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More comprehensive standards for monitoring glycosylation

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ABSTRACT

Biologics manufacturers must continually monitor the attachment of carbohydrates, called glycans, to their products, because any variability can impact safety and efficacy. To help the industry meet this challenge, the United States Pharmacopeial Convention (USP) offers glycan reference standards and validated methods for glycoprofiling using high-performance liquid chromatography (HPLC). The industry has recently adopted more advanced technologies for glycan analysis, including ultra-high performance liquid chromatography (UHPLC) and mass spectrometry. In this study, we confirm that USP's glycan reference standards are compatible with UHPLC by demonstrating comparable peak separation and glycan identification to HPLC methods. The improved resolving power and shorter run-times of UHPLC also allowed us to identify many of the minor glycan components present in USP's glycan reference standards. These more comprehensively characterized glycan reference standards will enable manufacturers to assess the micro-heterogeneity that can negatively impact the safety and efficacy of biological products.

1. Introduction

Glycosylation affects the stability, immunogenicity, clearance, and half-life of more than two-thirds of the biologics currently on the market [1–6]. Optimizing and controlling glycosylation of biologics is, therefore, essential to maintaining efficacy and for preventing adverse events [7,8]. Achieving product consistency and lot-to-lot reproducibility is difficult, however, because the addition of oligosaccharides or glycans, to a biologic can generate a diverse set of glycoforms. The resulting heterogeneity, including incomplete glycosylation (macro--heterogeneity) and variations in the structure of the attached glycans (micro-heterogeneity), must be tightly controlled, or the potency and pharmacokinetics of biologics will suffer [9–11].

The degree of macro- and micro-heterogeneity is mainly dependent on the host cell used for protein expression [12]. However, choosing a host-cell based solely on glycosylation is impractical. For example, the blockbuster cancer therapies pertuzumab (Perjeta®), rituximab (Rituxan®), and daratumumab (DARZALEX®) are made using Chinese hamster ovary (CHO) cells to achieve the high-yields necessary to meet demand even though these cells cannot replicate all forms of human glycosylation, such as α-2,6-sialylation [13]. CHO cells also produce the non-human glycans *N*-glycolylneuraminic acid (Neu5Gc) and galactose-α-1,3-galactose (α-gal) that can cause adverse events even when present at low levels [14–17]. Switching to a human-derived host-cell; choosing the right bioprocess, such as between batch, fed-batch, and perfusion production modes; and fine-tuning media composition, pH, temperature, and osmolality can address some of the problems mentioned above, but they cannot wholly eliminate glycosylation heterogeneity [18]. Also, modifying any of these parameters must be balanced against their effects on yield, aggregation, and other post-translational modifications, all of which can affect the efficacy of the product [19].

Other methods to produce more uniform and human-like glycoproteins in mammalian cells are available. Manufacturers can knock-out or increase the activity of specific enzymes to control the level of glycosylation. For example, removing α-1,6-fucosyltransferase or over-expressing α-2,3-sialyltransferase can improve serum half-life of monoclonal antibodies (mAbs) [20–25]. Also, unneeded glycosylation sites can be removed to minimize the potential for heterogeneity [26–28]. But even after these methods are applied, the risk of batch to batch heterogeneity in the glycosylation pattern remains. It is not surprising then that the U.S. Food & Drug Administration (FDA), the European Medicines Evaluation Agency (EMA), and the International Conference on Harmonization (ICH) all consider glycosylation a

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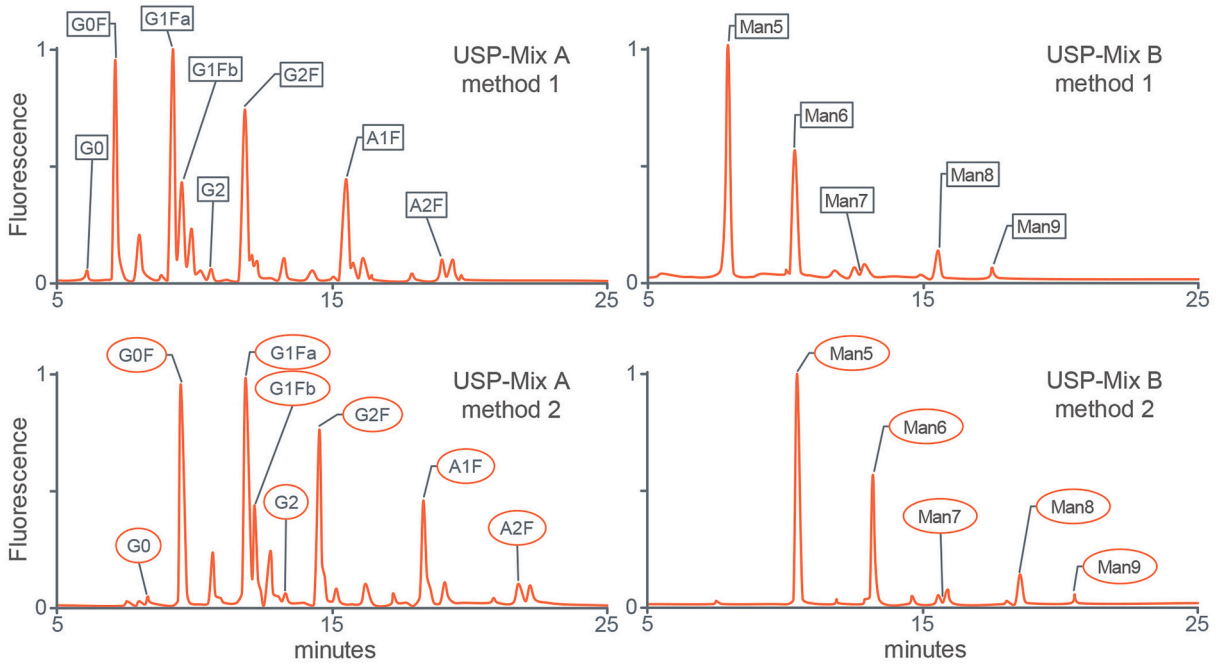


Fig. 1. HILIC separation of N-glycans in the USP Oligosaccharide System Suitability Mixture A (USP-Mix A, *left*) and B (USP-Mix B, *right*) using method 1 (*top*) and method 2 (*bottom*). The fluorescence (normalized) of the 2-AB label was monitored at 430 nm for 25 min at a flow rate of 0.4 mL/min.

potential Critical Quality Attribute (CQA) to be monitored and controlled during manufacturing [20,29,30].

