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Profiling Released High Mannose and Complex N-Glycan Structures from Monoclonal Antibodies Using *Rapi*Fluor-MS Labeling and Optimized Hydrophilic Interaction Chromatography

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APPLICATION BENEFITS

- Optimized LC method to improve the HILIC profiling of common mAb N-glycan species.
- Improved resolution of high mannose structures, in addition to sialylated species and N-glycan structures containing alpha-linked galactose units.
- The use of *Rapi*Fluor-MS™ High Mannose Standard in system suitability testing and peak identification.

WATERS SOLUTIONS

GlycoWorks™ *Rapi*Fluor-MS N-Glycan Kit

Intact mAb Mass Check Standard

*Rapi*Fluor-MS High Mannose Standard

ACQUITY UPLC® Glycan BEH Amide, 130Å, 1.7 µm

ACQUITY UPLC H-Class Bio System

Xevo® G2-XS QTof Mass Spectrometer

UNIFI® 1.7 Software

MassLynx® 4.1 Software

KEY WORDS

ACQUITY UPLC H-Class Bio System, ACQUITY UPLC Glycan BEH Amide Column, glycans, glycosylated protein, glycoprotein, Glycosylation, N-Linked glycans, HILIC, *Rapi*Fluor-MS labeling, Intact mAb Mass Check Standard, IgG, monoclonal antibody (mAb), high mannose N-glycans, *Rapi*Fluor-MS High Mannose Standard

INTRODUCTION

Glycan characterization is at the forefront of the biopharmaceutical industry, since most protein therapeutics possess N-glycosylation, if not also O-glycosylation. The most common therapeutic modality, an IgG monoclonal antibody (mAb), is, for instance, typically N-glycosylated at two conserved sites in its Fc domain. Not surprisingly, the nature of the N-glycans on a mAb can impact its circulation half-life and efficacy. As a result, it is particularly important that the N-glycans of a mAb be well characterized and routinely monitored.

A powerful strategy for such an analysis involves releasing N-glycans enzymatically, labeling them with a reagent to improve their detectability, and profiling them using hydrophilic interaction chromatography (HILIC).[1] Recent developments in released N-glycan profiling, made possible by the novel *Rapi*Fluor-MS labeling reagent, have eclipsed conventional N-glycan techniques by simplifying the steps in the procedure, reducing the overall sample preparation times, and enabling unprecedented sensitivities for both fluorescence and mass spectrometric detection.[2] However, regardless of how quickly a sample can be prepared or with what sensitivity it can be detected, it is imperative for N-glycan profiling to exhibit optimal chromatographic resolution. With there being many different sample types, it is often necessary to consider tailoring liquid chromatographic (LC) methods to ensure that robust and optimal chromatographic performance can indeed be achieved.

In this application note, we highlight the development of an LC method that optimizes the chromatographic resolution for the released N-glycans that are commonly found on mAbs, including the high mannose N-glycan structures that are known to negatively affect circulation half-life as well as indicate aberrant cell culture conditions. In this work, N-glycans from a mAb were rapidly released with PNGase F, labeled with *Rapi*Fluor-MS and profiled by HILIC using sensitive fluorescence and mass spectrometric detection. The separation method was optimized to improve the resolution of high mannose glycans, those terminated with N-glycolyl neuraminic acid (Sg) (a member of the broad class of sialic acids), and species containing alpha-linked galactose monosaccharides. Within this work, the utility of this newly developed LC method is demonstrated by means of system suitability testing with high mannose spiked samples.

EXPERIMENTAL

Sample description

The Intact mAb Mass Check Standard (p/n 186006552) was reconstituted in water to a concentration of 2 mg/mL. N-glycans were released from a 15-µg aliquot of this murine mAb and labeled with *Rapi*Fluor-MS using a GlycoWorks *Rapi*Fluor-MS N-Glycan Kit (p/n 176003606) following the instructions provided in its care and use manual (715004793). *Rapi*Fluor-MS-labeled N-glycans were prepared for injection at a concentration of 0.5 pmol/µL (as a mixture in a solvent composed of 90 µL SPE eluate, 100 µL dimethylformamide, and 210 µL acetonitrile).

*Rapi*Fluor-MS High Mannose Standard (p/n 186008317) was reconstituted in water to produce a 5 pmol/µL solution. A series of spiked samples were then prepared by mixing *Rapi*Fluor-MS labeled glycans from Intact mAb Mass Check Standard with the *Rapi*Fluor-MS High Mannose Standard and water. In this way, four spiked samples containing 0.45 pmol/µL of sample-derived N-glycans were prepared along with varying concentrations of the high mannose glycans. Spiking produced samples with mannose-5 (M5) at a relative abundance ranging from approximately 0.2% to 2%.

