Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

N-linked Glycan Characterization and Profiling: Combining the Power of Accurate Mass, Reference Glucose Units, and UNIFI Software for Confident Glycan Assignments

Ying Qing Yu
Waters Corporation, Milford, MA, USA

APPLICATION BENEFITS

The integrated UPLC/FLR/QTof MS analytical technologies available with the Waters Biopharmaceutical Platform Solution with UNIFI® improve a biopharmaceutical organization's ability to deliver well-characterized glycosylated biotherapeutics to market, from discovery through QC. The solution allows routine assignment of N-linked glycan structures using data from time-aligned FLR and MS channels along with database-driven assignment of glycans based on retention time. This enables the profiling of released glycans for individual analysis or to facilitate multi-batch or biosimilar/innovator comparability studies.

WATERS SOLUTIONS

UNIFI Scientific Information System

ACQUITY UPLC® H-Class Bio System

ACQUITY UPLC Glycan BEH Amide Column

ACQUITY UPLC FLR Detector

Xevo® G2-S QTof

GlycoWorks™ Reductive Amination Single Use Sample Preparation Kit

2-AB Dextran Calibration Ladder

2-AB Glycan Performance Test Standard

KEY WORDS

Biosimilar, etanercept, Waters Glycan GU Library, glucose units

INTRODUCTION

The Waters® Biopharmaceutical Platform Solution with UNIFI is comprised of industry-leading UPLC bioseparations columns and analytical instrumentation, along with optical detection and mass spectrometry, for comprehensive biopharmaceutical characterization and analysis. Data acquisition, processing, bioinformatics, and reporting tools are integrated and automated within UNIFI Scientific Information System's compliant-ready architecture.

In this application note, we detail a new workflow for a glycan assay, using FLR with mass confirmation, available in the latest version of the Biopharmaceutical Platform Solution with UNIFI. The practical use of this workflow for fluorescent labeled (2-AB) N-linked released glycan characterization is illustrated using a biosimilar/innovator biotherapeutic comparability study.

The analytical platform used for this study is comprised of an ACQUITY UPLC H-Class Bio System and an ACQUITY UPLC Fluorescent Detector in-line with a Xevo G2-S QTof Mass Spectrometer. This Glycan Application Solution with UNIFI enables the assignment and profiling of 2-AB labeled released N-linked glycans based on searches of calibrated retention time in glucose units (also known as GU) and accurate mass data within the Waters Glycan GU Library, which is integrated within UNIFI Scientific Information System version 1.7 and higher.

Accurate mass analysis proves a valuable technique for confirming GU based assignments and distinguishing cases where multiple glycan structures could be assigned to a single peak. Other complementary data for confirming these assignments (e.g. glycan DDA MS/MS data and exoglycosidase array studies) can also be collected on the Biopharmaceutical Platform Solution with UNIFI, and will be addressed in future application notes.

EXPERIMENTAL

LC conditions

System:	ACQUITY UPLC H-Class Bio System
Column:	ACQUITY UPLC Glycan BEH Amide Column, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742)
Column temp.:	40 °C
Mobile phase A:	50 mM Ammonium Formate (pH 4.4)
Mobile phase B:	Acetonitrile
Note:	LC-MS grade water and acetonitrile was used for this experiment

MS conditions

MS system:	Xevo G2-S QTof MS
Mode:	ESI+ in sensitivity mode
Capillary voltage:	3.0 kV
Cone:	80 V
Source temp.:	120 °C
Desolvation temp.:	300 °C
Desolvation gas flow:	800 L/h
Scan time:	0.5 s
Interval:	20 s

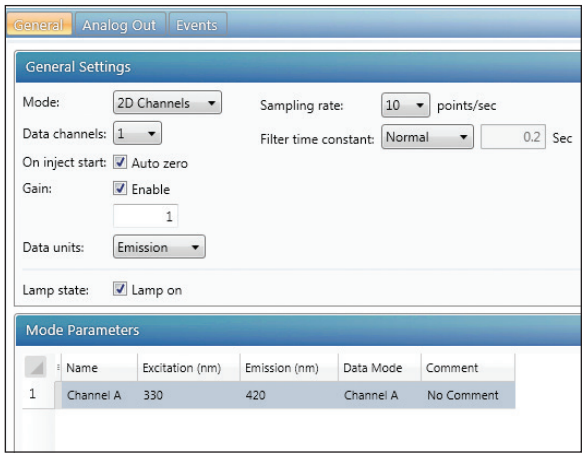
Data acquisition, processing, and reporting

UNIFI Scientific Information System

In this work, we illustrate the features of the platform for glycan analysis:

- The analytical workflow moves seamlessly from acquisition through data processing, with FLR and MS data channels being acquired and time-aligned automatically, for a routine and repeatable approach to data processing and reporting.
- The Waters Glycan GU Library allows confident assignment of the glycan structures based on retention time (in GU) with accurate mass confirmation.
- The streamlined workflow continues through reporting of quantitative (relative %) and qualitative analysis of N-glycan profiles, enabling scientists to easily communicate this critical information without exporting information to external data packages and thus reducing sources of data manipulation error. As a result, the laboratory's ability to maintain compliance and data integrity is enhanced.

