ROUTINE MONITORING OF N-GLYCANS USING A NOVEL LABELING REAGENT WITH FLUORESCENCE AND MASS DETECTION

Eoin FJ Cosgrave, Matthew A Lauber, Stephan M Koza, and ***Sean M McCarthy***
Waters Corporation, 34 Maple St, Milford, MA, 01757, USA

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

Introduction

The glycan profiles of biopharmaceuticals are commonly defined and controlled as critical quality attributes. The profile of glycans is determined during the development of a product and are closely monitored during manufacturing scale-up processes. Using current technologies the generation of released glycan profiles is complicated by laborious, time-consuming sample preparation. In addition, collection of orthogonal confirmatory data, such as mass data, can be challenging due to poor ionization efficiencies of labeled glycans and the need for scientists skilled in mass spectrometry.

To address these challenges, we have developed a new glycan labeling reagent with significantly improved fluorescence and mass spectrometric response. The new reagent enables researchers to prepare samples from submitted glycoprotein to UPLC-MS analysis in 30 minutes. In addition to reduced sample preparation times, the novel labeling reagent yields 14 times greater fluorescence response and 160 times greater MS response when compared to 2-AB. These improvements allow us to improve sample throughput, and routinely analyze samples with both FLR and mass detection.

We will present the use of the ACQUITY UPLC with both fluorescence and QDa mass detection for monitoring released glycan profiles of biotherapeutics. Our results demonstrate how highly resolving methods when coupled with fluorescence and mass detection allow for unambiguous assignment and monitoring of glycans. We will also discuss how rapid separation methods, when coupled with QDa mass detection, allow scientists to quickly assess samples for the presence of critical structures during bioprocessing without added complexity or specialized training.

RESULTS

FLR and QDa for Routine Identification of N-Glycans

Routine detection of N-glycans is made possible by *Rapi*Fluor-MS labeling, which provides both increased fluorescence and MS response in addition to a simplified sample preparation workflow. Routine identification, provided in part by mass data, is made possible by the availability of simplified mass detection instrumentation such as the ACQUITY QDa. However, spectral quality is paramount for N-glycan identification activities. To evaluate the ability of the QDa to generate meaningful spectra, the Waters *Rapi*Fluor-MS glycan performance standard was separated via HILIC using both fluorescence and mass detection. Figure 2 illustrates the resulting fluorescence (2A) and TIC (2B) chromatograms while Figure 3 captures the individual spectra obtained for each of the chromatographic peaks. As evidenced, the QDa together with *Rapi*Fluor-MS provides unambiguous data for glycan mass, therein simplifying routine detection and identification of complex N-glycans.

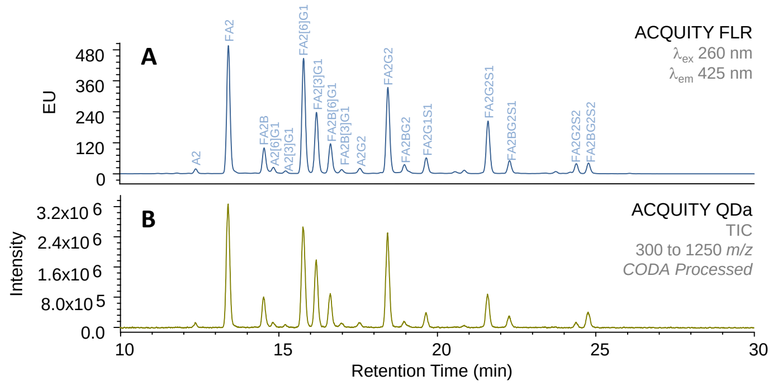


Figure 2. ***Rapi*Fluor-MS reagent provides sufficient MS response for detection by the QDa.** N-Glycans from 30 pmol of the Waters RapiFluor-MS glycan performance standard were analyzed using HILIC-FLR-QDa. All glycans detected by fluorescence (A) are also detected by the QDa (B).

