Automated High-Throughput Permethylation for Glycosylation Analysis of Biologics using MALDI-TOF-MS

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Supporting Information

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EXPERIMENTAL SECTION

Materials

Protein A Ceramic HyperD® F Affinity Chromatography Resin was obtained from PALL (Portsmouth, UK). Pierce™ Micro-Spin Columns and phosphate buffered saline was from Thermo Scientific (Hampshire, UK). Reagents for in-solution manual permethylation, i.e. dimethyl sulfoxide (DMSO), methyl iodide (MeI) and dichloromethane (DCM) were purchased from Sigma (Dorset, UK).All reagents for HT N-glycan release, Peptide N-Glycosidase F (PNGase-F); LudgerTag™ 2-aminobenzamide (2-AB) (LT-KAB-VP24) and LudgerTag™ procainamide (LT-KPROC-VP24) labeling kits; 96 well LudgerClean™ protein binding membrane (LC-PBM-96) plate; LudgerClean™ T1 cartridges (LC-T1-A6); LudgerClean™ CEX cartridges (LC-CEX-A6); anhydrous hydrazine (99.9%) and hydrazinolysis kit (LL-HYDRAZ-A2) for O-glycan release were obtained from Ludger (Oxfordshire, UK).

EXPERIMENTAL PROTOCOLS-SECTION 1.1

Sample preparation for automated and high-throughput (HT) permethylation

All steps for sample preparation were automated using a liquid handling robot with the exception of IgG4 affinity purification, O-glycan release, manual in-solution permethylation and sample spotting on the MALDI target plate.

IgG4 mAb affinity purification

Approximately 300 µL of mAb supernatants from five different bioreactor conditions were taken and affinity purification was performed manually as per the manufacturer's instruction from PALL. The five different bioreactor conditions used were classified as: Direct Gas Sparging - DGS; Silicone Membrane Aeration - SMA; Standard Culture Condition - SCC; Hypothermic Culture Condition (32 °C) - HCC; Control Temperature Condition (37 °C) - CTC.

N-glycan release

Fifty µg of human IgG glycoprotein was dispensed into a 100 µL PCR plate and dried down by vacuum centrifugation prior to release. 17.5 µL of pure water was added to each sample and the samples thoroughly mixed using pipette action. 5 µL of reaction buffer (250 mM sodium phosphate buffer; pH 7.5) and 1.25 µL of denaturation buffer (2% SDS in 1 M β-mercaptoethanol) were dispensed into each sample. The PCR sample plate was removed from the robot deck and sealed with a foil pierce seal using a plate sealer and incubated for 10 min at 100 °C to enhance denaturation of proteins. After cooling the samples to room temperature, the foil pierce seal was carefully removed and the PCR sample plate transferred back to the robot deck. 1.25 µL of Triton X-100 and 1 µL of PNGase F was added to each sample. Again the PCR sample plate was removed from the robot deck, sealed with a new foil pierce seal using a plate sealer, vortexed to mix the samples and incubated overnight at 37 °C in an off-deck incubator. After the incubation, the samples were allowed to cool. The foil seal was carefully removed and placed on the robot deck.

Hydrophilic liquid interaction chromatography (HILIC)-solid phase extraction (SPE) glycan enrichment

Following the enzymatic digestion, the released N-glycan samples were purified using a 96-well format robot-compatible HILIC SPE filter plate. 200 µL of 70% ethanol, 200 µL of water and 200 µL of ACN were used to wash and prime the membrane prior to N-glycan purification and enrichment. After the addition of each solvent, a vacuum was used to pull the solvent through the membrane. Once each solvent had been fully drained, the next reagent was added by the robot. Once each solvent had been fully drained, 180 µL of ACN was then added to each sample and mixed using pipetting action and transferred to the primed HILIC SPE filter plate. After allowing the samples to settle for 5 min to adsorb onto the plate, a low vacuum (between 0.05 and 0.1 bar pressure reduction) was used to elute the ACN to waste. Non-glycan contaminants were washed from the plate by applying 200 µL of ACN three times. Enriched glycans were eluted into a collection plate with 100 µL of 1% formic acid using a low vacuum. A second 100 µL of 1% formic acid was added into each sample well to elute any remaining enriched N-glycans. The collection plate containing the eluted samples was removed from the robot deck and the samples were manually transferred into a 300 µL PCR plate and dried down in a centrifugal evaporator.

O-glycan release

The O-glycans from recombinant human erythropoietin (rhEPO) and fetuin glycoproteins were released by hydrazinolysis. The samples were buffer exchanged into 0.1% TFA and dried prior to release. The dried samples were incubated with anhydrous hydrazine at 60 °C for 6 h. After incubation, the samples were cooled and dried to evaporate unreacted hydrazine. The samples were re-*N*-acetylated and cleaned up by passing them through CEX cartridges. The released O-glycans were eluted off the cartridges using water and dried down in a centrifugal evaporator prior to permethylation.

Manual in-solution permethylation

The manual in-solution method involved preparing a slurry with 250 mg of NaOH in 1 mL of anhydrous DMSO. The slurry needed to be vortexed constantly as 40 µL was removed and added to released and purified glycans in Eppendorf vials and were then incubated for 15 min at room temperature (RT). 20 µL of MeI was added and each sample was vortexed again and incubated for 1 h at RT. The step was repeated by adding an additional 20 µL of the NaOH-DMSO slurry and 20 µL of MeI and incubated for another hour. Next, the samples were subjected to liquid-liquid extraction (LLE) where the samples were extracted from reaction by the addition of 500 µL of DCM and 800 µL of water. The samples were vortexed and the top aqueous layer was discarded. The water addition step was performed at least 3 times and the aqueous layer was discarded to extract salts and render the pH neutral prior to mass spectrometry. Samples were then dried using the centrifugal evaporator prior to analysis using MALDI-TOF-MS.

MALDI-TOF-MS

Automated data acquisition was performed using the AutoXecute feature on the AutoFlex Speed MALDI-TOF-MS instrument from Bruker with a Smartbeam-II laser and system speed of 2 kHz for MS 200 Hz MS/MS. The instrument was used in high resolution reflectron positive (RP) mode. FlexControl 3.4 software build 119 from Bruker was used for acquiring data for all mass spectral measurements. A window of *m/z* 1300-6000 was used for N-glycans and *m/z* 450-800 was used for O-glycan data acquisition. 20,000 laser shots were accumulated at a laser frequency of 2000 Hz using a random walk style pattern with 50 shots per spot. Peptide calibration standard from Bruker Daltonics was used to calibrate the instrument and was also used as an external calibration standard for data acquisition of samples in the range of *m/z* 1300 – 6000. Permethylated heavy ^{13}C human IgG glycan standard from Ludger was used for calibration of the instrument and the heavy permethylated IgG glycan standard was also used as an external standard for IgG glycan samples in the *m/z* range of 1300-3500 using cubic calibration[1]. Data acquisition for each sample measurement / acquisition using MALDI-TOF-MS was performed in triplicate and averaged to provide relative peak intensities. Bruker Daltonics flexAnalysis software version 3.4 was utilized for data representation of $[M+Na]^+$ permethylated glycans using the snap algorithm.

EXPERIMENTAL PROTOCOLS-SECTION 1.2

Sample preparation for automated and HT fluorescent labeling

All steps for sample preparation were automated using a liquid handling robot and N-glycans were released from glycoproteins as mentioned in the previous section and dried in a vacuum centrifuge.

Protein Binding Membrane (PBM) clean-up

The released N-glycan samples were suspended in 20 µL of 1% formic acid and incubated at RT for 50 min for glycosylamine hydrolysis, prior to sample purification using a 96-well format robot compatible PBM clean-up plate. (Note: The addition of 1% formic acid solution to released N-glycan samples aids in the hydrolysis of the glycosylamine form of the N-glycans following PNGase F release. Hydrolyzing the glycosylamine promotes the formation of a reducing end which enables the glycans to be fluorescently labelled).

100 µL of methanol and 300 µL of water were used to wash and prime the membrane prior to N-glycan enrichment. After the sequential addition of methanol and water a vacuum was applied (0.1 to 0.2 bar pressure reduction), using the vacuum manifold integrated within the liquid handling robot, to pass the solvents through the membrane. Next, the acid hydrolyzed samples were transferred into the PBM plate from the PCR plate containing the released and hydrolyzed samples. The PCR plate was rinsed with 100 µL of water which was subsequently transferred to the PBM plate. Vacuum was applied and the flow-through was collected in a deep well collection plate. 100 µL of water was added again to the PBM plate and passed through by vacuum (0.1 to 0.3 bar) to retrieve any remaining sample. The combined flow-through was then transferred to a non-skirted 96-well PCR plate and dried down using a centrifugal evaporator.

Glycan labeling

Released and enriched N-glycans were fluorescently labeled using 2-AB labeling kit or procainamide labeling kit. Briefly, samples in 10 µL of water were incubated for 60 min at 65 °C with either 2-AB or procainamide labeling solution, both containing 2-picoline borane as reductant. The 2-AB labeled samples were cleaned using LC-T1 cartridges. The procainamide labeled samples did not undergo any post labeling clean-up. The labeled N-glycans were diluted to 78% ACN for UHPLC analysis.

HILIC UHPLC analysis

Procainamide and 2-AB labeled glycans were analyzed using a HILIC UHPLC with fluorescence detection. A Thermo Dionex UltiMate 3000 UHPLC with a BEH Glycan UPLC column of 1.7 µm, 150 x 4.5 mm dimension from Waters (UK) was used for the chromatographic separation of the labeled glycans. The UltiMate 3000 Fluorescence Detector was set at excitation wavelength of 250 nm and emission wavelength of 428 nm for 2-AB labeled glycans, while excitation of 310 nm and emission of 370 nm was set for procainamide labeled glycans. On average one sample would take a minimum of 30 min to run on the UHPLC. The data was analyzed using Chromeleon data software version 7.2 (Dionex, USA).

Glycan enrichment

We wanted to test if components from the protein or formulation would interfere with the MALDI analysis. We studied human IgG glycoprotein standards by releasing the N-glycans with PNGase F, and we used the PBM for clean-up prior to permethylation. The PBM plate clean-up step was later replaced by HILIC SPE clean-up and enrichment. A comparison of the two clean-up methods proved that the SPE clean-up with HILIC purification was a crucial step for glycan enrichment, as it removed most of the peptides, proteins, detergents, and salts.[2–4] SI Figure-S-10, shows that post HILIC SPE enrichment the IgG N-glycan spectrum is no longer compromised by a polymer ladder which most likely originates from triton X added during PNGase F digestion. This shows that the benefit of clean-up using HILIC SPE is the removal of Polyethylene glycol (PEG), which allows techniques such as LC-MS to be used without PEG interfering in the MS signal. Typically, enrichment of released glycans is performed using the conventional C18 Sep-Pak clean-up[5] which is a time consuming, multi-step and laborious process. To overcome this limitation we automated the HILIC SPE clean-up and reduced the final elution volume from approximately 3 mL to 200 µL. SI Figure S-11, demonstrates comparison of PNGase F released human IgG N-glycans permethylated with and without HILIC SPE clean-up using the liquid handling robot. Analysis showed that this automated and HT enrichment method is convenient, fast and requires minimal manual handling, while taking under 2.5 h of robot time for 96 samples. An additional advantage is that the reduced elution volume assists in faster sample drying and therefore increasing the sample processing efficiency.

ICH Q2 (R1) VALIDATION-SECTION-1.3

The methods for N-glycan release, glycan enrichment and permethylation were validated according to ICH Q2 (R1) guidelines (for Analytical Validation).The following validation characteristics were included in this validation study: Linearity, Working range, Specificity, Limit of Quantitation , Limit of Detection, Accuracy, Repeatability, Intermediate precision.

1) Linearity : Linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample. A linear relationship should be evaluated across the range of the analytical procedure. To analyze linearity we used 100 µg, 50 µg, 25 µg, 15 µg, 10 µg and 5 µg of starting material of IgG glycoprotein in triplicate. Post release, enrichment and permethylation samples were each dissolved in 10 µL 70% methanol. 0.5 µL of each sample was spotted along with equal volume of S-DHB matrix making the concentration of the sample spotted 1 µg, 0.5 µg, 0.25 µg, 0.15 µg, 0.1 µg and 0.05µg respectively. Each concentration was plotted for eight major peaks IgG N-glycans against their peak intensities. The R^2 values from the linear regression plot for 8 major peaks of IgG were all above 0.93. (See SI Figure S-1 for details).

2) Working range: The linear working range is normally derived from linearity studies and is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision within or at the extremes of the specified range of the analytical procedure. See SI Figure S-2, section 1 and 2 to visualize the decrease in peak intensities with decrease in concentration of the analyte. The sum of the intensities of the eight major N-glycans of IgG in a linear regression plot (Figure S-1) gave R^2 values above 0.93. From this plot it was determined that the working range was between 15 µg to 100µg for IgG glycoprotein samples.

3) Specificity: Specificity was demonstrated by verifying that the negative control (water blank) components do not interfere with permethylated glycans after N-glycan release, glycan enrichment and permethylation. (See Figure S-3 for comparison spectra of permethylated IgG N-glycans and water blank.)

4) Limit of Quantitation and Limit of Detection: The limit of quantitation (LOQ) of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The limit of detection (LOD) of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value. The same experimental set up used for the linearity experiment was used where 100 µg, 50 µg, 25 µg, 15 µg, 10 µg and 5 µg of starting material of IgG glycoprotein was released, enriched and permethylated. Approximately 1 µg, 0.5 µg, 0.25 µg, 0.15 µg, 0.1 µg and 0.05µg was spotted on the MALDI target. Signal to noise (S/N) ratios were extracted from flexAnalysis for five peaks which were above 5% relative intensities for all the above mentioned concentrations. From literature we know that LOQ = 10:1 and LOD = 3:1 of S/N ratios. Therefore from S/N ratios obtained, values above 10 were acceptable for LOQ and S/N value above 3 were acceptable for LOD. See Figure S-4 Section-A for table with S/N ratios, where all five peaks which were quantifiable were above 0.5 µg sample concentration and the LOD for all five peaks was up to 0.1 µg concentration. See Figure S-4 Section-B for a comparison of a zoomed in spectra of two human IgG N-glycans (*m/z* values of 2244.1 and 2285.1) at 0.5 µg and 0.25 µg concentration illustrating LOQ and LOD determination using S/N ratios and the illustration also allows for a rough visual evaluation of LOQ and LOD.

Accuracy, Repeatability (Intraday variation), Intermediate precision (Interday variability) : have been described in the manuscript under the **results and discussion section under subheading accuracy and repeatability.**

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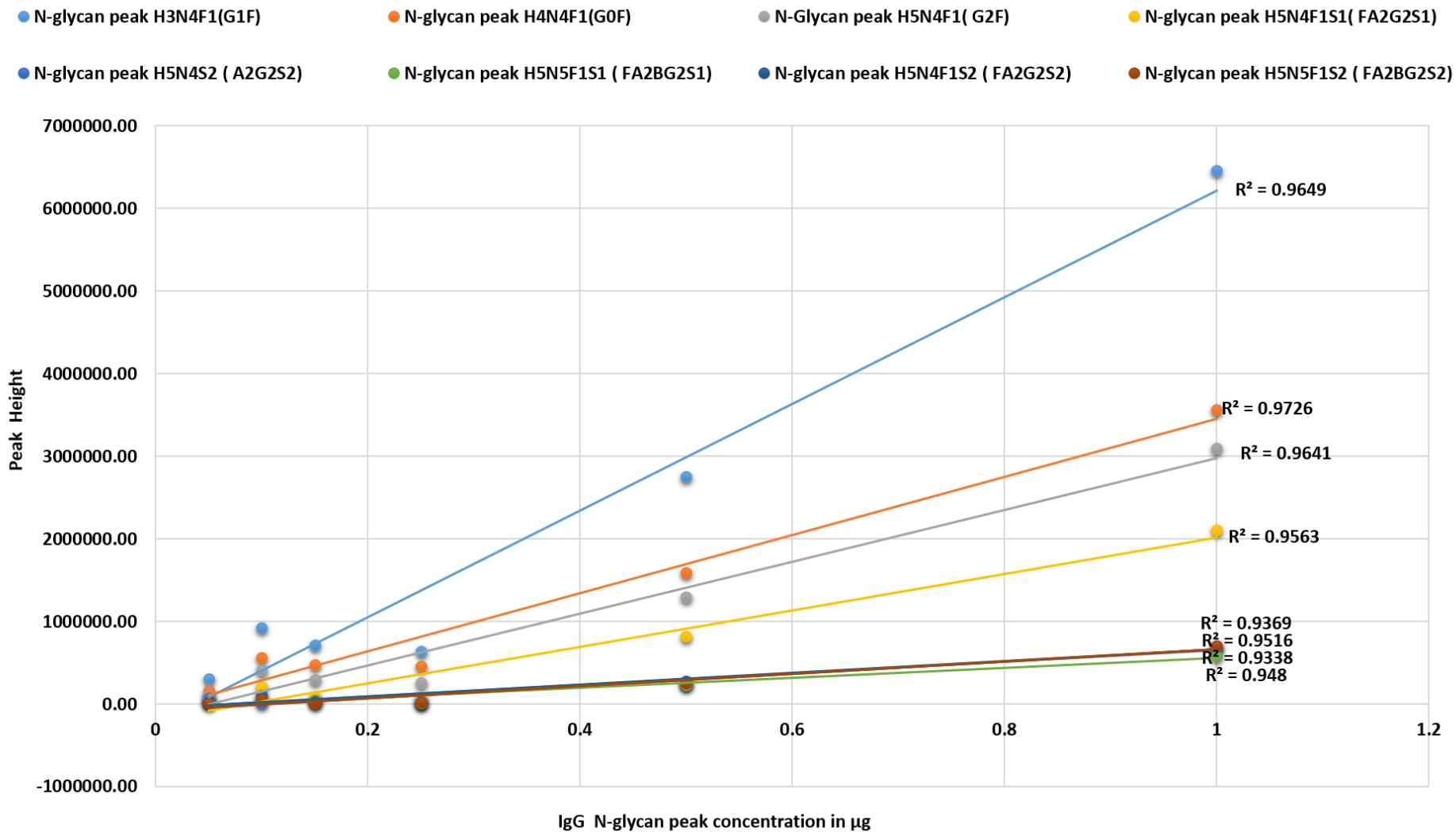


Figure S-1: Triplicate samples of IgG glycoprotein from different starting material concentrations (100 µg, 50 µg, 25 µg, 15 µg, 10 µg and 5 µg) were released, enriched and permethylated using the robot. Concentrations of (1 µg, 0.5 µg, 0.25 µg, 0.15 µg, 0.1 µg and 0.05µg) were spotted on the MALDI target and the peak intensities were plotted for eight major N-glycan peaks. A visual evaluation of the analyte signals as a function of the concentrations for IgG N-glycans, is shown in this linear regression plot to access linearity as a validation parameter.

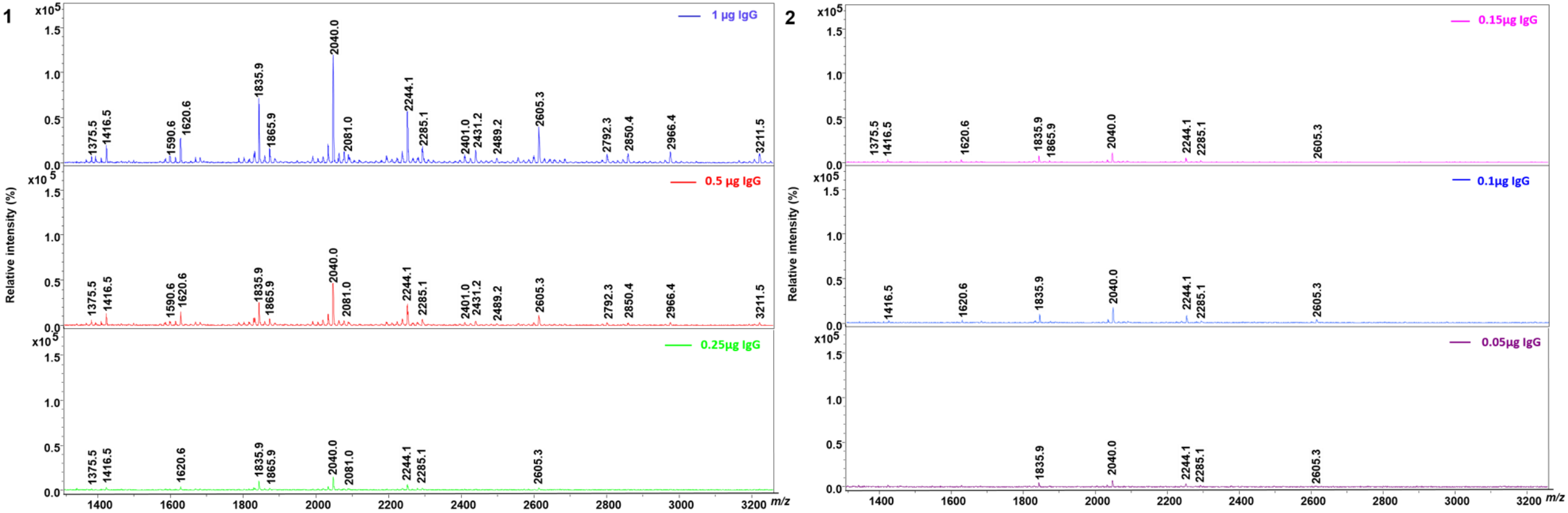


Figure S-2: Figure represents the comparison of relative intensities of permethylated N-linked glycans from released and enriched IgG glycoprotein for different concentrations spotted on the MALDI target (1 µg, 0.5 µg, 0.25 µg, 0.15 µg, 0.1 µg and 0.05µg). As the concentration of IgG decreases, the intensity of the signal of glycans in *m/z* range of 1400 to 3200 decreases, where Y axis is normalized.(1) Stacked MALDI-TOF-MS of permethylated human IgG showing sample concentration of 1 µg, 0.5 µg and 0.25 µg . (2) Stacked MALDI-TOF-MS of permethylated human IgG showing sample concentration of 0.15 µg, 0.1 µg and 0.05 µg. (The mass values shown in all the spectra are [M + Na]+ of permethylated glycans).

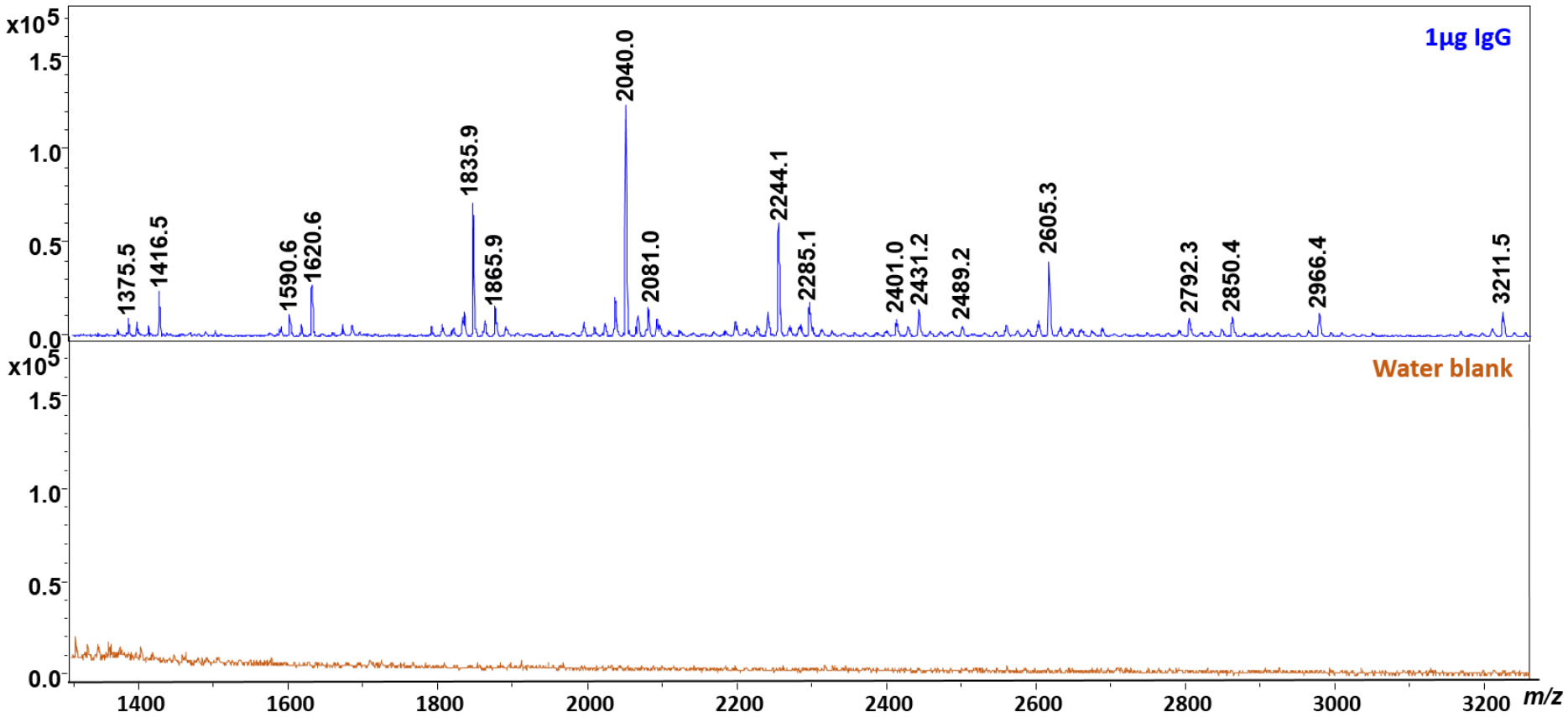


Figure S-3: MALDI-TOF-MS of permethylated N-glycans from human IgG compared with water blank (negative control) analyzed in parallel where the water blank underwent the same sample processing as the human IgG sample. Y axis is normalized to show that the negative control components do not interfere with permethylated glycans and demonstrate specificity of the method.

A

S/N ratios of permethylated MALDI-TOF-MS of human IgG N-glycans							
Glycan composition	m/z $[M+Na]^{+}$	1 µg	0.5 µg	0.25 µg	0.15 µg	0.1 µg	0.05 µg
H3N4F1	1835.9	104	58	17	19	19	8
H4N4F1	2040.0	179	92	24	27	32	10
H5N4F1	2244.1	84	42	10	12	14	5
H4N5F1	2285.1	25	12	4	4	4	3
H5N4F1S1	2605.3	58	24	4	4	8	2

B

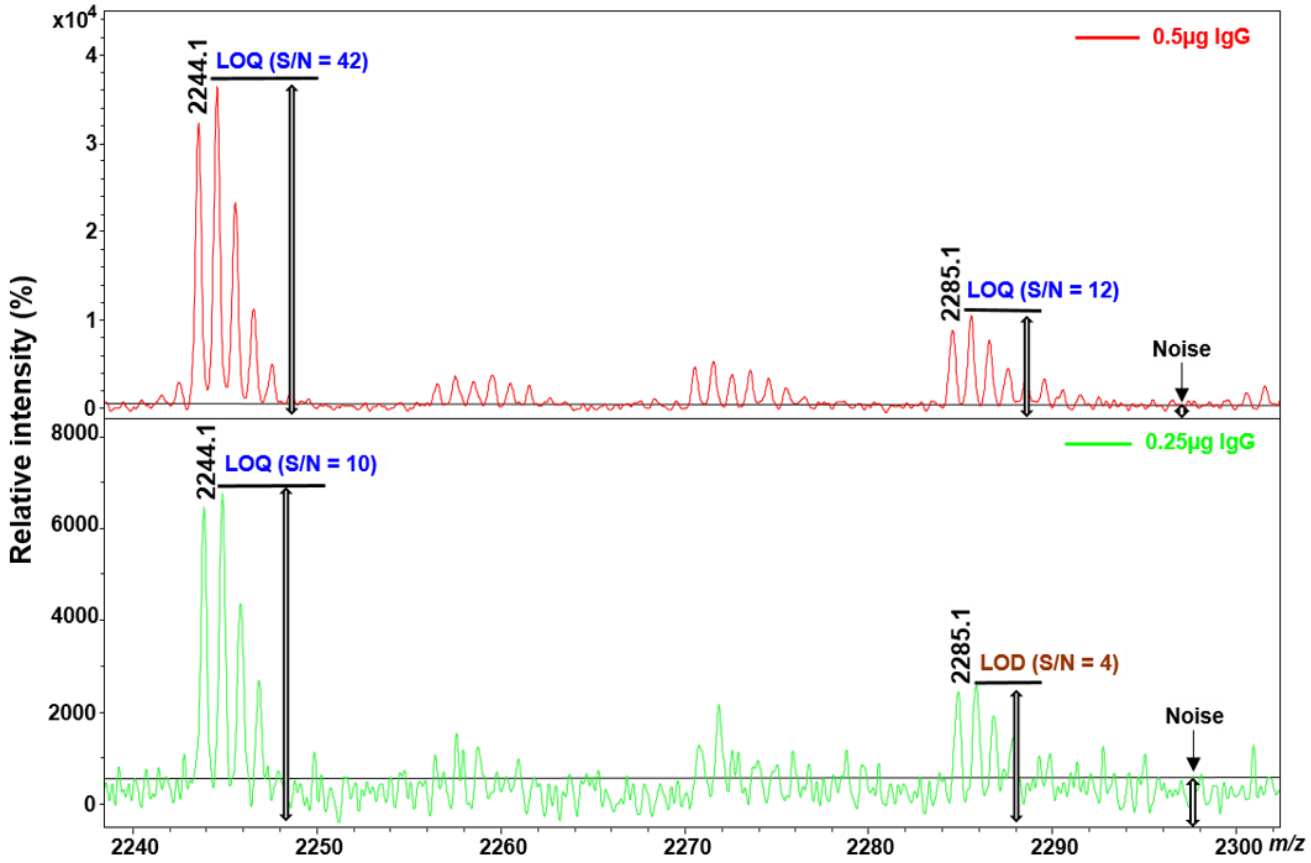


Figure S-4: (A) Table with signal to noise (S/N) ratios of permethylated human IgG glycan peaks above 5% relative areas. Samples with different concentrations (1 µg, 0.5 µg, 0.25 µg, 0.15 µg, 0.1 µg and 0.01µg) were spotted on the MALDI target, the S/N ratios gradually decrease with decrease in concentration which is evident from the table. S/N ratios in blue are acceptable for LOQ, brown are acceptable for LOD and not quantifiable and red S/N ratio are not acceptable for quantitation or for detection. (B) Zoomed in spectra of two human IgG N-glycans (*m/z* values of 2244.1 and 2285.1) at 0.5 µg and 0.25 µg concentration illustrating LOQ and LOD determination using S/N ratios. Both peaks with *m/z* values of 2244.1 and 2285.1 shown in the red spectrum (0.5 µg concentration) are quantifiable as they have S/N ratios of 42 and 12 respectively. While, the peak 2244.1 is quantifiable (S/N=10) and peak 2285.1 from the green spectrum (0.25 µg concentration) is only acceptable for detection and it is not quantifiable as the S/N is 4.

Note: LOQ = 10:1 and LOD = 3:1 of S/N ratio.

Table S-1: Peak numbers, glycan composition, relative intensities (RIs), standard deviation (SD) and coefficients of variation (CVs) for 2-AB labeled (HILIC UHPLC) data and permethylated (MALDI-TOF-MS) data for N-glycans from human IgG.

Peak no.	Glycan composition	2-AB labeled HILIC- UHPLC				Permethylated MALDI-TOF-MS			
		Glucose Units (GU)	Relative intensity (%)	SD	CV	$[M+Na]^+$ *m/z*	Relative intensity (%)	SD	CV
1	H3N4F1	5.79	19.90	0.02	0.12	1835.9	18.81	1.32	7.02
2	H3N5F1	6.14	4.39	0.03	0.77	2081.0	4.03	0.48	11.92
3	H4N4F1	6.52	20.47	0.07	0.34	2040.0	Isomer peaks 30.41	Isomer peaks 1.83	6.02
4	H4N4F1	6.64	8.64	0.07	0.81	2040.0			
5	H4N5F1	6.79	4.36	0.03	0.74	2285.1	6.13	0.75	12.23
6	H5N4F1	7.37	14.63	0.03	0.18	2244.1	14.57	0.76	5.22
7	H5N5F1	7.66	2.47	0.02	0.86	2489.2	2.22	0.54	24.35
8	H5N4S1	7.99	2.12	0.06	2.92	2431.2	3.98	0.21	5.28
9	H5N4F1S1	8.34	10.91	0.03	0.32	2605.3	8.07	0.33	4.09
10	H5N5F1S1	8.58	2.65	0.02	0.63	2850.4	3.55	0.55	15.48
11	H5N4S2	8.98	1.39	0.08	5.66	2792.3	2.42	0.68	28.14
12	H5N4F1S2	9.3	3.74	0.02	0.66	2966.4	3.03	0.81	26.74
13	H5N5F1S2	9.44	4.05	0.10	2.55	3211.5	2.78	0.91	32.68

Table S-2: Glycan composition, RIs, SDs and CVs for 6 human IgG N-glycan samples that were permethylated and analyzed on the same day using MALDI-TOF-MS to assess intraday variability.

MALDI-TOF-MS of permethylated human IgG				
Glycan composition	$[M+Na]^+$ *m/z*	Relative intensity (%)	SD	CV
H3N4F1	1835.9	17.83	0.98	5.49
H4N4F1	2040.0	28.64	1.53	5.33
H3N5F1	2081.1	4.22	0.91	21.62
H5N4F1	2244.1	13.39	0.67	5.04
H4N5F1	2285.1	5.83	0.65	11.16
H5N4S1	2431.2	3.49	0.53	15.28
H4N4F1S1	2401.2	3.09	0.43	14.00
H5N5F1	2489.2	1.74	0.35	20.35
H5N4F1S1	2605.3	9.08	0.71	7.85
H5N4S2	2792.3	3.23	0.45	13.85
H5N5F1S1	2850.4	2.65	0.58	21.80
H5N4F1S2	2966.4	3.48	0.51	14.78
H5N5F1S2	3211.5	3.32	0.34	10.17

A

MALDI-TOF-MS of permethylated human IgG				
Glycan composition	$[M+Na]^+$ *m/z*	Relative intensity (%)	SD	CV
H3N4F1	1835.9	18.02	2.55	14.16
H4N4F1	2040.0	28.63	4.03	14.08
H3N5F1	2081.1	4.48	2.11	47.03
H5N4F1	2244.1	13.68	1.56	11.40
H4N5F1	2285.1	6.19	2.57	41.53
H5N4S1	2431.2	3.33	1.57	47.14
H4N4F1S1	2401.2	3.14	1.75	55.55
H5N5F1	2489.2	1.91	1.25	65.45
H5N4F1S1	2605.3	8.12	2.06	25.42
H5N4S2	2792.3	3.74	2.45	65.40
H5N5F1S1	2850.4	2.36	1.14	48.13
H5N4F1S2	2966.4	3.10	1.68	54.09
H5N5F1S2	3211.5	3.30	1.54	46.47

B

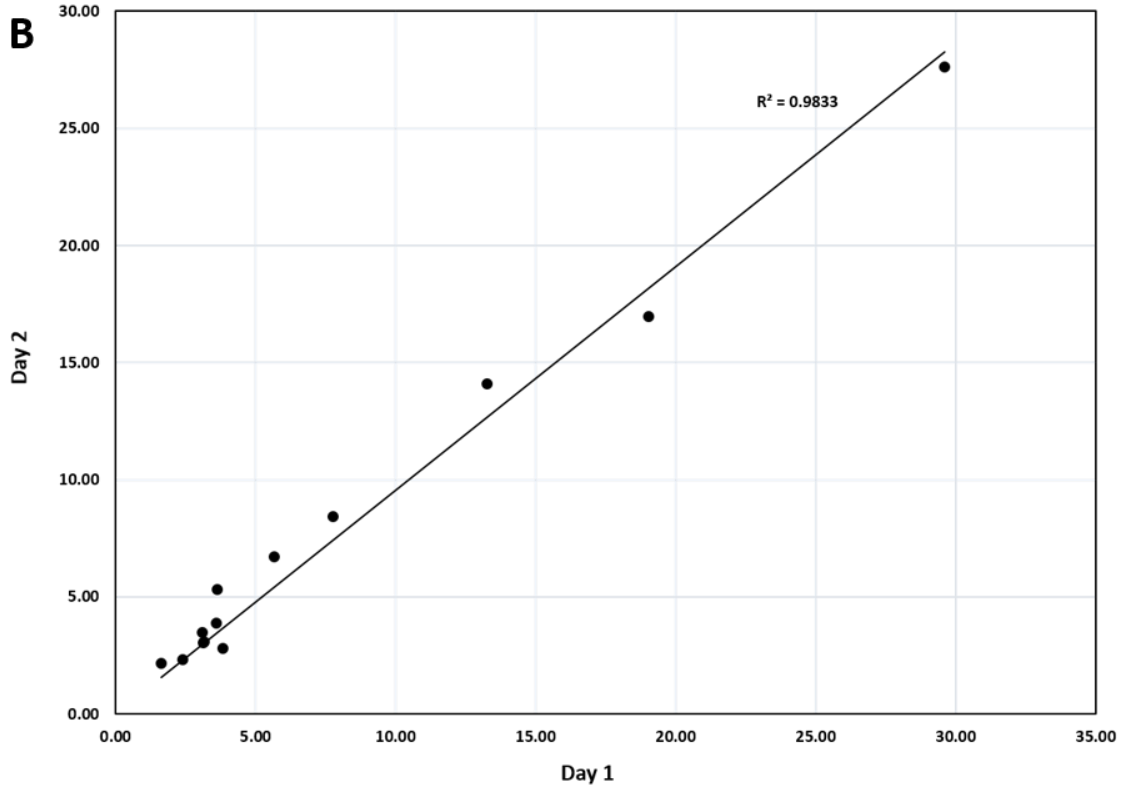


Figure S-5: (A) Table with RIs, SDs, CVs of N-Glycans from an average of 20 human-IgG samples that were permethylated on two different days (10 samples on day 1 compared to 10 samples from day 2). (B) Linear regression plot comparing the log-average relative intensity values for 20 human IgG samples (day 1 versus day 2). The R^2 value = 0.98, which is a value close to 1 and therefore shows a good correlation and a low variation between the two data sets.

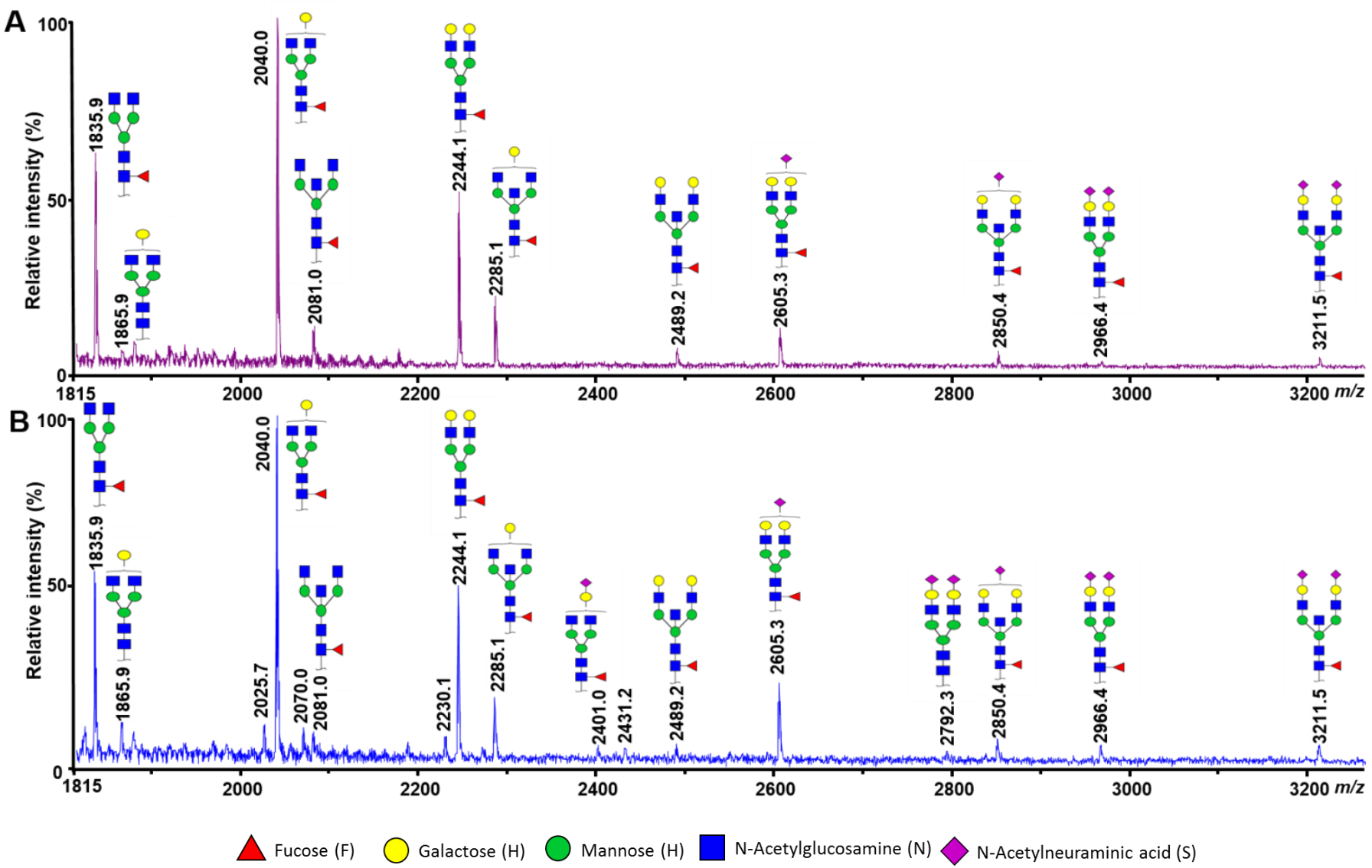


Figure S-6: MALDI-TOF-MS spectra of permethylated N-glycans from human IgG. (A) In-solution permethylation was performed manually which included longer sample preparation times along with labor intensive and repetitive extraction steps. (B) Automated high throughput permethylation was performed on the liquid handling robot eliminating manual extraction steps and faster processing times. The automated method provides comparable results to the manual in-solution method. Structures for glycans are depicted following the CFG notation: *N*-acetylglucosamine (blue square), fucose (red triangle), mannose (green circle), galactose (yellow circle), and *N*-acetylneuraminic acid (purple diamond).

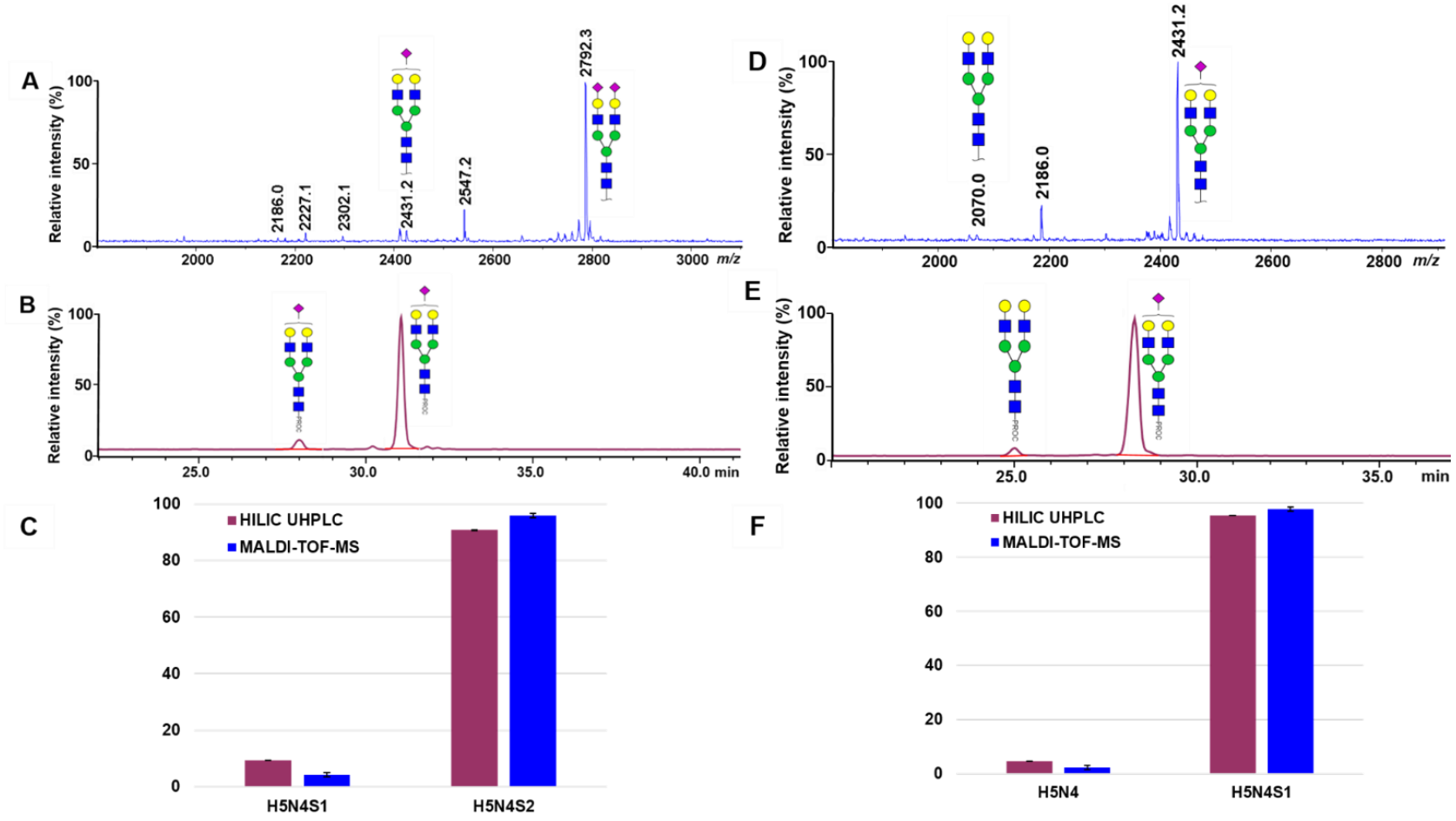


Figure S-7: Comparison of relative intensities of N-linked glycans from A2G2S2 (A, B and C) and A2G2S1 (D, E and F) glycan standards. (A) MALDI-TOF-MS spectrum after permethylation of A2G2S2 glycan standard. (B) HILIC UHPLC chromatogram with fluorescence detection after procainamide labeling of A2G2S2 standard. (C) Histogram comparing the relative peak intensities of biantennary, monosialylated structure (H5N4S1) and biantennary, disialylated structure (H5N4S2) using MALDI-TOF-MS and HILIC UHPLC after triplicate analysis. (D) MALDI-TOF-MS spectrum after permethylation of A2G2S1,N-glycan standard, (E) HILIC UHPLC chromatogram with fluorescence detection after procainamide labeling of A2G2S1 glycan standard. (F) Histogram comparing the relative peak intensities of biantennary, galactosylated asialylated structure (H5N4) and biantennary, monosialylated structure (H5N4S1) using MALDI-TOF-MS and HILIC UHPLC after triplicate analysis. Samples were permethylated and procainamide labeled using the liquid handling robot.

Note: Peaks with *m/z* value 2547.2 and 2186.0 are speculated to be a possible artefact due to PNGase A digestion / side reaction not identified in the UHPLC trace.

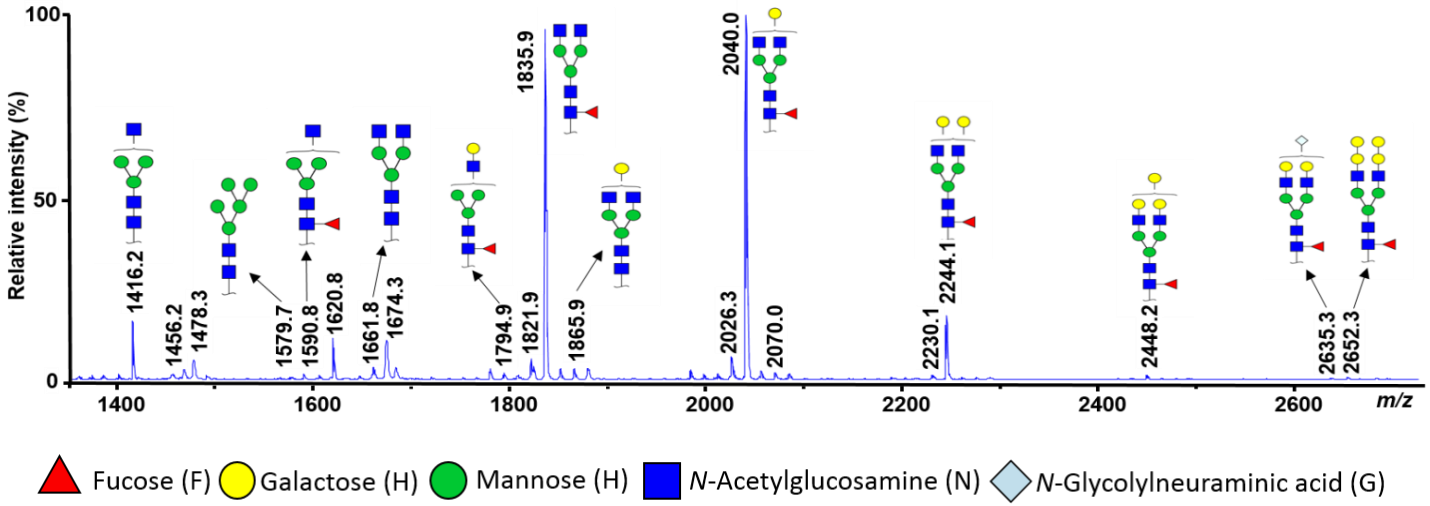


Figure S-8A: MALDI-TOF-MS spectrum of IgG1 mAb N-glycans permethylated on the liquid handling robot. The N-glycan structures in the spectrum were established and peak assignments were confirmed through data obtained from procainamide labeling and exoglycosidase digestion of the mAb.

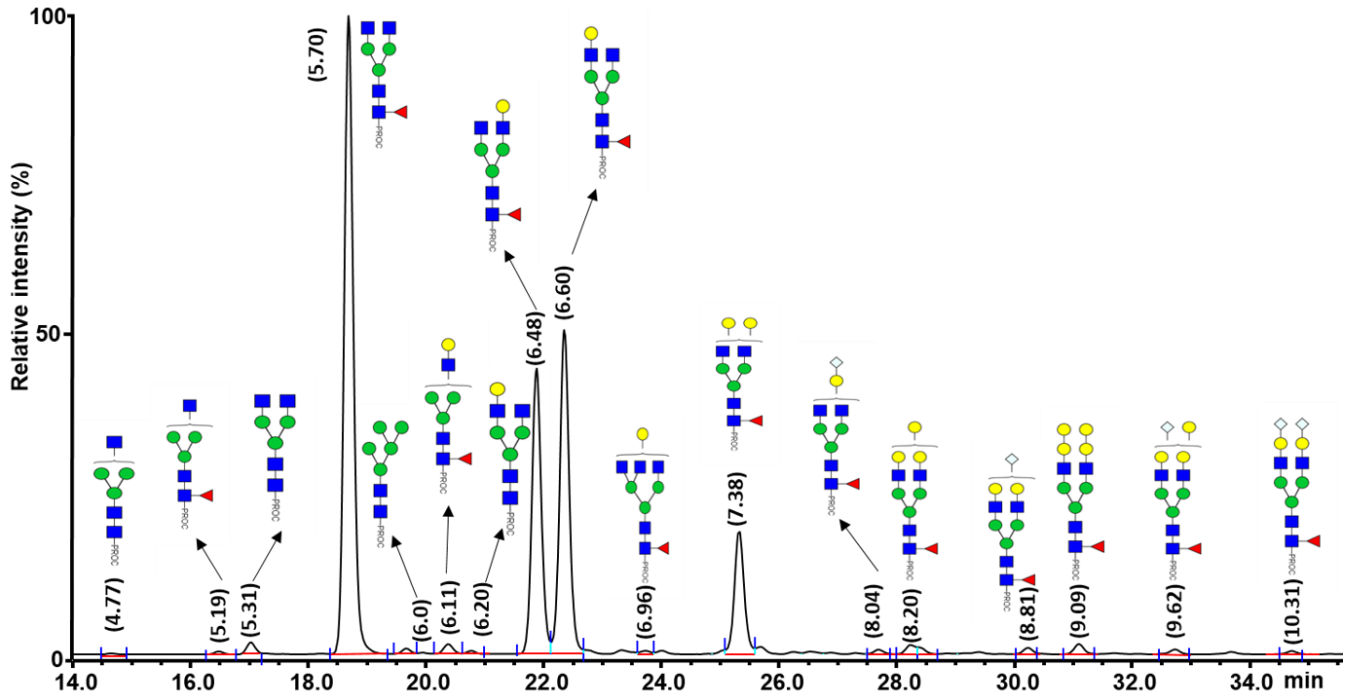


Figure S-8B: UHPLC profile of procainamide labeled IgG1 N-glycans . (Note: Measuring 96 samples on UHPLC takes a minimum of 48 hours).

Table S-3: Bioreactor culture condition, glycan compositions, RIs, SDs, CVs for 2-AB labeled (HILIC UHPLC) data and permethylated (MALDI-TOF-MS) data of N-glycans from IgG4 mAbs.

Culture condition	Glycan composition	2-AB labeled HILIC UHPLC			Permethylated MALDI-TOF-MS		
		Relative intensity (%)	SD	CV	Relative intensity (%)	SD	CV
DGS	H3N4F1	51.6	0.67	1.31	49.8	2.39	4.80
	H4N4F1	38.0	0.84	2.22	41.5	1.45	3.50
	H5N4F1	10.3	0.17	1.71	8.6	0.94	10.82
SMA	H3N4F1	52.7	0.22	0.42	51.9	1.05	2.02
	H4N4F1	36.3	0.30	0.83	38.6	1.11	2.88
	H5N4F1	10.7	0.09	0.88	9.5	0.37	4.00
SCC	H3N4F1	55.0	0.17	0.30	54.3	1.16	2.14
	H4N4F1	37.7	0.14	0.40	39.2	0.94	2.40
	H5N4F1	7.3	0.06	0.88	6.4	0.41	6.43
HCC	H3N4F1	60.0	0.12	0.21	61.0	2.77	4.54
	H4N4F1	33.7	0.15	0.44	33.0	1.76	5.36
	H5N4F1	6.3	0.03	0.56	6.0	1.08	18.25
CTC	H3N4F1	63.3	0.13	0.20	65.1	0.76	1.17
	H4N4F1	31.3	0.08	0.25	30.5	0.55	1.80
	H5N4F1	5.2	0.04	0.90	4.4	0.24	5.50

Key to bioreactor conditions: DGS - Direct Gas Sparging; SMA - Silicone Membrane Aeration; SCC - Standard Culture Condition; HCC - Hypothermic Culture Condition; CTC - Control Temperature Condition.

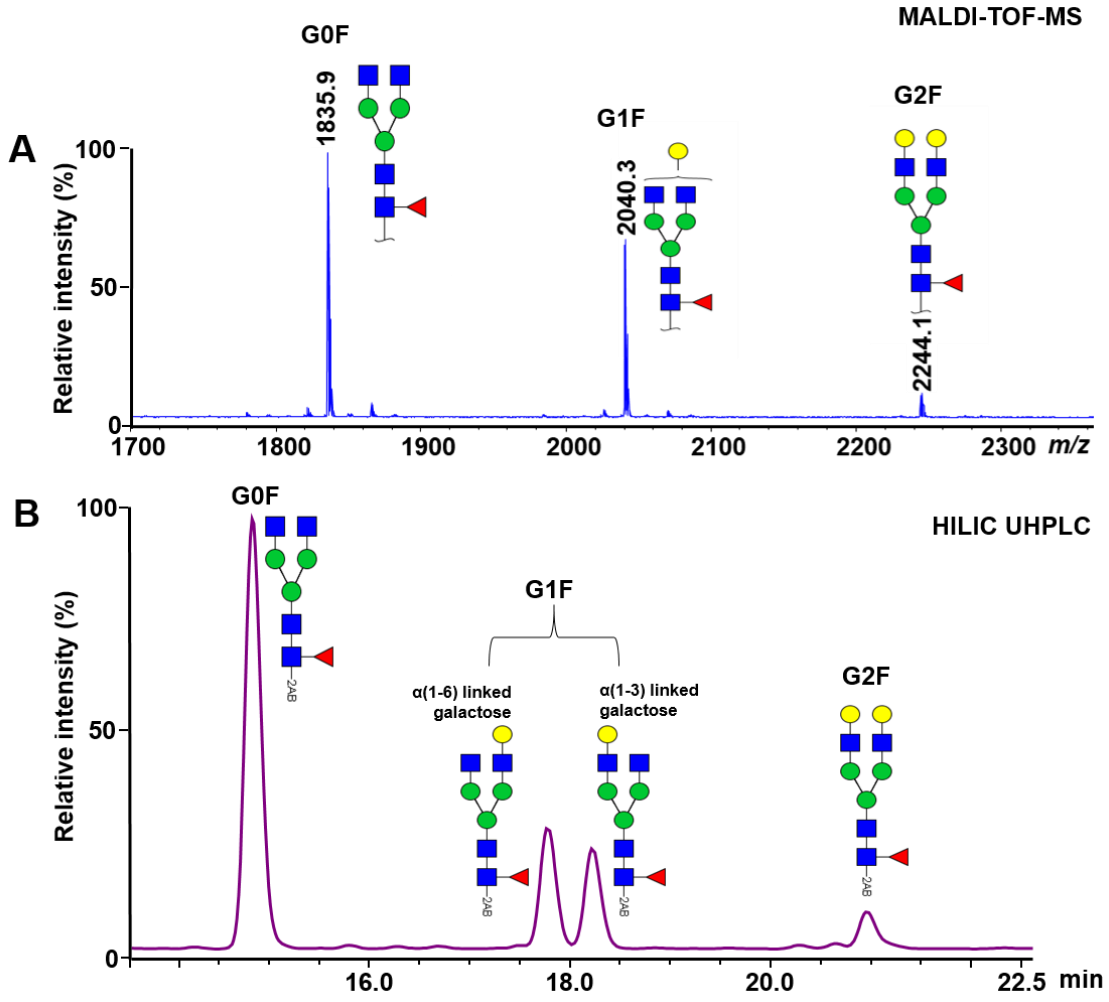


Figure S-9: Typical N-glycan trace from IgG4 mAb grown in stirred tank bioreactor with varied culture conditions (A) MALDI-TOF-MS analysis of IgG4 mAb after automated permethylation (B) HILIC UHPLC chromatogram of IgG4 mAb after automated 2-AB labeling. The isobaric G1F structures are not distinguished in the simple MALDI-TOF-MS profile while the HILIC UHPLC chromatogram clearly depicts different structural distribution of galactose.

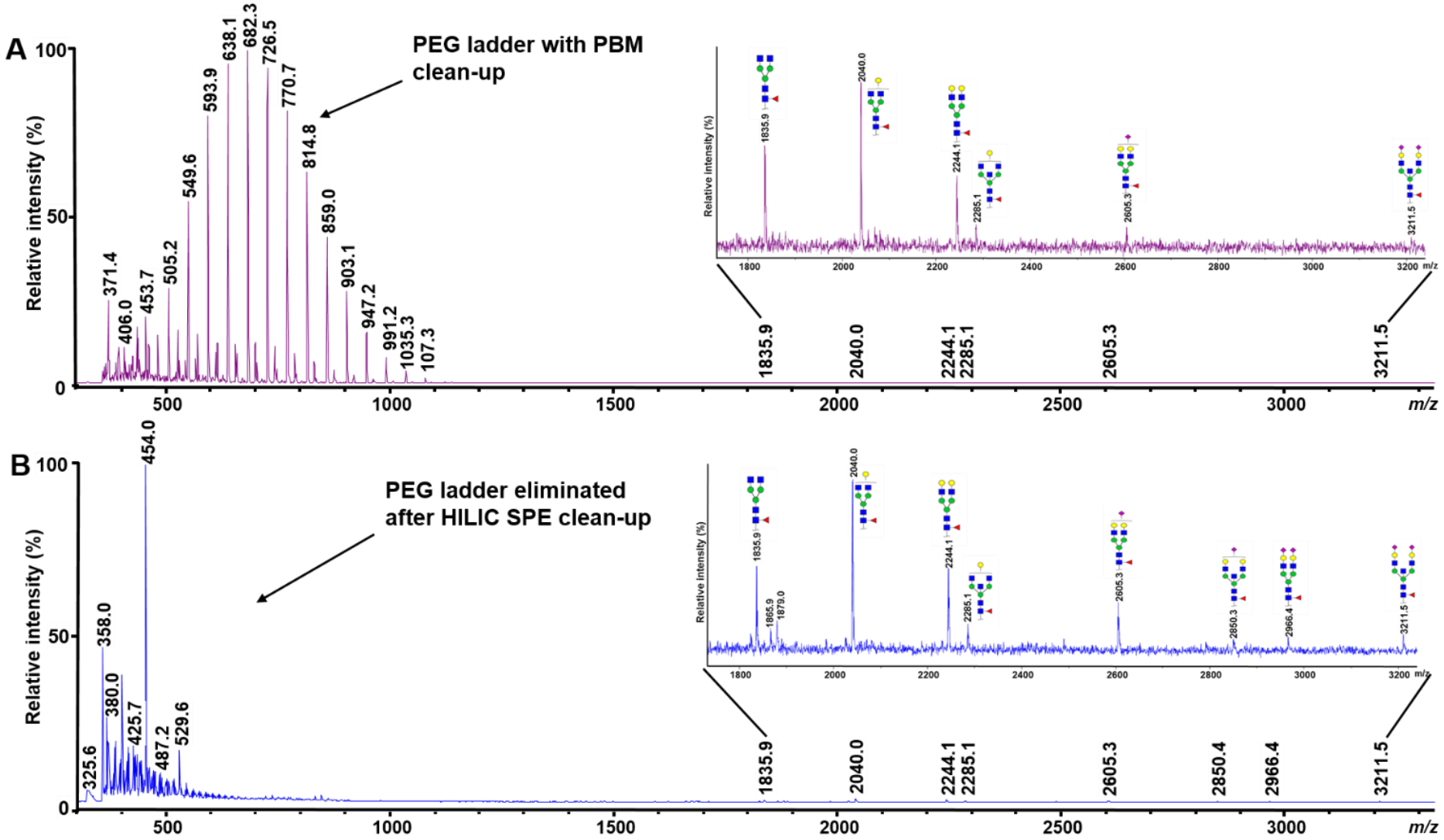


Figure S-10: Comparison of PNGase F released human IgG N-glycans with protein binding membrane (PBM) clean-up and with Hydrophilic Liquid Interaction Chromatography- Solid Phase extraction (HILIC SPE) clean-up with Y axis normalized. (A) Permethylated MALDI-TOF-MS of human IgG N-glycans after automated enzymatic release and PBM clean-up, showing a large polyethylene glycol (PEG) ladder. (B) Permethylated MALDI-TOF-MS of human IgG N-glycans after automated enzymatic release with HILIC SPE clean-up showing elimination of PEG ladder and salts.

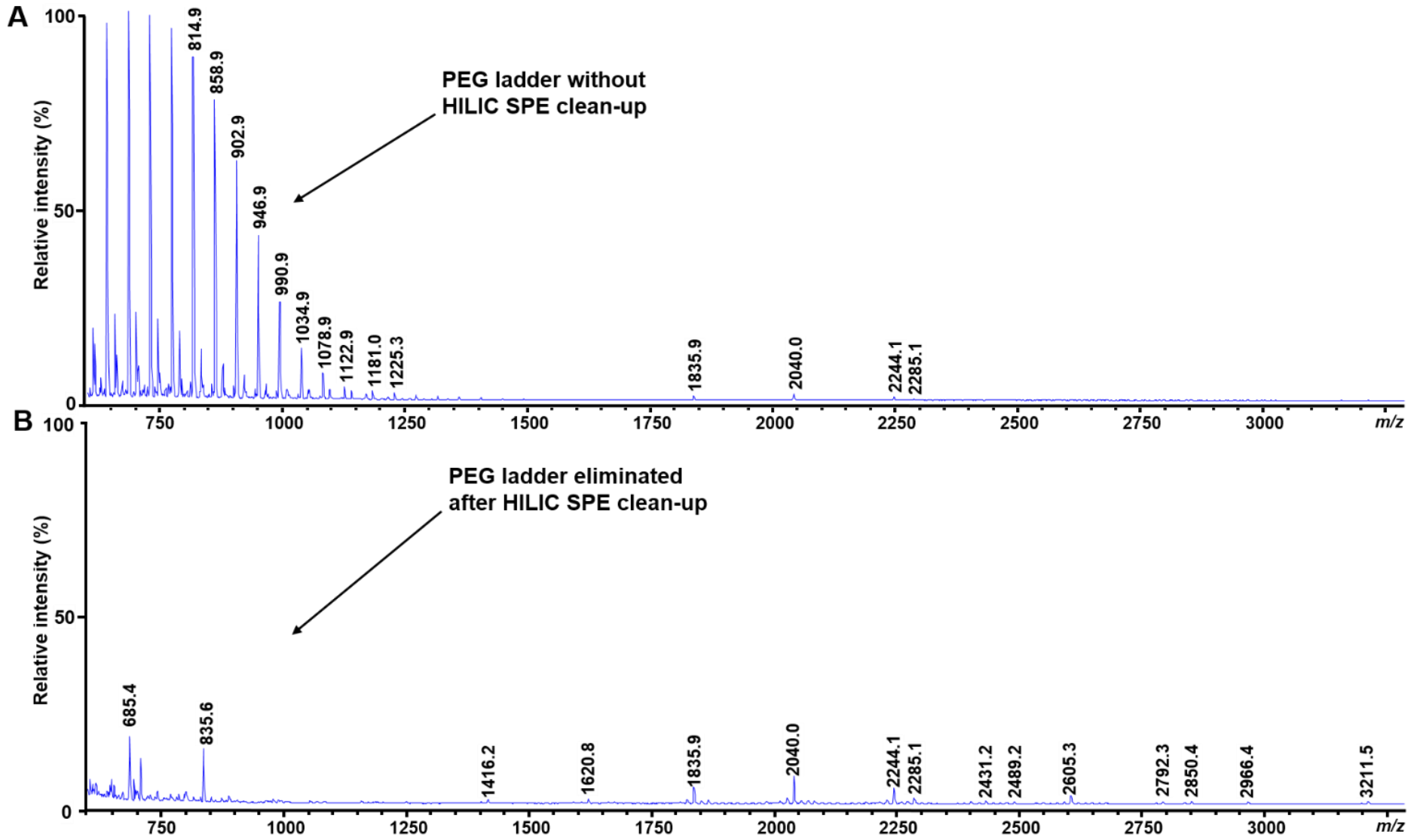


Figure S-11: Comparison of PNGase F released human IgG N-glycans with and without Hydrophilic Liquid Interaction Chromatography-Solid Phase extraction (HILIC SPE) clean-up where Y axis is normalized. (A) Permethylated MALDI-TOF-MS of human IgG N-glycans after automated enzymatic release and no HILIC SPE clean-up, showing a large polyethylene glycol (PEG) ladder. (B) Permethylated MALDI-TOF-MS of human IgG N-glycans after automated enzymatic release with HILIC SPE clean-up, showing elimination of PEG ladder and salts.

