[TECHNOLOGY BRIEF]

Released N-linked Glycan Analysis Using the Glycan Application Solution with UNIFI

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GOAL

To demonstrate two fit-for-purpose glycan analysis workflows for comprehensive N-linked glycan profiling and structural elucidation within the Waters® Glycan Application Solution with UNIFI®.

BACKGROUND

The vast majority of biotherapeutics are glycosylated. Glycans attached to the proteins play a critical role in the serum half-life, efficacy, and safety of the biotherapeutic drug. In recent years, Waters has launched a series of innovative analytical tools to address the challenges faced in N-glycan analysis. This began with the launch of the ACQUITY UPLC® BEH Amide Column (1.7 µm particle size) for enhanced chromatographic separations of glycans under HILIC mode.[1] Early in 2015, Waters then introduced a new GlycoWorks™ sample preparation kit that provides fast, easy N-glycan sample preparation from enzymatic glycan release to labeling and clean up. This kit includes a novel fluorescent labeling reagent, *Rapi*Fluor-MS,™ enabling highly sensitive mass spectral detection of the labeled glycans.[2,3] The advancements in sample preparation, chromatographic separation, and enhanced ESI MS is now further complemented with an equally enterprising Informatics solution – the UNIFI Scientific Information System – to streamline glycan data acquisition, processing, and reporting.[4]

Comprehensive N-linked glycan analysis using the Glycan Application Solution with UNIFI.

- ACQUITY UPLC H-Class Bio System
- ACQUITY UPLC Column Manager
- ACQUITY UPLC FLR Detector
- Xevo® G2-XS QTof MS
- UNIFI Scientific Information System
- GlycoWorks *Rapi*Fluor-MS N-Glycan Kit
- ACQUITY UPLC Glycan BEH Amide Column

UNIFI® SCIENTIFIC INFORMATION SYSTEM

Figure 1. Glycan Application Solution with UNIFI for RapiFluor-MS labeled glycan analysis.

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THE SOLUTION

Two workflows available with the Glycan Application Solution with UNIFI are featured: 1) Glycan FLR with MS confirmation for profiling and mass confirmation; 2) Glycan DDA workflow via exporting of processed MS/MS data to SimGlycan (Premier Biosoft) for identification and structural elucidation.

Workflow 1: Glycan FLR with MS confirmation

The heart of this workflow is a scientific library containing calibrated chromatographic retention times (in glucose units, GU[5]) and accurate mass values for fluorescently labeled glycan structures. N-glycan identification using the scientific glycan library is illustrated in Figure 2. The assignment is based on accurately matched retention times in GU (calibrated using a fluorescently labeled dextran ladder) and accurate mass measurements from a Xevo G2-XS QTof MS.[6] Currently, a comprehensive 2AB-glycan GU library containing 319 unique N-glycan structures from therapeutic proteins is available with the Glycan Application Solution with UNIFI. A new scientific library based on the *Rapi*Fluor-MS labeling technology is currently under joint development by Waters and NIBRT. UNIFI software also allows users to create customized glycan scientific libraries which can be constructed directly by entering experimental GU values and importing structures from GlycoWorkbench. In addition, this workflow automatically calculates relative percentage value for each glycan component based on integrated fluorescent intensity for robust quantitation.

Workflow 2: Glycan DDA

Figure 3 shows the Glycan DDA workflow: Glycan MS/MS information using a Data Dependent Acquisition (DDA) mode was first acquired, followed by peak processing to convert all ions to singly charged "ion sticks." The processed data can then be exported in either .mzML or .LCS file format into SimGlycan software for identification and fragment ion annotation.

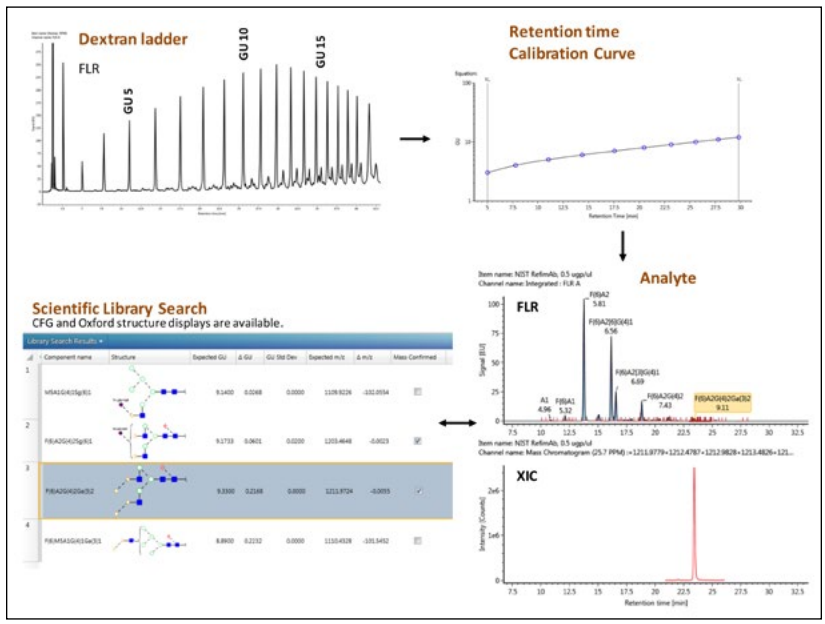

Figure 2. Workflow 1: Glycan FLR with MS confirmation.

