A Novel Approach to Glycan Method Development for Biotherapeutics using Superficially Porous Particle Technology

Introduction

Most of the new therapeutic drugs being developed currently are large molecules, such as polynucleotides and proteins. The proteins include monoclonal antibodies (mAb), fusion proteins, and antibody-drug conjugates (ADCs), and are produced in cell cultures (mouse, human, or combinations). For a protein to be targeted for a specific biotherapeutic function or application, extensive analysis and characterization is required. This includes analysis of protein structure, physicochemical properties, biological activity, immunochemical properties, purity and impurities. There is a need for a detailed understanding of all these structures and modifications to permit control of the manufacturing process.

Within biotherapeutic drug development the three main areas for separations are process support, characterization, and final release/QC. Process support can be rapid and low resolution, but more efficient separations can be developed and then "detuned" using the same column and mobile phase, thus not requiring a new qualification or validation. Characterization methods can be designed with high efficiency, requiring longer run times, but since they are only done on a limited basis, the increased time is negligible. These methods can then be adapted to final release/QC and stability to be faster when the highest resolution is secondary to time.

Usually, development of a new biotherapeutic entails developing a new method for analyzing various properties, such as glycosylation. Glycans are instrumental in cellular signaling, therefore a specific protein's glycosylation can influence the effectiveness of the biotherapeutic. Given the importance of glycosylation to overall performance of a glycoprotein, there is a need to completely characterize and then monitor these glycans for both research and manufacturing purposes. Traditional methods of analysis rely on cleavage of the glycans from the protein backbone, label with a UV- or fluorescence-based tag, and separation and identification of the glycans by HPLC/MS. Following identification, the subsequent characterization and routine monitoring can be done using HPLC/UV or HPLC/fluorescence.

However, since the glycans attached to any protein come from a known set of glycans, the goal of this work was to develop and optimize a universal method to separate and completely characterize all potential glycans from any protein. This may entail a long, comprehensive analysis, which is acceptable since the characterization is done only a few times during product development. A second goal was to develop a rapid, routine method to be used for monitoring and control during process development or final manufacturing.

Initial Scouting Work

The initial work to develop a broad ranging characterization method involved the release, labelling, and HPLC/UV/MS analysis for 13 proteins (Ipilimumab, Rituximab, Epoetin Zeta, Trastuzumab, Tocilizumab, Ecluzimab, Etanercept, Adalimumab, Abatacept, Panitumumab, Omailizumab, Ustekinumab, and Denosumab). These proteins were IgG1, IgG2, IgG2/4 fusion proteins. Released and labelled glycans from these 13 proteins and the dextran ladder were all analyzed on a HALO® Glycan column (2.1 x 150 mm, 2.7 µm, 90 Å pore size) using a gradient elution of 80-55% B in 60 min (A = 50 mM Ammonium formate, pH 4.4; B = Acetonitrile) at 60°C with a flow rate of 0.3 mL/min. Each sample consisted of a 5 µL injection dissolved in 70% acetonitrile/ammonium formate buffer.

The results of these analyses are shown in Figures 1A (UV 300 nm) and 1B (Total ion current MS: 600 – 3000 m/z).

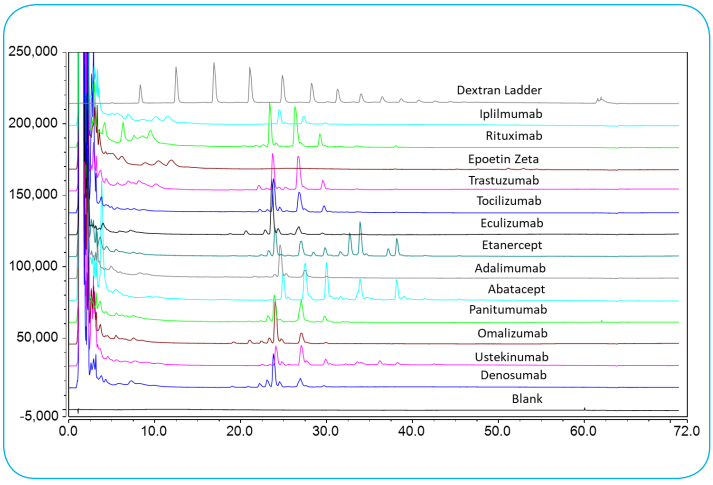

Figure 1A. PNGase F Released Procainamide (PAM)-Labeled mAb Glycan Analysis by HPLC/UV. Absorbance at 300 nm.

