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A Novel, High-Accuracy Glucose-Unit Based N-glycan Retention Library and Peak Assignment Tool for Compound Identification in LC or LC/MS Data

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Introduction

Glycosylation is a post-translational modification that impacts the stability, half-life, and efficacy of protein biotherapeutics.[1] Routine monitoring of these glycan profiles is therefore a necessary but often challenging task in pharmaceutical labs. Glycan structures contain complex branching patterns and a multitude of monosaccharide building blocks, which complicates structural elucidation. In some instances, chromatographic and/or mass spectrometry data alone may not be sufficient to completely deduce which glycans are present in a sample, particularly when isomeric structures are present.

Complementary techniques such as exoglycosidase digestions, MS^n, and analysis of analytical standards can aid in identifying glycan structures in unfamiliar samples, but these approaches can also be laborious or expensive. An alternate option in is to reference a library of glucose unit (GU) values for common glycans, which can often reduce the need for these aforementioned techniques. The practice of normalizing glycan retention times (RTs) to a glucose ladder has been well-established in the last several decades and aids in mitigating the effects of day-to-day or system-to-system retention time shifts.[2,3] By calibrating an LC system with a ladder sample that is run in parallel with glycan samples, a user can establish GU values for their sample peaks and match these with known GU values of common glycans.

To this end, we have developed a web application called the Glycan Peak Assignment Tool, which predicts RTs for common glycans analyzed with the Agilent AdvanceBio Amide HILIC column.[4] The tool contains an internal GU library for 115 InstantPC-labeled N-glycans and 105 2-AB-labeled N-glycans. Users enter RTs for the GU peaks of InstantPC maltodextrin or 2-AB glucose homopolymer, and the tool outputs predicted glycan RTs for the user's LC system. The tool also features a novel refinement option, which uses two glycan RTs in the user's data to further refine the predictions and reduce the number of putative matches to be considered for each peak. The tool is freely available via the link below and is compatible with data generated on any liquid chromatography system using the AdvanceBio Amide HILIC column.

https://www.agilent.com/biopharma/gpat

Experimental

Sample Preparation and LC/MS Data Collection

Glycans from the monoclonal antibody cetuximab (Erbitux) were released and labeled with InstantPC according to the kit instructions (part number GX96-IPC).

A set of standardized HILIC gradients was developed for the peak assignment tool as detailed below. Samples were analyzed on a 1290 Bio Infinity II LC/FLD system connected via a tee splitter to an 6545XT AdvanceBio LC/Q-TOF.

LC conditions	
Column	Agilent AdvanceBio Amide HILIC, 2.1 x 150 mm, 1.8 µm (p/n 859750-913)
Column Temp	60 °C
Mobile phases	A = 50 mM ammonium formate, pH 4.4, prepared from p/n G3912-00000 B = Acetonitrile
Flow rate	0.6 mL/min
Ladder standards	AdvanceBio InstantPC Maltodextrin (p/n GKPC-503) AdvanceBio 2-AB Glucose Homopolymer (p/n GKSB-503)
Injection volume	0.5 µL for InstantPC Maltodextrin 1 µL for all other samples
Fluorescence detection	InstantPC: λ_{Ex} 285 nm, λ_{Em} 345 nm 2-AB: λ_{Ex} 260 nm, λ_{Em} 430 nm

InstantPC – Gradient 1	
Time	%B
0	77
45	59
46	40
47	40
49	77
60	77

InstantPC – Gradient 2	
Time	%B
0	77
75	47
76	40
77	40
79	77
90	77

2-AB – Gradient 3	
Time	%B
0	74
50	54
51	40
52	40
54	74
64	74

2-AB – Gradient 4	
Time	%B
0	74
75	44
76	40
77	40
79	74
90	74

Experimental

6545XT AdvanceBio MS Conditions	
Drying gas temp.	150 °C
Drying gas flow	9 L/min
Nebulizer	35 PSI
Sheath gas temp.	300 °C
Sheath gas flow	10 L/min
Vcap	2500 V
Nozzle voltage	500 V
Data acquisition mode	2 GHz (Extended dynamic range) Standard mass range (*m/z* 3200)