ACQUITY UPLC FLR Detector settings



UPLC HILIC gradient table

	Time (min)	Flow Rate (mL/min)	Composition A (%)	Composition B (%)	Composition C (%)	Composition D (%)	Curve
1	0.00	0.400	30.0	70.0	0.0	0.0	Initial
2	2.06	0.400	30.0	70.0	0.0	0.0	6
3	34.80	0.400	47.0	53.0	0.0	0.0	6
4	36.00	0.250	80.0	20.0	0.0	0.0	6
5	39.00	0.250	80.0	20.0	0.0	0.0	6
6	40.00	0.400	30.0	70.0	0.0	0.0	6
7	45.00	0.400	30.0	70.0	0.0	0.0	6

Sample preparation and retention time calibration in GU values

The 2-AB Dextran Calibration Ladder (p/n 186006841) and the 2-AB Glycan Performance Test Standard (p/n 186006349) are glycan standards available from Waters Corporation. The 2-AB Dextran Calibration Ladder is used to calibrate and normalize labeled glycan retention times for exceptional day-to-day, system-to-system, and lab-to-lab reproducibility. This enables routine use of the Waters Glycan GU Library to produce primary glycan assignments.

The retention times for polyglucose 4–12 peaks were used to produce a fifth order polynomial calibration curve of GU vs. retention time. Peaks in experimental samples are automatically assigned and reported using this calibrated GU value. The 2-AB Glycan Performance Test Standard (p/n 186006349) contains a set of biantennary glycans, including high mannose and sialated structures, typical of many therapeutic mAbs commercializaed and in development today.

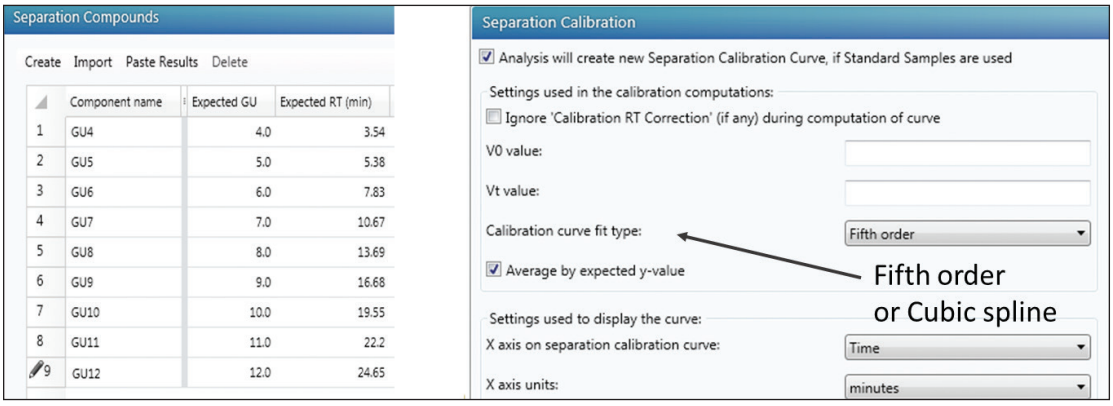
The GlycoWorks Reductive Amination Single Use Sample Preparation Kit (p/n 176003119) was used to generate 2-AB labeled released N-linked glycans from the innovator and a candidate biosimilar version of etanercept. The instructions were followed as detailed in the documentation package for the kit.

Fluorescent and MS chromatogram alignment

The fluorescent and MS chromatograms were aligned automatically during data acquisition using an experimentally derived value entered on the instrument console page. The time offset value depends on the length of the peak tubing (connection between the FLR and MS inlet) and the flow rate, and may vary system to system.

Critical settings

In the UNIFI processing method, settings are made for retention time calibration using 2-AB Dextran Calibration Ladder (p/n 186006841) (GU 4-12 is the typical range of calibration for mAb derived glycan samples). Both the fifth order and the cubic spine curve fit are applicable for retention time calibration.