Unfortunately, there are no standard limits for glycosylation. Instead, product batches must be comparable to a reference standard within previously approved limits. Maintaining comparability can be challenging because a manufacturer may need to change the source of raw material and the scale or site of production several times throughout a product's life-cycle, all of which can affect glycosylation [31]. Regardless of the nature of the change, the acceptance criteria for evaluating its effects must fall within limits set in the pre-approved comparability protocol [32]. However, biologics with heterogeneous glycosylation patterns tend to fall out of specification. Failure to account for product heterogeneity has resulted in delays in product licensing and, in some cases, the outright rejection of an application [33,34].

Maintaining comparability in bio-manufacturing is further complicated by the fact that characterizing glycan heterogeneity is technically demanding. N-glycans are highly branched carbohydrate structures composed of monosaccharide sugars such as fucose, galactose, mannose, N-acetylglucosamine, and sialic acid, and resolving their structural complexity requires high-resolution techniques [12]. One of the more robust methods separates N-glycans using high-performance liquid chromatography (HPLC) [12,35,36]. The identity and relative abundance of the N-glycan can be quantified by coupling the chromatography to mass spectral (MS) analysis to measure glycan ionization, fragmentation, and mass [37–39]. Analytical pipelines that include HPLC and MS can provide manufacturers with critical information regarding their products. Still, this approach is highly dependent on the use of a biologics performance standard containing a library of well-characterized N-glycans [33]. Without a valid standard, it is incredibly challenging to correlate chromatographic retention times of N-glycans with their structural identity.

The United States Pharmacopeial Convention (USP) provides N-

Table 1
GU values of major N-glycans in USP-Mix A and Mix B.

Glycan Library	Peak	GU 1 (%E)	GU 2 (%E)	Oxford Name
USP-Mix A	G0	5.35 (1.2%)	5.37 (0.8%)	*A2*
	G0F	5.81 (1.1%)	5.78 (1.6%)	*F(6)A2*
	G1Fa	6.62 (1.2%)	6.53 (2.5%)	*F(6)A2[6]G(4)1*
	G1Fb	6.75 (1.0%)	6.65 (2.4%)	*F(6)A2[3]G(4)1*
	G2	7.12 (1.1%)	6.92 (3.9%)	*A2G(4)2*
	G2F	7.54 (0.8%)	7.38 (2.9%)	*F(6)A2G(4)2*
	A1F	8.82 (0.3%)	8.65 (1.6%)	*F(6)A2G(4)2S(6)1*
	A2F	10.08 (0.4%)	9.92 (1.2%)	*F(6)A2G(4)2S(3,3/6)2*
USP-Mix B	Man5	6.14 (0.7%)	6.18 (1.3%)	*M5*
	Man6	7.03 (0.9%)	7.00 (0.4%)	*M6*
	Man7	7.92 (0.9%)	7.85 (0.0%)	*M7*
	Man8	8.84 (1.1%)	8.84 (1.2%)	*M8*
	Man9	9.54 (0.7%)	9.53 (0.6%)	*M9*

GU values were calculated for peaks G0, G0F, G1Fa, G1Fb, G2, G2F, A1F and A2F in USP-Mix A and peaks Man5, Man6, Man7, Man8, and Man9 in USP-Mix B from method 1 (GU 1) and the method 2 (GU 2). Differences between experimentally determined GU values and the theoretical GU values in the Waters 2-AB Glycan database are given as percent error (%E). The Oxford name for each N-glycan is given.

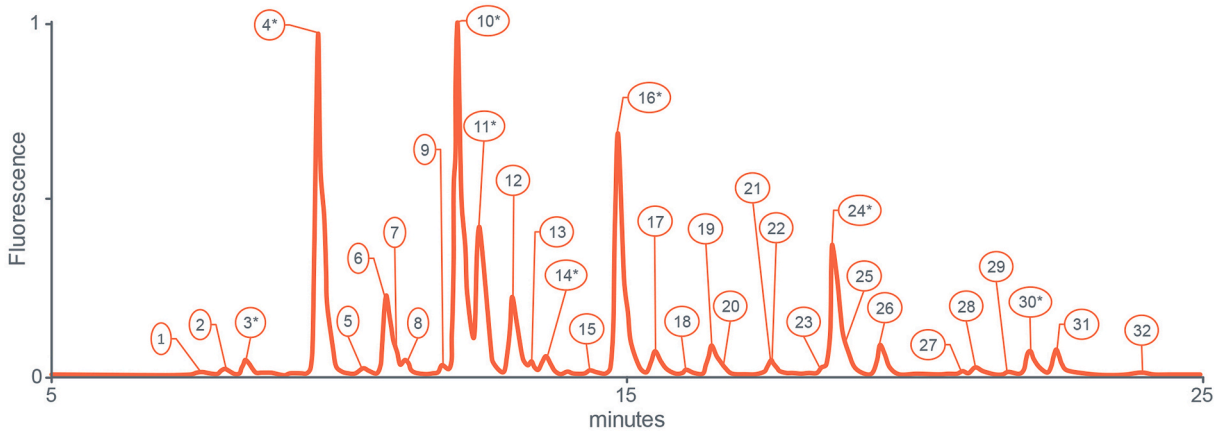


Fig. 2. HILIC separation of N-glycans in the USP Oligosaccharide System Suitability Mixture A (USP-Mix A) using method 2. GU values were determined for each peak (1–32). Peaks corresponding to the major N-glycans (G0, G0F, G1Fa, G1Fb, G2, G2F, A1F, and A2F) are denoted with an asterisk (*).

glycan libraries, including the Oligosaccharide System Suitability Mixtures A and B (USP-Mix A and B), for use as performance standards. USP-Mix A is composed of biantennary N-linked oligosaccharides released from human polyclonal immunoglobulin G (IgG). USP-Mix B consists of high-mannose N-linked oligosaccharides released from bovine ribonuclease B (RNase B). Both standards are validated for characterizing glycosylation of biologics, such as monoclonal antibodies and recombinant coagulation factors, using methods described in General Chapters <1084> Glycoprotein and Glycan Analysis, <212> Oligosaccharide Analysis, and <129> Analytical Procedures for Recombinant Therapeutic Monoclonal Antibodies published in the United States Pharmacopeia and National Formulary (USP 42-NF 37). The methods described in the USP-NF provide the standard protocols for assessing glycosylation; however, since their publication, analytical techniques such as ultra-high pressure liquid chromatography (UHPLC) combined with Quadrupole Time-of-flight (QToF) MS, have become more widely used by manufacturers.