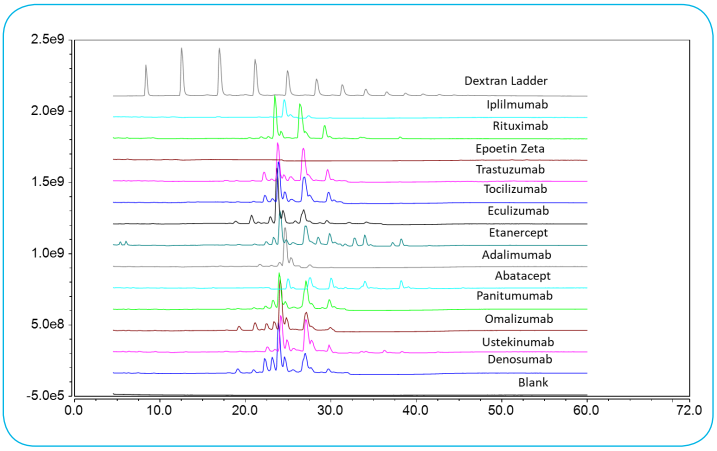

Figure 1B. PNGase F Released PAM-Labeled mAb Glycan Analysis by HPLC/MS. Total ion current 600 – 3000 m/z

The goal of this initial analysis was to determine which set of proteins could encompass the broadest variety of glycans seen from different proteins. The dextran ladder consisting of four to 20 glucose units, Denosumab, and Etanercept were chosen from those above; in addition, Ribonuclease B and Fetuin were added for additional diversity.

Denosumab is a human IgG2 monoclonal antibody with mostly neutral glycans (G0, G1, and G2 families) and low levels of sialylation. Etanercept is a fusion protein which fuses a TNF receptor to an IgG1 monoclonal antibody. It is heavily glycosylated with six N-glycans and up to 14 O-glycans. Ribonuclease B is a high mannose glycoprotein which is glycosylated at one site only (Asn34). Fetuin is a glycoprotein with three N-linked and three O-linked glycans which have high sialic acid content.

HPLC Optimization for Complete Characterization

Once the samples had been defined, the development and optimization of the HPLC method started, using principles first described by Snyder and Dolan (Snyder et.al). These principles stated that to optimize the separation of most or all compounds in a complex mixture, the approach is to run the same samples, mobile phase(s), and columns using four gradient separation conditions. In this case, the separation uses a HALO® Glycan column (2.1 x 150 mm, 2.7 µm, 90 Å, superficially porous particles) with a flow rate of 0.4 mL/min. The gradient was from 76% to 55% B and the four experiments that were run are detailed in Table 1.

Condition	Gradient Time (min)	Temperature (°C)
A	30	45
B	90	45
C	30	60
D	90	60

Table 1. Gradient time and temperature conditions for four runs.

Since the identity of each peak could be determined by the MS analysis after the LC/UV, the samples from release and labelling from all four proteins were combined for one sample injection at each condition. The dextran ladder sample was run in a separate injection.

The chromatogram observed for condition B (90 min gradient time, 45° C) for the mixed protein is shown in Figure 2 (UV at 300 nm and TIC).