*Rapi*Fluor-MS Dextran Calibration Ladder (p/n 186007982) was reconstituted with 100 µL of water to produce a 0.5 µg/µL solution. The GU assignments were calculated using a cubic spline fitting method and UNIFI 1.7 Software.

LC conditions for *Rapi*Fluor-MS Released N-Glycans

Chromatographic separations were performed using the following conditions, unless otherwise noted:

Universal N-Glycan Profiling Method

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	60 °C
Flow rate:	0.4 mL/min
Injection volume:	10 µL for DMF/ACN-diluted samples or 1 µL for aqueous samples
Column:	ACQUITY UPLC Glycan BEH Amide, 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Fluorescence detection:	Ex 265 nm / Em 425 nm, 2 Hz
Mobile phase A:	50 mM aqueous ammonium formate, pH 4.4 (LC-MS grade water; from a 100x ammonium formate concentrate (p/n 186007081)
Mobile phase B:	ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.0	0.4	25	75	
35.0	0.4	46	54	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	25	75	6
47.6	0.4	25	75	6
55.0	0.4	25	75	6

mAb N-Glycan Profiling Method

LC system:	ACQUITY UPLC H-Class Bio System
Sample temp.:	10 °C
Analytical column temp.:	45 °C
Flow rate:	0.5 mL/min
Injection volume:	10 µL (DMF/ACN-diluted samples), 1 µL (aqueous samples)
Column:	ACQUITY UPLC Glycan BEH Amide 1.7 µm, 2.1 x 150 mm (p/n 186004742)
Fluorescence detection:	Ex 265 nm / Em 425 nm, 2 Hz
Mobile phase A:	50 mM ammonium formate, pH 4.4 (LC-MS grade; from a 100x concentrate (p/n 186007081)
Mobile phase B:	ACN (LC-MS grade)

Time	Flow rate (mL/min)	%A	%B	Curve
0.00	0.5	20	80	
3.00	0.5	27	73	6
35.0	0.5	37	63	6
36.5	0.2	100	0	6
39.5	0.2	100	0	6
43.1	0.2	20	80	6
47.6	0.5	20	80	6
55.0	0.5	20	80	6

MS conditions for *Rapi*Fluor-MS Released N-Glycans

MS system:	Xevo G2-XS QTof
Ionization mode:	ESI+
Analyzer mode:	Resolution (~40 K)
Capillary voltage:	2.2 kV
Cone voltage:	75 V
Source temp.:	120 °C
Desolvation temp.:	500 °C
Source offset:	50 V
Desolvation gas flow:	600 L/Hr
Calibration:	NaI, 0.1 µg/µL from 100–2000 *m/z*
Acquisition:	700–2000 *m/z*, 0.5 sec scan rate
Lockspray™:	100 fmol/µL human Glu-fibrinopeptide B prepared in a solution composed of 70:30:0.1% water/acetonitrile/formic acid, sampled every 90 seconds
Data management:	MassLynx 4.1 Software, UNIFI 1.7 Software

RESULTS AND DISCUSSION

A Universal N-Glycan Profiling Method

The GlycoWorks *Rapi*Fluor-MS N-Glycan Kit facilitates the robust analysis of many, very diverse N-glycans. Accordingly, we first aimed to establish a separation method for an ACQUITY UPLC Glycan BEH Amide Column that can be universally applied to all types of N-glycans, from small biantennary structures up to highly sialylated, tetraantennary species. It is very useful to run this so-called 'universal N-glycan profiling method' when analyzing new samples. Given that this method is the basis of an upcoming glucose unit (GU) database for *Rapi*Fluor-MS labeled glycans, it will also be the technique recommended for future workflows involving GU based peak assignments.

Being a generic tool, the universal N-glycan profiling method is not optimized for any particular N-glycan sample, including the N-glycans obtained from the Intact mAb Mass Check Standard that is provided as a control sample in each GlycoWorks *Rapi*Fluor-MS N-Glycan Kit. Since this standard is a mAb expressed from a murine cell line, it is a relevant surrogate to many mAb therapeutics and will, in fact, produce a highly similar N-glycan profile. The universal N-glycan profiling method produces chromatograms where the mAb glycans elute in only the first half of the analytical gradient, as shown in Figures 1A and 1B. Using online, mass spectrometric detection, at least 14 different N-glycans can be readily identified (Table 1). In addition to having been eluted in a narrower retention window, several of these glycans are only partially resolved and consequently are difficult to monitor, and reliably quantitate, on an LC system that is not fully optimized for low extra-column dispersion. Such peaks, or those that are unresolved, require the use of MS detection and an analysis of extracted ion chromatograms (Figures 1C and 1D) to be deciphered.
Notable critical pairs exhibiting at least partial co-elution include M5/A2G1 and FA2G2Sg1/FA2G2Ga2. Given the significance of monitoring M5 and immunogenic glycans, like those containing the noted N-glycolyl neuraminic acid (Sg) and alpha-linked galactose monosaccharides, these separations were optimized for increased resolution between species containing these types of sugars.

