[APPLICATION NOTE]

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Rapidly Monitoring Released N-Glycan Profiles During Process Development Using *Rapi*Fluor-MS and the ACQUITY QDa Detector

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APPLICATION BENEFITS

- Rapid feedback on glycoprofiles during production to ensure product quality
- Reduced sample preparation times for released N-glycans
- Increased throughput for N-glycan analysis
- Specificity for N-glycan species by incorporating mass detection

WATERS SOLUTIONS

*Rapi*Fluor-MS™ Glycan Performance Test Standard (p/n 186007983)

ACQUITY® QDa® Detector

ACQUITY UPLC® H-Class Bio System (FTN)

ACQUITY UPLC Fluorescence Detector (FLR)

ACQUITY UPLC Glycan BEH Amide Columns

Empower®3 Chromatography Data Software

Waters® Fraction Manager – Analytical

KEY WORDS

Glycans, mass detection, H-Class, ACQUITY, QDa, *Rapi*Fluor-MS, IgG

INTRODUCTION

As glycosylated biotherapeutics move through the development pipeline, the glycoprofile and N-glycan species present are characterized. In addition, as new protein therapeutics progress through development, manufacturing conditions are carefully studied and evaluated during scale-up to ensure consistent safety and efficacy in preparation for clinical studies, and eventual commercialization. As part of this process, the critical quality attributes are often monitored closely to ensure production batches remain within defined acceptance criteria, and to identify those parameters that are critical, often as part of a quality-by-design (QbD) approach. In particular, the N-glycan profile is often monitored closely due to the importance of glycans on the safety and efficacy of protein biotherapeutics.

Monitoring of released N-glycan profiles has historically been burdened with labor intensive sample preparation, which often takes several hours to days. This makes monitoring of the impact of manufacturing conditions on N-glycan profiles challenging. In addition, the analysis of released and labeled N-glycans frequently requires long analysis times. When monitoring of specific structures is desired, users often rely on optical detection for identification and quantification.

In this application note we present the use of *Rapi*Fluor-MS, a novel reagent for rapidly labeling released N-glycans. *Rapi*Fluor-MS dramatically reduces overall released N-glycan sample preparation times to 30 minutes, while improving fluorescence signal by up to 14x and MS signal by up to 1000x, compared to traditional labeling techniques. In conjunction with reduced sample preparation times, we geometrically scaled a highly resolving chromatographic method to one having a total cycle time of 10 minutes. Finally, we incorporated the ACQUITY QDa Detector to monitor specific glycan species using selected ion recording (SIR), which provides a selective means of monitoring species, even if they co-elute. We will discuss how the combination of *Rapi*Fluor-MS and the ACQUITY QDa Detector provides a powerful solution for obtaining meaningful data rapidly and efficiently.

EXPERIMENTAL

Released N-glycans were prepared from commercially available trastuzumab following the protocol provided within the *Rapi*Fluor-MS sample preparation kit. High mannose species used in spiking studies were isolated from RNase-B following release and labeling with *Rapi*Fluor-MS. Mannose species were chromatographically separated and collected using the Waters Fraction Manager – Analytical. Collected samples were dried down using a CentriVap and reconstituted in water. For each analysis the mass load was approximately 32 pmol of released and labeled N-glycan on column. LC-MS grade acetonitrile and water were purchased from Pierce. Ammonium formate was prepared using Ammonium Formate Solution-Glycan Analysis (p/n 186007081) by pouring the entire contents of the solution into 1 L of water and mixed. The UPLC® system used was dedicated for applications which do not require non-volatile salts to reduce the likelihood of adduct formation in the mass detector.