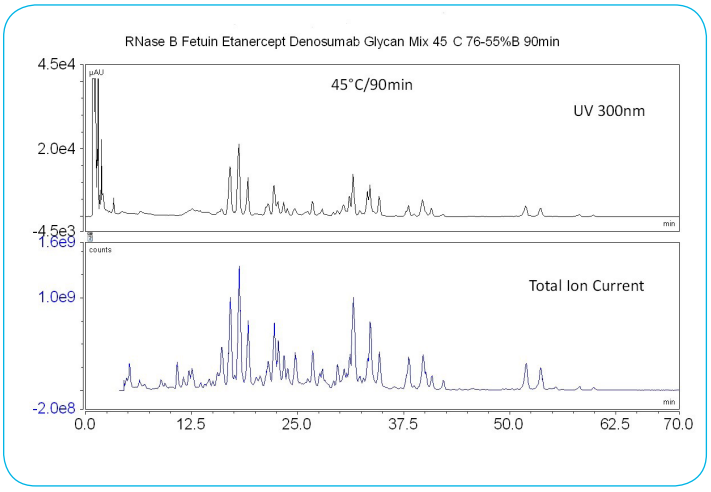

Figure 2. Separation at 45°C and 90 min gradient. Mixture of released and labelled glycans from four proteins (Denosumab, Etanercept, Ribonuclease B and Fetuin)

The chromatogram for the dextran ladder for condition B (90 min gradient time, 45° C) is shown in Figure 3.

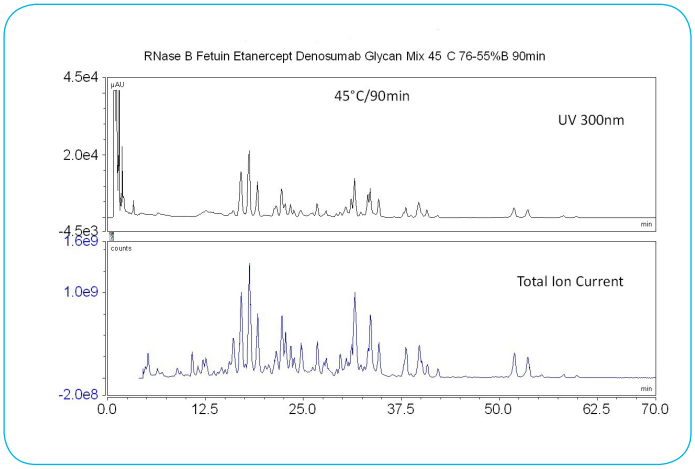

Figure 3. Separation at 45°C and 90 min gradient. Dextran ladder sample.

The retention time data for the dextran ladder samples is shown in Appendix Table 1. It is apparent that as the temperature increases, the retention time for each peak decreases (as expected) and that as the gradient time increases, the retention time increases (also as expected). A similar set of data was collected for all the glycan peaks and is shown in Appendix Table 2.

Once all the data had been collected and peaks identified, the optimization for a full characterization method was performed using DryLab®. The resulting map of resolution vs. conditions (gradient time and temperature) is shown in Figure 4. The indicated conditions for the optimal gradient conditions were 44° C, 90 min gradient time. A simulated extracted ion chromatogram is also shown in the bottom of Figure 4.

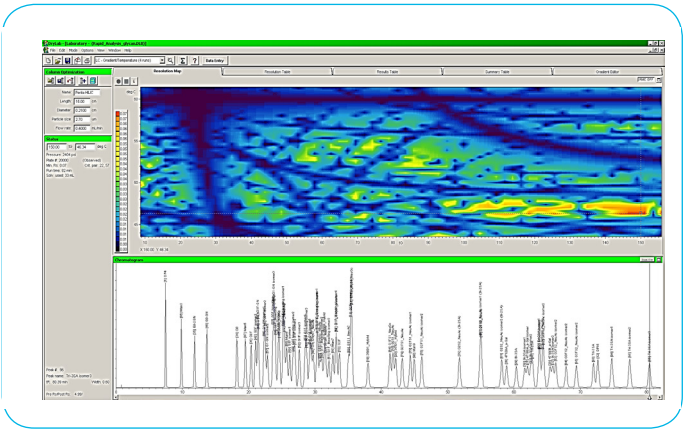

Figure 4. (Top) DryLab® resolution map for optimized characterization separation of glycans (including dextran ladder). (Bottom) Simulated extracted ion chromatogram of optimized separation.