RESULTS AND DISCUSSION

In this work, we provide specific details about the glycan UPLC-FLR/MS workflow (Figure 1) used with the Biopharmaceutical Platform Solution with UNIFI, including details of the analytical methods employed, the data review workflows employed, and reporting schemes required for efficient analysis of individual glycan samples and for more complex comparability studies.

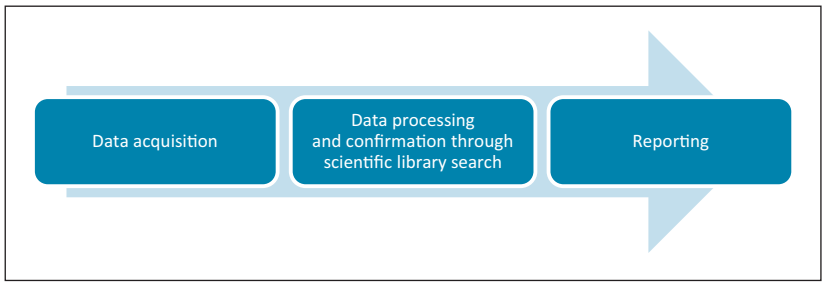


Figure 1. The glycan assay workflow, using FLR with MS confirmation.

Step 1: Data acquisition

Fluorescent-labeled glycans were separated using an ACQUITY UPLC H-Class Bio System with both FLR and MS detection, the latter using the Xevo G2-S QTof MS. The ACQUITY UPLC FLR Detector was directly interfaced with the QTof MS without any fluidic path modifications. The MS chromatogram is automatically time aligned with the FLR chromatogram as described above. An example of the UPLC-FLR/QTof MS chromatogram is shown in Figure 2.

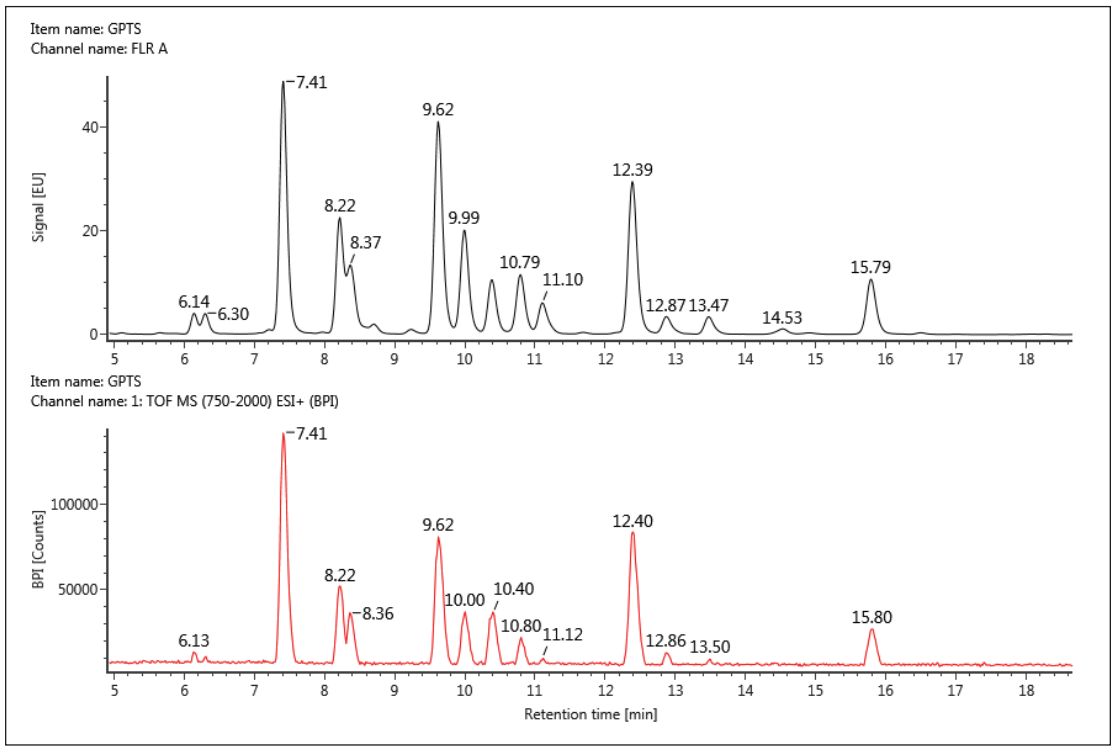


Figure 2. UPLC-FLR/MS chromatogram of 2-AB Glycan Performance Test Standard. The FLR chromatogram is shown at the top, and the BPI MS chromatogram is shown at the bottom. The BPI MS trace was time aligned with the FLR during data acquisition.