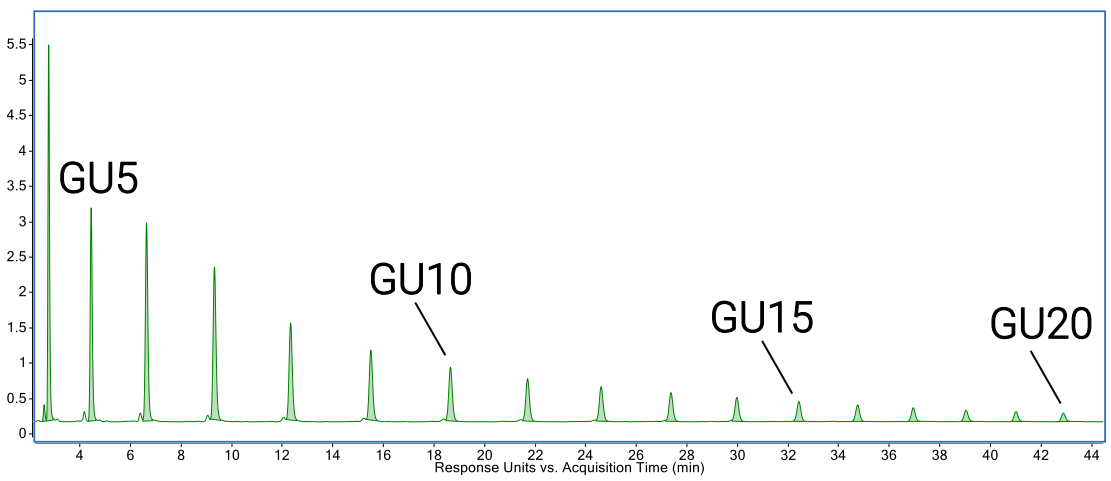

Figure 1. InstantPC maltodextrin fluorescence chromatogram showing peaks for GU 4–GU 20.

Glycan Peak Assignment Tool

The retention times for GU peaks 4–20 in the maltodextrin ladder were entered into the Glycan Peak Assignment Tool, along with retention times of InstantPC-G0F and InstantPC-G2S(3)2. The tool's resulting retention time predictions were used to assign chromatographic peak identities.

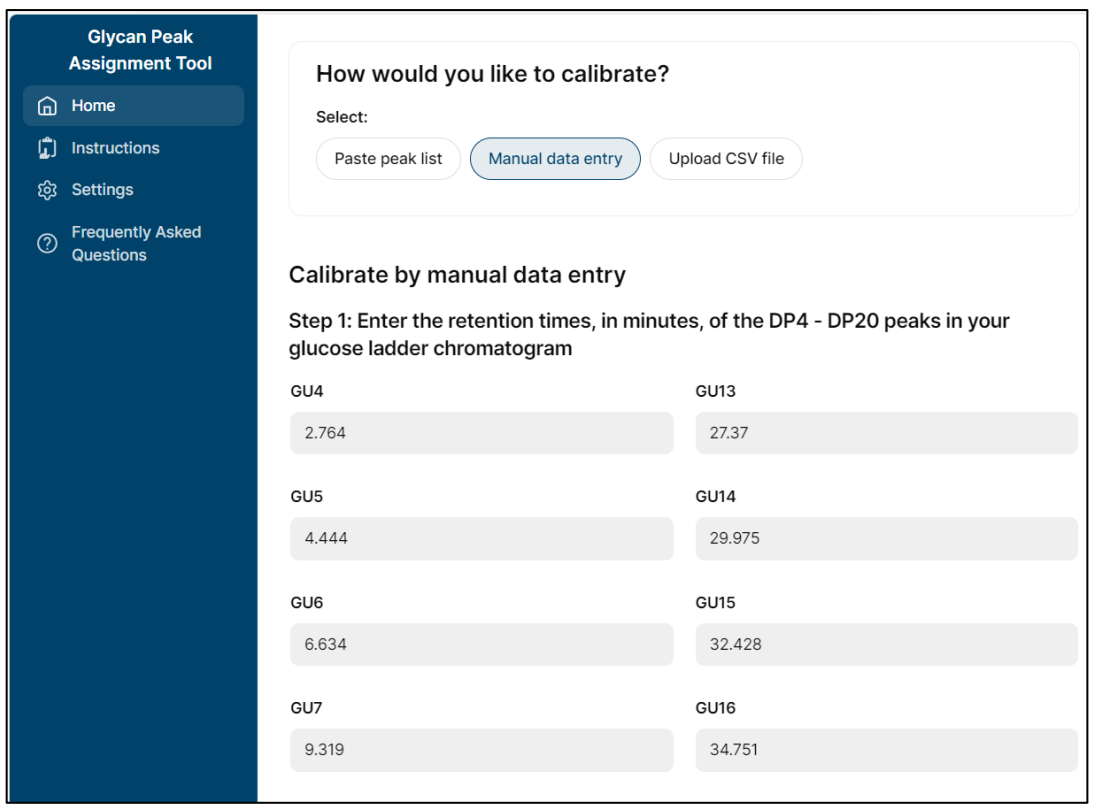

Figure 2. Glycan Peak Assignment Tool data entry page.