The actual separation of the glycans from etanercept as one example is shown in Figure 5. The conditions used were: 2.1 x 150 mm HALO® Glycan column, 0.4 mL/min, gradient from 77-65% B in 90 mins, 44° C. The column pressure was 158 bar. Although the gradient was programmed for 90 minutes, the run could be stopped in this case after 75 mins when all peaks have eluted.

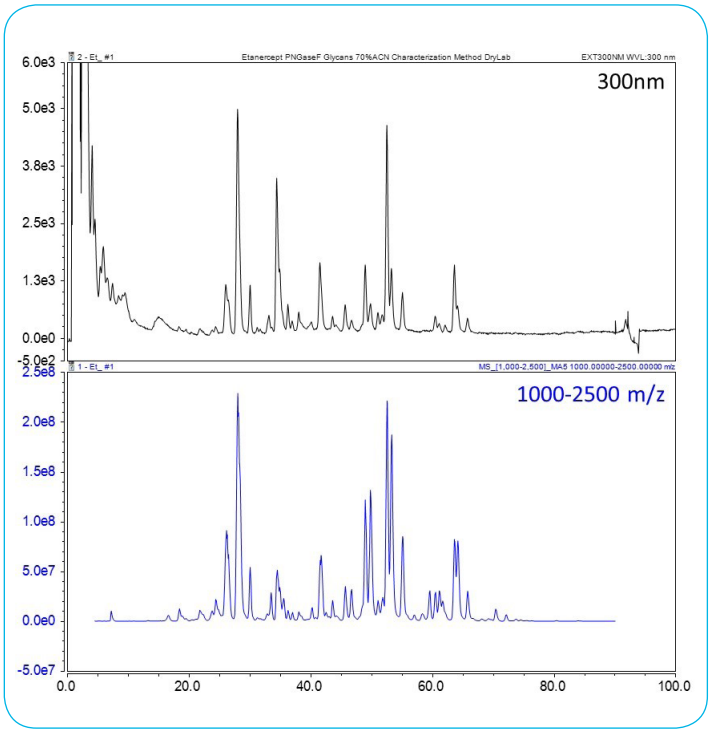

Figure 5. Optimized characterization separation showing glycans released and labelled from etanercept as one example protein.

This optimized characterization separation can, in theory, be used by other systems to obtain the same results. However, due to subtle differences in HPLC systems, including dead volumes and gradient mixing system, a second approach can also be used to assure that the chromatographic separation is optimized. The dextran ladder can be run using the optimized conditions and this is shown in Figure 6.

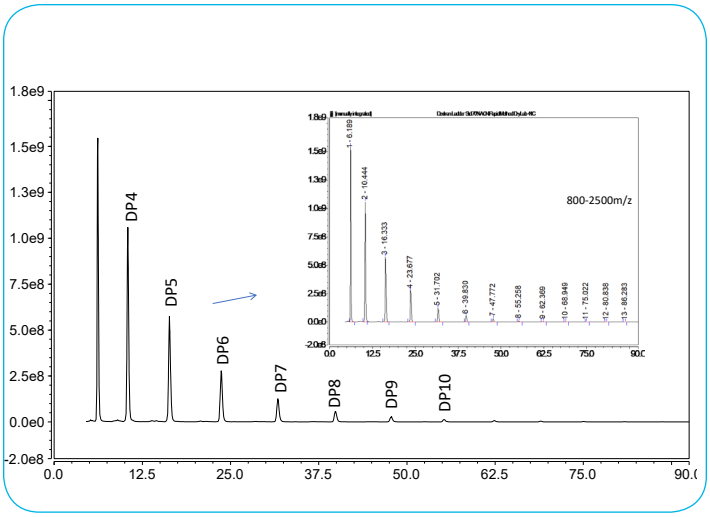

Figure 6. Optimized characterization separation of dextran ladder.