A 2-AB Dextran Calibration Ladder (p/n 186006841) was used as a retention time calibration standard. Typically, the samples are sandwiched in between dextran ladder injections. A fifth order curve, or cubic spline curve, for retention times vs. glucose unit values was automatically calculated using the average of all dextran ladders analyzed, and subsequently applied to the experimental glycan chromatograms during data processing. The benefit of using retention time calibration is to adjust the retention time shift to accommodate any variations in mobile phase preparation, instrument configuration, and other aspects of user and laboratory variability.

Figure 3 reviews the dextran ladder standard calibration result.

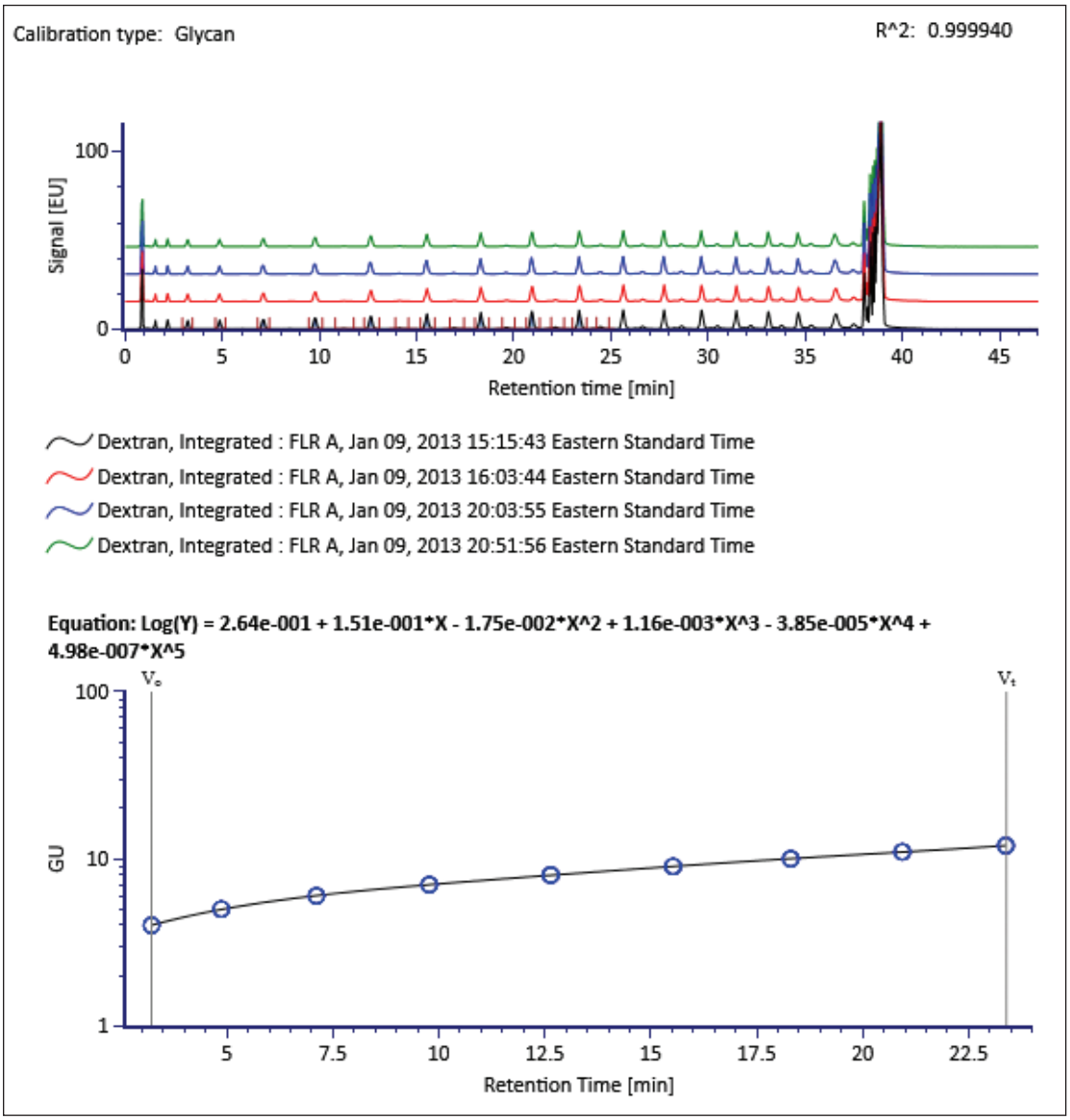


Figure 3. An example of dextran ladder calibration is shown. The top chromatogram shows the overlay of four injections of dextran ladder collected before and after two experimental sample runs. The bottom plot is the fifth order curve generated from these injections. The R^2 (0.999963) and overlayed data points highlight the excellent retention time correlations across these injections.