	Glycan	*Rapi*Fluor-MS labeled glycan composition	Mi (Da)	2+	3+
1	A2	$C_{67}H_{105}O_{37}N_9$	1627.66	814.84	543.56
2	FA2	$C_{73}H_{115}O_{41}N_9$	1773.72	887.87	592.25
3	M5	$C_{63}H_{99}O37N_7$	1545.61	773.81	516.10
4	FA1G1	$C_{71}H_{112}O_{41}N8$	1732.69	867.35	578.57
	A2G1	$C_{73}H_{115}O_{42}N_9$	1789.71	895.86	597.58
5	A2G1	$C_{73}H_{115}O_{42}N_9$	1789.71	895.86	597.58
6	FA2G1	$C_{79}H_{125}O_{46}N_9$	1935.77	968.89	646.26
7	FA2G1	$C_{79}H_{125}O_{46}N_9$	1935.77	968.89	646.26
8	FA2G2	$C_{85}H_{135}O_{51}N_9$	2097.82	1049.92	700.28
9	FA2G1Ga1	$C_{85}H_{135}O_{51}N_9$	2097.82	1049.92	700.28
10	FA2G2Ga1	$C_{91}H_{145}O_{56}N_9$	2259.88	1130.95	754.30
11	FA2G2Ga1	$C_{91}H_{145}O_{56}N_9$	2259.88	1130.95	754.30
12	FA2G2Sg1	$C_{96}H_{152}O_{60}N_{10}$	2404.92	1203.46	802.65
13	FA2G2Ga2	$C_{97}H_{155}O_{61}N_9$	2421.93	1211.97	808.32
14	FA2G2GaSg1	$C_{102}H_{162}O_{65}N_{10}$	2566.97	1284.49	856.66

Table 1. RapiFluor-MS labeled N-glycans from the Intact mAb Mass Check Standard, a murine monoclonal antibody.

