# A Streamlined Analytical Platform with RFMS Glycan GU Scientific Library Searching for Glycan Comparability Assessment of Innovator and Biosimilar Infliximab

*William R. Alley, Jr., Ying Qing Yu, and Min Du*
*Scientific Operations, Waters Corporation, Milford, MA 01757*

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

## INTRODUCTION

To analyze N-linked glycans derived from biotherapeutics, we have developed a streamlined UPLC-FLR-MS analytical platform. This platform utilizes *Rapi*Fluor-MS™ from Waters Corporation to rapidly label glycans to improve both FLR and MS sensitivities. This allows lower-abundance glycans to be successfully detected. Following data collection and processing, raw retention times are converted to Glucose Units (GUs), which are then searched against the RFMS Glycan GU Scientific Library for structural assignment, which simplifies that task. To confirm the GU structural assignments, accurate MS data (sub 2-ppm) is used.

We have applied this analytical approach to compare innovator and biosimilar infliximab and we pay particular attention to the possible immunogenic glycans, those with N-glycolylneuraminic acid and α-linked galactose-galactose pairs. In general, we have found that the major glycan structures on both mAbs are very similar, but there appear to be some slight differences in some lesser abundant glycans based on lower % relative fluorescence peak areas.

## EXPERIMENTAL WORKFLOW

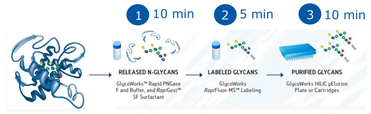



Figure 1. Outline of the sample preparation workflow used in this study.

Denature 15-µg aliquots of mAb with 1% *Rapi*gest at 90° C for 5 minutes

Release N-linked glycans using Rapid PNGase F for ~5 minutes at 55° C

Label glycans (released as glycosylamines) with *Rapi*Fluor-MS™

Purify the samples using a HILIC µ-elution plate. Dilute aqueous eluent with DMF and ACN.

UPLC-MS analysis

GlycoWorks™ *Rapi*Fluor™-

## UNIFI WORKFLOW

| General Workflow | Glycan Workflow |
|---|---|
| Automated LC-MS data collection | HILIC/FLR/MS data collection |
| Data processing | Calibrate RTs, assign GUs, |
| Review/reporting | GU Glycan Library Search |

## INSTRUMENT CONDITIONS

**LC Conditions**

| | |
|---|---|
| LC System: | ACQUITY UPLC H-Class Bio |
| LC Column: | ACQUITY BEH Glycan Amide, 2.1 x 150 mm, 130 Å pore size, 1.7 µm particles |
| Column Temp.: | 60° C |
| Sample Temp.: | 10° C |
| Fluorescence Detection: | Ex 265 nm/Em 425 nm |

**Gradient Conditions**

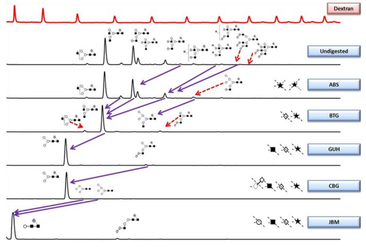
Mobile Phase A: 50 mM ammonium formate, pH = 4.5
Mobile Phase B: Acetonitrile

| Time (min) | Flow Rate (mL/min) | %A | %B |
|---|---|---|---|
| 0.0 | 0.400 | 25.0 | 75.0 |
| 35.0 | 0.400 | 46.0 | 54.0 |
| 36 | 0.300 | 70.0 | 30.0 |
| 41 | 0.300 | 70.0 | 30.0 |
| 42 | 0.200 | 25.0 | 75.0 |
| 47 | 0.400 | 25.0 | 75.0 |
| 55.0 | 0.400 | 25.0 | 75.0 |

**MS Conditions**

| | |
|---|---|
| MS System: | XEVO G2-XS QToF |
| Analyzer Mode: | ESI+, sensitivity mode, ~30k resolution |
| Capillary Voltage: | 3.0 kV |
| Cone Voltage: | 80 V |
| Source Temp.: | 120° C |
| Desolvation Temp.: | 250° C |
| *m/z* Range | 500-2000 |
| Scan Rate: | 2 Hz |
| Instrument Control: | UNIFI 1.8.2 |
| Scientific Library: | RFMS Glycan GU Scientific Library |