The adoption of more advanced technologies is driven in part by the demands of regulatory compliance. ICH Q11 states that risk assessment of glycosylated biologics will become more specific and rigorous with the availability of improved structural and functional information. In other words, increases in the resolution, speed, and ease of use of analytical equipment will be met with a corresponding increase in demand for more data to demonstrate batch to batch consistency of product glycosylation both pre- and post-market. However, for analytical methods to be acceptable to regulators, it must first be shown that they can accurately and precisely identify N-glycans using standards. Also, all of the commercially available glycan libraries only provide information on a subset of major N-glycans that may be present at distinct stages of the manufacturing process. Without validated standards that contain additional N-glycans, manufacturers cannot create an in-depth profile that includes the minor glycoforms that may be present; or validate the suitability of their equipment for such an analysis. The lack of more complete reference standards represents a significant bottleneck in a quality control strategy given that all the currently available analytical instruments are more than sufficient for detecting minor N-glycans.

Assessing minor N-glycans linked to a biologic during its production has clear benefits for a manufacturer [40,41]. First, the effect of minor glycoforms on activity is unpredictable. Therefore, the inability to determine how changes in the manufacturing process impact the presence of minor glycoforms creates a significant risk to batch-to-batch consistency that must be mitigated [31]. Second, a comprehensive understanding of the glycoprofile of a biologic and the process parameters that impact it may preempt the need for extensive ongoing monitoring [42]. As per ICH Q6B, the manufacture is required to justify the exclusion of any test for a specific quality attribute. Therefore, demonstrating the ability to control the heterogeneity of a more extensive selection of N-glycans during cell culture would be crucial to establishing *ratio decidendi* for opting out of expensive and time-consuming testing. Third, the comparable presence of minor glycoforms between a biosimilar and reference product provides significant evidence for the high degree of similarity required to support abbreviated clinical trials and interchangeability [43].

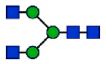
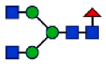
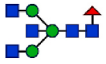
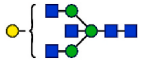
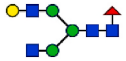
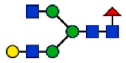
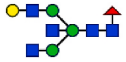
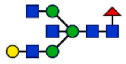
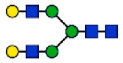
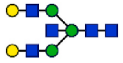
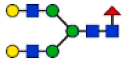
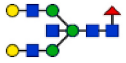
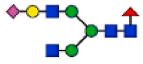
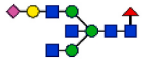
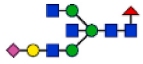
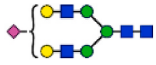
In this study, we show that the peak separation and identity of N-glycans in the reference standards USP-Mix A and B are comparable between previously validated methods and the most up-to-date protocols used in the industry today, including hydrophilic interaction liquid chromatography (HILIC), UHPLC and QToF. Next, we characterized and identified the minor N-glycans in both USP-Mix A and B. Analysis of the N-glycan composition of USP-Mix A showed that it has a better representation of sialylated, galactosylated, and fucosylated N-linked glycans than comparable commercial glycan libraries, which are instead dominated by asialylated biantennary oligosaccharides. This difference in composition has significant ramifications for manufacturers. For example, the degree of sialylated and fucosylated glycans constitutes a significant concern for manufacturers of immunosuppressive intravenous immunoglobulins (IVIG) because fluctuations in these glycoforms impact anti-inflammatory properties [30,44].

2. Materials and methods

2.1. Reagents

The USP System Suitability Mixtures A and B (USP-Mix A and B) were provided by USP (Rockville, MD). The Glyko® 2-AB Human IgG N-Linked Glycan Library was purchased from ProZyme® (Hayward, CA), and the 2-AB labeled IgG N-Glycan Library was purchased from Ludger Ltd (Oxfordshire, U.K.). The Glycan Labeling Kit was purchased from QA-Bio, Inc. (Burlington, ON). The GlycoClean™ S Cartridges were purchased from ProZyme® (Hayward, CA). The 2-AB Dextran Calibration Ladder, 2-AB Glycan Performance Test Standard, and 5 M ammonium formate (3.8% Formic Acid) were purchased from Waters Corporation (Milford, MA). Acetonitrile (ACN, ≥99.5% purity) and Acetic Acid were purchased from Thermo Fisher Scientific (Waltham, MA). All reagents are of analytical or HPLC grade.

Table 2
Identification of major and minor N-glycans in USP-Mix A.