The retention time data for all the glycans and the dextran ladder are contained in Tables 1 and 2 in the Appendix. The glycan peaks retention data can be normalized against the dextran ladder to provide a relative retention time for each glycan to the dextran peaks.

HPLC Optimization for Rapid Analysis Separation

The same data collected for the optimized complete characterization method can also be used and analyzed to develop a rapid, QC-type method to analyze for the major glycan peaks in any sample. As an example of this, the top glycan peaks (by UV peak area) observed in the four-run experiment were selected for a new analysis. The goal was to develop a rapid method that could be used for process support and/or final release testing, focusing only on the top glycans from a sample. The resulting map of resolution vs. conditions (gradient time and temperature) is shown in Figure 7. The indicated conditions for the optimal gradient conditions were 0.4 mL/min, 44° C, 20 min gradient time. A simulated extracted ion chromatogram is also shown in the bottom of Figure 7.

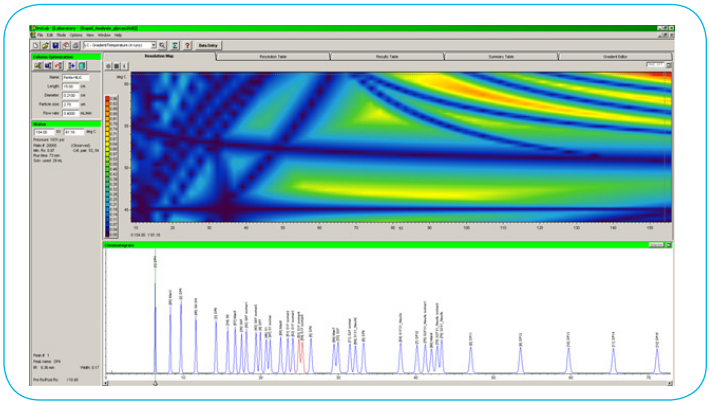

Figure 7. – (Top) DryLab® resolution map for optimized rapid separation of glycans (including dextran ladder). (Bottom) Simulated extracted ion chromatogram of optimized separation.

Figure 8 shows an example of the fusion protein etanercept using the rapid conditions.

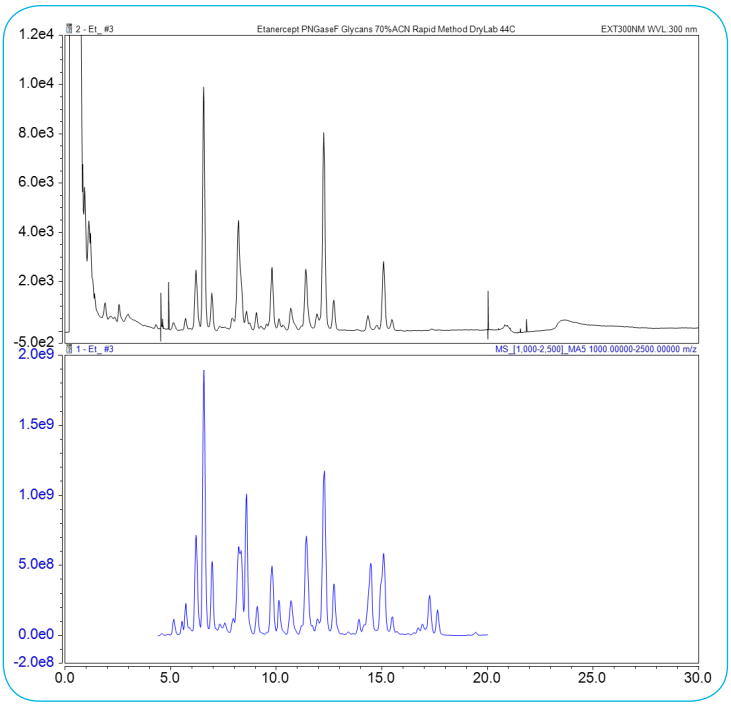

Figure 8. Optimized rapid separation showing glycans released and labelled from etanercept as one example protein.

