新規MSイオン化促進標識を使用したN型糖鎖の四重極MS検出ルーチンモニタリング
Routine Monitoring of *N*-Glycans Using a Novel MS Enhancing Labeling Reagent with Quadrupole Mass Detection

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INTRODUCTION

The *N*-glycan profiles of biopharmaceuticals are commonly defined and controlled as critical quality attributes. The profile of glycans is determined during development of biologics and closely monitored during manufacturing scaleup processes. During these analyses, scientists need to understand the presence or absence of structures, and ratios of critical structures.

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. Presented here is a novel labeling strategy which improves throughput while improving FLR and MS response by up to 14 and 1000x respectively.

METHODS

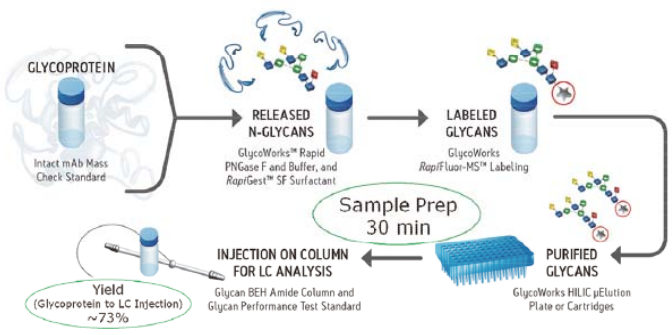
Glycan samples were prepared using a novel approach incorporating a rapid deglycosylation, rapid labeling using *Rapi*Fluor-MS (Waters Corp.), and a single SPE step reported by Lauber et.al.[1]. This streamlined process yields ready to analyze samples in 30 minutes. Labeled glycan samples were analyzed with LC/FLR/Mass Detection.

The separation was accomplished using HILIC chromatography using 50 mM ammonium formate, pH 4.4, and acetonitrile. Gradient times varied from 55 minutes to 10 minutes dependent the experimental goals. In each case, the gradient was from 75-54% acetonitrile.

The FLR detector was set with an excitation wavelength of 265 nm and emission wavelength of 425 nm.

The quadrapole mass detector settings were follows - full scan mass range: 500 – 1250 Da., cone voltage: 15V, capillary 1.5 kV, probe temperature 500 ℃.

Sample Preparation



N-Glycan Separation and Detection

Instrument: Waters ACQUITY UPLC H-Class Bio with FLR and QDa detector
FLR Settings: λ_{ex} = 265 nm, 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. Temp: 60 ºC

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

RESULTS AND DISCUSSION

*Rapi*Fluor-MS Glycans Detected by MS

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 personal use MS detector such as Acquity Qda (Waters Corp.) 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.

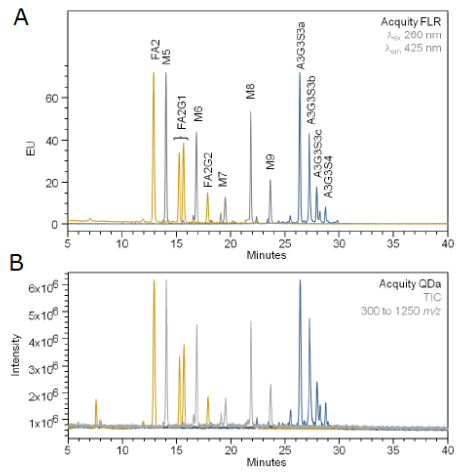


Figure 1. **The QDa can detect an array of *Rapi*Fluor-MS labeled *N*-glycans.** Glycans from human IgG (yellow profile), RNAse B (grey profile), and bovine fetuin (dark blue profile) were released with PNGase F, followed by labeling with *Rapi*Fluor-MS reagent. In-dividual glycan pools were then separated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

FLR and QDa for Routine Identification of N-Glycans

Routine detection of *N*-glycans with *Rapi*Fluor-MS labeling provides both increased fluorescence and MS response, as well as increased charge state populations as shown in Figure 2, which enables the routine collection of mass data. Routine monitoring is made possible by the availability of simplified mass detection instrumentation such as the ACQUITY QDa. We evaluated spectral quality with the *Rapi*Fluor-MS glycan performance standard was Figure 3 illustrates the resulting fluorescence (3A) and MS(3B) chromatograms. Figure 4 shows individual spectra obtained for each of the chromatographic peaks.

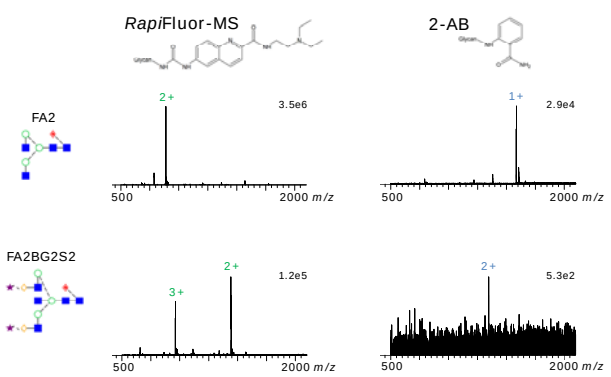


Figure 2. ***Rapi*Fluor-MS reagent improves MS response and charge state distribution.** Comparison of *Rapi*Fluor-MS and 2-AB labeled glycan MS response for selected structures. Data collected with ToF-MS.