Step 2: Data processing and scientific library search

The Waters Glycan GU Library contains retention times (in GU) and mass information for 2-AB labeled N-linked glycans from a list of diverse glycoproteins as well as bulk human serum. The total number of unique glycans is currently 300. GU value, molecular formula, glycan structure, and monoisotopic mass are associated with each glycan entry. Scientists can search for a particular glycan or focus a search on specific classes of glycans in the library search.

An example is shown in Figure 4. The reagent selected is 2-aminobenzamide (2-AB), since the experimental GU values in the Waters Glycan GU Library are from 2-AB labeled proteins. The search criteria functions as a filter to narrow the range of GU search tolerance and by using restrictions for many types of glycan attributes. The Waters Glycan GU Library is the default library delivered with the Biopharmaceutical Platform Solution with UNIFI; however, a user can create their own GU library to search instead or in addition to the Waters library.

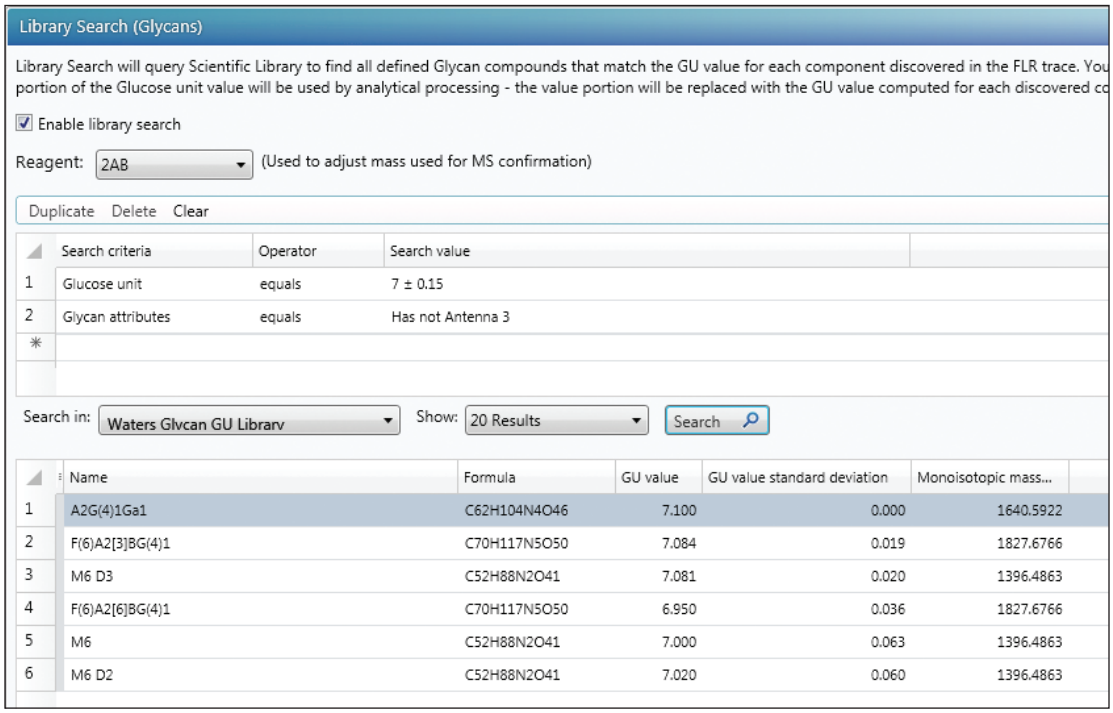


Figure 4. Library search settings for Waters Glycan GU Library.

