# A NOVEL COLUMN FOR ANALYSES OF *N*-LINKED GLYCANS OF GLYCOPROTEINS

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## INTRODUCTION

**Glycosylation is a post-translational modification of proteins. The sugar chains on glycoproteins can mediate biological activity and are, therefore, associated with safety and efficacy attributes of many biopharmaceuticals. The relative amounts of the individual glycan structures must be monitored at all stages of biopharmaceutical research and development.**

**In the typical HPLC workflow, *N*-linked glycans are released from glycoproteins and are labeled with 2-aminobenzamide (2-AB). The derivatized oligosaccharides have been separated using HPLC in HILIC mode on amide-phase chromatography columns. The HPLC columns separate many glycan structures, but with the advent of sub-2 micron particles, and appropriate instrumentation, increased resolution and sensitivity are achieved. The isomeric structures that are present on most glycoprotein drugs can be quantitated separately.**

### METHODS

#### Instrument

| | |
|---|---|
| Pump: | Waters® ACQUITY® Binary Solvent Manager |
| Injector: | ACQUITY Sample Manager |
| Detector: | ACQUITY Fluorescence Detector |

#### Typical Chromatography Conditions

| | |
|---|---|
| Sample: | 2-AB labeled *N*-linked glycans |
| Sample Concentration: | 10 pmol/µL |
| Sample Volume: | 1.5 µL |
| Injection Mode: | Partial Loop |
| Column: | ACQUITY UPLC® BEH Glycan, 1.7µm 2.1 x 150 mm column |
| Eluent A: | 100 mM Ammonium Formate, pH 4.5 |
| Eluent B: | Acetonitrile |
| Weak Needle Wash: | Acetonitrile/100 mM Ammonium Formate, pH 4.5 (90:10 v/v) |
| Strong Needle Wash: | Acetonitrile/100 mM Ammonium Formate, pH 4.5 (10:90 v/v) |
| Seal Wash: | Methanol/Water (10:90 v/v) |
| Temperature: | 60°C |
| Fluorescence: | λex = 330 nm, λem = 420 nm |

Gradient:

| Time (minutes) | Flow rate (mL/min) | Buffer A % | Buffer B % |
|---|---|---|---|
| Init | 0.5 | 25 | 75 |
| 46.5 | 0.5 | 40 | 60 |
| 48 | 0.25 | 100 | 0 |
| 49 | 0.25 | 100 | 0 |
| 50 | 0.5 | 25 | 75 |
| 63 | 0.5 | 25 | 75 |

### Typical Sample Preparation

GLYCOPROTEIN → Denature (Waters *Rapi*Gest™ SF) Reduce and Alkylate Digest with PNGase F

Glycans and Protein → Separate glycans from protein (MassPREP™ HILIC µElution Plate) Dry Glycans Heat glycans in DMSO/Acetic acid/ 2-aminobenzamide and $NaCNBH_4$

Labeled Glycans 2-AB reagent → Separate labeled glycans from 2-AB (MassPREP™ HILIC µElution Plate)

Pure 2-AB labeled Glycans

### Hydrophilic Interaction Chromatography (HILIC)

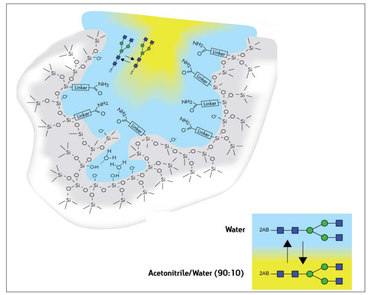


*Figure 1. The primary mechanism of hydrophilic interaction liquid chromatography (HILIC) is the partitioning of a polar analyte between bulk mobile phase and the immobilized water layer. Analytes may also hydrogen bond to the amide stationary phase directly or interact by an ion-exchange mechanism.*

## RESULTS

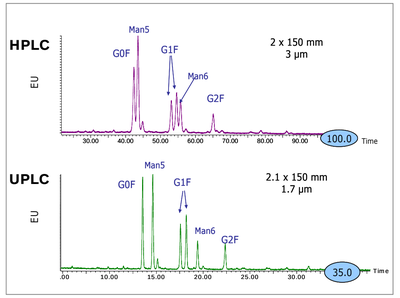


*Figure 2. The 2-AB labeled glycans from a murine monoclonal antibody were analyzed using HPLC with fluorescence detection (top). The direct comparison of the "best" HPLC separation with the UPLC analysis (bottom) clearly shows the improved resolution, sensitivity, and speed that is possible with Waters UPLC-Based, Glycan Separation Column.*

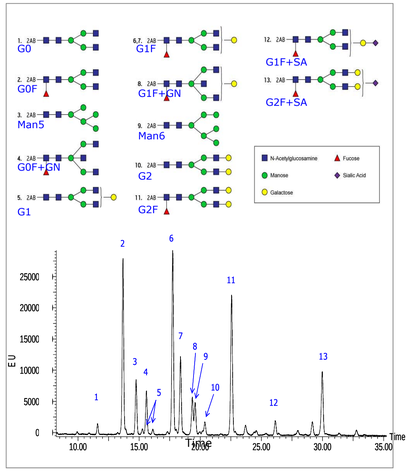


*Figure 3. The 2-AB labeled glycans released from pooled human IgGs are typical of the analytical challenge associated with biopharmaceuticals. The N-linked glycans in this mixture include the high mannose, neutral, and sialylated complex structures shown above.*