Peak	GU (%E)	*m/z*	ppm	N-glycan	Oxford Name	CFG
1 2	5.18 (N/A) 5.29 (0.8%)	1380.523/1 1380.539/1	−13.26 −1.59	G0FN	F(6)A1	
3*	5.35 (1.2%)	1437.565/1	−1.53	G0	*A2*	
4*	5.81 (1.1%)	1583.615/1	−3.73	G0F	*F(6)A2*	
5	6.17 (0.3%)	1542.588/1	−3.76	Man4G0FN	*F(6)M4[3]A1*	
6	6.17 (1.2%)	1787.715/2	−4.59	G0FB	*F(6)A2B*	
7 8	6.17 (1.4%) 6.31 (0.9%)	1600.625/2 1600.625/2	−1.37 −1.37	G1	*A2[3/6]G(4)1*	
9	6.49 (1.1%)	1803.701/2	−0.78	G1B	*A2[6]BG(4)1*	
10*	6.62 (1.2%)	1746.682/2	−0.92	G1Fa	*F(6)A2[6]G(4)1*	
11*	6.75 (1.0%)	1746.682/2	−0.92	G1Fb	*F(6)A2[3]G(4)1*	
12	6.87 (1.2%)	1949.763/2	−1.33	G1FBa	*F(6)A2[6]BG(4)1*	
13	6.98 (1.5%)	1949.763/2	−1.33	G1FBb	*F(6)A2[3]BG(4)1*	
14*	7.12 (1.1%)	1762.665/2	−5.90	G2	*A2G(4)2*	
15	7.30 (1.2%)	1965.744/2	−5.70	G2B	*A2BG(4)2*	
16*	7.54 (0.8%)	1908.733/2	0.00	G2F	*F(6)A2G(4)2*	
17	7.69 (0.8%)	2111.825/2	−5.97	G2FB	*F(6)A2BG(4)2*	
18	7.88 (0.0%)	2037.782/2	−2.75	G1FSa	*F(6)A2[3]G(4)1S(6)1*	
19	8.04 (1.7%)	2037.782/2	−2.75	G1FSb	*F(6)A2[6]G(4)1S(6)1*	
20	8.12 (1.2%)	2240.846/2	−4.11	G1FBSa	*F(6)A2[6]BG(4)1S(3)1*	
21	8.40 (2.4%)	2240.846/2	−4.11	G1FBSb	*F(6)A2[3]BG(4)1S(3)1*	
22	8.40 (0.2%)	2053.783/2	−5.75	G2S	*A2G(4)2S(6)1*	

(*continued on next page*)

Table 2 (*continued*)

Peak	GU (%E)	*m/z*	ppm	N-glycan	Oxford Name	CFG
23	8.66 (1.5%)	2256.861/2	−4.96	G2BS	*A2BG(4)2S(3)1*	
24*	8.82 (0.3%)	2199.829/2	−1.00	G2FS	*F(6)A2G(4)2S(6)1*	
25	8.91 (1.2%)	2402.899/2	−3.83	G2FBS	*F(6)A2BG(4)2S(6)1*	
26	9.05 (0.4%)	2402.899/2	−3.83		*F(6)A2BG(4)2S(3)1*	
27	9.53 (N/A)	2490.913/2	−4.58	G2FS2	*F(6)A2G(4)2S2*	
28	9.68 (0.1%)	2344.873/2	−2.90	G2S2	*A2G(4)2S(6,6)2*	
29	9.86 (0.6%)	2547.933/2	−4.87	G2BS2	*A2BG(4)2S(6,6)2*	
30*	10.08 (0.4%)	2490.913/2	−4.58	G2FS2	*F(6)A2G(4)2S(3/6,6)2*	
31	10.22 (0.5%)	2694.011/2	−2.82	G2FBS2	*F(6)A2BG(4)2S(3/6,6)2*	
32	10.82 (0.7%)	2856.027/2	−10.36	G3FS2	*F(6)A3G(4)3S(3/6,3)2*	

The identities of the major and minor N-glycans in USP Oligosaccharide System Suitability Mixture A (USP-Mix A). Peaks corresponding to the major N-glycans (G0, G0F, G1Fa, G1Fb, G2, G2F, A1F, and A2F) are denoted with an asterisk (*). GU values for all peaks (1–32) were calculated and differences from the theoretical GU values expressed as a percent error (%E). The *m/z* value is expressed as mass (m) in daltons (Da) divided by the charge (z). The mass measurement error for each reading is given in part per million (ppm).

2.2. Labeling of N-glycans

The USP-Mix A and B were labeled with 2-AB according to the manufacturer's protocol (QA-Bio, Inc., 2017). Briefly, 150 μL of glacial acetic acid (QA-Bio, Inc.) was added to a vial of 350 μL of DMSO (QA-Bio, Inc.). The solution was mixed by pipette action. Then 100 μL of the DMSO-acetic acid solution was added to a vial of containing 5 mg of LudgerTag™ 2-AB Dye (QA-Bio, Inc.). The solution was mixed until complete dissolution of dye. The solubilized dye was added to a vial of LudgerTag™ Sodium Cyanoborohydride (QA-Bio, Inc.) and mixed by pipette action. The labeling solution was incubated at 70 °C for up to 2 min and then cooled at room temperature for 10 min. Within 1 h of preparation, 10 μL of labeling solution was added to a vial of 20 μg of each N-glycan library (U.S. Pharmacopeia). The labeling reactions were mixed and incubated at 65 °C for 2 h then cooled at room temperature for 10 min.

Unbound 2-AB reagent was removed using the GlycoClean™ S Cartridges (ProZyme®). Briefly, each cartridge was washed with 1 mL of ultrapure water (Milli-Q®, Millipore Corporation, Billerica, MA)

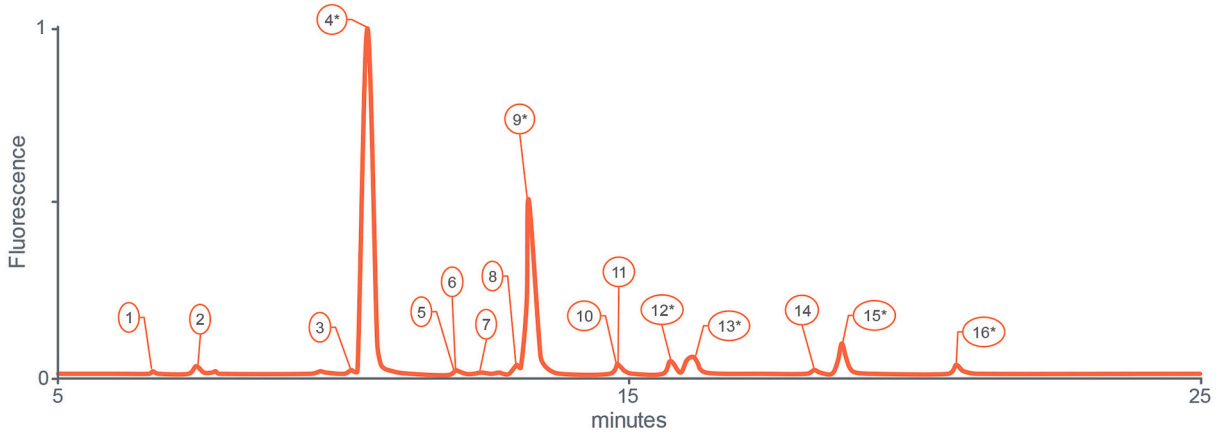
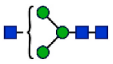
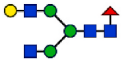
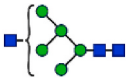
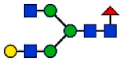
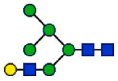
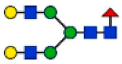
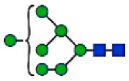
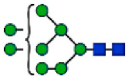
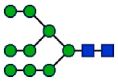