Waters Glycan GU Library

UNIFI Scientific Information System's automated data processing encompasses calculation of the GU value for integrated FLR peaks, determination of accurate mass values associated with the peaks, and the resulting scientific library search. The assignment is based on the following logic:

1. All glycans with experimental GU values (experimental vs. library) within the database GU search tolerance are associated with an FLR peak.
2. Among the potential assignments, those with accurate mass confirmation are given priority of assignment, with closest GU value assigned as the default candidate.
3. Since FLR is more sensitive than mass spectrometry, the very low-level glycans may have good FLR signal, but no or low MS signals. The assignment of these glycans may only be based on the GU value difference from the database.
4. In the case of coeluting glycans or glycans with identical GUs, the glycan that is most abundant (by mass spectrometry signal) gets the default assignment. Less abundant glycans (if present) are still represented in the alternative assignments (with mass confirmed checked).
5. When a GU value is not found in the library within the given search tolerance, such peaks are marked as "Discovered" components. Further investigation is needed to identify these peaks, and once the structures of these glycans are verified, a new library entry can be created.
6. Glycans that are structural isomers tend to have close GUs and identical mass. In such cases, the matching isomers will be marked as mass confirmed; the one that has the closest GU value will be highlighted as the top assignment. These may require glycosidase treatment or MS/MS analysis for direct assignment.

Figure 5 is a screen capture from UNIFI Scientific Information System's review tab, detailing the processed library search results. The FLR peaks are integrated and assigned with the best match. After reviewing the search result, an assignment can be changed to another glycan that has a similar GU value. This change is tracked by audit trail within the software.

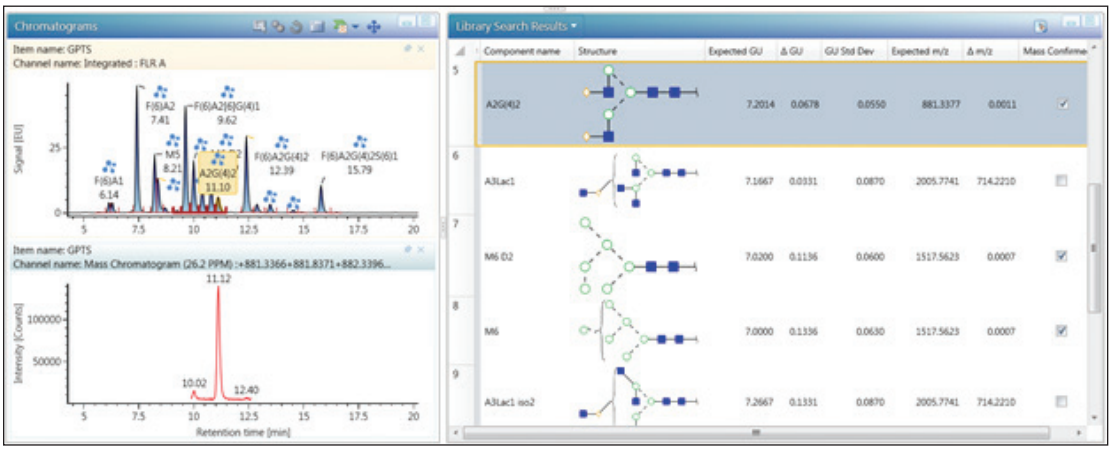


Figure 5. Waters Glycan GU Library search result from the review window. On the left is the processed FLR chromatogram and XIC MS of a highlighted glycan; on the right is the library search result of the selected glycan peak. Information such as the structure (with linkage assignment), expected GU, expected mass, ΔGU and Δmass are listed. In addition, the "Mass Confirmed" box is checked off if the mass of any of the candidate glycans is observed.

Practical application: Using UNIFI Scientific Information System to compare N-glycan profiles of an innovator biotherapeutic and a biosimilar candidate

Etanercept (trade name Enbrel) is a biotherapeutic mAb fusion protein for the treatment of rheumatoid arthritis and other autoimmune diseases; it is also one of the highest revenue biotherapeutics on the market today. Many biotechnology companies are actively working to creating biosimilar versions of etanercept.

In this study, we compared the 2-AB labeled N-glycan profile from one biosimilar candidate to that of the innovator using the Glycan Application Solution with UNIFI and its FLR/MS workflow. We observed that the biosimilar's N-glycan profile is highly similar to that of the innovator's, but some points of difference can be detected.

For example, high mannose structures were observed at higher abundance in the biosimilar candidate, including some extended mannose (e.g. Man 6 and Man 8) structures detected only in the biosimilar candidate (Figure 6). We also observed that the biosimilar candidate contains the following glycans, F(6)A2[6]BG(4)1, F(6)A2[3]G(4)1S(3)1, F(6)A3G(4)3S(3,3)2, and A2G(4)2S(6)1 in relative abundance that is greater than 0.1%, however, these sialylated glycans are either absent or below the 0.1% threshold in etanercept.

The cause of the N-glycan profile differences is most likely due to the variations in cell culture conditions. Bioassays and clinical experience are likely required to establish the extent to which these differences would affect the safety or efficacy of the biosimilar candidate.

