NIST Special Publication 260-224

Certification of Standard Reference Material® 3655

Glycans in Solution (Frozen)

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National Institute of Standards and Technology
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Abstract

Standard Reference Material (SRM) 3655 is intended primarily for use as a calibration standard for the measurement of enzymatically released N-linked glycans. Applications of SRM 3655 include the benchmarking and comparability of analytical techniques, as a material for ensuring system suitability, and for analytical method validation. This material may also be used to value-assign in-house calibrators or control materials. SRM 3655 consists of thirteen (13) aqueous solutions of glycans commonly associated with monoclonal antibody therapeutics. Each solution contains a purified, free-reducing glycan at a defined mass fraction. A unit of SRM 3655 consists of thirteen 0.5 mL vials each containing approximately 0.2 mL of solution, frozen and stored at -80 °C. The tube is labeled and is sealed with a color-coded screw cap with a Teflon liner. This publication documents the production, analytical methods, measurement results, and statistical evaluations involved in characterizing these glycan materials.

Keywords

Glycans; N-linked glycosylation; monoclonal antibody therapeutics; Standard Reference Material (SRM)

Technical Information Contact for this SRM

Please address technical questions about this SRM to srms@nist.gov where they will be assigned to the appropriate Technical Project Leader responsible for support of this material. For sales and customer service inquiries, please contact srminfo@nist.gov.

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1. Purpose and Description

The quantitative and qualitative makeup of N-linked glycans associated with monoclonal antibodies is considered a critical quality attribute of the therapeutic antibody production process – an understanding of which helps ensure the desired product quality, safety, and efficacy. Generally, this analysis is completed using semi-quantitative approaches that rely on comparisons of chromatographic peak heights or peak areas for the major glycans, using a framework which is not calibrated to a known quantity (uncertain traceability chain). This approach may not account for potential biases associated with enzymatic glycan release from the protein backbone, purification through multiple stages, chemical labeling or derivatization, variable ionization efficiency (LC-MS), instrumental drift, or stability of in-house quality control (QC) materials.

NIST SRM 3655 Glycans in Solution (Frozen) is intended to support these biomanufacturing efforts by providing an International System of Units (SI)-traceable, homogeneous, and stable material that can be used to calibrate the measurement of enzymatically released N-linked glycans. This primary reference material will facilitate comparability between monoclonal antibody (mAb) products, batch releases, laboratories, analytical methods, or over time, and may be useful to value-assign in-house control materials.

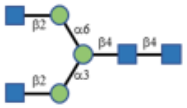
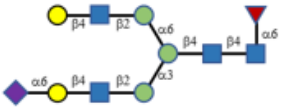
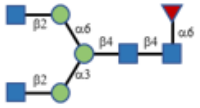
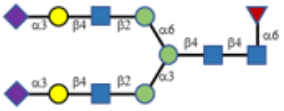
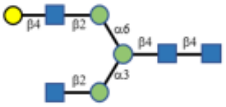
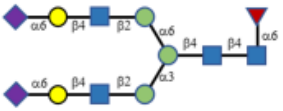
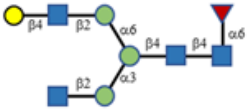
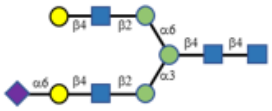
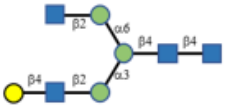
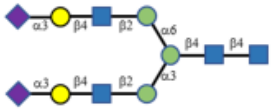
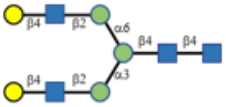
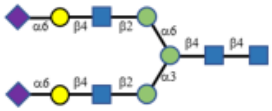
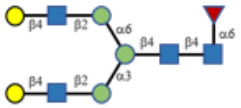
2. Background

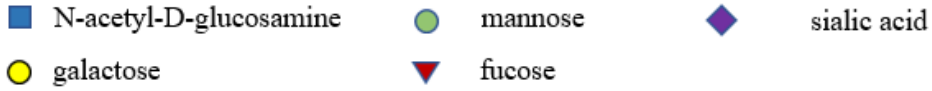
National Institute of Standards and Technology (NIST) SRM 3655 Glycans in Solution (Frozen) was developed within NIST's Material Measurement Laboratory's Biomanufacturing Initiative. This is the first SRM offered by NIST having certified, quantitative values of N-linked glycan mass fractions. SRM 3655 consists of 13 individually vialed, pure glycans in water solutions intended to support the biomanufacturing and protein therapeutics communities. This SRM is designed to facilitate measurements of released N-linked glycans with an estimate of quantitative trueness and limited bias, ensured through the material traceability to the International System of Weights and Measures (SI). It is intended to support the biopharmaceutical industry in effort to assess the consistency of the biomanufacturing process, and to provide a clearer understanding of a drug product.

3. Material Acquisition

Glycan powders were provided through a collaboration with Dr. Geert-Jan Boons and his laboratories at the Complex Carbohydrate Research Center (Univ. of Georgia) and Utrecht University (The Netherlands). The glycans for SRM 3655 were produced through chemoenzymatic synthesis and sent to NIST in purified powder form. Glycans were synthesized through a multistep chemoenzymatic approach. The details of the chemoenzymatic work (1-5) are outside the scope of this Special Publication. Aqueous solutions of each glycan were prepared at NIST gravimetrically, aliquoted, and frozen. Solutions were characterized for homogeneity, stability, and glycan mass fraction. Table 1 describes the 13 glycan structures using glycan short names and cartoon structures designated by CFG nomenclature agreement (Consortium for Functional Glycomics). Roughly 9 mg to 10 mg of powder for each glycoform was provided to NIST in several discrete tubes (corresponding to discrete syntheses). One glycan, G1aF, was provided to NIST in smaller quantity, and hence has a reduced mass fraction in the SRM unit. Lyophilized powders were shipped at room temperature and were subsequently stored in the dark at 4° C prior to bulk sample preparation.

Table 1: Thirteen glycan structures provided in SRM 3655

glycan	structure	glycan	structure
G0		G2FS1(6)	
G0F		G2FS2(3)	
G1a		G2FS2(6)	
G1aF		G2S1(6)	
G1b		G2S2(3)	
G2		G2S2(6)	
G2F			



4. SRM Preparation

Frozen, bulk glycan solutions were prepared gravimetrically from powder in pure LC-MS grade water. Solutions were removed from the -80 °C freezer and allowed to thaw individually overnight on ice for aliquoting on the subsequent day. A thawed solution was kept in an ice bath and thoroughly mixed for one hour using a magnetic stir bar prior to aliquoting. The solutions were continually mixed using a stir bar at a medium-low speed setting (3 of 10) throughout the aliquoting process. The wide-mouth mixing vessel remained capped during the aliquoting process, except when five-mL volumes were repeatedly removed for pipetting. Evaporative losses during aliquoting were kept to a minimum by constant aliquoting on-ice with capped vessels – evaporative losses were determined experimentally to be statistically negligible through gravimetric analysis. An Eppendorf repeater pipette with a 5 mL tip (Eppendorf Combitip) was used to dispense the glycan solution. Roughly 1000 aliquots of approximately 200 µL each were prepared in 0.5 mL microtubes (Sarstedt part # 72.785) and capped using one of 13 different color, PTFE, O-ring-lined screw caps (see Table 2). Aliquots were prepared 25 at a time from a filled repeater pipette and capped before beginning the next batch. Each glycan fill was completed in ≈ 1.5 h from the first to the 1000th aliquot. After all aliquots were prepared, each glycan lot was refrozen at -80 °C and stored in 81-place cardboard freezer boxes. SRM 3655 is an aqueous solution stored at < -80 °C until use.

Table 2: Color-coding of polytetrafluoroethylene (PTFE) screw caps used to identify glycan parts within a unit of SRM 3655.

Glycan	Unit Part Name	PTFE Cap Color
G0	A	light yellow
G0F	B	black
G1a	C	red
G1b	D	blue
G1aF	E	brown
G2	F	pink
G2F	G	dark yellow
G2S1(6)	H	orange
G2S2(3)	I	light green
G2S2(6)	J	dark green
G2FS1(6)	K	purple
G2FS2(3)	L	clear/colorless
G2FS2(6)	M	white

5. Certification Measurements of SRM 3655

Glycan mass fractions are traceable to amount-of-substance units of the SI (kg) based on masses of measurands, purity assessment, and appropriate uncertainties. All sample and calibrant preparations were performed gravimetrically using a calibrated microbalance under controlled temperatures and humidity. Certified values of mass fractions were determined through a combination of results from two orthogonal measurement techniques – 1) gravimetric preparation of pure compound solutions corrected through purity assessment, and 2) liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis using multiple-reaction monitoring (MRM).

Gravimetry

Gravimetric preparation of glycan powders in working solutions was completed in aluminum weigh boats and performed on a Mettler Toledo XP205 microbalance at 22 °C and 22 % relative humidity. Powders were directly transferred into high density polyethylene (HDPE), wide mouth, 500 mL mixing vessels (VWR laboratory bottles, cat. # 414004-114). Careful consideration was paid to temperature and humidity, and losses due to electrostatic effects. LC-MS grade Honeywell Chromasolv water was added gravimetrically to powders using a Mettler Toledo MS300 2TS balance. Approximately 200 mL of water was used to reconstitute each powder directly in the HDPE mixing vessels on the same day that the powder was weighed. Glycan solutions were lightly rocked end-over-end for one hour, shaken gently at room temperature for 90 minutes, and stored at -80 °C until aliquoted. Calculation of the gravimetric mass fractions was determined from the ratio of the powder mass to the total mass, after accounting for powder purity and applying an appropriate uncertainty budget.

LC-MS/MS (MRM)

Calibration of mass spectrometry-based measurements was achieved separately for each glycan using reconstituted powders that were previously value-assigned in-house by gravimetry and corrected for purity. An internal standard (raffinose) was used to normalize for analytical instrument variability, and the same internal standard solution was spiked gravimetrically into samples and calibration solutions. Although an exact-matched stable isotope-labeled (SIL) internal standard (IS) is ideal, cost and availability of SIL-IS for many of these glycoforms constrained us to use raffinose as an a non-exact-matched IS. For pure solution SRMs, quantitative bias using non-exact matched IS is relatively small as compared to matrix-based measurements. Calibration was achieved by bracketing the expected target mass fractions. MRM assays were qualified for consistency based on repeatability of the peak areas over the course of the analyses.

Samples were selected by stratified sampling from among the SRM fill lot and analyzed for homogeneity and certified for quantity. For each glycan, analysis was performed on up to five separate days using discrete calibrator preparations from stock. Each day of analysis consisted of six or seven vials selected from the SRM lot and five calibrators, all prepared gravimetrically and independently (day-to-day) and analyzed in replicate. The mid-point of the calibration range was set to be equivalent to the expected mass fraction of the SRM measurand, and the remaining calibrators were used to bracket that ratio in a linear fashion at ± 10 % and ± 20 % of the mid-point. Homogeneity was determined based on variability observed ‘between-days’ and ‘between-boxes’. In general, more variability was observed ‘between-days’ as compared to ‘between-boxes’. However, both sources of variability are statistically insignificant when combined with ‘between-method’ variability (gravimetry and LC-MS/MS). After statistical analysis was performed considering all sources of variability, the fill lot for each glycoform was

determined to be chemically homogenous. A rigorous analysis of homogeneity data (see Supplemental file) was performed by Blaza Toman of the NIST Statistical Engineering Division using Hierarchical Bayes estimation, and variability from orthogonal measurements was combined using the NIST Consensus Builder [6]. The uncertainties provided with the measured glycan certified mass fraction values are the expanded uncertainties about the mean and cover the measurand with approximately 95 % confidence, consistent with the ISO/JCGM Guide [7]. The expanded uncertainty is calculated as $U = ku_c$, where u_c is the combined uncertainty, and k is a coverage factor corresponding to approximately 95 % confidence for the analyte [8]. For the certified values shown below, $k = 2$.