Fig. 3. HILIC separation of N-glycans in the USP Oligosaccharide System Suitability Mixture B (USP-Mix B) using method 2. GU values were determined for each peak (1–16). Peaks corresponding to the major N-glycans (Man5, Man6, Man7, Man8 and Man9) are denoted with an asterisk (*).

Table 3
Identification of major and minor N-glycans in USP-Mix B.

Peak	GU (%E)	*m/z*	ppm	N-glycan	Oxford Name	CFG
1	4.86 (0.4%)	1234.480/1	−2.43	G0N	*A1*	
2	5.11 (2.7%)	1193.458/1	−1.09	Man4	*M4*	
3 4*	6.05 (N/A) 6.14 (0.7%)	1355.511/1 1355.511/1	−1.11 −1.11	Man5	*M5*	
5	6.61 (1.3%)	1746.682/2	−0.92	G1Fa	*F(6)A2[6]G(4)1*	
6	6.61 (0.3%)	1559.591/2	−0.92	Man5G0N	*M5A1*	
7	6.82 (0.0%)	1746.682/2	−0.92	G1Fb	*F(6)A2[3]G(4)1*	
8 9*	6.93 (N/A) 7.03 (0.4%)	1517.554/1 1517.554/1	−5.67 −5.67	Man6	*M6*	
10	7.53 (0.1%)	1721.640/2	−5.00	Man5G1	*M5A1G(4)1*	
11	7.53 (0.9%)	1908.733/2	0.00	G2F	*F(6)A2G(4)2*	
12* 13*	7.78 (N/A) 7.92 (0.9%)	1679.606/1 1679.606/1	−5.60 −5.60	Man7	*M7*	
14 15*	8.62 (N/A) 8.84 (1.2%)	1842.690/2 1841.662/1	−8.03 −3.42	Man8	*M8*	
16*	9.54 (0.7%)	2004.751/2	11.37	Man9	*M9*	

The identities of the major and minor N-glycans in USP Oligosaccharide System Suitability Mixture B (USP-Mix B). Peaks corresponding to the major N-glycans (Man5, Man6, Man7, Man8 and Man9) are denoted with an asterisk (*). GU values for all peaks (1–16) were calculated. Differences from the theoretical GU values expressed as a percent error (%E). The *m/z* value is expressed as mass (*m*) in daltons (*Da)* divided by the charge (*z*). The mass measurement error for each reading is given in part per million (*ppm*).

followed by five washes with 1 mL of 30% acetic acid solution (v/v) (Thermo Fisher Scientific). Each cartridge was then cleaned with four washes of 1 mL of acetonitrile (Thermo Fisher Scientific). The labeling reactions were spotted onto freshly washed cartridge membranes and incubated at room temperature for 15 min to allow for absorption into the wet membrane. The membrane was washed with 1 mL of acetonitrile, followed by six washes with 1 mL of 96% acetonitrile solution (v/v) (Thermo Fisher Scientific). The flow-through was discarded. The cartridge was placed over a collection vessel, and the labeled N-glycans were eluted with three volumes of 0.5 mL of ultrapure water (Milli-Q®). Each elution was allowed to drain completely. The eluted samples were dried by centrifugal evaporation without heating in an HT-4X centrifugal vacuum evaporator (Genevac Ltd, U.K.). The dried samples were reconstituted in 500 μL of ultrapure water (Milli-Q®). The sample was split among 50 μL aliquots that were all stored in a freezer at −30 °C.

2.3. Separation of N-glycans

2-AB labeled N-glycans were separated by hydrophilic interaction chromatography (HILIC) using an ACQUITY UPLC® Glycan BEH Amide, 130 Å column (2.1 × 150 mm) with 1.7 μm bridged ethylene hybrid (BEH) particles (Waters Corporation). The column was connected to an ACQUITY ultra-performance liquid chromatography (UPLC) H-Class Bio System (Waters Corporation) consisting of a quaternary solvent manager, sample manager, and fluorescence detector (FLR). The FLR excitation and emission wavelengths were set to 330 and 420 nm for 2-AB. The instrument is coupled to the Synapt G2 Q-Tof MS and is under the