Results and Discussion

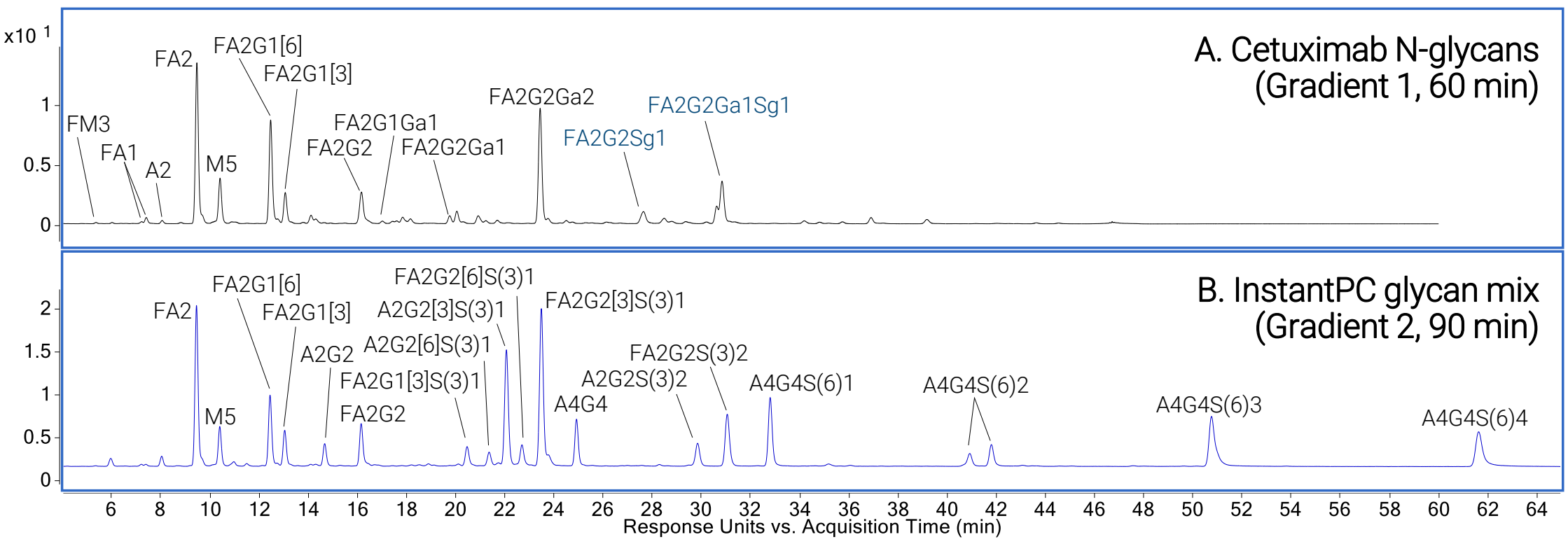

Figure 3. Chromatographic peak annotations based on the Glycan Peak Assignment Tool's output for InstantPC-labeled cetuximab N-glycans analyzed with Gradient 1 (A) and an InstantPC-labeled N-glycan reference mixture analyzed with Gradient 2 (B). Compounds annotated in blue (NGNA-sialylated glycans) are not present in the GU library and were identified by MS.

Results and Discussion

Retention Time Prediction Accuracy

Actual and predicted RTs for the InstantPC-labeled N-glycan mix are shown in the table below. RT predictions show <2% error in all cases, reduced to <1% when using the optional refinement feature. In cases where multiple candidate structures are predicted to elute near an observed peak, MS data can serve as orthogonal information to aid in deducing the specific glycan present. For example, in Figure 4, the peak assignment tool shows 3 potential structures, but the MS data only matches one of them. The tool provides m/z values for $[M+2H]^{2+}$ of all library glycans.