For each measurand and the IS, two fragmentation transitions were monitored by MRM (Table 3). Additionally, both alpha and beta anomeric forms of each glycan were quantified. Anomers were separated chromatographically on a porous graphitized carbon (PGC) stationary phase and could be treated as unique datapoints. Peak area repeatability and peak area ratio repeatability were monitored as quality controls of the analytical platform. The coefficient of variation for peak area repeatability ranged from ≈ 1.3 % to ≈ 5.2 %, while the CV for peak area ratio repeatability ranged from ≈ 0.3 % to ≈ 1.5 % among measurements. Details of the instrumental MRM method are described below on pages 18 and 19, section 6, and in Table 3 below. Raffinose was monitored with fragmentation transitions at *m/z* 527.1→ *m/z* 365.1 and *m/z* 527.1→ *m/z* 203.0 with a collision energy voltage of 35 V. MRM dwell time was set to 200 ms, and the Fragmentor voltage was set at 380 V. Peak areas were integrated using MassHunter (Agilent) Quantitative Analysis (version 10.0) software and manually inspected. Data analysis was performed in Microsoft Excel.

Table 3 – Fragmentation parameters for MRM analysis of glycans using an Agilent 6460 QQQ-MS

glycan structure	precursor ion *(m/z)*	product ion *(m/z)*	collision energy (V)
G0	659.4	528	18
		204	20
G0F	732.5	528	12
		204	12
G1a/b	740.4	366	12
		204	12
G1aF	813.4	366	10
		204	10
G2	821.4	366	15
		204	15
G2F	894.5	366	15
		204	15
G2FS1(6)	1039.8	657	20
		366	20
G2FS2(3/6)	1185.6	1039.9	12
		657	12
G2S1(6)	967.1	657	15
		366	15
G2S2(3/6)	1112.4	657	16
		366	16

Certification

A mean mass fraction for each glycan was determined on up to five separate days, from 6 to 7 boxes, for two anomeric peaks, using two fragmentation transitions, and ratioed to two different raffinose transitions – in all up to 280 total datapoints per glycan were considered. Type B uncertainty budgets were determined for each glycan powder by considering the following sources of uncertainty: uncertainty in weighing out solids, weighing out solutions, assuming densities for calibrators and samples, estimating water impurity of calibrators, estimating chemical impurity of calibrators, peak integration, use of non-exact-matched internal standards. The total Type B budget was combined with Type A uncertainties (analytical measurement uncertainties) to estimate an overall quantitative uncertainty for each glycan from SRM 3655. Total type B uncertainties ranged from 0.5 % to 6.7 % among glycans.

A combined-method mean mass fraction was determined by consensus of the gravimetry and LC-MS/MS results. The data from both methods has been deemed acceptable for use in determining the certified values for SRM 3655. Final certified mass fraction values are provided in Table 4 as well as in the Certificate of Analysis for SRM 3655 (https://www-s.nist.gov/srmors/certificates/3655.pdf). For convenience, glycan values are provided in amount-of-substance concentrations (micromoles per liter). The amount-of-substance concentrations were calculated from the mass fraction values using the density of water at 22 °C (0.9977 g/mL ± 0.00083 g/mL) [9] and the average, relative molecular masses and uncertainties [10] of each glycan.

Table 4 – Certified Values for Glycans in SRM 3655

Glycan[(a)]	Part	Cap Color	Mass Fraction[(b)] (µg/g)	Molar concentration[(c)] (µmol/L)
G0	A	light yellow	37.1 ± 0.4	28.1 ± 0.4
G0F	B	black	40.6 ± 3.7	27.7 ± 2.5
G1a	C	red	37.7 ± 1.0	25.4 ± 0.7
G1b	D	blue	35.3 ± 1.3	23.8 ± 0.9
G1aF	E	brown	13.1 ± 0.3	8.0 ± 0.2
G2	F	pink	33.6 ± 1.5	20.4 ± 0.9
G2F	G	dark yellow	37.0 ± 4.8	20.7 ± 2.7
G2S1(6)	H	orange	31.1 ± 3.3	16.1 ± 1.7
G2S2(3)	I	light green	31.9 ± 2.5	14.3 ± 1.1
G2S2(6)	J	dark green	22.7 ± 8.5	10.2 ± 3.8
G2FS1(6)	K	purple	31.3 ± 2.3	15.0 ± 1.1
G2FS2(3)	L	clear	29.9 ± 1.8	12.6 ± 0.8
G2FS2(6)	M	white	29.3 ± 1.6	12.3 ± 0.7

[(a)] Symbol Nomenclature for Glycans (SNFG).

[(b)] The results are expressed as the certified value ± the expanded uncertainty. Each result is the combined mean of the gravimetric and the LC-MS/MS values. The uncertainty provided with each value is an expanded uncertainty about the mean to cover the measurand with approximately 95 % confidence: It expresses both the observed difference between the results from the methods and their respective uncertainties, incorporating uncertainty components for purity correction and gravimetry, consistently with the ISO/JCGM Guide and with its Supplement 1.

[(c)] The amount-of-substance concentrations (µmol/L) were obtained by multiplying the certified values in mass fraction units by the density of the water solution at 22 °C and dividing by the average, relative molecular masses with uncertainties for each of the compounds.

6. Purity analysis of calibrator powders

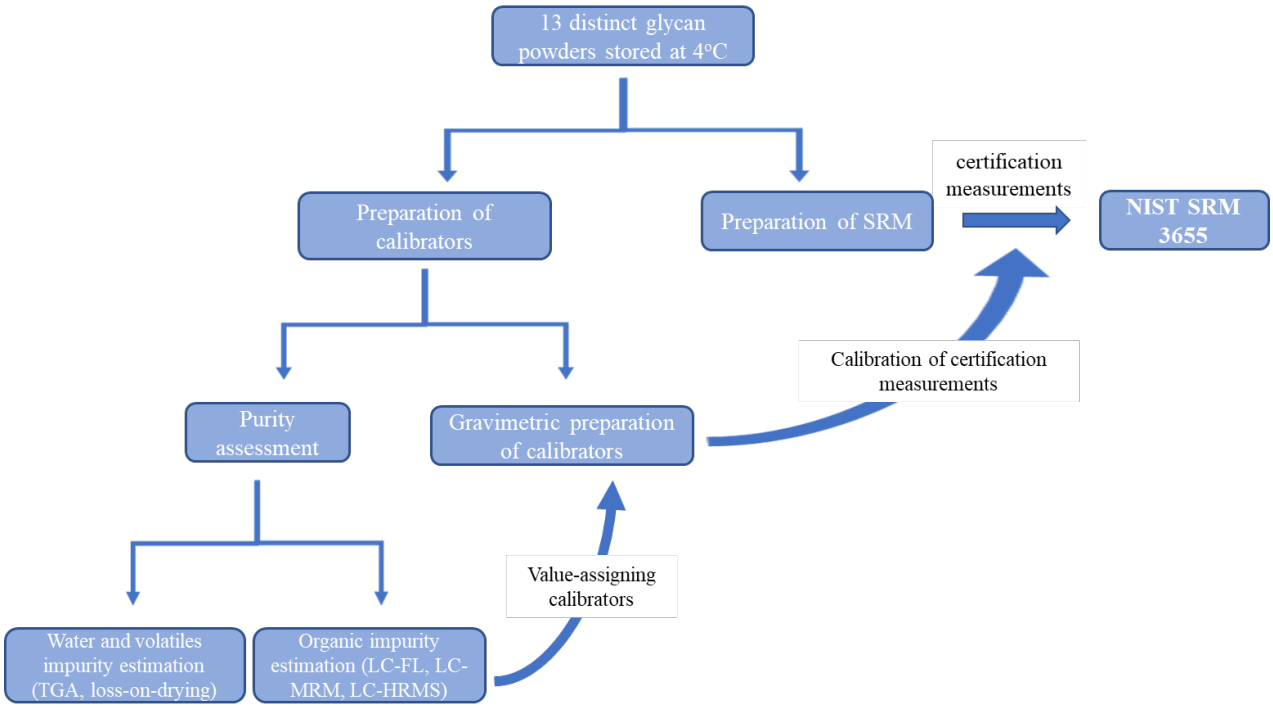
A systematic purity analysis based on mass balance was performed on a subset fraction of each glycan powder that was removed initially from the bulk material, and is independent from reconstitution of the SRM solution. Purity estimates for were extrapolated to the remaining (bulk) glycan powder. Solutions are directly traceable to the SI and were made from < 1 mg subset powders (Table 5) which were subsequently aliquoted and frozen at -80 °C. Aliquots were later selected through random sampling for use as calibrators in the certification analysis by LC-MS/MS (MRM). Remaining aliquots were stored at -80 °C for future use to calibrate long-term assessments of the glycan mass fractions, spanning the life of the SRM.

Table 5 – Gravimetric preparation of calibrator stocks from powder

glycan structure	mass powder (mg)	mass of water (g)	concentration (g/g)
G0	0.7179	5.4057	0.0001328
G0F	0.4482	4.84207	0.00009256
G1a	0.3534	4.15946	0.00008496
G1aF	0.3849	4.02532	0.00009562
G1b	0.6539	5.41656	0.0001207
G2	0.6773	5.44706	0.0001243
G2F	0.3155	3.94815	0.00007991
G2FS1(6)	0.4946	5.07536	0.00009745
G2FS2(3)	0.6238	5.13264	0.0001215
G2FS2(6)	0.3909	4.02828	0.00009704
G2S1(6)	0.909	6.31582	0.0001439
G2S2(3)	0.4747	5.03468	0.00009429
G2S2(6)	0.4173	4.22539	0.00009876

Figure 1 below illustrates the plan for preparation of SRM 3655 from powder, demonstrating how the purity analysis and calibration ties into the certification measurements.

Figure 1 – SRM 3655 preparation flowchart



Water impurity

Purity analysis included an examination of water and residual solvent impurities using thermogravimetric analysis (TGA) and loss-on-drying analysis, in addition to estimation of organic and related chemical contamination. For the TGA experiments, samples were weighed as-is, with no drying or addition of reagents on Mettler Toledo UMX5 milligram balance with a resolution of 0.1 µg, directly into the TGA crucibles. Thermogravimetric measurements were made using an Instrument Specialists Incorporated TGAi 1000. The TGA had been calibrated using a 10 mg (nominal) weight whose mass had been verified using a balance with a NIST traceable calibration. The oven of the TGA was purged with dry nitrogen gas running at a flow rate of 20 mL/min. The analysis on the TGA oven was performed from room temperature up to 160 °C at a heating rate of 10 K/min. A baseline correction was used for apparent fluctuations in weight due to buoyancy variations in the sample chamber caused by changes in the temperature of the oven and purge gas. After a smooth baseline was run, a portion of the glycan sample was weighed out into the sample crucible using an analytical microbalance with resolution of 0.1 µg. Samples were analyzed in replicate. A traditional loss-on-drying method was used to corroborate the TGA mass loss for most of the samples. Uncertainty estimates for the individual measurements were calculated using a Monte Carlo method. For any given glycan, the combined uncertainty, u_c, was determined by adding the uncertainty for each measurement and the overall standard deviation in quadrature. The expansion factor, k, was multiplied by u_c to express the expanded uncertainty, U. The average water and volatile content for the glycan powders is given in Table 6.