Summary and Conclusions

The development and optimization of a method to separate and identify 79 glycans has been accomplished using four scouting runs and then analyzing the data using DryLab® optimization software. Two final methods were developed. The first uses a long gradient time (90 mins) and is designed for the best separation for characterization of glycans from various proteins. The second uses a shorter gradient time (20 mins) and is designed for rapid analysis of the major glycans to support process testing or QC release. Since the methods use the same column and mobile phase and only involve changes to gradient time and flow rate, the two chromatograms can be related to each other easily in the case of a new protein.

Reference

Dolan, J., Snyder, L., Djordjevic, N., Hill, D., & Waeghe, T. (1999). Reversed-phase liquid chromatographic separation of complex samples by optimizing temperature and gradient time: I. Peak capacity limitations. Journal of Chromatography A, 857 (1-2), 1-20.

APPENDIX – SUPPLEMENTAL DATA

Table 1 Retention time data as a function of gradient conditions for dextran ladder sample.

PEAK NUMBER	DEXTRAN NUMBER	30MIN 45° C	90MIN 45° C	30MIN 60° C	90MIN 60° C
1	DP4	6.25	7.27	5.56	6.25
2	DP5	8.27	10.68	7.47	9.23
3	DP6	10.29	14.82	9.47	12.98
4	DP7	12.20	19.35	11.41	17.33
5	DP8	13.99	24.05	13.20	21.90
6	DP9	15.63	28.62	14.86	26.41
7	DP10	17.11	32.99	16.39	30.79
8	DP11	18.47	37.04	17.79	35.01
9	DP12	19.72	40.88	19.06	38.86
10	DP13	20.87	44.42	20.23	42.50
11	DP14	21.93	47.72	21.33	45.89
12	DP15	22.91	50.82	22.32	49.10
13	DP16	23.82	53.71	23.24	52.06
14	DP17	24.65	56.39	24.11	54.75
15	DP18	25.44	58.95	24.93	57.32
16	DP19	26.18	61.32	25.66	59.81
17	DP20	26.88	63.55	26.39	62.07

Table 2 Retention time data as a function of gradient conditions for glycans released and labelled from four proteins.

PEAK NUMBER	GLYCAN	30MIN 45° C	90MIN 45° C	30MIN 60° C	90MIN 60° C
1	Man3	7.47	9.36	6.60	7.98
2	G0-2GN	8.42	11.07	7.47	9.50
3	G0-GN	9.11	12.55	8.19	10.79
4	G0	10.65	16.04	9.72	14.02
5	Man5	11.17	17.06	10.18	14.83
6	G0F	11.29	17.61	10.32	15.38
7	G0F isomer1	11.52	18.12	10.52	15.84
8	G1F-GN	11.72	18.43	10.67	16.11
9	G1-GN	11.72	18.47	10.67	16.11
10	G1-GN isomer1	11.91	19.06	10.84	16.55
11	G0F isomer2	11.96	19.17	10.92	16.76
12	G1-GN isomer2	12.17	19.50	11.06	16.96
13	G0F bisecting	12.24	19.96	11.32	17.77
14	G0F_Bisecting	12.24	19.96	11.32	19.70
15	G1	12.31	20.13	11.31	17.71
16	G1-GN isomer3	12.37	20.15	11.23	17.47
17	G1 isomer	12.47	20.57	11.46	18.07
18	35000_Hybrid	12.57	20.70	11.60	18.31
19	G1F	12.69	21.14	11.65	18.51
20	G0F bisecting isomer1	12.71	21.08	11.76	18.73
21	G1F isomer1	12.90	21.73	11.89	19.23
22	Man6	12.95	21.52	11.98	19.11
23	G1F isomer2	13.11	22.27	12.09	19.67
24	46101_a-Gal	13.13	14.44	12.13	12.41
25	G0F bisecting isomer2	13.17	22.17	12.19	19.70
26	G1F isomer3	13.28	22.73	12.23	20.10
27	G0F bisecting isomer3	13.51	23.45	12.53	20.68
28	G1F isomer4	13.56	23.38	12.51	20.69
29	G1F isomer5	13.66	23.80	12.64	21.01
30	G1F_Bisecting	13.70	23.90	12.82	21.53
31	G1(-GN)S1_NeuAc	13.83	24.14	12.99	21.89
32	G1F_Bisecting isomer1	13.93	24.51	13.00	22.03
33	G0F bisecting isomer4	13.95	24.56	12.99	21.90
34	G1F(-GN)S1_NeuAc	14.16	24.87	13.28	22.56
35	G1F_Bisecting isomer2	14.18	25.04	13.22	22.61
36	36000_Hybrid	14.26	25.31	13.29	22.76
37	G1F_Bisecting isomer3	14.39	25.73	13.41	23.13
38	G1F_Bisecting isomer4	14.58	26.38	13.59	23.78
39	G1F(-GN)S1_NeuAc	14.64	26.43	13.76	24.10
40	Man7	14.65	26.19	13.72	23.76
41	G2F	14.75	26.77	13.68	24.00
42	G1S1_NeuAC	15.03	27.60	14.20	25.42
43	G2F isomer	15.17	27.91	14.08	25.03
44	36100_Hybrid	15.21	27.93	14.36	25.80
45	G1F(-GN)S1_NeuGc	15.21	27.95	14.39	25.80
46	G2F_Bisecting	15.32	22.67	14.35	19.68