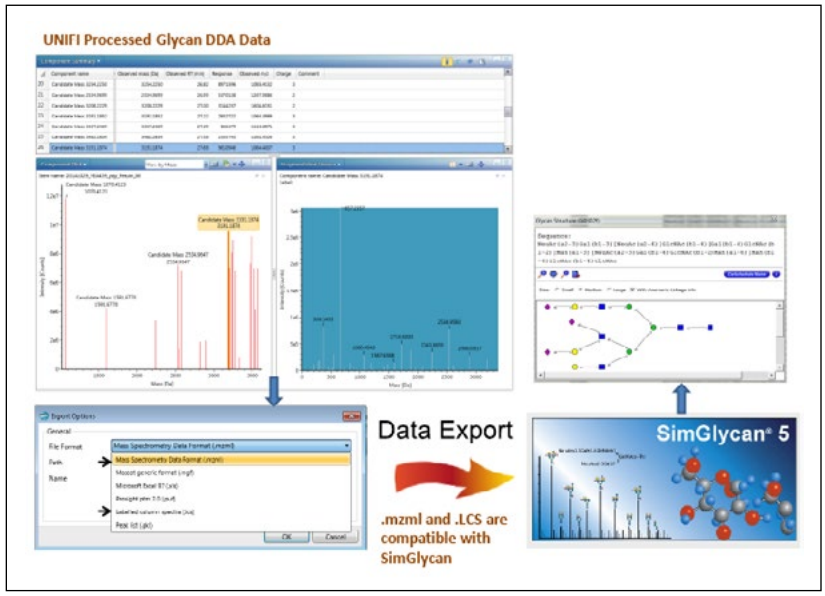

Figure 3. Workflow 2: Glycan DDA workflow in UNIFI Software. Collision-induced dissociation (CID) of glycans in data dependent acquisition (DDA) mode is processed and exported to SimGlycan.

Analytical method for Glycan Application Solution in UNIFI

Sample preparation: N-glycans were prepared using the GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003713)

System: Biopharmaceutical Platform with UNIFI

LC settings for *Rapi*Fluor-MS labeled glycans

Column: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7µm, 2.1 mm x 150 mm (p/n 186004742)

Column temp.: 60 °C

Mobile phase A: 50 mM ammonium formate (pH 4.4, LC-MS grade)

Mobile phase B: 100% acetonitrile (LC-MS grade)

Gradient:

Time (min)	Flow rate (mL/min)	A (%)	B (%)	Curve
0.0	0.4	25%	75%	6
35.0	0.4	46%	54%	6
36.5	0.2	80%	20%	6
39.5	0.4	25%	75%	6
43.1	0.4	25%	75%	6
55.0	0.4	35%	75%	6

Fluorescent: λex = 265 nm, λem = 425 nm

Xevo G2-XS QTof MS settings

Capillary voltage: 3.0 kV

Sample cone: 30 V

Source temp.: 120 °C

Desolvation temp.: 300 °C

Desolvation gas: 800 L/hr

Recommend settings for DDA

Charge state recognition: 2+, 3+, and 4+

Collision energy ramping

Low mass start: 10 V, low mass end: 15 V

High mass start: 44 V, high mass end: 50 V

MS scan: 0.5 sec, MS/MS scan: 0.5 sec

SUMMARY

Released glycan analyses are traditionally done using either optical or MS only analytical systems, and the data interpretation can be very challenging due to a lack of integrated analytical systems. The Glycan Application Solution with UNIFI features two independent analytical glycan workflows. These workflows allow scientists to characterize and profile glycans using both optical (fluorescent) and MS (MS) data within an integrated UPLC/FLR/QTof MS system. Combined with the novel *Rapi*Fluor-MS glycan labeling technology and the sophisticated UNIFI Software, scientists are now able to identify and quantify low abundant, potentially immunogenic glycan structures with higher confidence.

References

1. "Separation of 2-aminobenzamide labeled glycans using hydrophilic interaction chromatography columns packed with 1.7 um sorbent." *J. Chrom. B.* 878 (2010) 403–408.
2. "Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Labeling Reagent that Facilitates Sensitive Fluorescence and ESI-MS Detection." *Anal. Chem.* 2015, 87 (10), 5401–5409.
3. "Applying a Novel Glycan Tagging Reagent, *Rapi* Fluor-MS, and an Integrated UPLC-FLR/QTof MS System for Low Abundant N-Glycan Analysis." Waters Application Note (p/n 720005383EN).
4. "UNIFI Scientific Information System." Waters literature. (p/n 720004686EN).
5. Mattew P. Campbell, Louise Royle, Catherine M. Radcliffe, Raymond A. Dwek and Pauline M. Rudd. "GlycoBase and autoGU: tools for HPLC-based glycan analysis." Bioinformatics Applications Note, vol. 24, no, 9, 2008, 1214–1216.
6. "Biopharmaceutical Platform Solution with UNIFI: A Holistic Workflow for Acquiring, Processing, and Reporting Fluorescent-Labeled Glycans." Waters Application Note (p/n 720004619en).

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