Table 6. Average water content of the glycan samples along with the standard deviation, the combined uncertainty (u_c), number of replicates (n), the coverage factor (k), and the expanded uncertainty (U). The combined uncertainty is calculated by adding the standard deviation and the estimates of uncertainty for each measurement in quadrature.

glycan	average water content (%)	standard deviation (%)	u_c (%)	n	k	U
G0	7.30	0.61	0.63	4	3.182	2.01
G0F	5.62	0.12	0.22	4	3.182	0.70
G1a	6.96	1.27	1.28	4	3.182	4.08
G1b	5.48	1.35	1.36	4	3.182	4.34
G2	6.35	0.27	0.32	4	3.182	1.03
G2F	6.69	1.23	1.25	3	4.303	5.36
G2FS1(6)	7.23	0.66	0.69	4	3.182	2.19
G2FS2(3)	7.50	0.35	0.37	4	3.182	1.19
G2FS2(6)	7.22	0.48	0.51	4	3.182	1.61
G2S1(6)	7.52	0.61	0.62	3	4.303	2.67
G2S2(3)	7.32	1.56	1.56	3	4.303	6.72
G2S2(3)	7.07	1.90	1.91	3	4.303	8.19

Organic impurity

Estimates of chemical impurities within the "pure" calibrator powders were determined through three orthogonal procedures: 1) fluorescence detection (LC-FL) with 2-AB labeling and quantitative impurity estimation, 2) targeted analysis of structurally similar glycan impurities by multiple-reaction monitoring using LC-MS/MS, and 3) non-targeted, high-resolution (HR) analysis of the glycan powders using liquid chromatography coupled to an Orbitrap mass spectrometer (LC-HRMS) for estimation and identification of impurity content.

1) LC-Fluorescence: For LC-FL analysis, calibrators were derivatized using 2-aminobenzamide (2-AB) according to a standard protocol, briefly described here. Five (5) mg of 2-AB powder (Aldrich, A89804) was dissolved in 100 µL of 30/70 acetic acid (99.5 %) / dimethylsulfoxide (DMSO, Sigma, D2650), and all 100 µL was added to 6 mg of sodium cyanoborohydride ($NaCNBH_3$, Aldrich, 156159). Glycan calibrators were dried in a glass autosampler insert to dryness without added heat in a speedvac (ThermoSavant SPD 1010). Ten (10) µL of the 2-AB solution was added to the dried glycans in the glass insert. Glycans were derivatized by heating in an oven at 37 °C for 16 h, and then dried to dryness in a speedvac. Derivatized glycans were reconstituted for LC-FL analysis in 100 µL of acetonitrile (ACN) without customary clean-up steps to ensure labeled impurities were retained.

Chromatographic separation was achieved on a Waters XBridge BEH amide column (2.5 µm, 2.1 x 150 mm) at a flow rate of 200 µL/min. Derivatized glycans were separated using a normal phase gradient consisting of 50 mmol/L NH_4HCO_2 (mobile phase A) and 0.1 % (v/v) formic acid in ACN (mobile phase B) as follows: starting conditions – hold at 3 % 'A' for 1 min., ramp to 50 % 'A' in 19 min., ramp to 95 % 'A' in 5 min. Gradient elution is followed by a column wash at 95 % 'A' and column re-equilibration at 3 % 'A'. Column temperature was maintained at 40 °C; autosampler plate temperature control was set at 5 °C. Glycans were detected using a fluorescence detector on a Dionex (Thermo) Ultimate 3000 stack with excitation wavelength of 320.0 nm and emission wavelength of 420.0 nm at 5 Hz. The fluorescence cell was coupled in-line with a Thermo Orbitrap Elite mass spectrometer. LC-MS/MS data was used to confirm glycan identity.

Several peaks observed in blanks and samples eluting before 15 min. or after 39 min. have been identified and attributed to column bleed, solvent background, and plasticizers. The dominant early eluting peak is due to unbound 2-AB, as anticipated. In this work, 2-AB-derivatized glycans elute prior to their non-derivatized analogs in HILIC mode. For each non-sialylated glycan, a LC-FL trace is provided (Figure 2a-g).

In general, it can be established from LC-FL analyses that glycan powders of non-sialylated glycoforms are highly pure. Glycan powders of G0, G0F, G1a, G1b, G1aF, G2, and G2F were observed to contain little to no impurities, run in triplicates. Detectable contaminants are established at low levels (< 0.1 % - 0.2 % relative abundance), based on integrated peak area ratios. In contrast, sialylated glycan powders were observed to have significant impurities due to structurally related, desialylated glycoforms (missing either one or two sialic acid residues). It is known that sialic acids may be unstable during common rapid derivatization reactions at high temperatures, and so a milder derivatization for longer time periods (37 °C) was used in this work. Stability analysis demonstrated that these sialylated glycans are stable at up to 37 °C for up to 28 days. The LC-FL data was confirmed by two other analytical techniques, as described below. Figures 3a-g show representative LC-FL traces for each sialylated glycoform. Fluorescence detection was considered as the primary analytical method used to estimate structurally similar, de-sialylated impurities in the glycan calibrators. Fluorescence signal from 2-AB derivatized glycans is expected to have lower quantitative bias when compared to relative peak area ratios from ESI-MS, where signal intensity is contingent on ionizability and fragmentation efficiency and necessitates calibrators and internal standards. 2-AB derivatization occurs at the reducing terminus, which, being structurally identical among these glycoforms, is subjected to less quantitative bias.