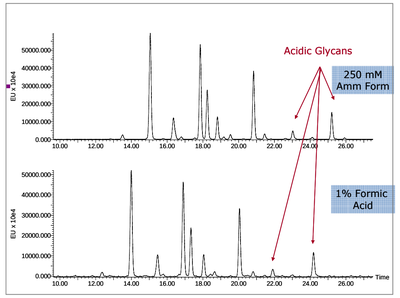


*Figure 4. 2-AB labeled IgG glycans are typically analyzed with ammonium formate buffer. Changing the mobile phase modifier from ammonium formate to formic acid may be used for LC/MS applications as long as high ionic strength is maintained for retention sialylated (acidic) glycans.*

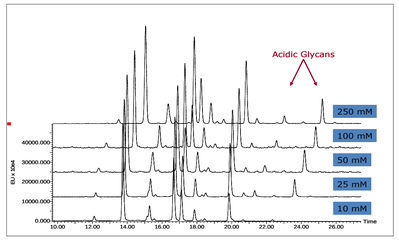


*Figure 5. Decreasing the ionic strength of ammonium formate results in decreased retention of glycans with particularly significant loss of retention and yield of sialylated (acidic) glycan structures.*

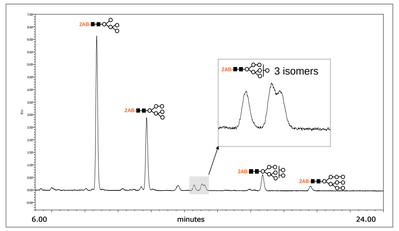


*Figure 6. The 2-AB labeled high mannose glycans released from bovine ribonuclease B were separated under similar conditions as the IgG glycans. The Man7 region (zoom) shows clearly that there are three isomers present.*

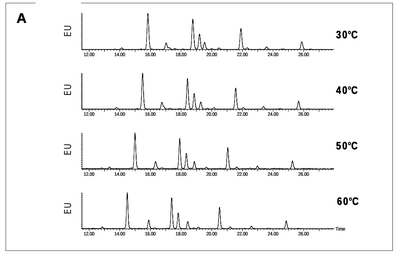


*Figure 7A. 2-AB labeled IgG glycans were analyzed at increasing temperatures. Increasing the column temperature to 60°C narrowed peak widths and decreased retention without significant effects on selectivity.*

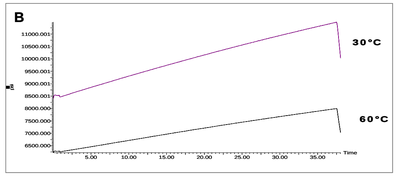


*Figure 7B. Changing the temperature of the glycan column separation results in significant changes in backpressure.*

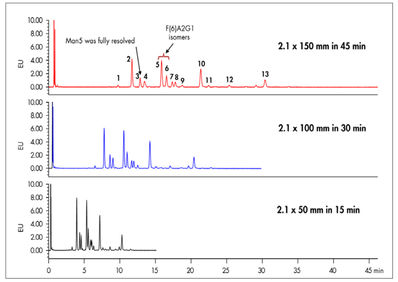


*Figure 8. If highest resolution is not required, shorter columns and much shorter run times are possible. In all cases the Man5 glycan is fully separated from G0F, and the G1F isomers can be quantitated.*

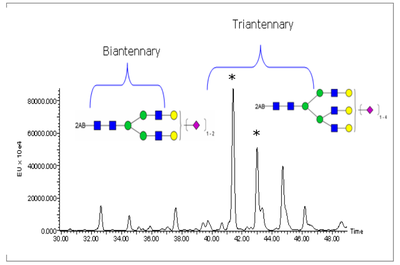


*Figure 9. In this example, sialylated 2-AB fetuin oligosaccharides were separated using the same mobile phase and gradient conditions that were used for the IgG glycans. Note that the isomeric triantennary structures (*) were particularly well-resolved.*

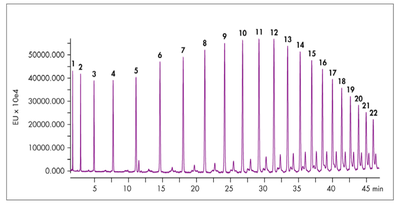


*Figure 10. Neutral 2-AB labeled oligosaccharides, represented by this series of homologous glucose oligomers, also separate on the ACQUITY UPLC BEH Glycan column. The separation gives good peak shape and identification from 1 to more than 22 Glucose Units.*

## CONCLUSION

- **Waters has developed a glycan analysis solution using the new ACQUITY UPLC BEH Glycan column containing 1.7µm particles.**
- **The Waters ACQUITY UPLC Glycan Separation Technology columns when operated with the ACQUITY UPLC instrument and fluorescence detector provide a high resolution and rapid method for separating and profiling glycans.**
- **The 2-AB labeled glycans are separated in HILIC mode on an amide stationary phase.**
- **Positional and linkage isomers can be resolved.**
- **The same column and mobile phase can be used for 2-AB labeled neutral and charged oligosaccharides such as highly sialylated glycans.**

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