## CREATING THE RFMS GLYCAN GU SCIENTIFIC LIBRARY

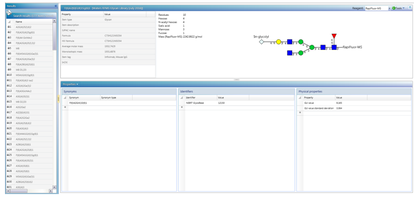



This figure shows how the library was created. Glycans were first labelled with RFMS and analyzed by UPLC to determine their Glucose Unit (GU) value. The glycans were then subjected to a battery of exoglycosidase digestions to selectively remove specific monosaccharides to determine their linkages and locations. The structures were verified through accurate mass measurements. This work was done jointly with Mark Hilliard and Pauline Rudd of NIBRT.

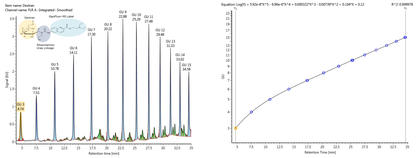
## THE RFMS GLYCAN GU SCIENTIFIC LIBRARY

- 177 glycans tailored to biopharma
- Created from: etanercept, infliximab, trastuzumab, erythropoietin, NIST mAb, human IgG, mouse IgG, bovine fetuin, yeast invertase
- Contains high mannose, complex, and hybrid glycans
- Contains glycans with N-glycolylneuraminic acid and N-acetylneuraminic acid, and α-linked galactoses
- GU values were averaged from at least 10 injections per sample

## EXAMPLE LIBRARY ENTRY

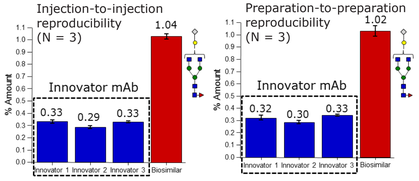


## CALIBRATING RETENTION TIMES



The RFMS GU Glycan Scientific Library search is based on glucose units (GU) rather than raw retention times. We have found GU values to be more stable across different instruments. To convert retention times to GUs, a dextran ladder labeled with RFMS is first analyzed as a separation calibration. The resulting equation is then used to convert retention times of unknown samples.

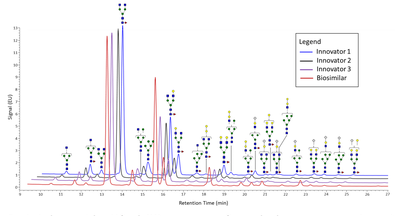
## QUANTITATION REPRODUCIBILITIES




The injection-to-injection and preparation-to-preparation reproducibilities were monitored through triplicate injections of one sample and triplicate preparations of all samples. For this particular glycan of interest, the %RSDs were less than 2%.

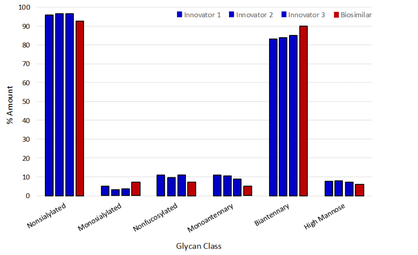
## RESULTS

### COMPARING INNOVATOR AND BIOSIMILAR INFLIXIMAB



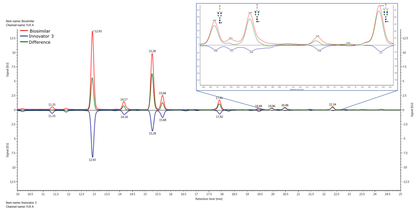

In total, we identified 23 mass-confirmed glycans present on the innovator mAb and 21 on the biosimilar. All of the glycans on the biosimilar were present on the innovator molecule.

### COMPARING GLYCAN CLASSES




While some slight differences were observed for different classes of glycans (sialylated and biantennary glycans being slightly elevated for the biosimilar), overall the different classes were present at comparable levels on each mAb.

### IDENTIFYING DIFFERENCES IN ABUNDANCE LEVELS




This mirror plot shows that most of the higher abundance glycans are present at very comparable levels. However, some differences were noted for some lower abundance glycans, particularly those with N-glycolylneuraminic acid and α-linked galactoses.