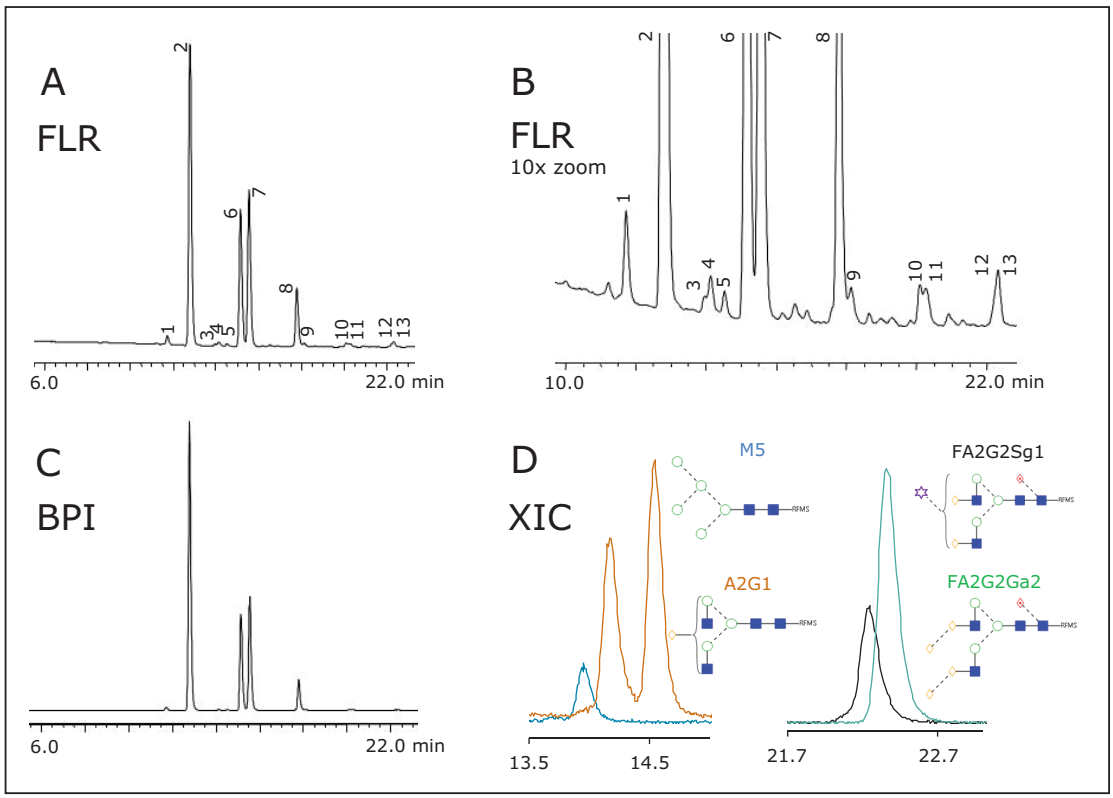


*Figure 1. (A) Fluorescence (FLR) chromatogram of RapiFluor-MS labeled N-glycans from Intact mAb Mass Check Standard obtained using the universal N-glycan profiling method and a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column. (B) Fluorescence chromatogram scaled with a 10x zoom to show low abundance N-glycans (C) Base peak intensity (BPI) chromatogram of Rapi*Fluor*-MS labeled N-glycans obtained with the universal N-glycan profiling method (D) Extracted ion chromatograms (XICs) of critical pairs to highlight issues with partial co-elution. N-glycan samples corresponding to 0.38 µg of the Intact mAb Mass Check Standard were analyzed in each experiment.*

Developing a Higher Resolution mAb N-Glycan Analysis Method

The significant co-elution of the members of these critical glycan pairs necessitated the development of an LC method specifically tailored for N-glycans released from mAbs. Modifying a HILIC separation by reducing the slope of the gradient can produce more resolution between labeled N-linked glycans with similar partition coefficients. As a result, the first change made to the method was to reduce the gradient slope while retaining the overall run time. A new gradient running from 26%–37% mobile phase A (versus 25% to 46%) indeed showed improvement in peak resolution. In another step, the retention time of the glycans was reduced by increasing the flow rate of the separation from 0.4 to 0.5 mL/min. This flow rate adjustment shifted the mAb N-glycan profile to be well within the gradient window, while at the same time, the maximum pressure of the analysis was maintained well below the pressure limit of the system. Increasing the flow rate of the separation also yielded an improvement in the resolution of the critical pairs (Figures 2A and 2B), but caused an average of a 90% increase in peak widths. This peak broadening was attributed to poor band formation when the injected sample approached the head of the column. To improve band formation, the eluent strength at the onset of the separation was reduced. The gradient was changed to have two segments, an initial ramp from 20% to 27% mobile phase A over 3.2 minutes followed by 27% to 37% mobile phase A over 31.8 minutes. Indeed, these changes facilitated better band formation at the head of the column and obtaining correspondingly sharper glycan peaks (Figure 2C). Despite improving the method in multiple ways, the two noted critical pairs of *Rapi*Fluor-MS labeled N-glycans remained only partially resolved. One final adjustment to the running conditions proved highly effective in improving the resolution of these critical pairs. Lowering the column temperature from 60 °C to 45 °C increased their separation to the point that near baseline resolution could be achieved (Figure 2D). With these final conditions, half-height resolution of the M5/A2G1+FA1G1 and the FA2G2Sg1/FA2G2Ga2 peaks were found to be 1.61 and 1.13, respectively. The extent of separation has been improved for all major species throughout the mAb profile, except for the alpha-linked galactose isomers of the FA2G2Ga1 glycan eluting at approximately 25 minutes.