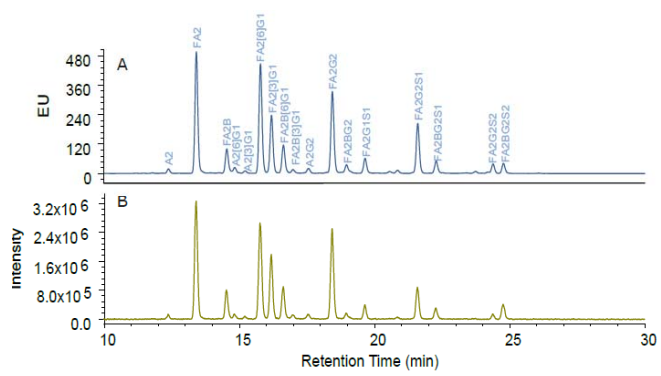


Figure 3. ***Rapi*Fluor-MS reagent provides sufficient MS response for de-tection by the QDa.** *N*-Glycans from 30 pmol of the Waters *Rapi*Fluor-MS glycan performance standard were analyzed using HILIC-FLR-QDa. All glycans detected by fluorescence (A) are also detected by the QDa (B).

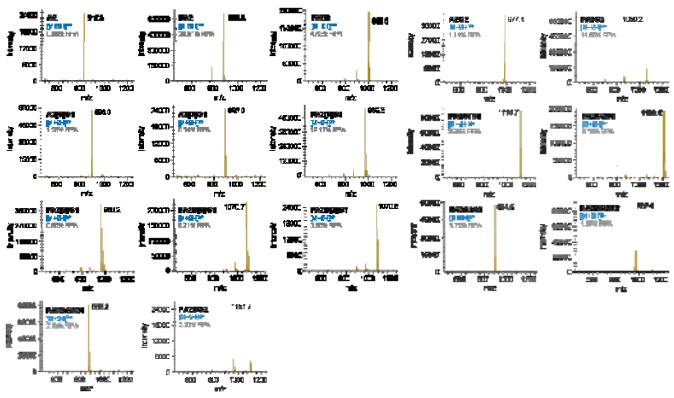


Figure 4. **Spectral quality of QDa-detected *Rapi*Fluor-MS labeled *N*-glycans.** Individual spectra obtained from each chromatographic peak provide unambiguous information with respect to the composition of each structure.Spectra are based on 30 pmol of *N*-glycan material. Each spectral plot provides 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.

UPLC-FLR-QDa Workflow Simplifies High Throughput Glycan Monitoring

To explore the application of mass data in high throughput analyses, we released and labeled *N*-glycans from trastuzumab. Purified M5 was added in varying concentrations to simulate changes in glycosylation that might occur during bioprocess development. 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 shown in the FLR profiles, relative peak area increased from Figure A through to F. The source of this increase would be un-known without additional characterization efforts. with mass detection using 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).

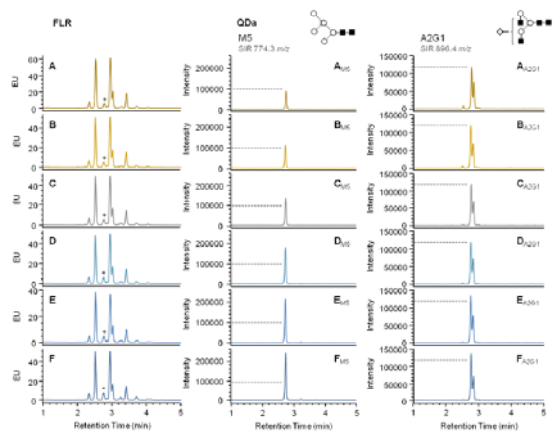


Figure 5. **Selected ion recording (SIR) identifies co-eluting glycan structures.** Left: fluorescence profiles of trastuzumab N-glycans with increasing M5 (A to F). The asterisk denotes the retention time for co-eluting glycans M5 and A2G1. Middle: SIR of M5 for each of the glycan samples A to F. Right: SIR for the co-eluting structure, A2G1. Use of QDa SIR enables the quick determination of glycan structure responsible for changing peak area in fluorescence profiles.

SUMMARY

· *Rapi*Fluor-MS labeled glycans demonstrate dramatically improved fluorescence and mass spectrometric properties for small neutral to large charged glycans.

· Improved mass detection allows detectors such as the QDa to provide valuable mass information when added to current HILIC-FLR workflows.

· 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.

REFERENCES

1. Matthew A. Lauber, Michael F. Morris, Darryl W. Brousmiche, Stephan M. Koza, *Anal.Chem.*,2015,**87**,5401-5409