Figure 2a

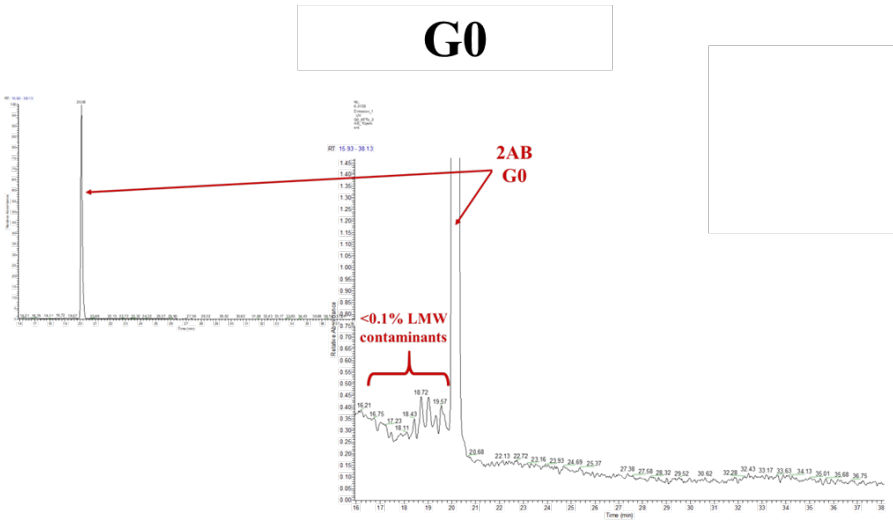


Figure 2b

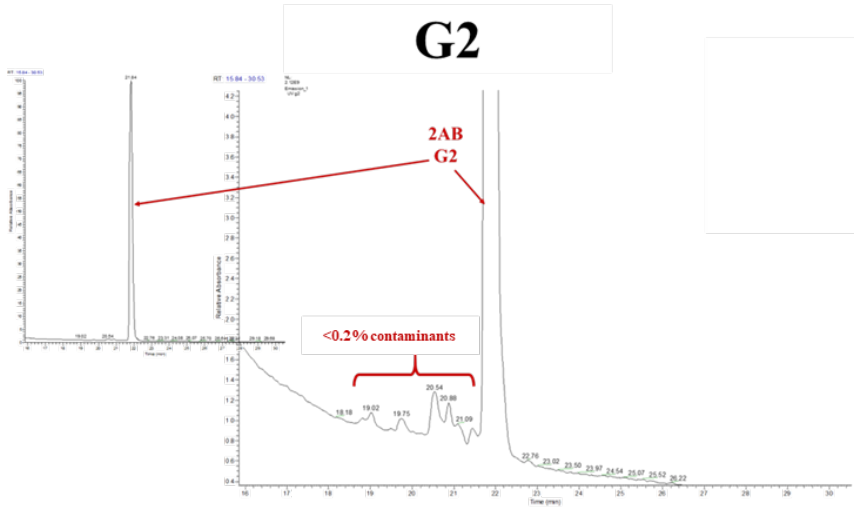


Figure 2c

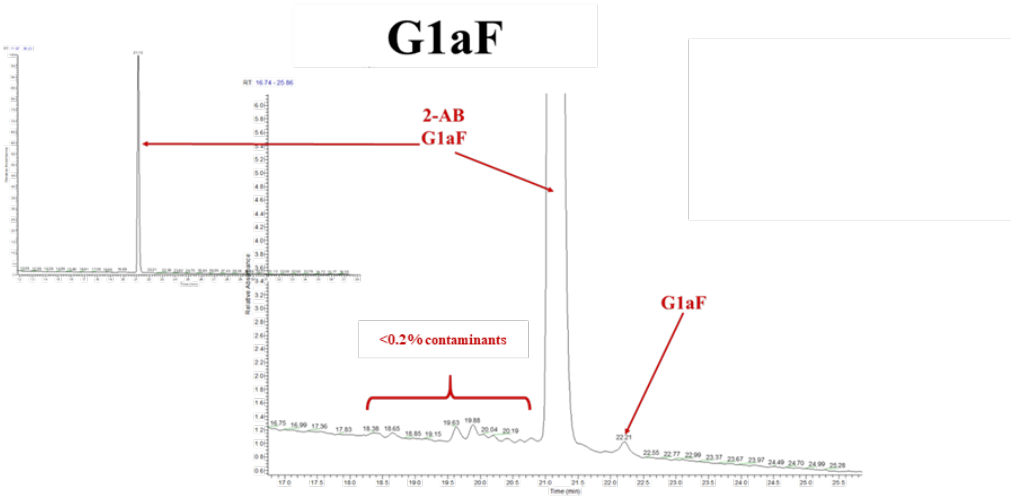


Figure 2d

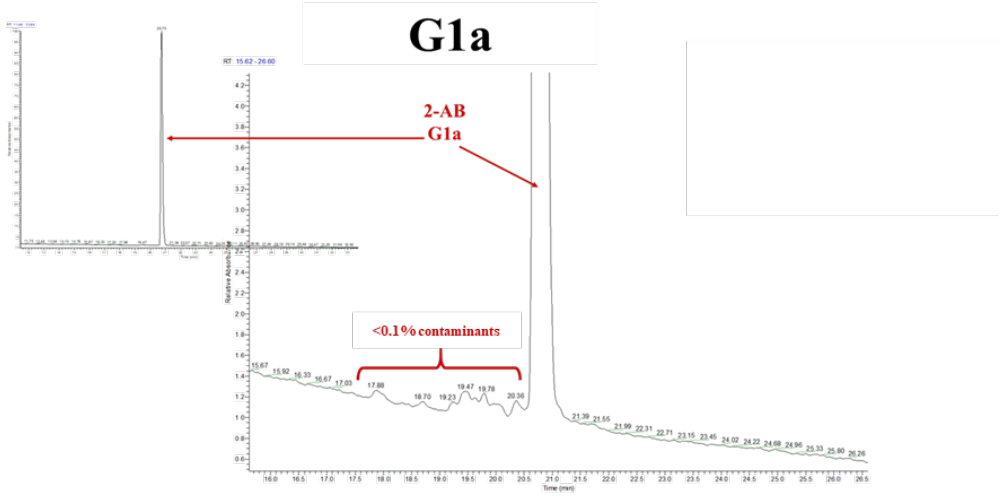


Figure 2e

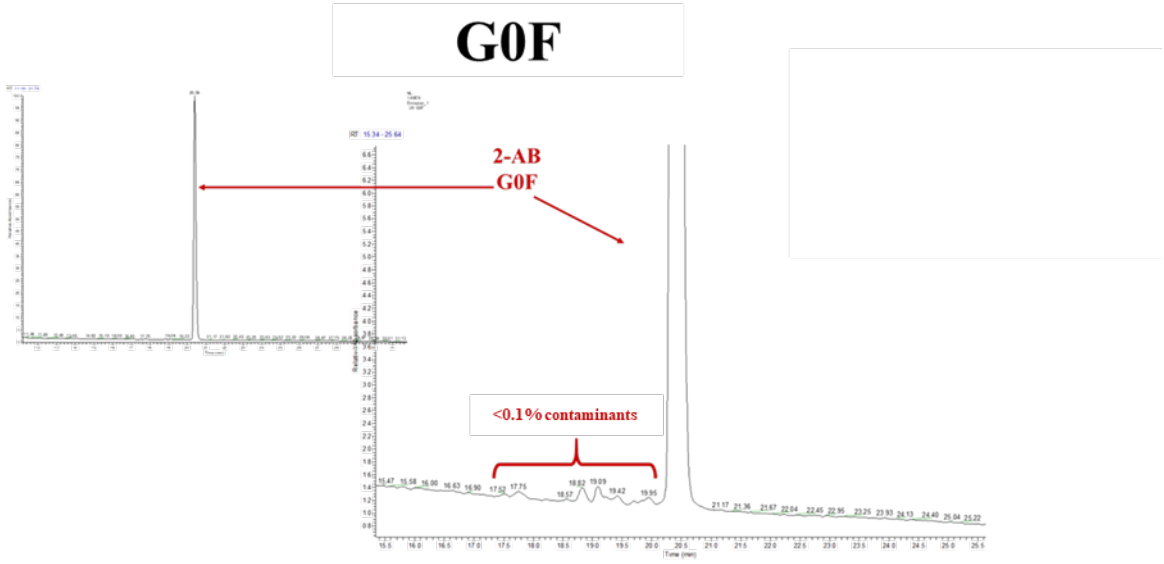


Figure 2f

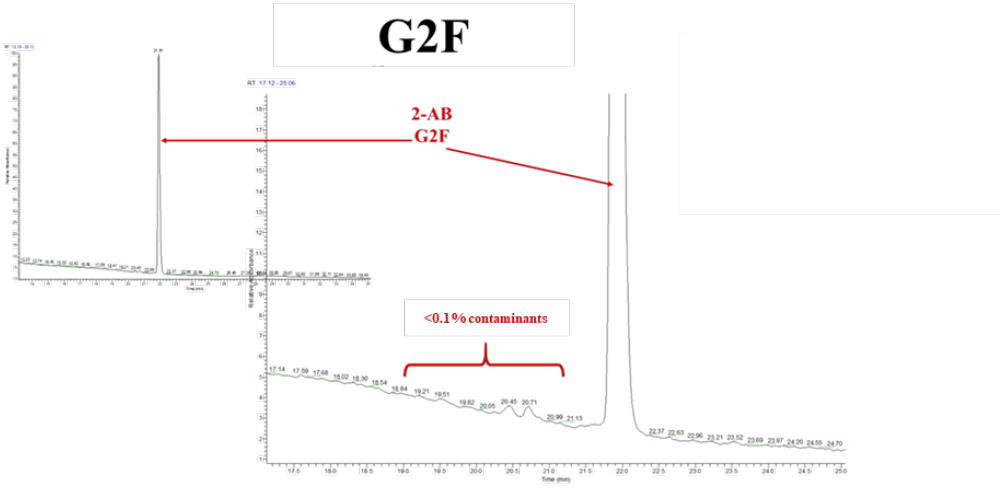


Figure 2g

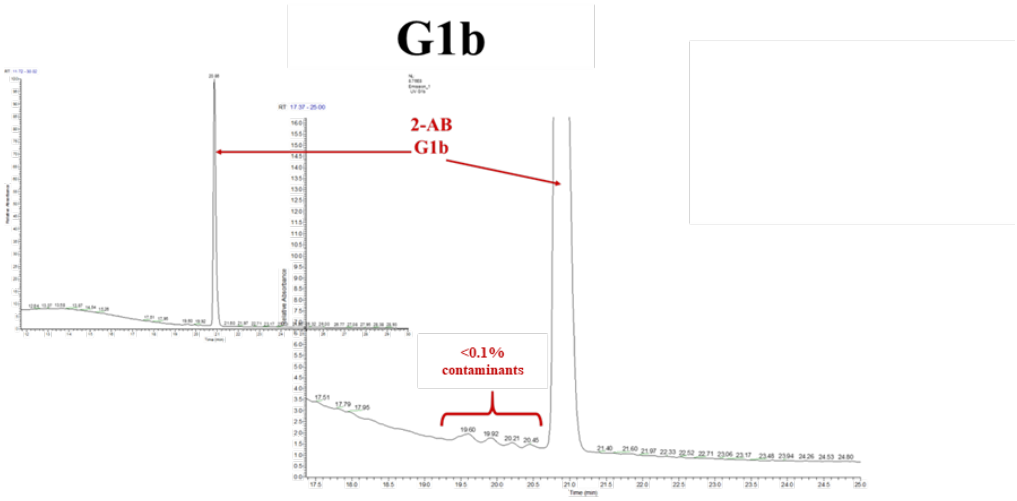


Figure 3a

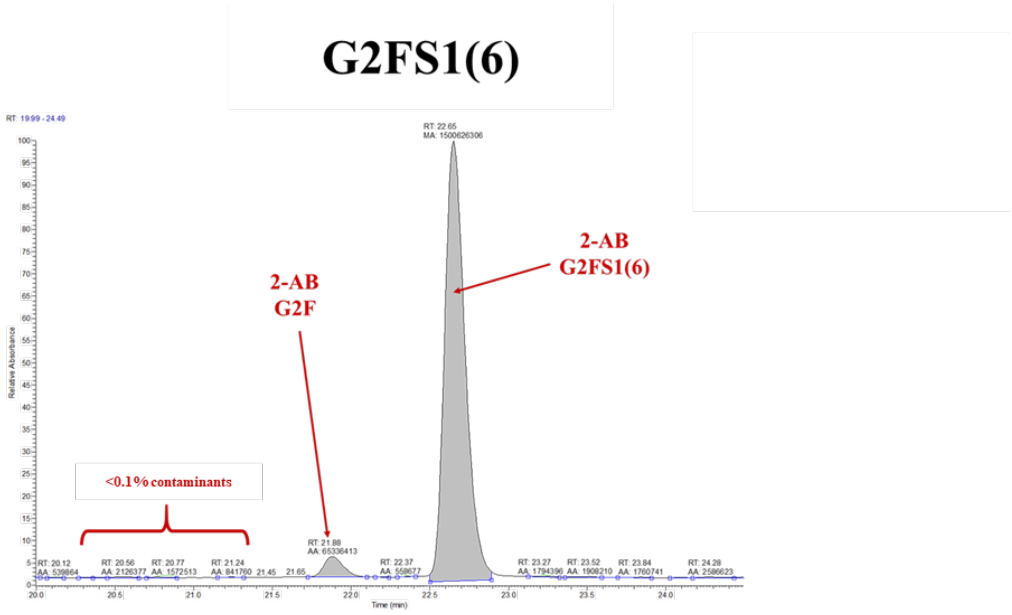


Figure 3b

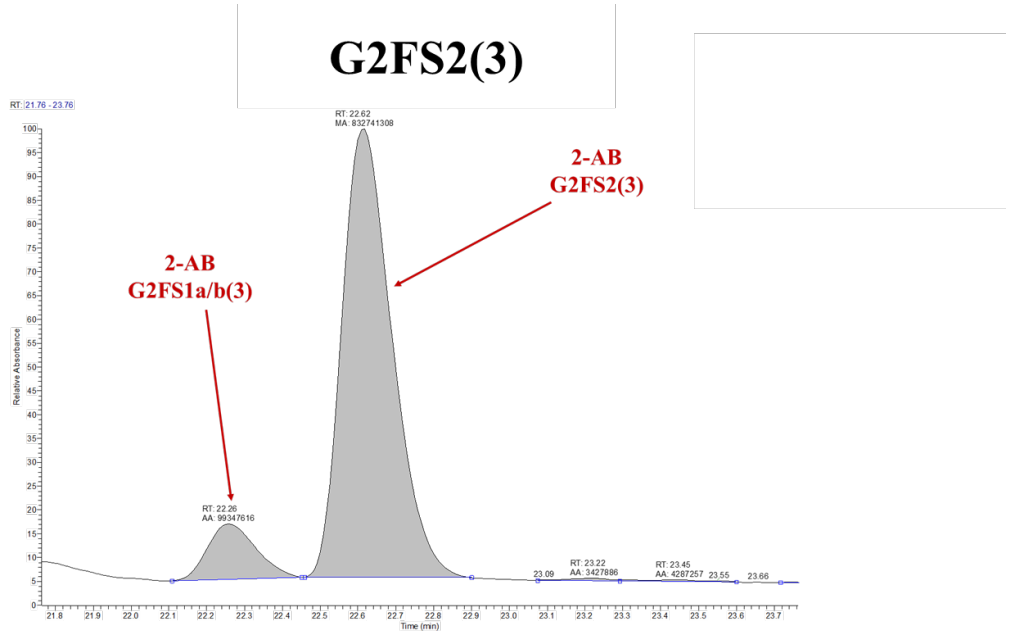


Figure 3c

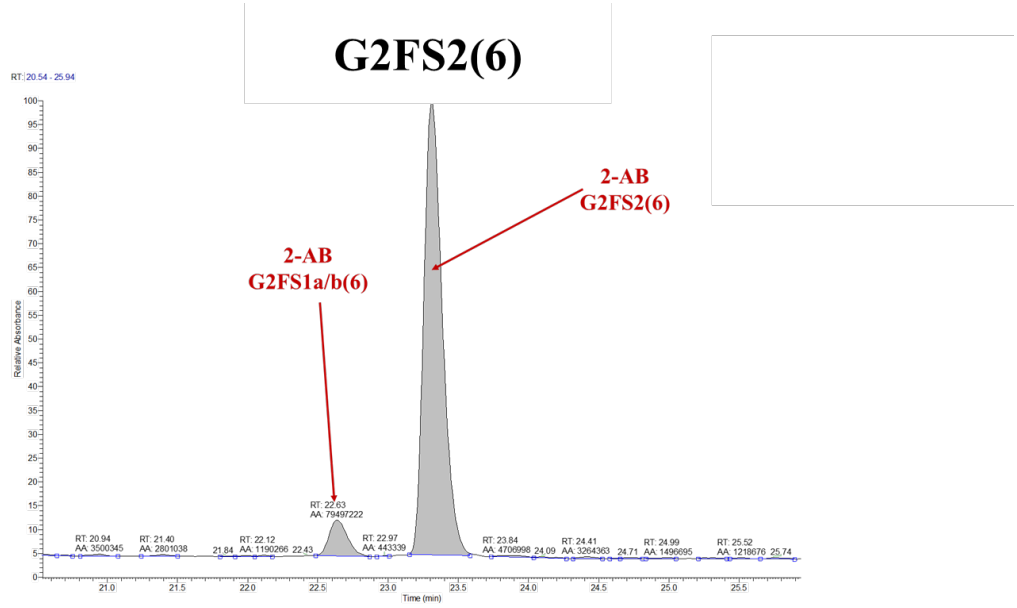


Figure 3d

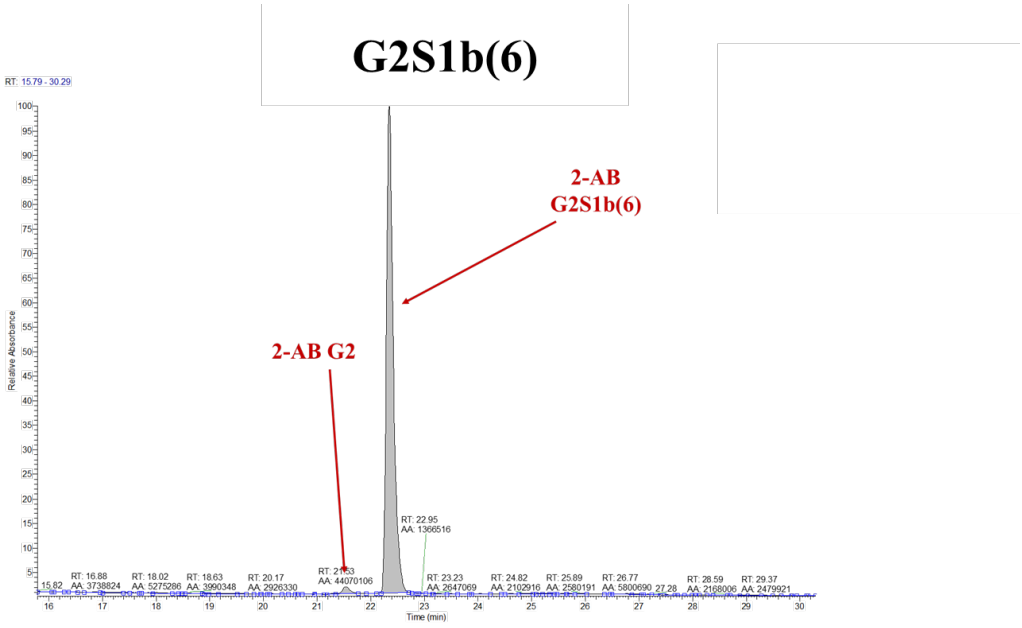


Figure 3e

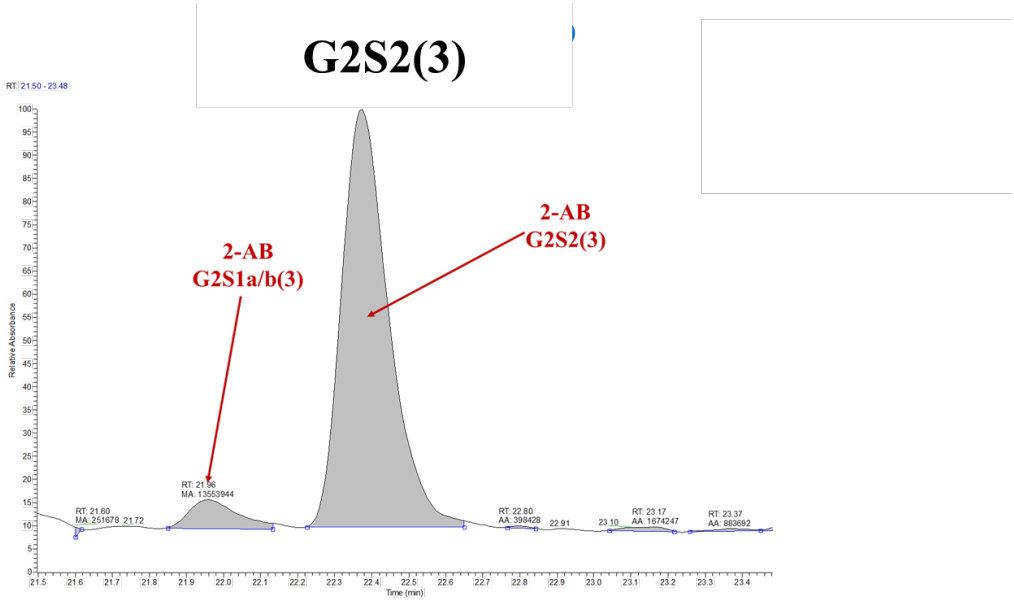


Figure 3f

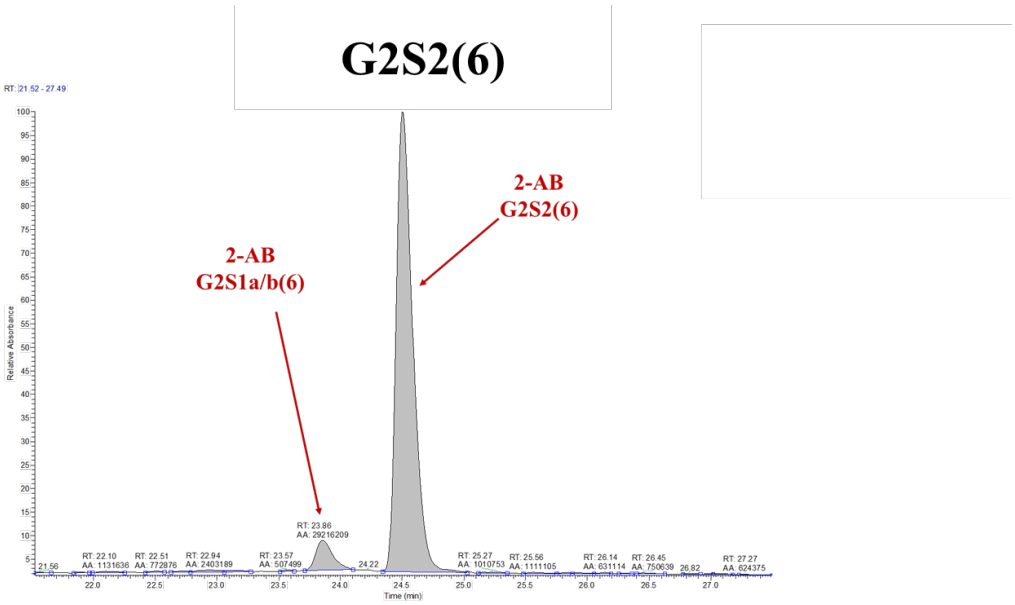


Table 7 provides an estimate for structurally-similar, desialylated contamination observed in sialylated calibrators. A weighted mean of all impurity estimates from this analysis, and others, was used in the calibrator value-assignment.

Table 7 – Impurity estimates based on peak area ratios – LC-FL data

glycan	contaminant	impurity, %
G2FS1(6)	G2F	4.2
G2FS2(3)	G2FS1a/b(3)	10.7
G2FS2(6)	G2FS1a/b(6)	7.3
G2S1(6)	G2	1.5
G2S2(3)	G2S1a/b(3)	6.7
G2S2(6)	G2S1a/b(6)	6.2

2) LC-MRM: Purity analysis by MRM was performed in triplicate for each glycan calibrator. Twenty fragmentation transitions were monitored during the assay, representing transitions from suspected or known contaminating glycoforms (Table 3). Blank injections were monitored between samples to ensure no sample carryover. Glycans are known to readily fragment in-source. Because glycans are chromatographically resolved in this work, in-source fragmentation (ISF) can be distinguished from impurities based on retention time. ISF will not affect quantification when applied through a proper calibration framework. Targeted assays using MRM-MS offer an orthogonal approach for detecting and estimating impurity content in the glycan calibrators, specifically for known impurities. Higher S/N ion chromatograms and better sensitivity are observed relative to non-targeted MS, at the expense of detecting only a subset of all conceivable compounds.

For MRM analysis, an Agilent Infinity 1290 UPLC system was coupled in-line with an Agilent 6460 triple quadrupole (QQQ) mass spectrometer with Jet Stream equipped with a standard micro-flow source. A similar chromatographic separation was applied to all LC-MS analyses. LC separation was achieved on a Hypercarb (porous graphitized carbon) analytical column (2.1 x 100 mm, 3 µm particles; part #35003-102130) at a flow rate of 200 µL/min. Glycans were separated using 50 mmol/L NH_4HCO_2 (mobile phase A) and 0.1 % (v/v) formic acid in ACN (mobile phase B) using serial isocratic and gradient elutions, as follows: starting conditions – hold at 3 % 'B' for 3 min., ramp to 8 % 'B' in 1 min., hold at 8 % 'B' for 12 min. (isocratic), ramp to 20 % 'B' in 20 min. (gradient). Gradient elution is followed by a column wash at 95 % 'B' and column re-equilibration at 3 % 'B'. Column temperature was maintained at 30 °C; autosampler plate temperature control was set at 5 °C.

Tandem mass spectrometry was performed without scheduling, in positive polarity mode using unit resolution of the $[M+2H]^{2+}$ of the precursor ions. Multiply-charged precursors allowed for QQQ-MS to be used notwithstanding a limited *m/z* scan range. Oxonium ions were determined experimentally to be

the predominant product ions formed (in every case with exception of G2FS2(3/6) which also fragmented to the +2 ion of G2FS2(3/6) – NANA + H_2O). The two most intense fragment ions for each precursor were selected for MRM. Fragmentation and ionization parameters were determined experimentally. MRM transition dwell time was set to 100 ms, and Fragmentor voltage was set to 130 V. Table 3 describes details of the MRM functions used during tandem mass spectrometry analysis. Source conditions were held constant throughout: source temperature = 200 °C, gas flow = 5 L/min, nebulizer gas = 138 kPa (20 psi), sheath gas temperature = 250 °C, sheath gas flow = 11 L/min, capillary voltage = 3500 V, nozzle voltage = 1500 V, ΔEMV = +350 V.

LC-MRM assays confirm the results determined from non-targeted LC-FL detection. Glycan calibration powders of the non-sialylated glycoforms were found to be exceptionally pure. Figure 4(a-g) details the MRM mass chromatograms characterizing non-sialylated glycoforms. G1a appears to show an inconsiderable amount (< 0.1 %) of G1aF contamination; G2 contains < 1 % G2F contamination, and G2F contains an insignificant amount (< 0.1 %) of G2 contamination. It should be noted that minor contamination of afucosylated powders (G1a or G2) with fucosylated species (G1aF or G2F) was unanticipated, and an explanation has not been identified.

Figure 4a

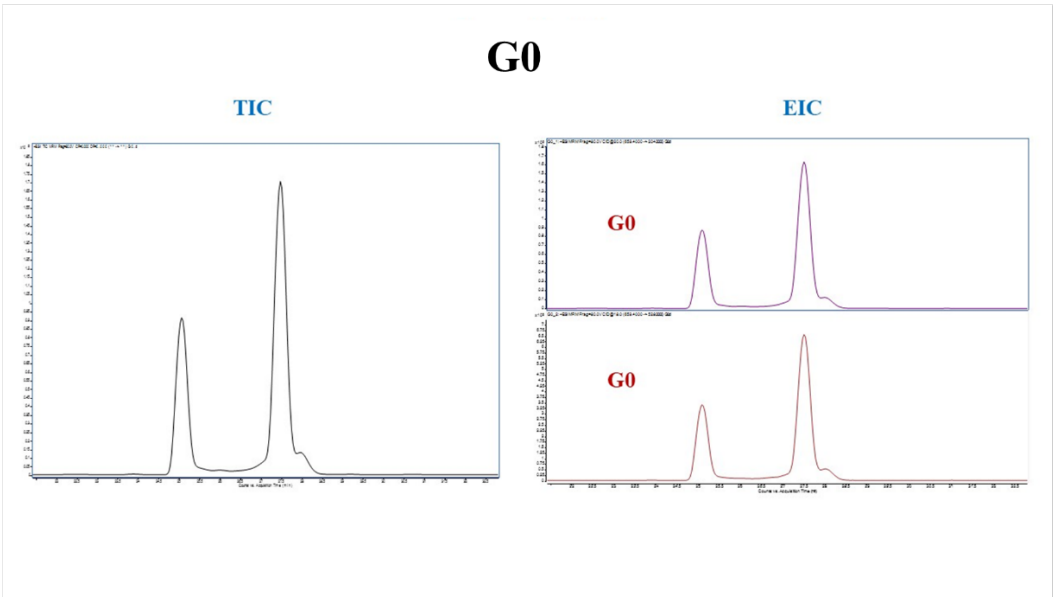


Figure 4b

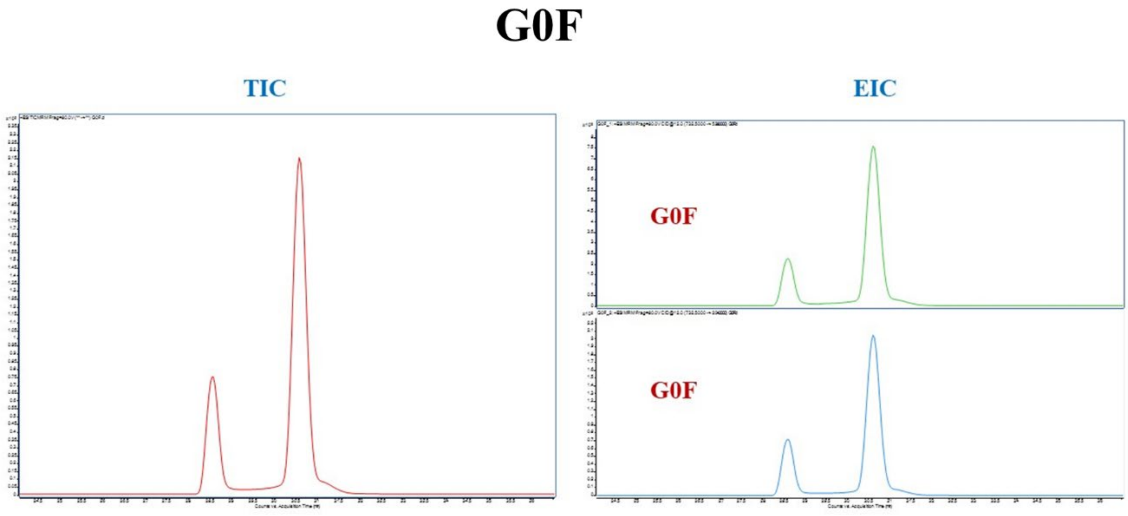


Figure 4c

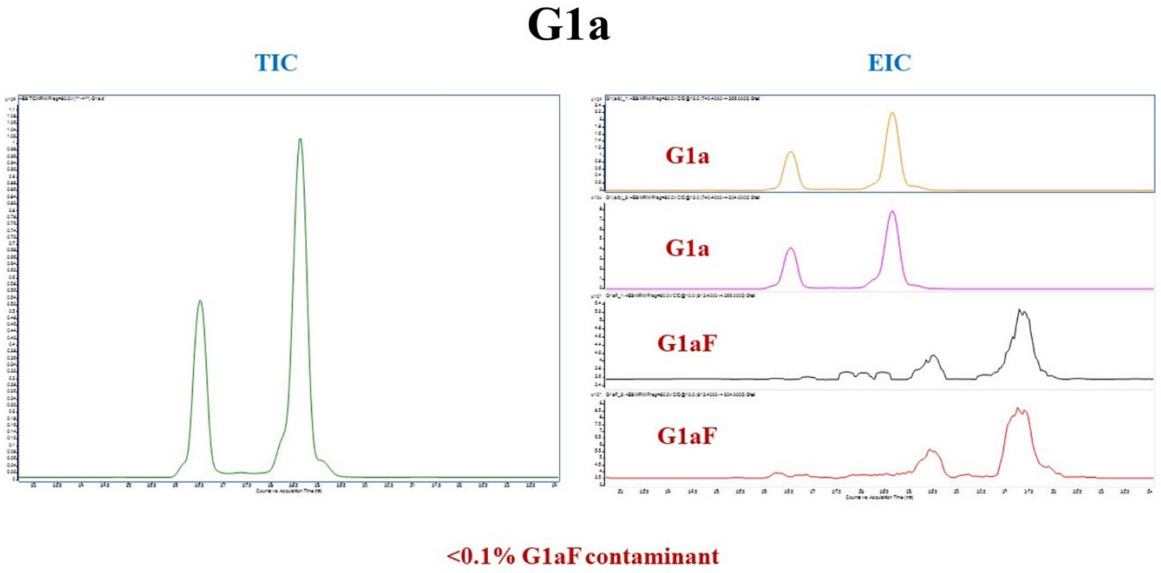


Figure 4d

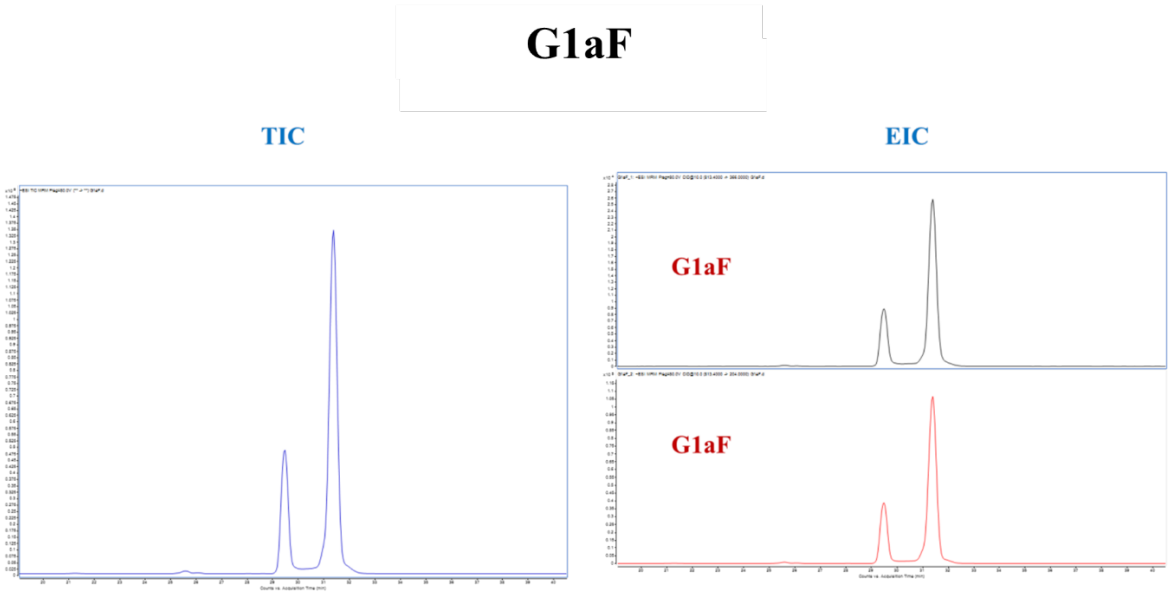


Figure 4e

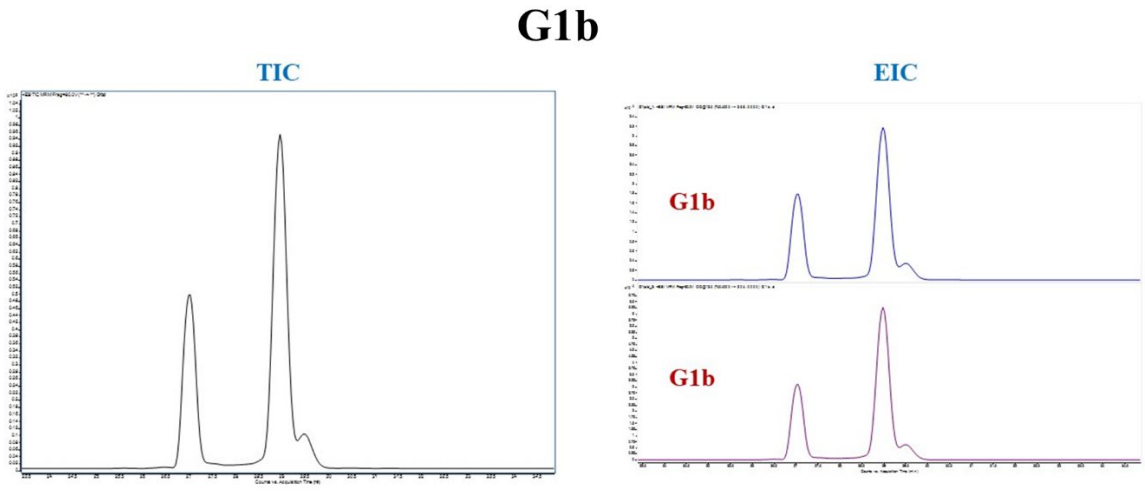
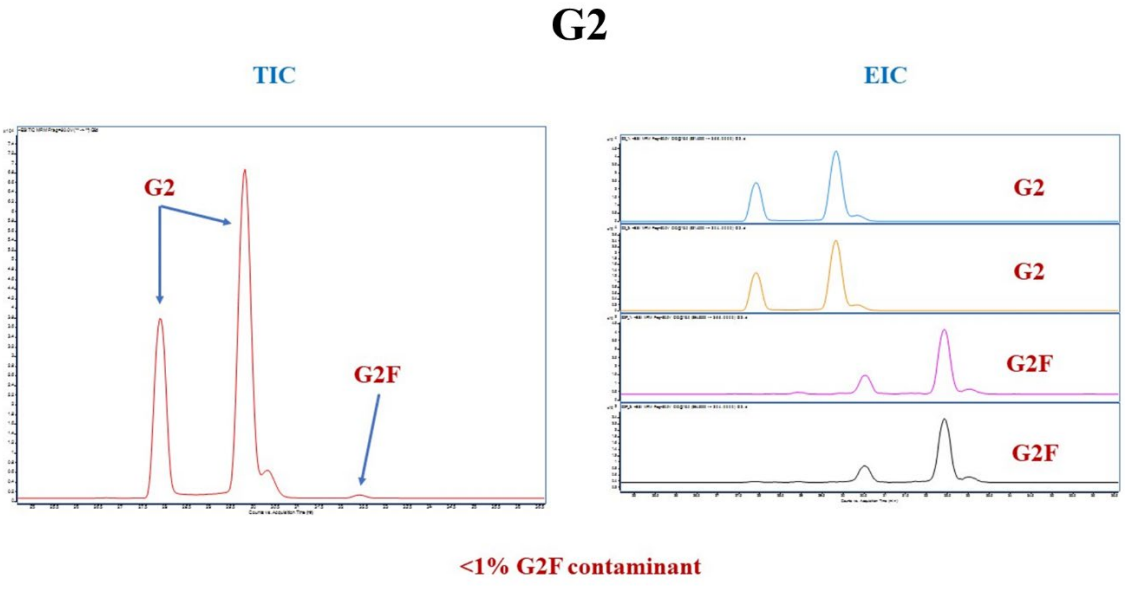
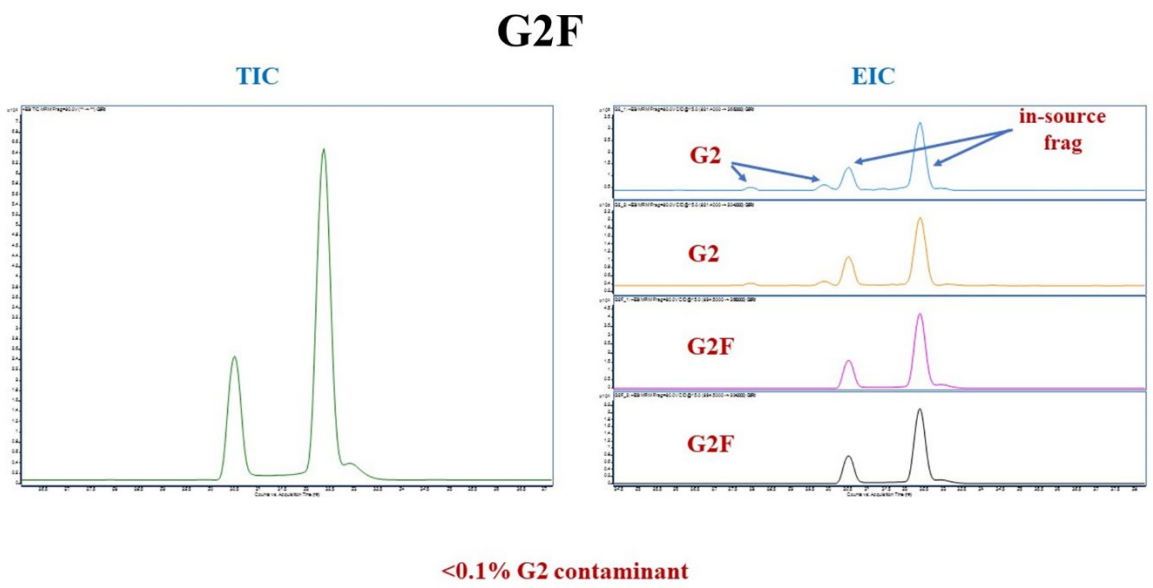


Figure 4f



<1% G2F contaminant

Figure 4g



Powders of the sialylated glycan calibrators appear to have impurities due to de-sialylated glycoforms (Table 8). Figure 5(a-f) details MRM mass chromatograms from the sialylated calibrators. The data support the results as determined from LC-FL analyses, however, they differ slightly in amplitude. ESI-MRM quantification without proper calibration can be biased by variable ionizabilities of different MRM transitions. A weighted mean of these results along with LC-FL and LC-HRMS data, with associated uncertainties, was applied as a correction factor to mass fraction of the calibrators used for quantification of the SRM.