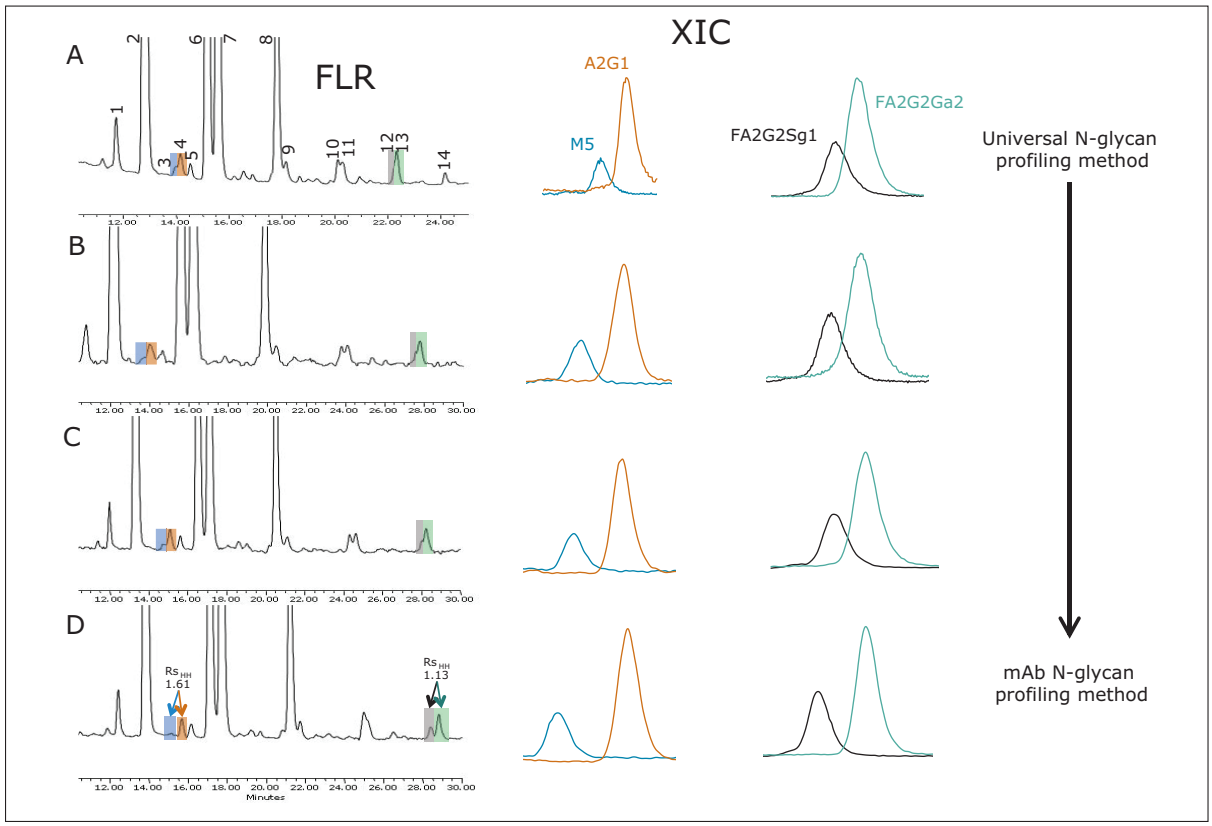
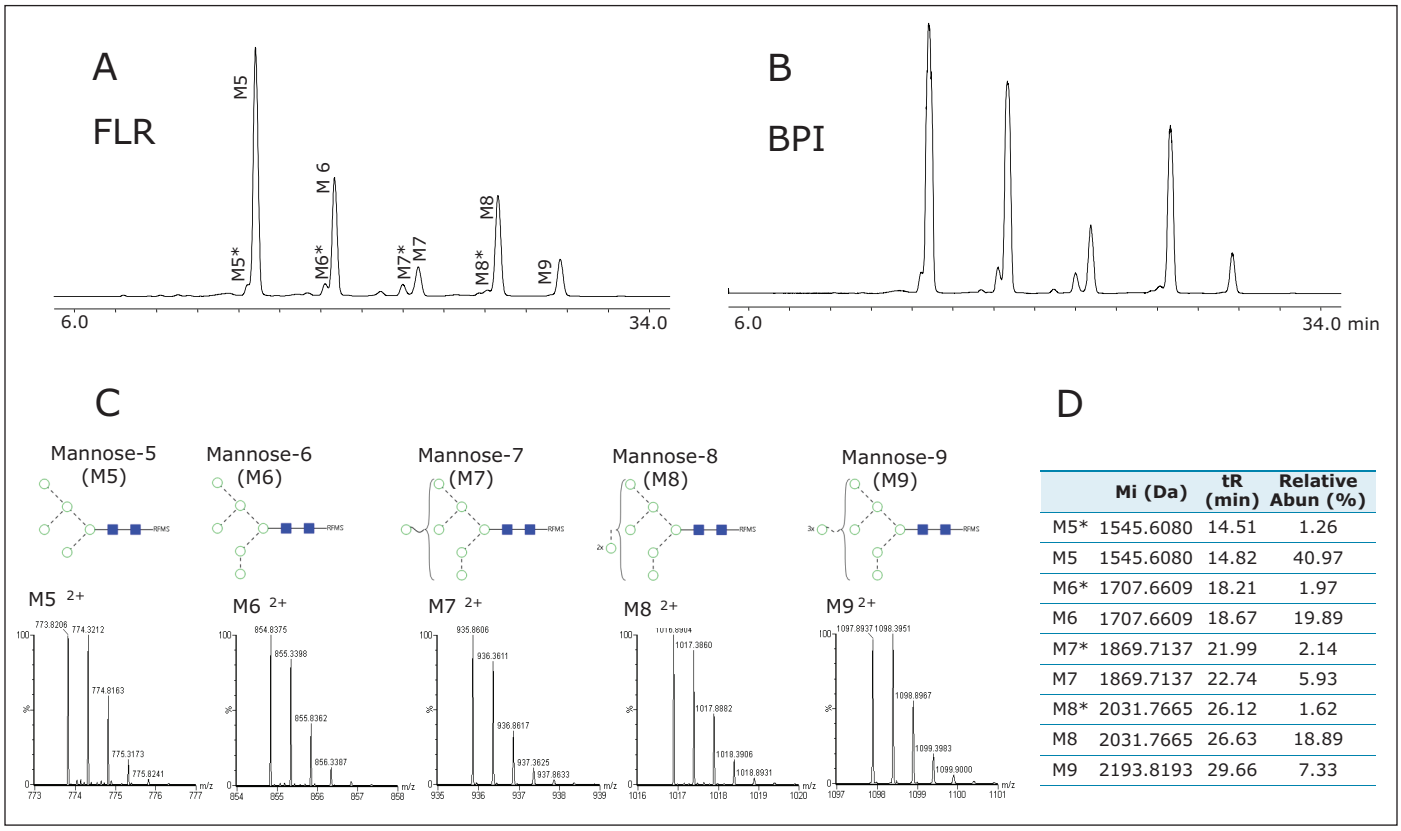