PEAK NUMBER	GLYCAN	30MIN 45° C	90MIN 45° C	30MIN 60° C	90MIN 60° C
47	35001_Hybrid	15.82	29.61	14.84	27.06
48	46100_a-Gal	16.40	31.59	15.54	29.16
49	G1FS1_NeuGc	16.40	31.59	15.54	29.16
50	G2S1_NeuAc	16.40	31.59	15.54	31.32
51	35101_Hybrid	16.69	32.21	15.86	29.96
52	G2FS1_NeuAc	16.84	32.79	15.92	30.28
53	G2FS1_NeuAc isomer1	17.09	33.54	16.17	31.09
54	Man9	17.37	34.03	16.54	31.75
55	G2FS1_NeuAc isomer2	17.48	34.65	16.53	32.08
56	36101_Hybrid	18.00	27.64	17.19	25.34
57	G2S2_NeuAc (Bi-2SA)	18.66	38.12	17.91	36.18
58	G2FS2_NeuAc	19.21	39.82	18.45	39.87
59	G2S2_NeuAc isomer1 (Bi-2SA)	19.41	40.14	18.68	38.24
60	G2S2_NeuAc isomer2 (Bi-2SA)	20.13	42.16	19.42	40.32
61	Bi-3SA	20.38	43.34	19.64	41.37
62	47100A_a-Gal	20.41	42.74	19.66	40.67
63	Bi-3SA isomer1	20.75	44.22	19.93	42.02
64	Tri-2SA	20.75	44.74	20.36	42.59
65	Bi-3SA isomer2	20.99	44.75	20.40	43.51
66	G3FS2_NeuAc isomer2	21.07	45.70	20.33	43.80
67	47100A_a-Gal isomer	21.14	44.73	20.44	42.82
68	Tri-2SA isomer1	21.14	45.50	21.02	43.61
69	Bi-3SA isomer3	21.19	45.52	20.68	44.26
70	G2F2S2_NeuAc	21.19	45.52	20.67	46.10
71	G3FS2_NeuAc isomer2	21.46	46.84	20.68	44.87
72	Bi-3SA isomer3	21.67	46.79	21.12	45.52
73	G3FS2_NeuAc isomer2	21.82	47.82	21.13	45.95
74	47100B_a-Gal	21.90	46.85	21.24	45.05
75	G3FS2_NeuAc isomer2	22.21	48.83	21.48	46.88
76	Tri-3SA	22.60	50.24	22.01	48.78
77	Tri-3SA isomer1	23.22	51.97	22.63	50.43
78	Tri-3SA isomer2	23.84	53.66	23.29	52.25
79	Tri-3SA isomer3	24.48	55.50	23.94	54.04

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