LC conditions

LC system:	ACQUITY UPLC H-Class Bio
Detectors:	ACQUITY UPLC FLR ACQUITY QDa
Columns:	High resolving method: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 mm x 150 mm (p/n 186004742) High throughput method: ACQUITY UPLC Glycan BEH Amide, 130Å, 1.7 µm, 2.1 mm x 50 mm (p/n 186004740)
Column temp.:	60 °C
Sample temp.:	10 °C

FLR settings

Data rate:	5 points/sec
Excitation wavelength:	265 nm
Emission wavelength:	425 nm

QDa settings

Sample rate:	5 points/sec
Mass range:	500 – 1250 Da
Cone voltage:	15 V
Capillary voltage:	1.5 kV
Probe temp.:	400 °C
Mode:	Positive ion
Mobile phase A:	Acetonitrile (Pierce, LC-MS Grade)
Mobile phase B:	50 mM ammonium formate, pH 4.4, (LC-MS Grade, ammonium formate concentrate)
Mobile phase C:	Acetonitrile (LC-MS Grade)
Mobile phase D:	Acetonitrile (LC-MS Grade)

Gradient table high resolution method:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.400	75	25	0	0
35.0	0.400	54	46	0	0
36.5	0.200	0	100	0	0
39.5	0.200	0	100	0	0
42.5	0.200	75	25	0	0
47.4	0.400	75	25	0	0
55.0	0.400	75	25	0	0

Gradient table high throughput method:

Time	Flow (mL/min)	%A	%B	%C	%D
Initial	0.800	75	25	0	0
5.8	0.800	54	46	0	0
6.1	0.400	0	100	0	0
6.6	0.400	0	100	0	0
7.1	0.400	75	25	0	0
8.0	0.800	75	25	0	0
10.0	0.800	75	25	0	0

Data management

Empower 3 Chromatography Data Software (CDS)

RESULTS AND DISCUSSION

During characterization of released N-glycans, a highly resolving method is often used to provide accurate identification and quantification of the species present in samples. While these methods can be effectively scaled, there is a corresponding loss in resolution as overall run time decreases when using the same chromatograph and particle size column. Often, some loss in resolution will be tolerated if the benefit of speed is achieved, however critical structures must remain clearly identifiable. As shown in Figure 1, moving from a higher resolving 55 min method to a 10 min high throughput method preserves much of the resolution between N-glycan species, however there is loss of resolution between the indicated peaks when moving to the shorter method. This loss of resolution complicates accurate monitoring by optical detection as there is no ability to discriminate between two species.

Due to the improved MS response, we introduced the use of the ACQUITY QDa Detector as part of the detector stream to selectively monitor each of the species present in the sample. By using the SIR function of the ACQUITY QDa Detector we were able to collect independent chromatographic traces for each of the components to overcome the challenge of using optical only detection. As shown in Figure 2, we can clearly discriminate between different glycoforms by using selected ion recording. For each species the corresponding peak, or peaks for species with resolved positional isomers, can be clearly identified and integrated for quantification.

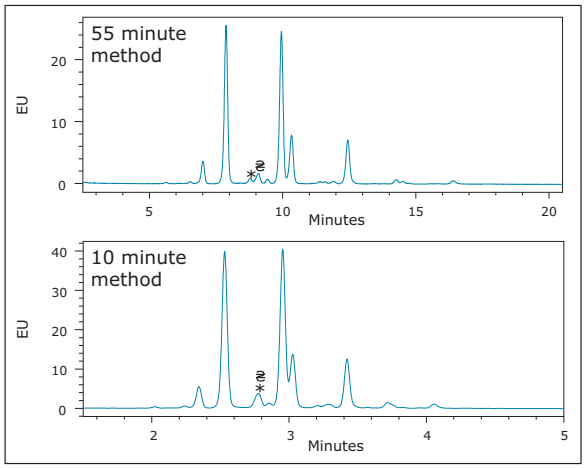

Figure 1. A high resolution separation (top chromatogram) was scaled to a high throughput method by scaling the gradient geometrically while reducing column length and flow rate. While resolution is reduced, selectivity remains constant.