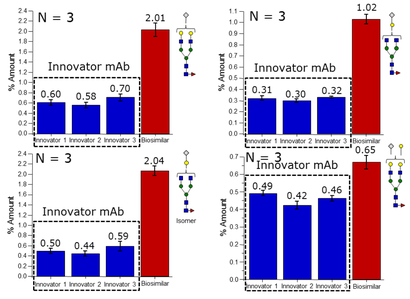
## GLYCANS WITH N-GLYCOLYLNEURAMINIC ACID

### Example Library Search

| Component name | Structure | Expected GU | ΔGU | GUStd Dev | Expected m/z | Δm/z | Mass Confirmed |
|---|---|---|---|---|---|---|---|
| F(6)A2(6)G(4)1Sg(6)1 | | 8.1627 | 0.0011 | 0.0500 | 1122.4384 | 0.0005 | True |
| M4A1 | | 8.1557 | 0.0081 | 0.0500 | 1122.9486 | 0.5107 | False |
| F(6)A2[3]BG(4)1Sd(6)1 | | 8.2239 | 0.0601 | 0.0500 | 1215.9806 | 93.5427 | False |
| FA2G2F1 | | 8.2365 | 0.0727 | 0.1000 | 1122.9486 | 0.5107 | False |
| F(6)A3G(4)3 | | 8.2393 | 0.0755 | 0.1000 | 1232.4857 | 110.0478 | False |
| F(6)A2G(4)2Ga(3)1 | | 8.2457 | 0.0819 | 0.1000 | 1130.9460 | 8.5081 | False |
| F(6)A2G(4)2Ga1 iso | | 8.2995 | 0.1357 | 0.0500 | 1130.9460 | 8.5081 | False |

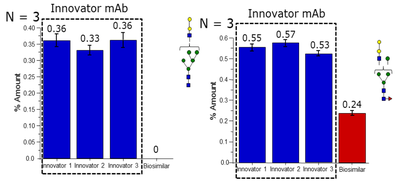
This figure shows the results of the library search for a GU value of ~ 8.16 ± 0.2. Within this GU window, only one glycan could be mass confirmed and was measured at sub-2 ppm mass accuracy. This glycan was determined to be F(6)A2(6)G(4)1Sg(6)1.

### Summary Plots for Glycans with N-Glycolylneuraminic Acid



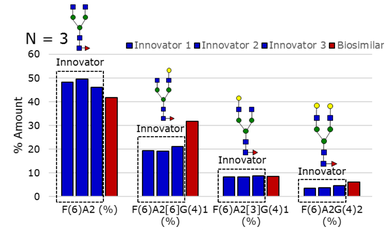

N-glycolylneuraminic acid is not present on human glycans and can induce an immunogenic response. This is an important monosaccharide to monitor. These summary plots show that the complex glycans terminated with N-glycolylneuraminic acids are elevated in the biosimilar product when compared to multiple batches of the innovator drug. These types of glycans were present at low abundances in both mAbs.

### Summary Plots for Glycans with α-linked Galactoses




Glycans with α-linked galactoses are nonhuman in nature and their presence in the human body has been shown to result in a severe immunogenic response. Therefore, this is another important group to monitor. This plot shows two glycans with α-linked galactose units were present at higher abundance levels in the innovator samples. Interestingly, we observed a glycan with α-linked galactoses and N-glycolylneuraminic acid that was elevated on the biosimilar mAb.

### Summary Plots for the 4 Most Abundant Fucosylated Glycans




Fucose levels can influence the antibody-dependent cell-mediated cytotoxicity of a therapeutic mAb. The overall level of fucosylation was slightly elevated in the biosimilar product and was due its higher level of the F(6)A2[6]G(4)1 glycan, even though F(6)A2 was slightly lower. The other more abundance fucosylated glycans were at approximately the same level in both mAbs.

## CONCLUSIONS

- We have developed a streamlined platform for glycan analysis, from sample preparation to data collection and analysis using a scientific library for glycan structural determination.
- Overall, the glycan profiles were very similar, especially for the abundant structures.
- Some differences were observed for some lower-abundance glycans, including a complex glycan terminated with an N-glycolylneuraminic acid (elevated in the biosimilar sample) and a hybrid glycan possessing α-linked galactose units, which was observed only on the innovator samples.

Symbols: ■ N-acetylglucosamine; ● Mannose; ○ Galactose; ▶ Fucose; ◇ N-glycolylneuraminic acid; ◆ N-acetylneuraminic acid;

***TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS***