Supplemental Material for

High Throughput Analysis of Fluorescently Labeled N-Glycans Derived from Biotherapeutics Using an Automated LC-MS Based Solution

Ximo Zhang, Corey E. Reed, Robert E. Birdsall, Ying Qing Yu, and Weibin Chen

Waters Corporation, 34 Maple Street, Milford, MA 01757, USA

Author for correspondence: Ximo Zhang
e-mail: ximo_zhang@waters.com
Table S1. A standard gradient condition for N-glycan UPLC-FLR/MS analysis using a 2.1 x 150 mm Acquity Glycan BEH Amide column (1.7 µm particle size, 130 Å pore size)

Mobile Phase A: 50 mM ammonium formate, pH=4.4

Mobile Phase B: acetonitrile

| Time  | Flow rate (mL/min) | MPA | MPB |
|-------|--------------------|-----|-----|
| 0.00  | 0.400              | 25  | 75  |
| 35.00 | 0.400              | 46  | 54  |
| 35.50 | 0.200              | 80  | 20  |
| 39.50 | 0.200              | 80  | 20  |
| 43.10 | 0.200              | 25  | 75  |
| 47.60 | 0.400              | 25  | 75  |
| 55.00 | 0.400              | 25  | 75  |
Figure S1. Separation of N-glycan Performance Test standard on three columns using the developed 5-min gradient method. Retention times of peak 1-4 are listed in Table S2.
Table S2. Retention times of peak 1-4 in Figure S1. Three consecutive injections were performed on three columns to evaluate the retention time variation. Results demonstrated that retention times of all peaks are reproducible for both intra- and inter-column injections.

|       | Peak 1 (min) | Peak 2 (min) | Peak 3 (min) | Peak 4 (min) |
|-------|--------------|--------------|--------------|--------------|
| Column 1 | Run 1 | 1.85 | 2.15 | 2.47 | 2.90 |
|        | Run 1 | 1.85 | 2.15 | 2.47 | 2.91 |
|        | Run 1 | 1.85 | 2.15 | 2.47 | 2.90 |
| Column 2 | Run 2 | 1.86 | 2.16 | 2.49 | 2.92 |
|        | Run 2 | 1.86 | 2.16 | 2.49 | 2.92 |
|        | Run 2 | 1.85 | 2.15 | 2.48 | 2.91 |
| Column 3 | Run 3 | 1.88 | 2.18 | 2.52 | 2.94 |
|        | Run 3 | 1.89 | 2.19 | 2.52 | 2.95 |
|        | Run 3 | 1.89 | 2.19 | 2.52 | 2.95 |
| Standard deviation | 0.02 | 0.02 | 0.02 | 0.02 |
Figure S2. Investigation into two co-eluted peaks at retention time 2.06 min in Figure 3B. The two co-eluted peaks were tentatively assigned to be FA2B and A2G1 based on the accurate mass and were further confirmed by the fragmentation data. By aligning the XICs of the fragment and parent ion, the source of fragmentation can be determined. (A) MS full scan spectra showing two co-eluted peaks at m/z 895 and m/z 989 (z=2); (B) TIC of the two co-eluted glycans shown as one peak; (C) XIC of FA2B at m/z 989; (D) XIC of A2G1 at m/z 895; (E) MS fragmentation spectra showing multiple fragment ions; (F) XIC of m/z 1571 in fragmentation spectra showing a retention time of 2.06 min, suggesting the fragment ion was from FA2B; (G) XIC at m/z 1425 in fragmentation spectra showing a retention time of 2.09 min, suggesting the fragment ion was from A2G1; (H) XIC at m/z 366 showing retention times at both 2.06 and 2.09 min, suggesting this fragment ion was from both FA2B and A2G1. Fragment ions in (F) and (G) were identified as FA1 and A1, which could be the fragmentation under high energy from FA2B and A2G1, respectively.