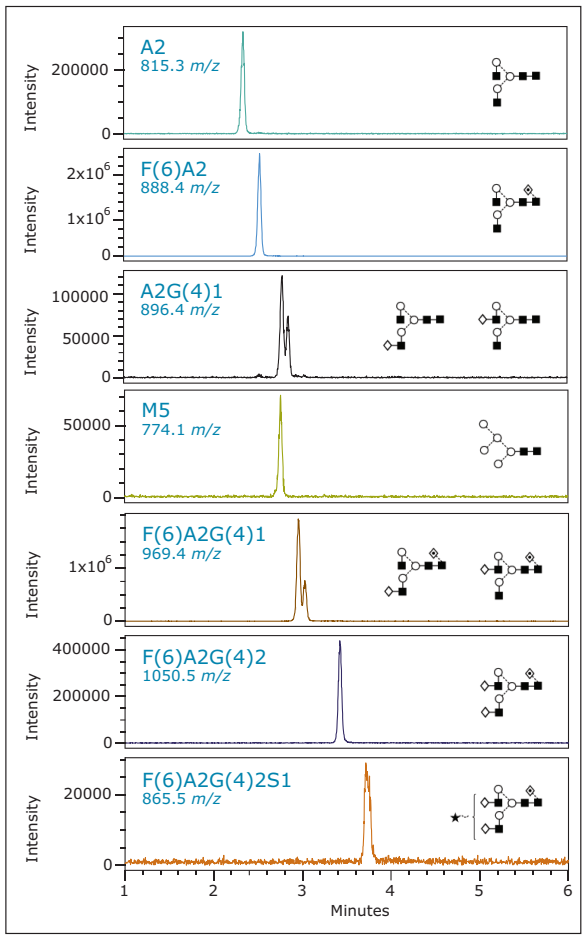

Figure 2. Selected ion chromatograms for N-Glycan species separated using high throughput method. Co-eluting M5 and A2G(4)1are easily discriminated by mass detection.

With a high throughput method developed and the ability to selectively monitor particular species with mass detection, we investigated the linearity of response for the target N-glycan species Mannose 5 (M5). As described in the experimental section, the *Rapi*Fluor-MS labeled M5 species was isolated from the labeled N-glycan pool of RNAse-B. After collection, the collected material was dried and reconstituted in water. The reconstituted sample was added to a *Rapi*Fluor-MS labeled released N-Glycan sample from trastuzumab at various levels. We investigated the linearity of the response by selectively monitoring the peak area of M5 in relation to the volume added to the sample. As shown in Figure 3, the chromatographic reproducibility was quite good. In addition, the peak area for each volume added was highly linear (Figure 4), strongly indicating that the mass detector provides a response suitable for quantification.

After determining the linearity of response for spiked M5 species, we simulated a bioreactor process in which the relative amount of M5 was increasing. For this study we selected the A2G1 species as the reference for relative quantification and spiked in increasing amounts of M5. As shown in Figure 5, the abundance of A2G1 (right column) remains largely constant over the course of the study while the M5 species (middle column) increases as expected with increased spiking levels. In addition, the FLR trace (left column) demonstrates an increase in peak area for these two species (labeled peak), however in the absence of mass information the precise cause of this increase cannot be determined.

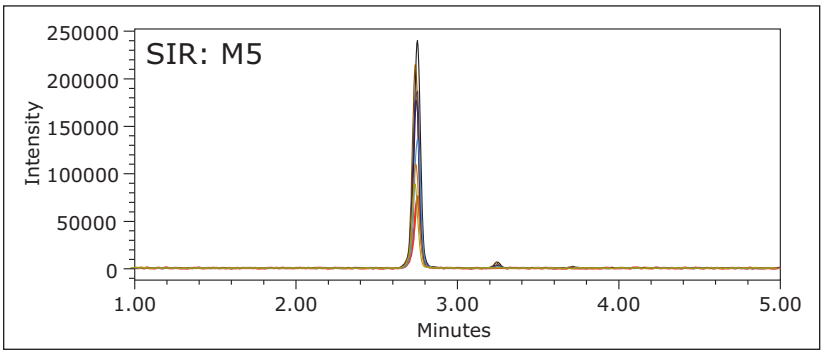

Figure 3. Overlay of chromatograms over a range of M5 spike levels. Spiked amounts ranged from 1-6 µL of reconstituted M5. Absolute concentrations were not determined.

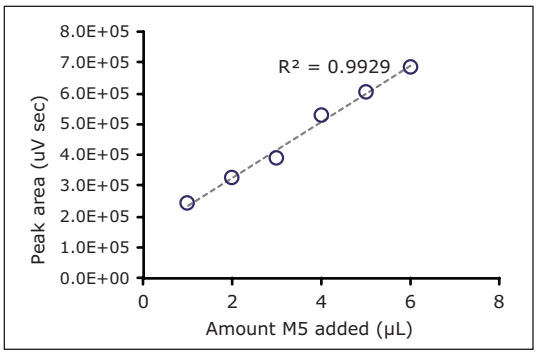

Figure 4. Plot of amount of M5 added vs. peak area for spike M5 samples (data shown in Figure 3).