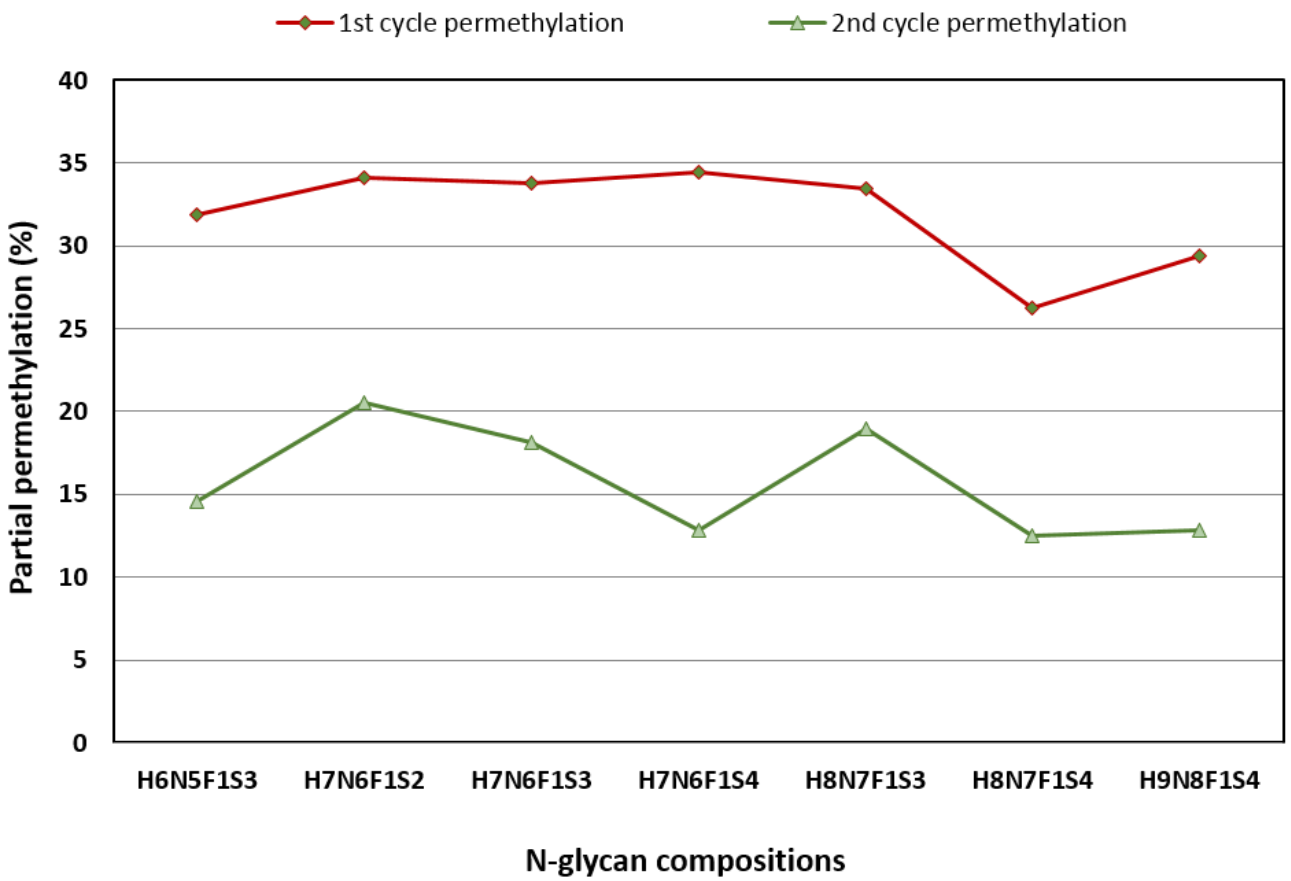
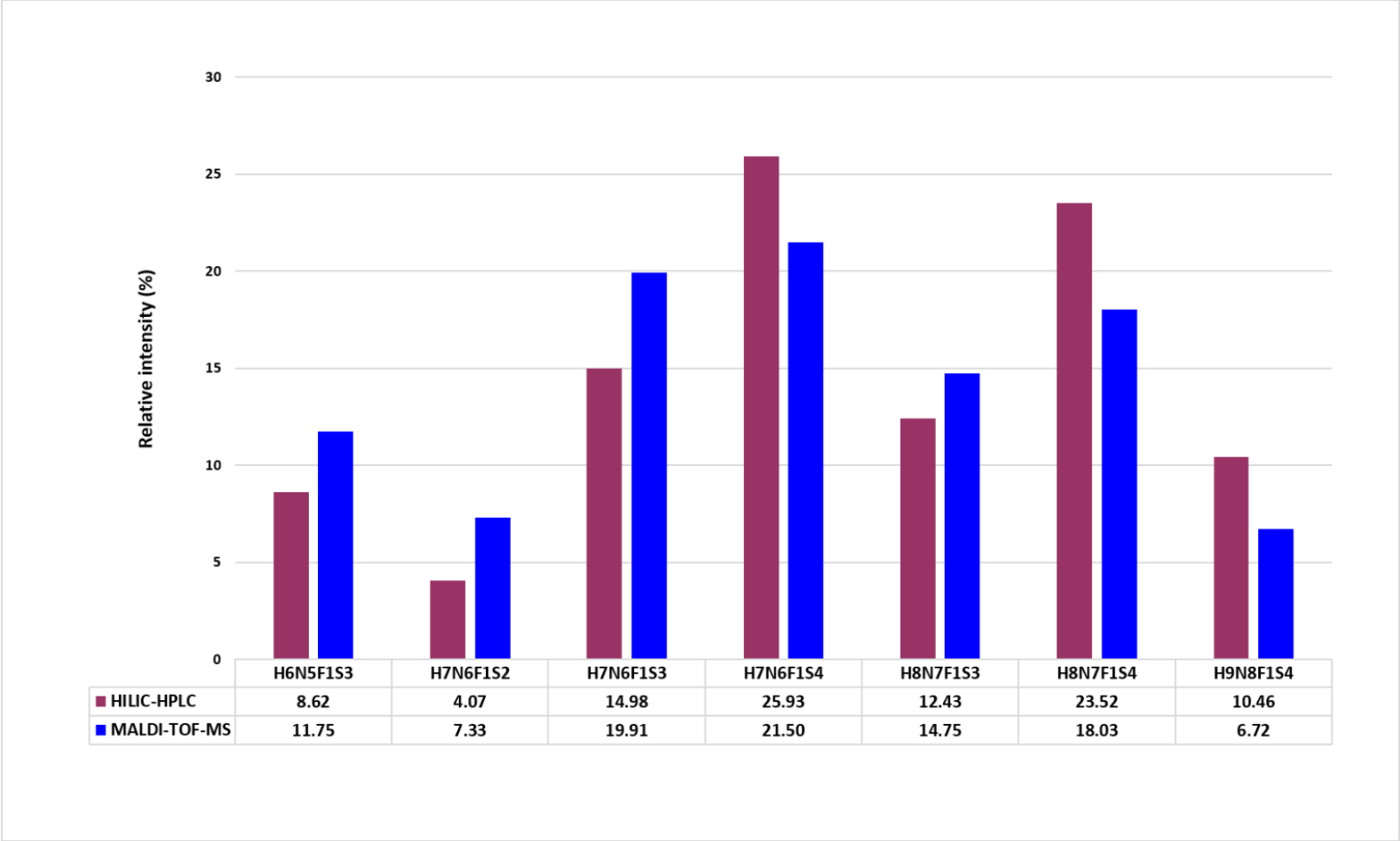


Figure S-12: Efficiency of automated HT permethylation as a function of reduction of partial permethylation after two cycles of derivatization for N-glycans derived from rhEPO.



	H6N5F1S3	H7N6F1S2	H7N6F1S3	H7N6F1S4	H8N7F1S3	H8N7F1S4	H9N8F1S4
HILIC-HPLC	8.62	4.07	14.98	25.93	12.43	23.52	10.46
MALDI-TOF-MS	11.75	7.33	19.91	21.50	14.75	18.03	6.72

Figure S-13: Histogram comparing the relative intensities of major 7 N-linked glycans from rhEPO with mauve bars representing HILIC-HPLC data and blue bars depicting MALDI-TOF-MS data.