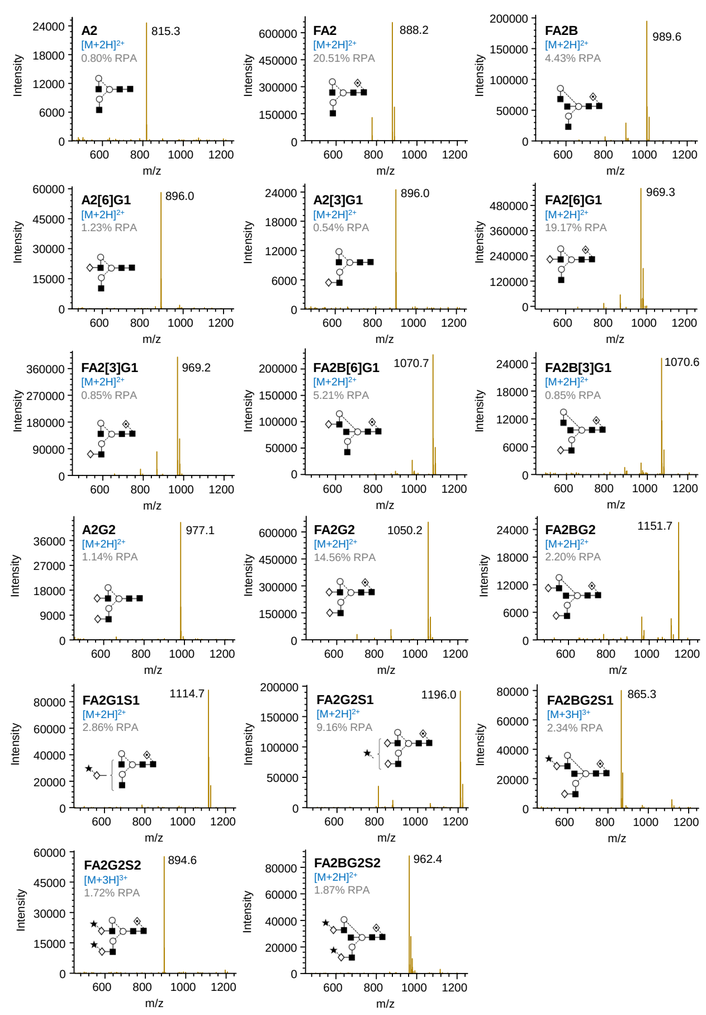
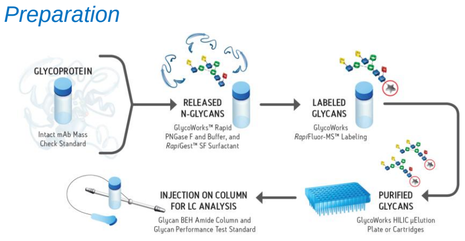


Figure 3. **Spectral quality of QDa-detected *Rapi*Fluor-MS labeled N-glycans.** Individual spectra obtained from each chromatographic peak provide unambiguous information with respect to the composition of each structure. Spectra are based on 30 pmol of N-glycan material. Each spectral plot provides the glycan name, the detected ion, the relative peak area, and the glycan structure. Arm specific glycan structural assignments are made possible based on the known elution order of individual glycan isomers.

METHODS

Sample Preparation



N-Glycan Separation and Detection

Instrument	Waters ACQUITY UPLC H-Class Bio with FLR and QDa detector
FLR Settings	λ_{ex} = 265 mn, λ_{em} = 425 nm, Date rate = 5 Hz
Mobile Phase A	Acetonitrile, LC-MS grade
Mobile Phase B	50 mM ammonium formate, pH 4.4, LC-MS grade
Column	ACQUITY UPLC Glycan BEH Amide column, 130 Å, 1.7 µm
Column Temp	60 ºC
QDa Settings	Cone 15V, Capillary 1.5kV, Source 600ºC, 500-1250 M/Z

High Resolution Method Gradient
2.1 mm x 150 mm column

Time (min)	Flow (mL/min)	% B
0	0.4	25
35	0.4	46
36.5	0.2	100
39.5	0.2	100
42.5	0.2	25
47.5	0.4	25
55	0.4	25

High Throughput Method Gradient
2.1 mm x 50 mm column

Time (min)	Flow (mL/min)	% B
0	0.8	25
5.8	0.8	46
6.1	0.4	100
6.6	0.4	100
7.1	0.4	25
8	0.8	25
10	0.8	25