Figure 2. (A) Fluorescence (FLR) chromatogram and extracted ion chromatograms (XICs) obtained with the universal N-glycan profiling method and a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column. (B) FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60 °C column temperature, and a 35 minute gradient from 26% to 37% H_2O (C) FLR chromatogram and XICs obtained with a 0.5 mL/min flow rate, 60 °C column temperature, and a two-step gradient of 20% to 27% H_2O in 3.2 min followed by 27% to 37% H_2O in 31.8 min. (D) FLR chromatogram and XICs obtained as listed in (C) except with a column temperature of 45 °C (the mAb N-glycan profiling method). N-glycan samples corresponding to 0.38 µg of the Intact mAb Mass Check Standard were analyzed in each experiment. RsHH denotes peak resolution measured at half-height (HH).

Using the *Rapi*Fluor-MS High Mannose Standard to Demonstrate System Suitability

The resolution gains afforded by the new mAb N-glycan profiling method allows for better monitoring of high mannose structures. To this end, we have employed a new proficiency standard, the *Rapi*Fluor-MS High Mannose Standard, to demonstrate its ability to precisely monitor high mannose structures. This new standard contains *Rapi*Fluor-MS-labeled M5, M6, M7, M8, and M9. When separated on its own, it is evident that the *Rapi*Fluor-MS High Mannose Standard is a relatively simple mixture (Figure 3).



	Mi (Da)	tR (min)	Relative Abun (%)
M5*	1545.6080	14.51	1.26
M5	1545.6080	14.82	40.97
M6*	1707.6609	18.21	1.97
M6	1707.6609	18.67	19.89
M7*	1869.7137	21.99	2.14
M7	1869.7137	22.74	5.93
M8*	2031.7665	26.12	1.62
M8	2031.7665	26.63	18.89
M9	2193.8193	29.66	7.33

Figure 3. (A) Fluorescence (FLR) and base peak intensity (BPI) chromatograms (B) of the Waters RapiFluor-MS High Mannose Standard as obtained using a 2.1 x 150 mm ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column and the mAb N-glycan profiling method. Asterisks denote isomers of M5, M6, and M7. (C) ESI mass spectra of each major component. (D) Representative chromatographic data. Approximately 5 pmoles of high mannose N-glycans were analyzed in these experiments. An asterisk (*) denotes a linkage isomer.

As a result, it can potentially be used to support the identification of high mannose species when MS detection may not be available. Moreover, this high mannose glycan mixture is well suited for use in spiking studies, which can be performed to establish system suitability. In this work, *Rapi*Fluor-MS labeled N-glycans from Intact mAb Mass Check Standard were spiked with varying concentrations of the *Rapi*Fluor-MS High Mannose Standard (as outlined in the experimental section). Four *Rapi*Fluor-MS labeled glycan samples were prepared with M5 relative abundances ranging from 0.2% to 2.0% and analyzed as illustrated in Figure 4A. In these samples, M5, M6, and M8 are readily detected, while M7 and M9 are not due to their lower relative abundances in the spiking standard. The high resolution of the method allows for better integration of the high mannose glycan species. This can be clearly demonstrated by plotting the fluorescence peak areas of M5, M6, and M8 as functions of the spiking level. The linearity of these data ($R^2 \geq 0.974$) underscores the suitability of this technique for monitoring high mannose structures (Figure 4B). These spiking experiments also demonstrate that the Intact mAb Mass Check Standard is effectively free of high mannose species and that the M5 previously monitored during the development of the separation is near the limit of quantitation of this method.