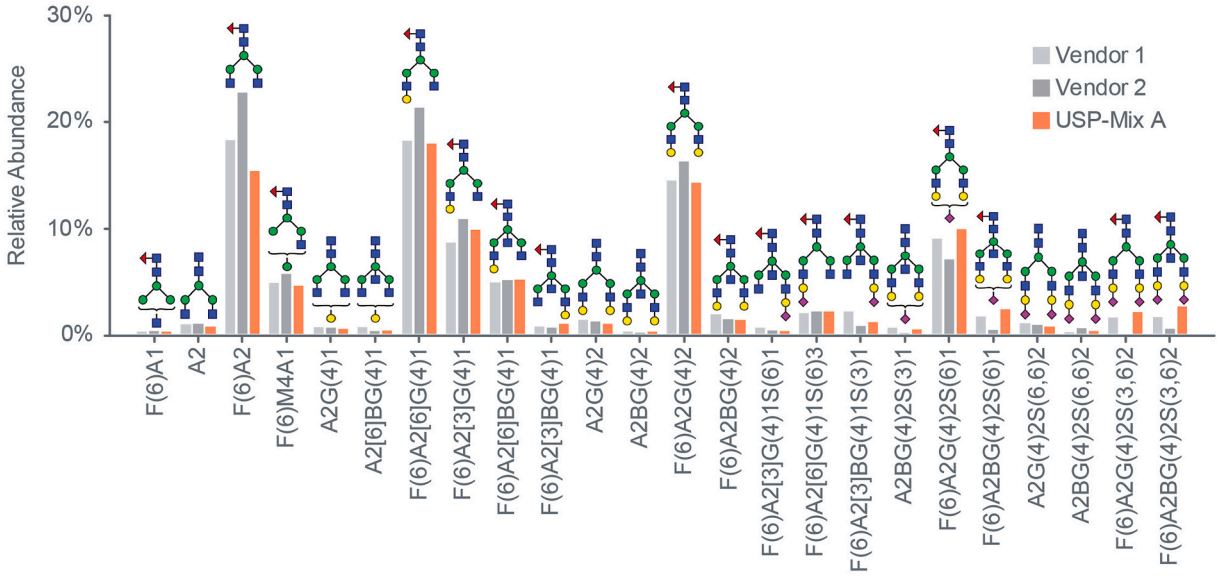


Fig. 4. Comparison of the relative abundance of 24 glycans in the 2-AB labeled commercial N-glycan libraries (*light & dark gray*) and the USP-Mix A (*orange*).

control of the MassLynx v4.1 software (Waters Corporation). Mobile phase A was 25% ACN/75% 50 mM Ammonium Formate, pH 4.4 (v/v). Mobile phase B was 75% ACN (v/v). The samples were placed in the sample manager and incubated at 5 °C.

Two UHPLC methods were used to separate all samples. Method 1 was developed for the analysis of 2-AB labeled N-glycans using the Empower workstation and the GlyoBase 3+ database for glycan unit assignment [45]. It uses a linear gradient of 70–53% ACN (v/v) at a separation temperature of 40 °C for 33 min. Method 2 was developed for the analysis of RapiFluor-MS (RFMS), a fluorescent labeling agent developed to decrease the time required for N-glycan sample preparation [39]. It uses a linear gradient of 75–54% ACN (v/v) at a separation temperature of 60 °C for 35 min. Both methods use a flow rate of 0.4 mL/min. The system was calibrated using a Waters 2-AB Dextran Calibration Ladder, which has an average molecular weight of 4500 Da for the glucose homopolymer. The ladder was solubilized in 200 μL 75%:25% mobile phase B:mobile phase A (v/v). A cubic spline curve for retention times versus glucose unit (GU) values were automatically calculated using the average of all dextran ladders analyzed. The observed GU range was from 2 to 30. The calibration curve was used to convert retention times of separated N-glycans into GU values. 50 μL aliquots of 2-AB labeled USP-Mix A and B were thawed and dried by centrifugal vacuum evaporation. The samples were then solubilized in 10 μL of 75%:25% mobile phase A:mobile phase B (v/v). All samples were maintained at 5 °C prior to injection.

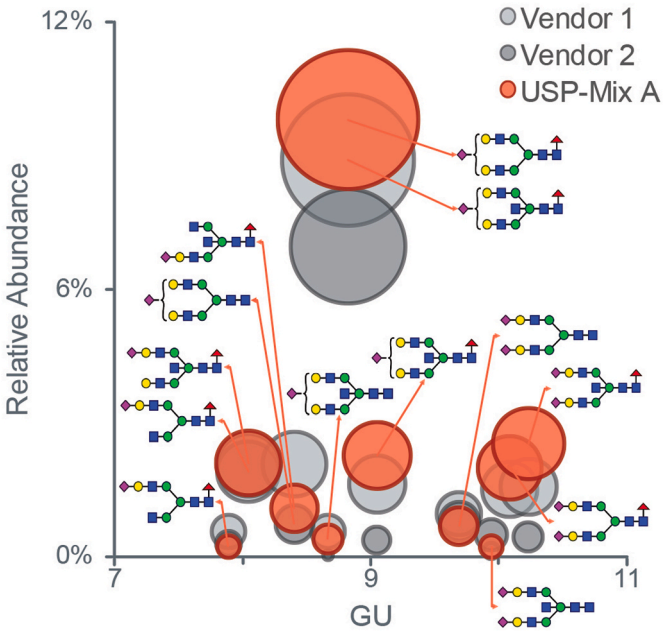


Fig. 5. Comparison of the relative abundance of sialylated N-glycans in the 2-Ab labeled commercial N-Glycan libraries (*light & dark gray*) and the USP-Mix A (*orange*).

2.4. Mass spectral analysis of N-glycans

2-AB labeled samples display poor ionization efficiency that can interfere with the identification of glycans by MS. Therefore, each sample was injected at a 5-fold excess to that recommended in USP General Chapter <212>. The analysis was performed in both positive and negative ion modes. The source temperature at 120 °C, desolvation temperature at 500 °C, and the cone gas rate at 25 L/h. In positive ion mode, the mass spectra were recorded from 700 to 2400 m/z. The capillary voltage was set to 2.5 kV, and the sampling cone voltage was set to 20 V. A source offset of 20 V was also used. The desolvation gas flow was to 700 L/h, and the nebulizer was set to 6.5 bar. In negative ion mode, the mass spectra were recorded from 700 to 2000 m/z. The capillary voltage was set to 2.2 kV, and the sampling cone voltage was set to 40 V. A source offset of 80 V was used. The desolvation gas flow was 600 L/h, and the nebulizer was set to 6.0 bar. The fluorescent and mass spectrometry chromatograms were aligned automatically during data acquisition.

2.5. Data analysis

For GU determination, the chromatographic peaks resulting from the UHPLC-FLR analysis were processed with Empower 3 (Waters Corporation) using an automated method with a traditional integration algorithm, after which each chromatogram was manually corrected to maintain the same intervals of integration for all samples. The chromatograms were all separated in the same manner into individual peaks.

Peaks were automatically assigned GU values based on the calibration curve. Unknown N-glycans were identified by using their experimentally determined GU and *m/z* values to search the Waters 2-AB Glycan GU library within the UNIFI software (Waters Corporation). Thresholds for database searching were ±0.2 GU and ±10 ppm. The relative quantity of each identified N-glycan was determined as the ratio of the area of its FLR elution peak to the sum of peak areas for all N-glycans identified.