UPLC-FLR-QDa Workflow Simplifies High Throughput Glycan Monitoring

Addition of mass detection to an existing glycan workflow permits rapid and unambiguous identification of glycans. To explore the application of mass tracking in high throughput analyses, we released and *Rapi*Fluor-MS-labeled N-glycans from the commercial monoclonal antibody, trastuzumab. Purified *Rapi*Fluor-MS labeled M5 was then added in varying concentrations to trastuzumab glycans to simulate potential changes in glycosylation that might occur during bioprocess development. To increase throughput, a high resolution HILIC method was geometrically scaled to a smaller column with a higher flow rate, resulting in a 6-fold decrease in analysis time. As a consequence, some peaks that were previously resolved now co-eluted as was the case with M5 and A2G1. This co-elution is denoted by the asterisked peak in the FLR profiles of Figure 4.

As shown in the FLR profiles, the relative peak area is increasing from figure A through to F. In the absence of mass information, the cause of this increase would be unknown without additional characterization efforts. However, with mass detection available through the QDa together with selected ion recording (SIR), the cause of the increase can be quickly identified. In this example, M5 is clearly increasing (2nd column) while its co-eluting partner A2G1 maintains a consistent level (3rd column).

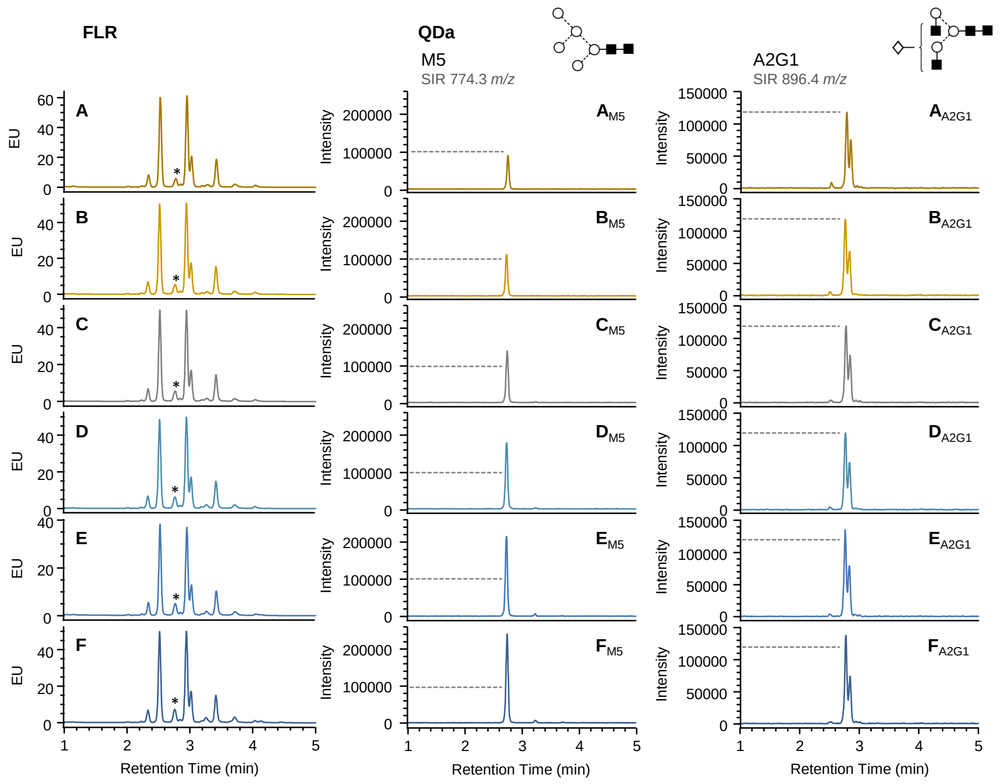


Figure 4. **Selected ion recording (SIR) identifies co-eluting glycan structures.** *Rapi*Fluor-MS labeled M5 was spiked into aliquots of *Rapi*Fluor-MS labeled trastuzumab to simulate potential bioprocess development challenges. The left column illustrates the fluorescence profiles of trastuzumab N-glycans with increasing M5 (A to F). The asterisk denotes the retention time for co-eluting glycans M5 and A2G1. The middle column illustrates the SIR profile of M5 through each of the glycan samples A to F, where an obvious increase in M5 level is observed. The right column illustrates the SIR profile for the co-eluting structure, A2G1, where a consistent level of this glycan is observed within the trastuzumab sample. Use of SIR with the QDa enables the quick determination of what glycan structure is responsible for changing peak areas in fluorescence profiles.