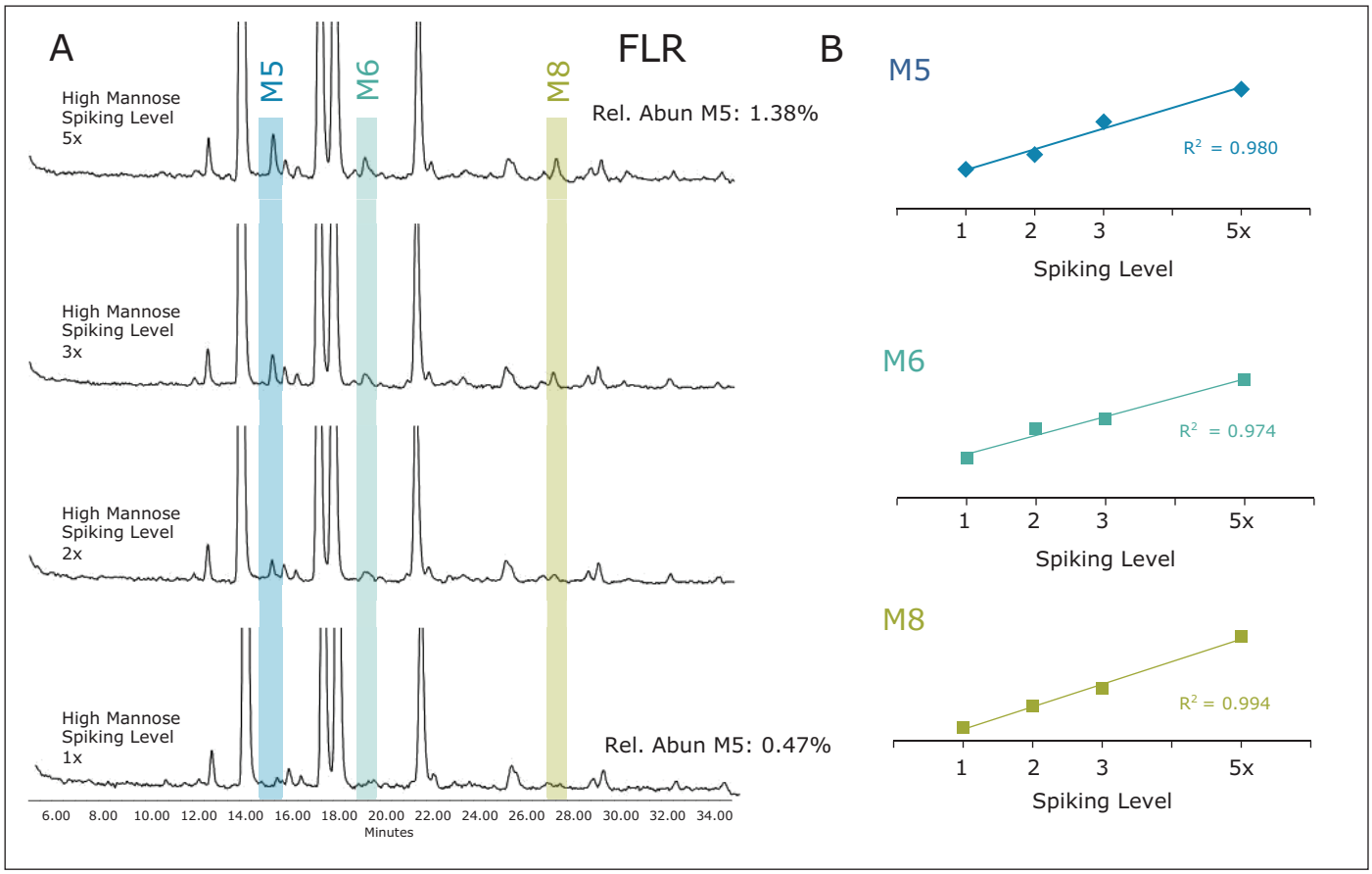


Figure 4. (A) Fluorescence (FLR) chromatogram obtained for RapiFluor-MS N-glycans prepared from Intact mAb Mass Check Standard spiked with varying concentrations of the RapiFluor-MS High Mannose Standard. Top to bottom: spiking levels are 5x, 3x, 2x, and 1x. (B) Peak area of high mannose structures at different spike concentrations. N-glycan samples corresponding to 0.34 µg of the Intact mAb Mass Check Standard were analyzed in each experiment. Separations were performed using the mAb N-glycan profiling method and an ACQUITY UPLC Glycan BEH Amide 130Å 1.7 µm Column.

GU Values from the Universal N-Glycan Profiling Method Versus the mAb N-Glycan Profiling Method

As mentioned earlier, the universal N-glycan profiling method is a generic tool for all N-glycan sample types. It will also be the method recommended for workflows involving assignment of new glycan peaks based on matching GU values to data in an upcoming *Rapi*Fluor-MS GU database, which is currently being constructed in collaboration with the National Institute for Bioprocessing Research and Training (NIBRT).

