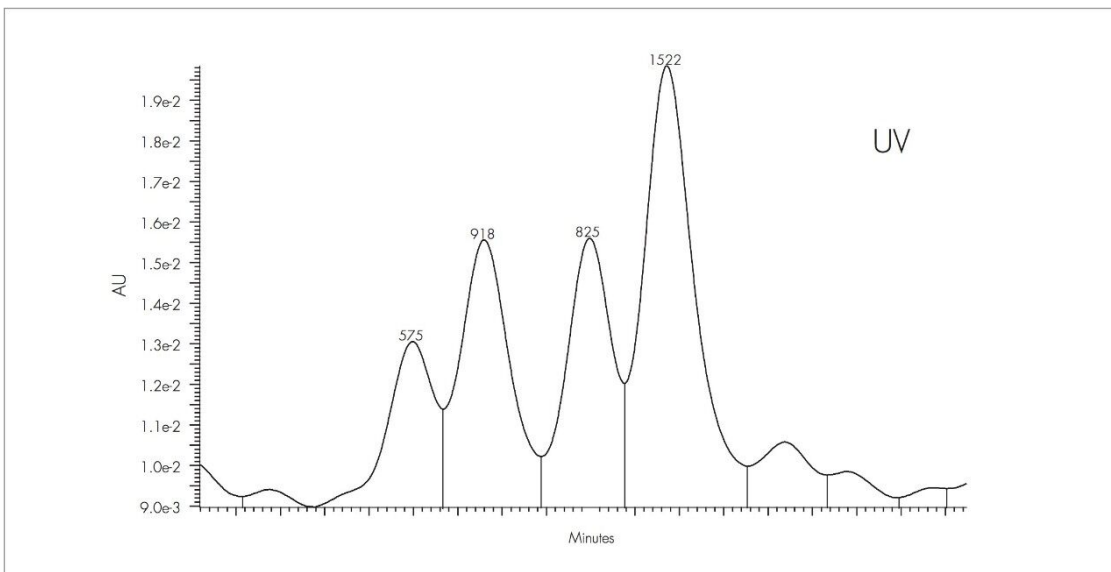


Figure 5. Detection of Four Glycopeptide Species in the UV Trace.

## Conclusion

The Waters UPLC Peptide Analysis Solution was described as a tool for peptide mapping protein characterization. The technique has proven useful for improved resolution and speed in peptide mapping. The significantly improved peptide resolution is now shown to provide separation of glycopeptides with mixed glycan structures, even the separation of glycopeptide positional isomers. Quantitation of the relative amounts of glycan structures can thus be approached within the peptide map without the cumbersome procedures required for released glycan analysis.

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