Figure 5a

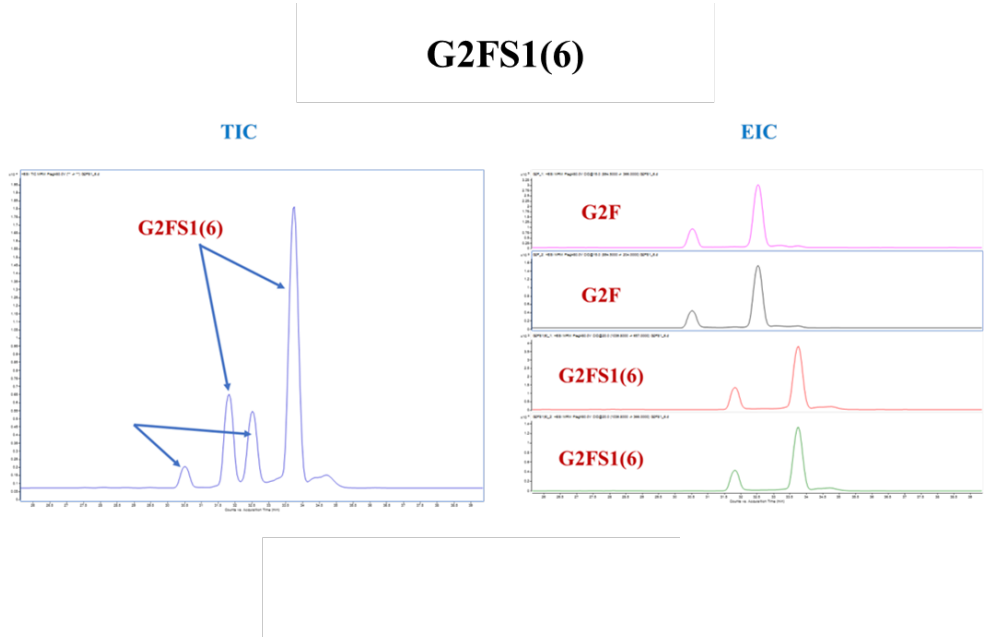


Figure 5b

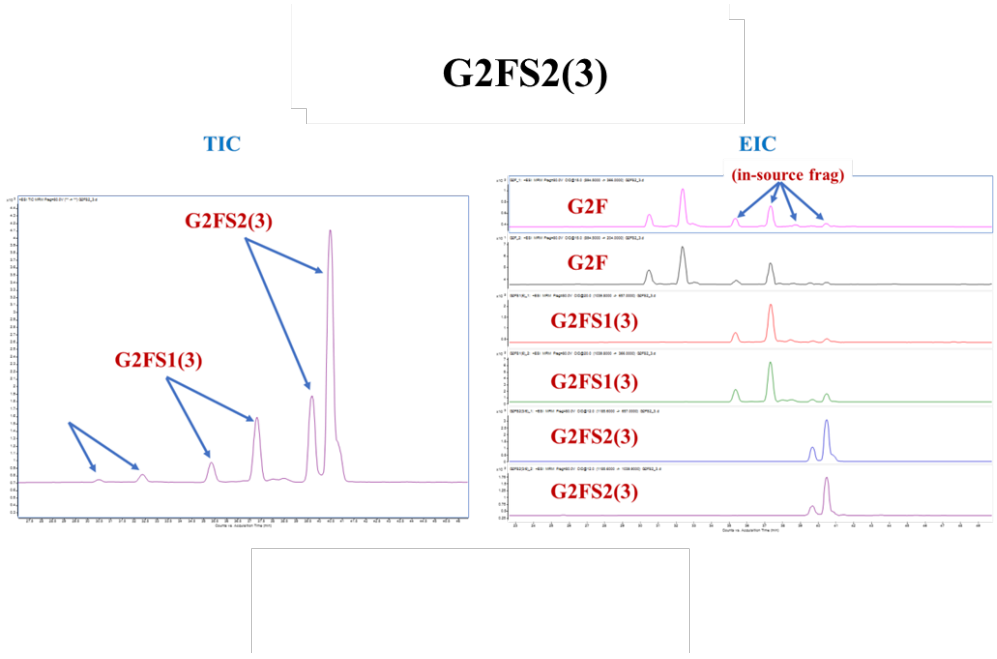


Figure 5c

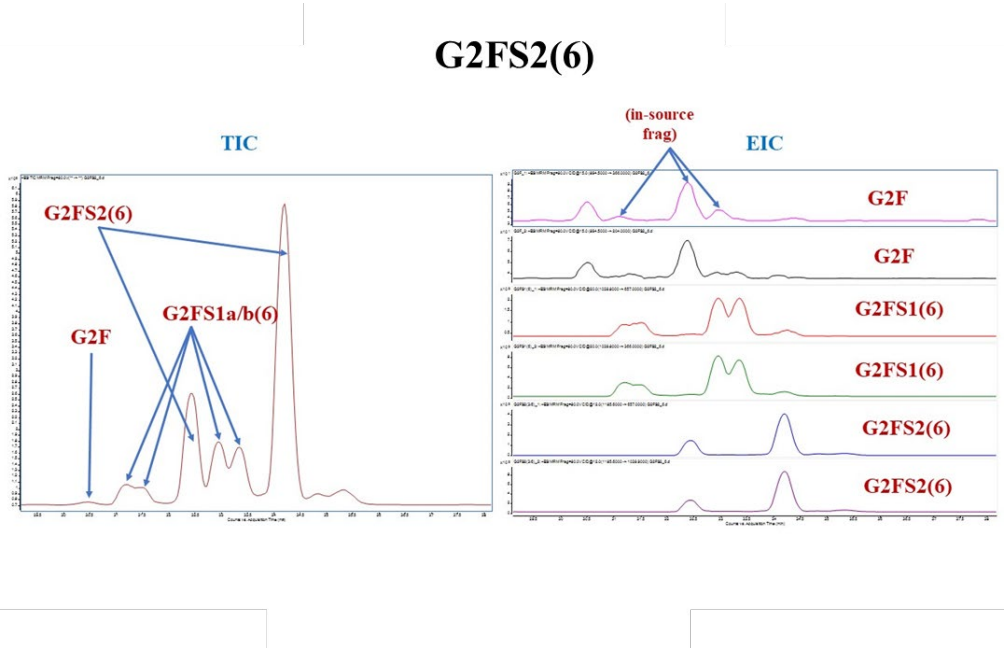


Figure 5d

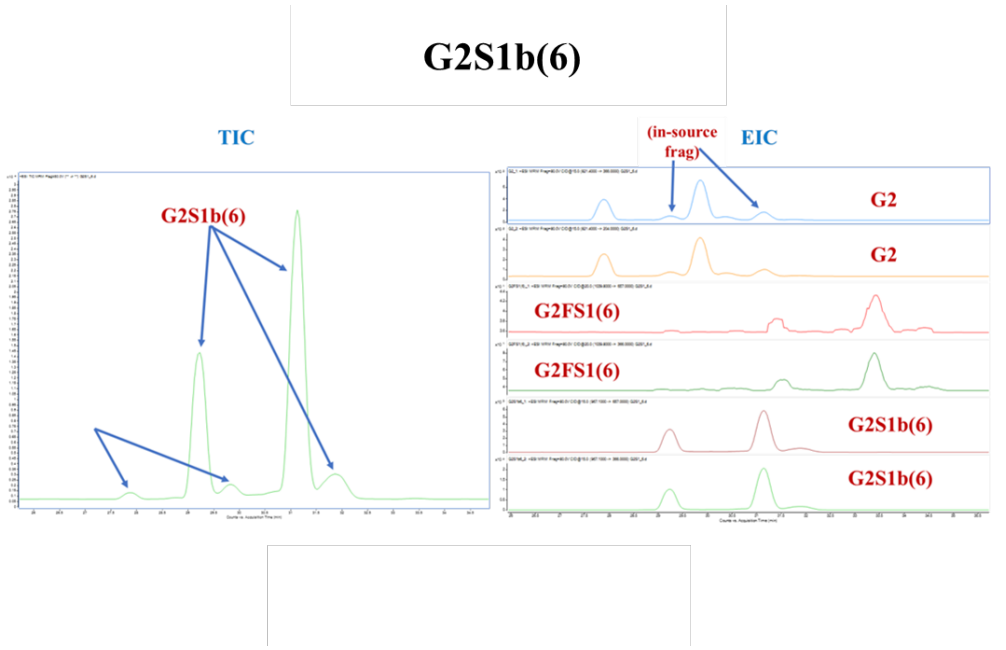


Figure 5e

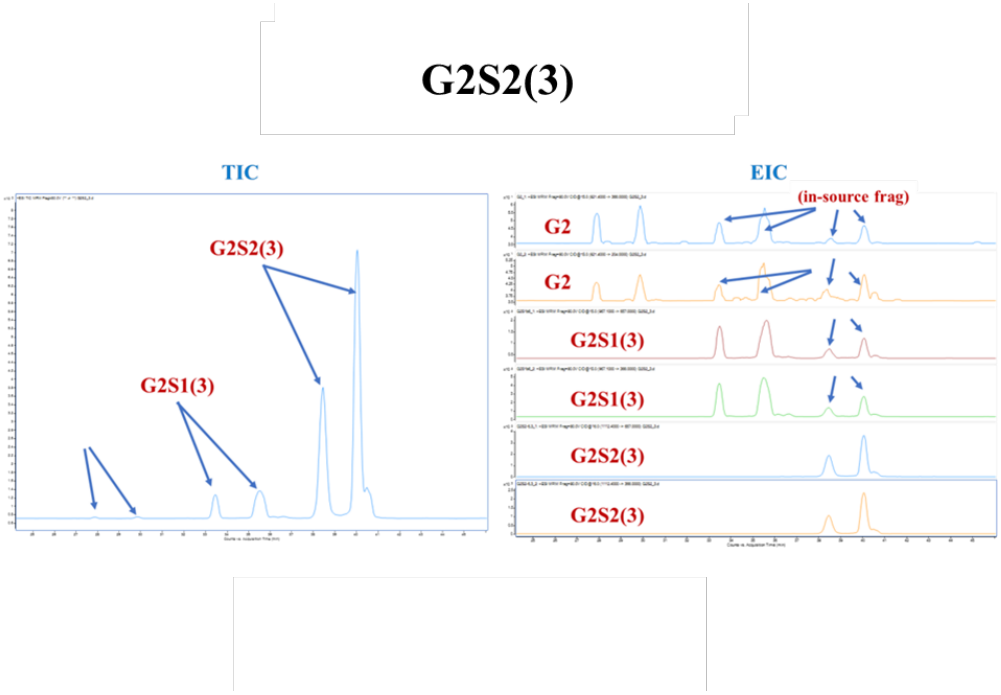
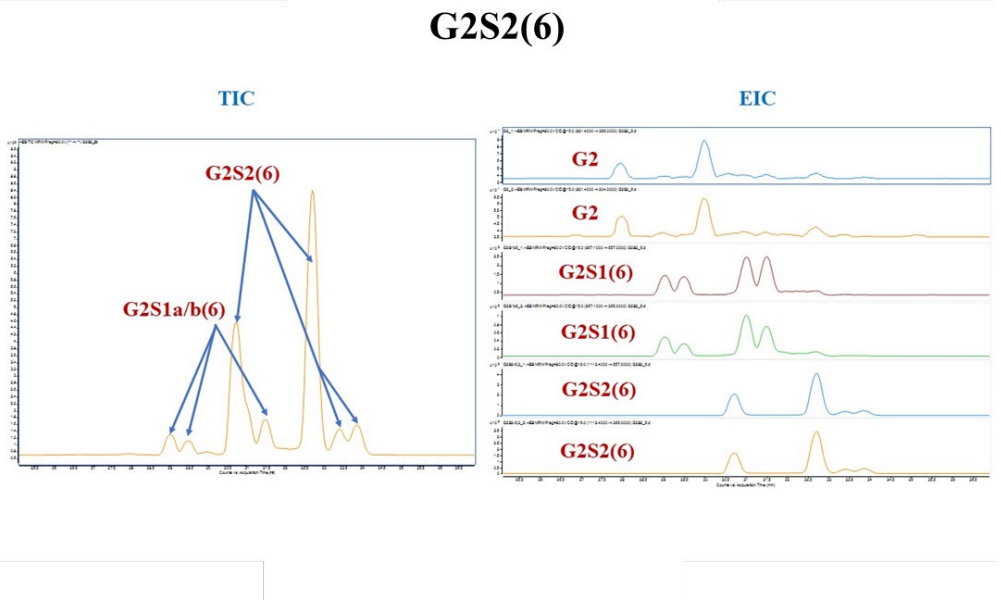


Figure 5f



Impurity estimates for powders of the sialylated glycoforms as determined through MRM TIC peak area ratios are provided in Table 8.