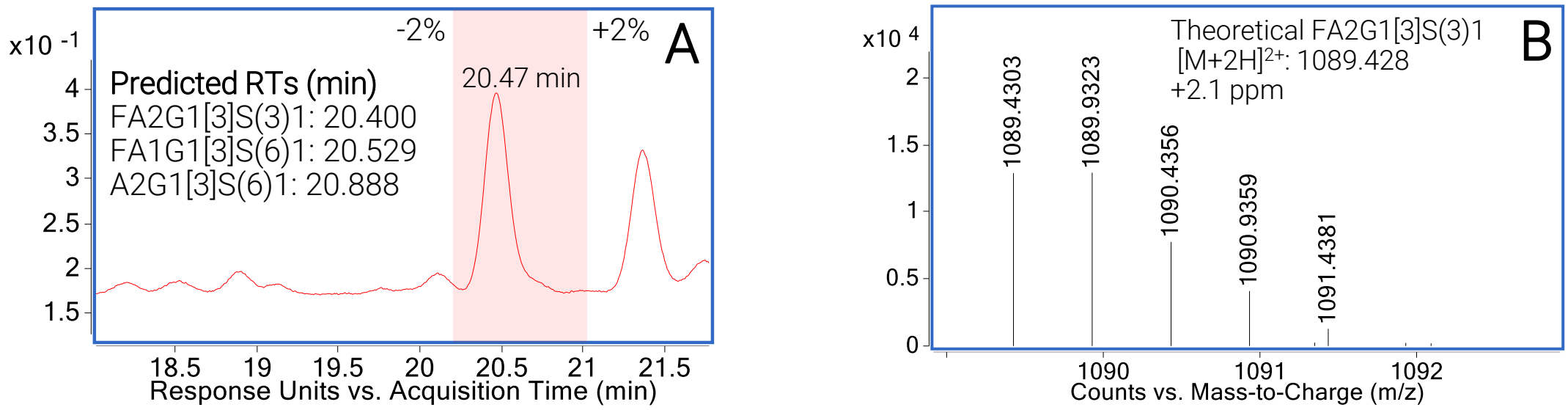

Figure 4. Three candidate InstantPC N-glycans have predicted RTs within approximately 2% of the peak at 20.47 min (A). MS data confirms that the true peak identity is FA2G1[3]S(3)1 (B).

Glycan	Actual RT (min)	Predicted RT – no refinement (min)	Predicted RT – with refinement (min)	% error – no refinement	% error – with refinement
A1	5.97	5.95	5.96	-0.40%	-0.15%
A2	8.04	8.03	8.05	-0.14%	0.08%
FA2	9.46	9.44	9.46	-0.21%	0.00%
M5	10.40	10.37	10.39	-0.32%	-0.12%
FA2G1[6]	12.45	12.46	12.48	0.07%	0.25%
FA2G1[3]	13.05	13.07	13.09	0.20%	0.36%
A2G2	14.67	14.72	14.75	0.35%	0.50%
FA2G2	16.16	16.22	16.24	0.35%	0.49%
FA2G1[3]S(3)1	20.47	20.69	20.40	1.10%	-0.33%
A2G2[6]S(3)1	21.36	21.60	21.31	1.09%	-0.26%
A2G2[3]S(3)1	22.07	22.30	22.02	1.07%	-0.21%
FA2G2[6]S(3)1	22.70	22.92	22.64	0.98%	-0.25%
FA2G2[3]S(3)1	23.49	23.72	23.44	0.98%	-0.19%
A4G4	24.92	25.04	25.06	0.49%	0.56%
A2G2S(3)2	29.85	30.33	29.85	1.62%	0.00%
FA2G2S(3)2	31.05	31.51	31.04	1.48%	-0.04%
A4G4S(6)1	32.80	32.96	32.73	0.49%	-0.21%
A4G4S(6)2 Iso 1	40.92	41.22	40.86	0.75%	-0.15%
A4G4S(6)2 Iso 2	41.80	42.06	41.71	0.63%	-0.22%
A4G4S(6)3	50.76	51.24	50.81	0.94%	0.11%
A4G4S(6)4	61.63	62.51	62.10	1.43%	0.76%

Conclusions

- The Glycan Peak Assignment Tool is a freely-available web application that uses non-confidential calibration data to predict N-glycan RTs using the AdvanceBio Amide HILIC column.
- The tool uses internal GU libraries to predict glycan RTs with a high degree of accuracy and can be used in combination with MS data to confidently deduce glycan structures with detailed linkage information.

References

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https://www.agilent.com/en/promotions/asms

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