With this in mind, it is important to recognize that GU values, regardless of labeling strategy, are method specific. So although the mAb N-glycan profiling method can be used with GU values, it will not generate GU values that are meaningful for searching a NIBRT database based on the universal N-glycan profiling method. GU values still have merit for glycan analyses, even if they are not used for database matching. Use of GU values minimizes subtle retention time variations between runs and between different instruments by expressing chromatographic retention in terms of standardized GU values.[3] To assign GU values, a dextran ladder, consisting of glucose multimers of increasing length, is used as an external calibrant. The retention times of the glucose multimers are then used via cubic spline fitting to convert glycan retention times into GU values. Chromatographic data collected from separations of the *Rapi*Fluor-MS Dextran Calibration Ladder are provided in Table 2, one set of data obtained with the universal N-glycan profiling method and the other with the new, mAb N-glycan profiling method. Not surprisingly, differences between the methods led to shifts in the retention times of the individual glucose multimers. Therefore, GU values derived for the mAb N-glycans are also shifted, as shown in (Table 2). For the most strongly retained species, the FA2G2Ga1Sg1 glycan, there is, in fact, a GU shift of +0.37. Clearly, it is important to give consideration to how GU values are generated and how they are to be used. The universal N-glycan profiling method is the appropriate method for GU database searching. Nevertheless, GU values can be used along with the mAb N-glycan profiling method as replacements to standard retention times to improve the robustness of data reporting.

		Universal N-Glycan Profiling	mAb N-Glycan Profiling
Component	Name	Glucose Units	Glucose Units
1	A2	5.49	5.54
2	FA2	5.82	5.91
3	M5	6.19	6.24
4	FA1G1 + A2G1	6.23	6.37
5	A2G1	6.38	6.49
6	FA2G1	6.69	6.72
7	FA2G1	6.85	6.86
8	FA2G2	7.43	7.69
9	FA2G1Ga1	7.55	7.81
10	FA2G2Ga1	8.25	8.57
11	FA2G2Ga1	8.30	8.60
12	FA2G2Sg1	9.06	9.39
13	FA2G2Ga2	9.11	9.49
14	FA2G2Ga1Sg1	9.88	10.25

Table 2. Glucose unit values for the RapiFluor-MS labeled N-glycans from the Intact mAb Mass Check Standard and the RapiFluor-MS High Mannose Standard, as obtained with the universal N-glycan profiling method versus the mAb N-glycan profiling method. Glucose unit (GU) values were assigned using cubic spline fitting and UNIFI 1.7 Software.

CONCLUSIONS

The N-linked glycosylation of mAbs can impact their circulation half-life and efficacy. Therefore, it is particularly important for the N-glycans of a mAb to be well characterized and routinely monitored. By labeling mAb N-glycans with *Rapi*Fluor-MS, high sensitivity detection by both fluorescence and MS is made possible. The sample loading condition, gradient steepness, flow rate, and separation temperature of the universal N-glycan profiling method were adjusted to create a mAb N-glycan profiling method that was able to better resolve the Man5/A2G1+FA1G1 and FA2G2Sg1/FA2G2Ga2 critical pairs. The mAb N-glycan profiling method yielded a half-height resolution of 1.61 for M5/A2G1+ N-glycans and FA2G2Sg1/FA2G2Ga2 of 1.13. By improving the resolution of these critical pairs of N-glycans, we have provided additional separation space for monitoring high mannose structures. To this end, the *Rapi*Fluor-MS High Mannose Standard was used in a series of spiking experiments to demonstrate the quantitative performance of this new gradient for analyzing high mannose N-glycan structures. Using the mAb N-glycan profiling method in conjunction with the new *Rapi*Fluor-MS High Mannose Standard and the *Rapi*Fluor-MS Dextran Ladder allows for the easier adoption of this system solution.

References

1. Rudd, P. M.; Guile, G. R.; Kuster, B.; Harvey, D. J.; Opdenakker, G.; Dwek, R. A., Oligosaccharide sequencing technology. *Nature* 1997, 388 (6638), 205–7.
2. Lauber, M. A.; Yu, Y. Q.; Brousmiche, D. W.; Hua, Z.; Koza, S. M.; Magnelli, P.; Guthrie, E.; Taron, C. H.; Fountain, K. J., 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), 5401–9.
3. Campbell, M. P.; Royle, L.; Radcliffe, C. M.; Dwek, R. A.; Rudd, P. M., GlycoBase and autoGU: tools for HPLC-based glycan analysis. *Bioinformatics* 2008, 24 (9), 1214–6.

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