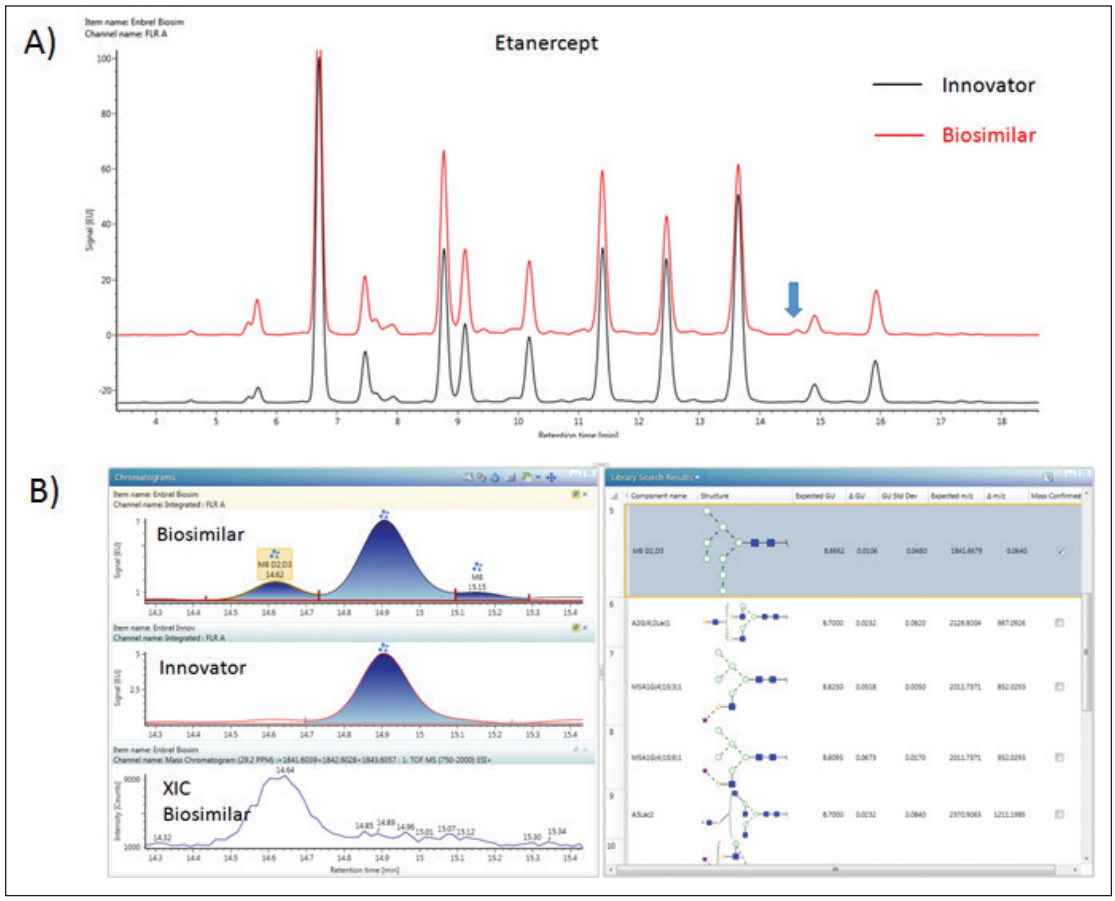


Figure 6. A) Overlay chromatogram of the N-glycans from the innovator and a biosimilar candidate etanercept. The blue arrow highlights the Man8 glycan that is only observed in the biosimilar candidate. B) The Waters Glycan GU Library search result shows that the marked peak from (A) is assigned to Man8. The library search result for the highlighted peak, Man8, XIC of Man8 provides further evidence for the correct structural assignment display in the chromatogram window.

Reporting

UNIFI Scientific Information System includes application-specific reporting templates. The default glycan assay report templates provide a sound basis for reporting details on individual samples as well as comparative data. The templates can also be readily modified to suit the reporting needs of a specific glycan analysis project.

Figure 7 gives an example of the type of information captured by a generic glycan analysis report using the innovator/biosimilar etanercept N-glycan analysis as an example.