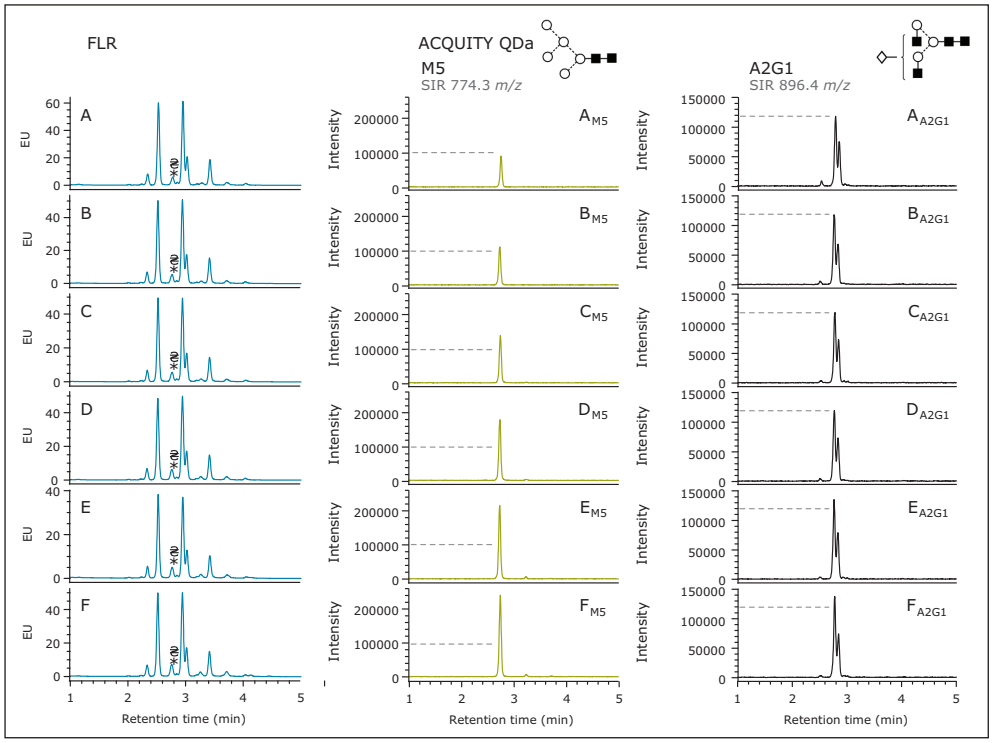

Figure 5. SIR for co-eluting glycan structures. Left: fluorescence profiles of trastuzumab N-glycans with increasing M5 (A to F). The indicators denote the retention times for co-eluting glycans M5 and A2G1. Middle: SIR of M5 for each of the glycan samples A to F. Right: SIR for the co-eluting structure, A2G1. Use of ACQUITY QDa SIR enables the quick determination of glycan structure responsible for changing peak area in fluorescence profiles.

CONCLUSIONS

For routine high throughput assays, *Rapi*Fluor-MS with the ACQUITY UPLC H-Class Bio System and the ACQUITY QDa Detector provides a novel approach for accurately monitoring released N-glycan species. Reduced sample preparation times and greatly improved MS response when coupled with the ACQUITY UPLC and ACQUITY QDa enable the ability to more closely monitor released N-glycan profiles, something which has previously not been possible. While FLR detection was used in this example, for high throughput methods requiring only relative quantification this may not be needed as each species can be monitored with the ACQUITY QDa Detector. As discussed here, complete sample preparation and analysis can be completed in 40 minutes. In addition to reproducible sample preparation, separation and quantification are reproducible and quantitative, allowing scientists to make meaningful decisions rapidly.

Reference

1. Lauber MA, Brousmiche DW, Hua Z, Koza SM, Guthrie E, Magnelli P, Taron CH, and Fountain KJ. Rapid Preparation of Released N-Glycans for HILIC Analysis Using a Novel Fluorescence and MS-Active Labeling Reagent. 2015, Waters Application Note P/N 720005275EN.

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