Preparation Scale-Up of Complex Biological Samples for Deep N-glycomic Analysis by CE-LIF and CESI-MS

Andras Guttman[1,2] and Marton Szigeti[2]

1 SCIEX, Brea, CA, US

2 Horváth Csaba Memorial Laboratory of Bioseparation Sciences, University of Debrecen, Hungary

Introduction

Complex biological samples, including bioprocessing specimens in the pharmacological sector, contain proteins in a wide concentration range of pg/mL to mg/mL levels.[1] Most of these proteins are post-translationally modified, including the attached carbohydrates, which allegedly influence their activity, physicochemical properties and effector functions.[2] Therefore, deciphering the glycan moieties of such proteins is important in both the biomedical and biopharmaceutical arenas. For deep N-glycomic analysis of biological and bioprocessing samples using high-performance glycoanalytical tools—such as capillary electrophoresis with laser-induced fluorescence (CE-LIF) detection or its hyphenated format, capillary electrophoresis with electrospray ionization mass spectrometry (CESI-MS)—efficient and precipitation-free sample preparation methods are needed to provide the sufficient amount of analyte for high sensitivity.

In this technical note, an efficient and scalable sample preparation workflow is introduced to accommodate deep N-glycomics analysis of complex biological samples containing up to milligrams of proteins. The protocol includes the capture of glycoproteins by amine coated magnetic beads along with a novel temperature gradient denaturation process to avoid precipitation. The workflow also helps to significantly decrease the concentration of free low molecular mass sugar contamination, minimizing their inhibition effect on endoglycosidase digestion-based oligosaccharide removal. Most importantly, application of the new sample preparation scale-up workflow (Figure 1) is made possible to provide the appropriate amount of N-linked carbohydrates for deep glycomic analysis by CE-LIF and CESI-MS, even in negative ionization mode for the latter.

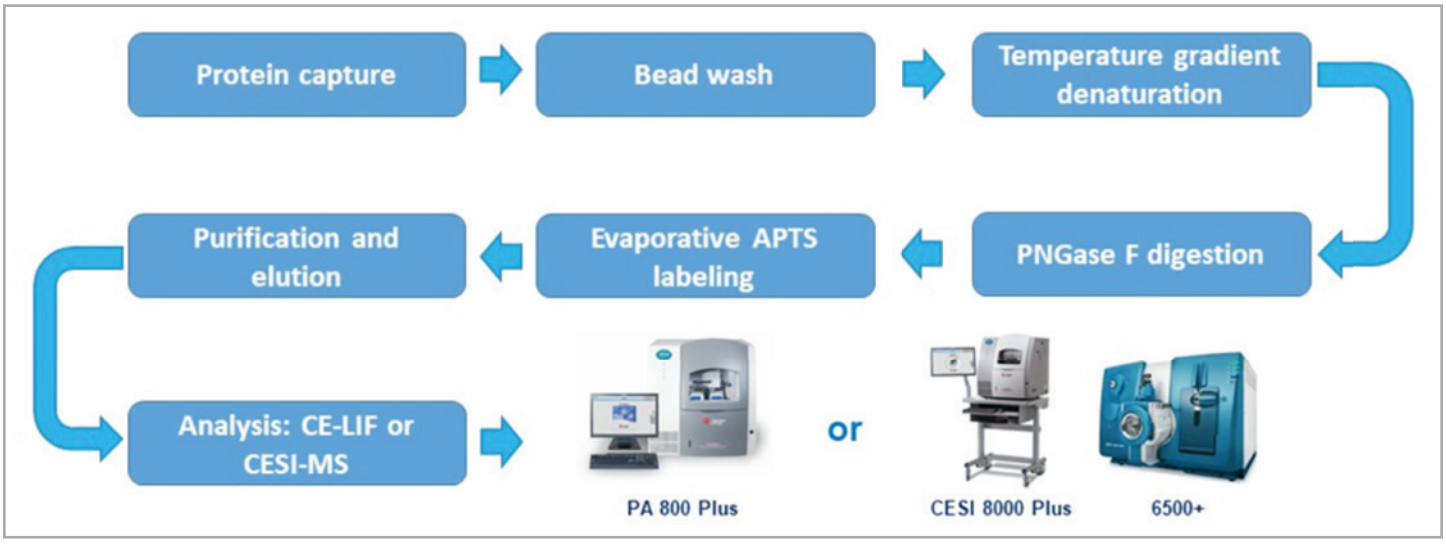


Figure 1. Sample preparation scale-up workflow for deep N-glycomic analysis of complex biological samples by CE-LIF and CESI-MS.