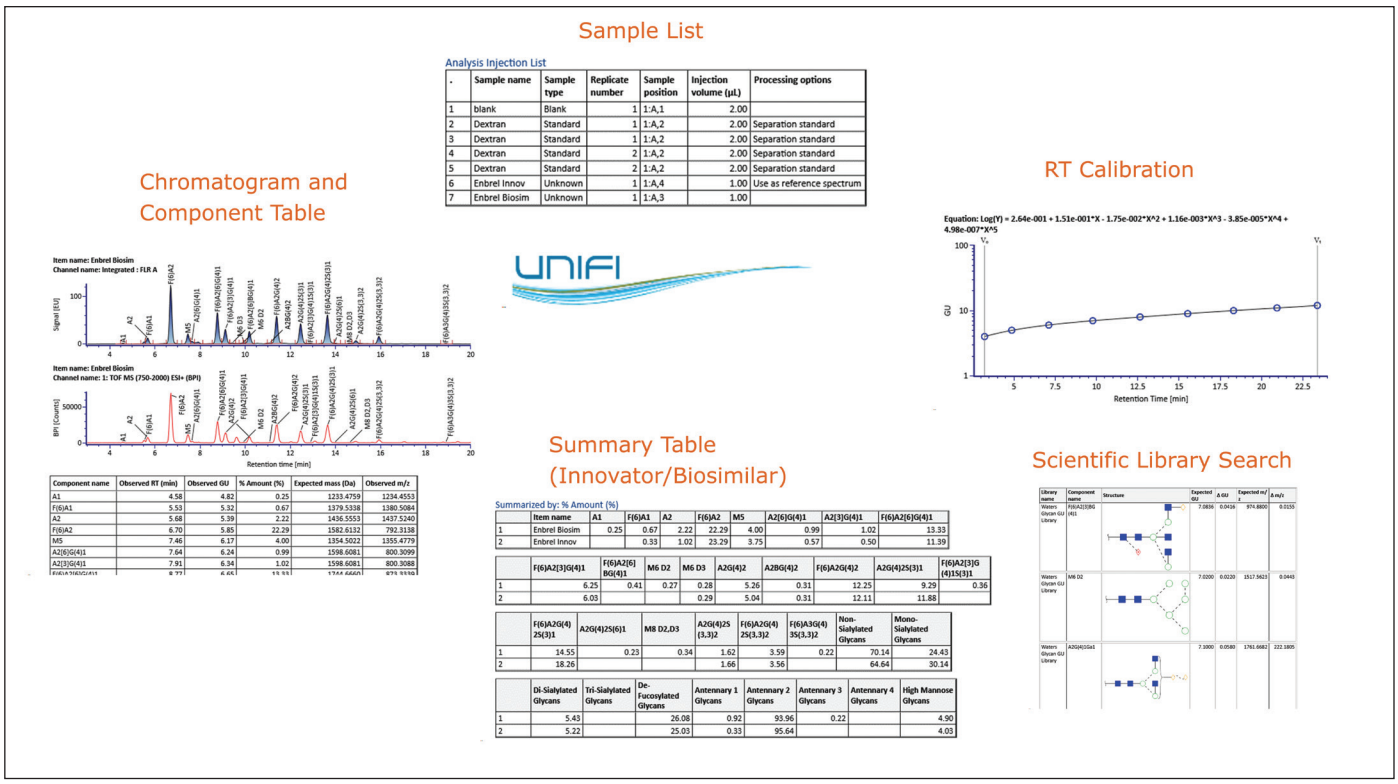


Figure 7. An example of a UNIFI report for the N-glycan profiling and comparison between innovator/biosimilar etanercept glycans. Key information such as sample list, retention time calibration curve, the scientific library search result for each identified glycan, summary table on relative % amount for innovator/biosimilar N-glyans, UPLC-FLR/MS chromatograms, and component tables. The report template is customized to fit the analysis type.

CONCLUSIONS

Glycan characterization has remained a challenging aspect of biotherapeutic characterization compared to techniques such as intact mass or peptide map analysis, which most labs consider routine today. The addition of the glycan UPLC-FLR/MS workflow and use of the experimentally derived Waters Glycan GU Library within the Glycan Application Solution with UNIFI have addressed the desire for compliant-ready, automated, high-confidence glycan structure assignments by enabling rapid acquisition, review, and communication of individual glycan profile results, and the larger sets of glycan analyses used for comparability studies.

Additional capabilities with the Biopharmaceutical Platform Solution with UNIFI, such as glycan/glycopeptide DDA MS/MS analysis and the ability to execute exoglycosidase arrays, certainly complement this new workflow, providing additional orthogonal results that enable the characterization of even the most complex biotherapeutic glycoproteins.

Waters

THE SCIENCE OF WHAT'S POSSIBLE.®

Waters Corporation
34 Maple Street
Milford, MA 01757 U.S.A.
T: 1 508 478 2000
F: 1 508 872 1990
www.waters.com