Table 8 – Impurity estimates based on peak area ratios – LC-MS/MS (MRM) data

sialylated glycan	single sialic acid loss, $\sum(\alpha6 + \alpha3)$	double sialic acid loss	total impurity
G2FS1(6)	19.9	N/A	19.9
G2FS2(3)	18.5	4.6	23.1
G2FS2(6)	26.0	0.5	26.5
G2S1(6)	4.7	N/A	4.7
G2S2(3)	12.3	0	12.3
G2S2(6)	13.1	0	13.1

3) LC-HRMS: For non-targeted LC-MS analysis, a Dionex UltiMate 3000 LC stack (Thermo) was used in-line with a Thermo QExactive Hybrid Quadrupole-Orbitrap MS system. Data was acquired in positive polarity at a resolution of 17,500. Analyses were done in replicates using differing precursor *m/z* scan ranges (*m/z* 160.0 to *m/z* 2400.0 or *m/z* 500.0 to *m/z* 3500.0), unique chromatographic columns (same column chemistry), different instrument tuning and calibration, differing calibrator aliquots, and different gradient elution profiles. A "Top 5" data-dependent acquisition (DDA) experiment was designed with dynamic exclusion turned off. AGC targets for full and MS^2 scans were set to $1e^6$ and $1e^5$ with maximum IT of 100 ms and 50 ms, respectively. Normalized collision energy was static at 30 V. All precursor charge states greater than +3 and isotopes were excluded. HESI source conditions were as follows: sheath gas flow rate = 35; aux gas flow rate = 10 and heater temperature = 300 °C; spray voltage = 3500 V; capillary temperature = 250 °C; S-lens RF = 30.0.

LC separation was achieved on a Hypercarb (porous graphitized carbon) analytical column (2.1 x 100 mm, 3 µm particles; part #35003-102130) at a flow rate of 200 µL/min. Glycans were separated using 50 mmol/L NH_4HCO_2 (mobile phase A) and 0.1 % (v/v) formic acid in ACN (mobile phase B) using serial isocratic and gradient elutions, as described above in *LC-FL*. Initial starting conditions – hold at 3 % 'B' for 3 min., ramp to 8 % 'B' in 1 min., hold at 8 % 'B' for 12 min. (isocratic), ramp to 20 % 'B' in 20 min. (gradient). Elution profiles were varied through replicate injections by either shortening or extending the isocratic or gradient portions of the chromatography. All injections were followed by a column wash at 95 % 'B' and column re-equilibration at 3 % 'B'. Column temperature was maintained at 30 °C; autosampler plate temperature control was set at 5 °C.

Detection of compounds in this analysis assumed solubility in water, affinity to porous graphitized carbon, and ionizability by electrospray. Non-targeted analysis identified several impurities that were determined to be from column or mobile phase contamination, or plasticizers. Due to the non-

reduced GlcNAc free hydroxyl group, glycoforms were observable in both anomeric forms (α and β anomers). High confidence in glycan identity is based on MS^1 exact mass in Orbitrap analysis, MS^2 spectral matching, chromatographic resolution of all glycan measurands, and prior probabilities based on the specificity of chemoenzymatic schemes used for synthesis. In general, it can be established from the nontargeted LC-MS analyses that glycan powders of non-sialylated glycoforms are highly pure, supporting the previous work. Glycan powders of G0, G0F, G1a, G1b, G1aF, G2, and G2F were observed to contain little to no impurity above noise. Sialylated glycan powders were observed to have impurities from structurally related, desialylated glycoforms (missing either one or two sialic acid residues). An estimate of glycan impurity for sialylated glycan powders was achieved using normalized, relative peak area ratios (Table 9). As discussed above, this data was combined into a weighted mean with other analytical approaches to estimate calibrator purity.

Table 9 – Impurity estimates based on peak area ratios – LC-HRMS data

Sialylated glycan	Estimate of desialylated impurity, (relative %)
G2FS1(6)	4.5
G2S1(6)	1.2
G2S2(3)	3.5
G2S2(6)	5.4
G2FS2(3)	7.3
G2FS2(6)	6.3

Total impurity estimates

All sources of impurities were considered and weighted sums were combined into an overall impurity estimate. Those estimates are provided in Table 10 below.

Table 10 – Total impurity estimate for each glycoform calibrator used to value-assign SRM 3655

		G0	G0F	G1a	G1aF	G1b	G2	G2F	G2FS1(6)	G2FS2(3)	G2FS2(6)	G2S1(6)	G2S2(3)	G2S2(6)
source of impurity	water	7.30	5.62	6.96	6.86	5.48	6.35	6.69	7.23	7.50	7.22	7.52	7.32	7.07
	sialic acid missing	0.0	0.0	0.0	0.0	0.0	0.0	0.0	8.0	13.1	11.1	2.1	6.9	8.0
	other impurity	0.1	0.1	0.2	0.2	0.1	1.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
	total:	**7.4**	**5.7**	**7.2**	**7.1**	**5.6**	**7.6**	**6.9**	**15.3**	**20.7**	**18.4**	**9.7**	**14.3**	**15.2**

7. Homogeneity Analysis

The homogeneity assessment was made at the time of the certification analysis. A stratified sampling plan was devised to test for homogeneity across all boxes of SRM vials within the production lot. There was no statistical trend in the data when vial-to-vial differences were plotted against the material fill order.

8. Stability Analysis

Stability was assessed prior to the certification analyses. LC-MS/MS analysis was used to examine glycan degradation under temperature conditions encountered during shipment from NIST to the end-user (-20 °C up to 37 °C). There was no apparent trend in the data, which suggests that routine shipping conditions will not affect the composition of the material over a one-month period. A freeze-thaw analysis was performed for each glycan by alternating units from -80 °C to room temperature. No apparent degradation was observed for selected glycans for up to five (5) freeze-thaw cycles.

9. Instructions for Storage and Use

Storage: SRM 3655 is shipped frozen on dry ice in polypropylene vials. Upon receipt, material should be stored frozen below –70 °C, in the original unopened vial, until ready for use.

Instructions for Use: Vials of the SRM to be analyzed should be removed from the freezer and allowed to stand at room temperature (20 °C to 25 °C) until thawed. After the material is thawed, it may be gently vortexed and centrifuged to clear material from the cap threads. Material should be used promptly (within several hours) after opening. Unused material should be discarded. All certification measurements were performed under the conditions described. The certified values were established based on proper storage below −70 ºC.

10. Certificate of Analysis

In accordance with ISO Guide 31: 2000, a NIST SRM certificate is a document containing the name, description, and intended purpose of the material, the logo of the U.S. Department of Commerce, the name of NIST as a certifying body, instructions for proper use and storage of the material, certified property value(s) with associated uncertainty(ies), method(s) used to obtain property values, the period of validity, if appropriate, and any other technical information deemed necessary for its proper use. A Certificate is issued for an SRM certified for one or more specific physical or engineering performance properties and may contain NIST reference, information, or both values in addition to certified values. A Certificate of Analysis is issued for an SRM certified for one or more specific chemical properties. Note: ISO Guide 31 is updated periodically; check with ISO for the latest version. [https://www.nist.gov/srm/srm-definitions]

For the most current version of the COA for NIST SRM 3655 Glycans in Solution (Frozen), please visit: https://www-s.nist.gov/srmors/view_cert.cfm?srm=3655.

11. Acknowledgments

The authors would like to thank Eric Kilpatrick and David Bunk for their assistance in SRM preparation. The authors are also grateful to Pamela Marino (NIH-CFG) for her advice and impact.

NIST Disclaimer

Certain commercial equipment, instruments, and materials are identified to describe adequately the work presented herein. Such identification does not imply recommendation or endorsement by NIST, nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

12. References

[1] Li, T.; Huang, M.; Liu, L.; Wang, S.; Moremen, K.W.; Boons, G.J.; *Divergent Chemoenzymatic Synthesis of Asymmetrical-Core-Fucosylated and Core-Unmodified N-Glycans*; Chem. Eur. J., Vol. 22, pp. 18742–18746 (2016).

[2] Chinoy, Z.S.; Friscourt, F.; Capicciotti, C.J.; Chiu, P.; Boons, G.J.; *Chemoenzymatic Synthesis of Asymmetrical Multi-Antennary N-Glycans to Dissect Glycan-Mediated Interactions Between Human Sperm and Oocytes*; Chem. Eur. J., Vol. 24, pp. 7970–7975 (2018).

[3] Wang, Z.; Chinoy, Z.S.; Ambre, S.G.; Peng, W.; McBride, R.; de Vries, R.P.; Glushka, J.; Paulson, J.C.; Boons, G.J.; *A General Strategy for the Chemoenzymatic Synthesis of Asymmetrically Branched N-Glycans*; Science, Vol. 341, pp. 379−383 (2013).

[4] Gagarinov, I.A.; Li, T.; Toraño, J.S.; Caval, T.; Srivastava, A.D.; Kruijtzer, J.A.; Heck, A.J.; Boons, G.J.; *Chemoenzymatic Approach for the Preparation of Asymmetric Bi-, Tri-, and Tetra-Antennary N-Glycans from a Common Precursor*; J. Am. Chem. Soc., Vol. 139, pp. 1011−1018 (2017).

[5] Liu, L.; Prudden, A.R.; Bosman, G.P.; Boons, G.J.; *Improved Isolation and Characterization Procedure of Sialylglycopeptide from Egg Yolk Powder*; Carbohydrate Research, Vol. 452, pp. 122–128 (2017).

[6] NIST Consensus Builder. Available at: https://consensus.nist.gov/app/nicob

[7] JCGM 100:2008; *Evaluation of Measurement Data — Guide to the Expression of Uncertainty in Measurement* (GUM 1995 with Minor Corrections); Joint Committee for Guides in Metrology (2008); available at https://www.bipm.org/en/publications/guides (accessed Oct 2021); see also Taylor, B.N.; Kuyatt, C.E.; *Guidelines for Evaluating and Expressing the Uncertainty of NIST Measurement Results*; NIST Technical Note 1297; U.S. Government Printing Office: Washington, DC (1994); available at https://www.nist.gov/pml/nist-technical-note-1297 (accessed Oct 2021).

[8] JCGM 101:2008; *Evaluation of Measurement Data — Supplement 1 to the "Guide to the Expression of Uncertainty in Measurement" — Propagation of Distributions Using a Monte Carlo Method*; Joint Committee for Guides in Metrology (JCGM) (2008); available at https://www.bipm.org/en/publications/guides (accessed Oct 2021).

[9] Tanaka, M.; Girard, G.; Davis, R.; Peuto, A.; Bignell, N.; *Recommended Table for the Density of Water Between 0 °C and 40 °C Based on Recent Experimental Reports*; Metrologia, Vol 38, pp. 301–309 (2001).

[10] Lafarge, T.; Possolo, A.; *The NIST Uncertainty Machine*; NCSLI Measure The Journal of Measurement Science, Vol. 10(3), pp. 20−27 (2015).

Supplemental File – Statistical analysis of data

Statistician report
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