*Rapi*Fluor-MS™ Glycans Detected by the QDa

N-Glycosylation is a non-template driven process that generates a vast array of glycan structures that vary in size, charge, and extent of branching depending on the protein and expression system. To evaluate the capacity of the QDa to detect glycans both within and beyond its mass range, three glycoproteins (human IgG, Rnase B, and bovine fetuin) were selected to provide typically observed glycans ranging from neutral bi-antennary structures, such as FA2 at ~1774 Da, to tetra-sialylated structures, such as A3G3S4 at ~3482 Da. N-glycans from each protein were released and labeled with *Rapi*Fluor-MS. Labeled glycans were then separated via HILIC and detected using both an Acquity FLR and Acquity QDa. As is evidenced in figure 1, all glycans are chromatographically resolved. More importantly, each glycan observed in fluorescence (figure 1a) is also observed by the QDa (figure 1b), indicating an ability of the QDa to detect glycans across the spectrum of possible structures.

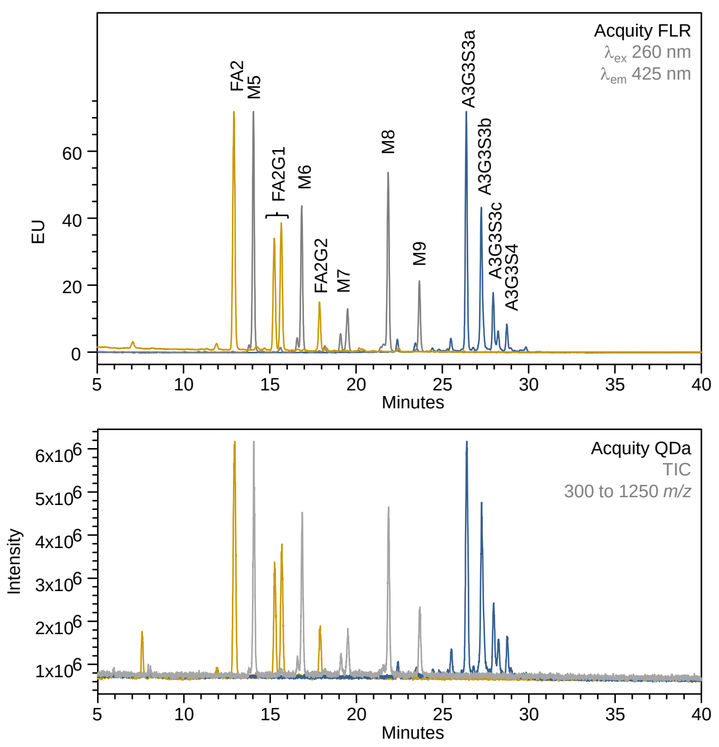


Figure 1. The QDa can detect an array of *Rapi*Fluor-MS labeled N-glycans. Glycans from human IgG (yellow profile), RNAse B (grey profile), and bovine fetuin (dark blue profile) were released with PNGase F, followed by labeling with *Rapi*Fluor-MS reagent. Individual glycan pools were then separated via HILIC and detected with both fluorescence (A) and mass detection (B).

Summary

- *Rapi*Fluor-MS labeled glycans demonstrated dramatically improved fluorescence and mass spectrometric properties
- Improved mass detection allows detectors such as the QDa to provide valuable mass information when added to current HILIC-FLR workflows.
- All glycans of interest from small neutral to large charged glycans are observed in FLR and QDa detectors.
- Mass data for glycan structures with <0.5% RPA is possible with QDa.
- Ease of use of the QDa permit its use in high throughput analytical environments where mass information allows for faster decision making.
- Rapid sample preparation together with rapid analysis has the potential to significantly reduce the analytical burden of labs tasked with high throughput glycan analysis.

to download a copy of this poster, Visit www.waters.com/posters