3. Results and discussion

In this study, we characterized two glycan libraries, USP-Mix A and B, using UHPLC-FLR. USP-Mix A consists of N-glycans released from human polyclonal immunoglobulin G (IgG). USP-Mix B contains N-glycans derived from bovine ribonuclease B (RNase B). Both glycan libraries were labeled with the 2-AB fluorescent tag to enable their detection and quantitation at the femtomole level [26]. The 2-AB tag was chosen over other available fluorophores, including 2-aminopyridine (2-AP), 2-aminoanthranilic acid (2-AA), 2-aminoacridone (AMAC) and RFMS, because its fluorescence intensity is directly correlated to the number of moles of labeled N-glycan present in the sample allowing the relative abundance of individual N-glycans to be estimated from the area of their corresponding peaks, and it is capable for labeling free glycans [36,46–49].

3.1. System suitability test for glycan separations

The Waters 2-AB Glycan Performance Standards were run to validate the suitability of the proposed workflow for assessing glycan mixtures independently of the sample libraries to be tested. The performance standards were separated based on their size and hydrophilicity using HILIC-UHPLC-FLR (method 1). HILIC was used for system calibration and validation because of the ease with which glycan retention times can be assigned GU values if proper chromatographic reference standards are used [50] The GU value can then be used to determine the identity of individual N-glycans because it is directly related to the number and linkage of constituent monosaccharides [37].

The elution times of the N-glycans in the performance standard were converted to GU values using the calibration curve. Experimentally determined GU values were all within ±0.15 GU of those in the database. Next, the *m/z* values for all major N-glycans were measured. Almost all of the *m/z* values were within ±5 ppm of the manufacturer's assignment. For Man5, F(6)A2B, and A2G(4)2), the error was ±10 ppm due to interference from co-eluting N-glycans. The identity of all the N-glycans in the Waters 2-AB Glycan Performance Standards was confirmed.

3.2. Bridging study

The USP has published two validated HPLC methods that use HILIC for glycan analysis in USP General Chapter <212>. Since the publication of these HPLC methods, there have been significant advances in the use of UHPLC technology. In general, HPLC methods have less peak resolution and much longer run-times than UHPLC methods. Waters Corporation has published two HILIC-UHPLC methods (methods 1 and 2) for use with the ACQUITY UPLC® Glycan BEH Amide column. Method 1 was developed for the analysis of 2-AB labeled glycans and was used to determine the G.U. values in the Waters 2-AB database. Method 2 was developed for the analysis of RapiFluor-MS labeled N-glycans. Both methods have a similar gradient slope; however, method 2 calls for a higher column temperature than method 1. The increase in temperature reduces the viscosity of the mobile phase. The result is a decrease in back pressure at a constant flow rate that gives improved peak resolution without the need to increase run times [51,52]. The better peak resolving capacity of method 2 makes it useful for mapping minor glycans in USP-Mix A and B; however, method 2 is not validated for identifying 2-AB labeled N-glycans.

Therefore, to use method 2 for mapping of minor N-glycans, we needed to compare GU value assignments of 2-AB labeled glycans (USP-Mix A and B) using both methods 1 and 2 (Fig. 1). The glycan profiles of UPS-Mix A and B produced using methods 1 and 2 were similar to each other as well as to the HPLC method described in the USP General Chapter <212>. GU values for the major peaks were calculated as described, and the GU values for the major peaks were determined from the chromatograms produced from both UHPLC methods (Table 1). We hypothesized that the GU values calculated by the two methods would agree based on the similar gradient slopes. However, to ensure that GU values would match, we normalized them using the same 2-AB labeled dextran standards. Using the GU values, we determined the identity of each N-glycan and confirmed that they matched with those listed in the certificate of analysis (COA) accompanying both USP-Mix A and B. The major peaks in USP-Mix A correspond to the N-glycans G0, G0F, G1Fa, G1Fb, G2, G2F, A1F, and A2F. The major peaks in USP-Mix B correspond to the N-glycans Man5, Man6, Man7, Man8 and Man9. The results of this bridging study demonstrated that method 2 is comparable to method 1 for identifying major N-glycans in both USP-Mix A and B.

3.3. Identification of minor glycans

USP-Mix A was re-analyzed using method 2 to assess the presence of minor N-glycans (Fig. 2). The combination of HILIC and MS allowed assignment of GU values to 32 chromatographic peaks and identification of 30 N-glycans with significant diversity in monosaccharide composition, linkage, and branching pattern (Table 2). All calculated values were within ±0.2 GU of the corresponding values in the database with a 1.1% S.E. The mean mass accuracy error was 3.12 ± 1.8 ppm, with all values within the ppm cutoff of ±10 ppm. Only two peaks (1 and 27) could not be definitively identified. The GU and *m/z* values of peak 1 (5.18 GU & 1380.5228 m/z) were similar to that of peak 2 (5.29 GU & 1380.5389 m/z). Therefore, peak 1 likely corresponded to an isoform of F(6)A1 found in peak 2 or FA1. Similarly, the GU and *m/z* value of peak 27 (9.53 GU & 1245.4564 m/z) were similar to that of peak 30 (10.08 GU & 1245.4564 m/z). Therefore, peak 27 was likely an isoform of F(6)A2G(4)2S(3,6)2 or F(6)A2G(4)2S(3,3)2, herein referred to as FA2G2S2.

The analysis was repeated for USP-Mix B. A total of 16 N-glycans isoforms were assigned to 14 of the 16 chromatographic peaks (Fig. 3). GU values were within ±0.2 GU of the corresponding values in the database with a 1.2% S.E. The mean mass accuracy error (ppm) was 3.81 ± 3.1 ppm. Only 1 of the 16 m/z values exceeded the ±10 ppm threshold (Table 3). Identification of the N-glycans in peaks 5, 6, 12, and 13 was ambiguous. The N-glycans in peak 5 and 6, F(6)A2 [6]G(4)1 and M4A1G(4)1), had the same GU value but different *m/z* values. The N-glycans in peak 10 and 11, M5A1G(4)1 and F(6)A2G(4)2), had the same GU value but different *m/z* values. Also, it was not possible to distinguish M5A1G(4)1 from M4A1G(4)1Ga(3)1 with the current approach since both have the same GU and *m/z* values in the N-glycan database. Finally, peaks 3, 8, 12, and 14 were satellite peaks eluting just before peaks 4, 9, 13, and 15. The satellite peaks had the same GU and *m/z* values as their corresponding main peaks and were likely isoforms of Man5, Man6, Man7, and Man8.

Structure characterization of isomers is possible, however. For example, Zhu et al. observed the presence of multiple structure isoforms of high mannose glycans, and characterized the isoforms of Man7 by direct analysis of permethylated glycans from ribonuclease B with ion mobility spectrometry-tandem mass spectrometry (IMS-MS/MS) [53]. More recent studies have revealed 18 isomeric structures of high mannose glycans (Man4-Man9) by applying an on-line porous graphitic carbon liquid chromatography (PGC-LC)-electronic excitation dissociation (EED) MS/MS method to analyze glycans released from ribonuclease B [54]. The peak assignments in this article are consistent with the observations from previous studies. Finally, the analysis for each glycan library was also performed using negative ion mode to rule out any

incorrect identifications. If a glycan detected in positive mode is due to fragmentation from a parent glycan, then it is unlikely that the same fragment would appear in negative mode since the source voltage and parameters were different. Negative ion analysis resulted in identical glycan assignments (data not shown).

3.4. Comparison of glycan reference standards

Two commercial libraries (Vendor 1 and Vendor 2) with N-glycan composition similar to that of USP-Mix A were analyzed using method 2. The identities of the major peaks from the libraries were independently assigned as described and agreed with the manufacturer's assignments (data not shown). To assign minor peaks, we reasoned that since USP-Mix A and both commercial libraries were all sourced from human IgG that similar GU values correspond to the same N-glycan. Therefore, USP-Mix A minor peak assignments were used to assign the minor N-glycan peaks in the other libraries.

The relative abundance of 24 N-glycans in USP-Mix A, and both commercial N-glycan libraries was determined by calculating individual peak area in relation to the total N-glycan elution profile. The relative abundance of individual glycans was compared for all three libraries (Fig. 4). The vendor N-glycan libraries were dominated by asialylated biantennary oligosaccharides, whereas the USP-Mix A library had a more uniform distribution of partially galactosylated, partially fucosylated biantennary and triantennary N-glycans.

Sialylated N-glycans made up over 25% of the USP-Mix A library (Fig. 5). Vendor 1 and 2 contained only 22% and 10% of N-glycans, respectively. This result suggests that USP-Mix A would be beneficial for the detection of sialylated glycans that are important in the development of monoclonal antibodies. For example, the presence of sialylated glycans on the Fc region of immunosuppressive immunoglobulins (IVIG) reduces the antigen-dependent and complement-dependent cellular cytotoxicity that underlies their anti-inflammatory properties [55] However, high levels of sialylation can also result in a reduction in efficacy. For example, increased sialylation reduces the efficacy of IVIG products that depend on binding to FcγRIIIa on natural killer (NK) cells or to cell-surface antigens [56].

4. Conclusions

In this study, we validated the use of USP Oligosaccharide System Suitability Mixtures A and B Reference Standards (USP-Mix A and B) with two UHPLC methods (method 1 and 2) that have much shorter run times than comparable HPLC protocols. Both methods were suitable for the identification of all N-glycans previously identified using HPLC methods, as described in USP General Chapter <212>. Next, we used UHPLC method 2 to characterize previously unidentified peaks in both USP-Mixes A and B. A total of 30 N-glycans were identified in USP-Mix A, including the eight major N-glycans listed in the certificate of analysis (COA) as well as 22 N-glycans that were previously uncharacterized. The total was similar to that previously reported from the analysis of released N-glycans from IgGs [57]. In USP-Mix B, a total of 14 N-glycans were identified, 9 of which were previously uncharacterized.

The N-glycan composition of USP-Mix A was compared to two commercial libraries also derived from IgG release. All three libraries had a similar distribution of major N-glycans, but USP-Mix A had a better overall representation of sialylated glycans than the two commercial libraries. Characterizing sialylation is not only vital for established drug products such as IVIGs but is also critical for developing the next generation of cancer therapies. Cancer cells are known to have a higher density of glycan structures terminating in sialic acid than healthy cells [58]. This coat of sialic acid activates Siglec receptors on the surface of T cells, macrophages, and NK cells, causing immune suppression [59].

Consequently, inhibiting the interaction between Siglecs and sialic acid could unleash an immune response more potent than that generated by checkpoint inhibitors. Indeed, drugs targeting Siglecs are just now beginning to enter clinical trials [60]. However, the road to a safe and effective therapy based on this technology remains long and uncertain. There are currently over a dozen different Siglecs to choose from, and each one interacts with more than one sialic acid. To develop inhibitors of these receptors requires a complete understanding of all the possible interactions. A reference standard rich in sialylated N-glycans would be an essential component to characterizing receptor preference and ensuring drug specificity.

USP-Mix A and B are valuable tools for many different projects, but their most important function is to demonstrate a complete understanding of and control over the glycosylation of a biological product. This use is critical, especially as regulators improve guidelines and increase expectations for manufacturers to validate the performance of essential components of their processes. The results from this study demonstrate that USP-Mixes A and B not only support compendial procedures but can also be used to evaluate the performances of advanced methods like UHPLC, as well as the instruments and analysts involved in glycoprofiling. Finally, using well-characterized reference standards with harmonized analytical methods plays an essential role in characterizing N-glycan heterogeneity in biopharmaceuticals. To expand the number of available reference standards available for benchmarking glycoproteins, we will continue to characterize more glycan libraries, including USP glycan Mix C and D, as well as various glycoprotein reference standards using modernized analytical procedures.

Disclaimer

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CRediT authorship contribution statement

Jingzhong Guo: Writing - original draft, Conceptualization, Investigation, Methodology, Visualization, Formal analysis, Data curation. **Huiping Tu:** Writing - review & editing, Supervision, Visualization. **Maheswara Rao B:** Investigation. **Anjali Kumari Chillara:** Investigation. **Edith Chang:** Writing - review & editing, Supervision. **Fouad Atouf:** Project administration, Supervision.

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