Key features of the C100HT Biologics Analyzer System

- An efficient sample preparation scale-up workflow is introduced for deep N-glycomics analysis of complex biological samples and bioprocessing specimens
- Protein precipitation is prevented by the novel temperature gradient denaturing protocol
- The free sugar content in the complex biological samples is significantly decreased, enabling improved endoglycosidase digestion efficiency
- The fluorophore labeling yield is enhanced by the modified evaporative labeling protocol

Methods

Chemicals: The Fast Glycan Labeling and Analysis Kit (SCIEX, Brea, CA) included the tagging dye of 8-aminopyrene-1,3,6-trisulfonic acid (APTS), HR-NCHO separation gel buffer and the purification components. The amine functionalized magnetic beads were from chemicell (Berlin, Germany). PBS, acetic acid, ammonium acetate, isopropanol, sodium-cyanoborohydride (1 M in THF), tetrahydrofuran, HPLC grade water, acetonitrile, human IgG1 and human serum were from Sigma-Aldrich (St. Louis, MO). The PNGase F enzyme was from Asparia Glycomics (San Sebastian, Spain).

Temperature gradient denaturation: Glycoproteins from 50 µL of undiluted human serum (or 50 µL of 30 mg/mL of hIgG1 solution) were captured by 20 µL (50 mg/mL) of well-suspended amine-functionalized magnetic beads mixed with 50 µL of 1 M sodium-cyanoborohydride (in THF) and 1.0 mL of 0.1x PBS. After discarding the supernatant, the beads were washed with 1.0 mL of HPLC grade water and re-suspended in 20 µL of HPLC grade water. The captured proteins were denatured on the beads by applying a temperature gradient of 5.0 °C/min, with a linear increase from 30 °C to 80 °C and an additional 5.0 min isothermal incubation step at 80 °C in a PCR machine (Model PTC-100, Marshall Scientific, Hampton, NH) in the presence of 10 µL of premixed denaturation solution (Fast Glycan Labeling and Analysis Kit, SCIEX) with the addition of 12.5% glycerol.

Endoglycosidase digestion: The PNGase F digestion was accomplished on the beads with the addition of 3 IUB (International Union of Biochemistry) units of enzyme (2.0 µL) followed by incubation at 50 °C for 1.0 h and finished by precipitating the deglycosylated proteins onto the surface of the amine coated beads by 120 µL of ice-cold acetonitrile. The supernatant was dried and labeled with 8-aminopyrene-1,3,6-trisulfonic acid (APTS) utilizing a modified evaporative labeling protocol of 1 h at 50 °C with closed lid vials and 1 h at 55 °C with open lid vials.[3] The excess APTS dye was removed by following the sample preparation and analysis protocol of the Fast Glycan Labeling and Analysis Kit (SCIEX). The purified samples were analyzed by CE-LIF and CESI-MS.

Capillary Electrophoresis with Laser-Induced Fluorescence (CE-LIF): A PA 800 Plus Pharmaceutical Analysis System (SCIEX) was used for all CE-LIF analysis utilizing a solid-state laser with 488 nm excitation and 520 nm emission wavelengths and employing the HR-NCHO separation gel buffer. The effective lengths of the 50 µm ID bare fused-silica capillaries were 50 cm and 20 cm, depending on the application. The respective electric field strengths were 500 and 1000 V/cm, in reversed polarity mode. The separation temperature in all analyses was 30 °C, and the samples were injected using a 5 s/3–5 psi water pre-injection followed by electrokinetic sample injection by 1–6 kV/1–3 s and the injection of the bracketing standard by 1 kV/1 s. The 32 Karat™ Software 10.1 (SCIEX) was used for data acquisition.

CESI-MS: All CESI-mass spectrometry tests used the CESI 8000 Plus High-Performance Separation - ESI Module, connected to a QTRAP® 6500+ LC-MS/MS System (both from SCIEX) through an OptiMS capillary cartridge (91 cm total length, 30 µm ID, 150 µm OD). All separations were done using 10 mM of ammonium acetate, 0.1% acetic acid (pH 4.5), and 20% isopropanol containing MS friendly background electrolyte. The applied voltage was 20 kV in reversed polarity mode with an additional 2 psi forward pressure at both the separation and the conductive capillaries. The cartridge temperature was set to 20 °C. Injection: 1 psi / 5 s water pre-injection followed by 10 kV / 20s sample introduction.

Important:

- A separation current above 5 µA might cause permanent damage to the separation capillary.
- Generally, please do not apply >2000V to generate electrospray as it may result in capillary damage.

Mass Spectrometry: MS analysis used a QTRAP® 6500+ System in negative ionization mode. The ESI voltage was set to -1,600 V for 1 min to stabilize the spray and to -1,400 V for high-sensitivity detection. The orifice plate temperature was 80 °C with 5 psi curtain gas pressure for spray stabilization at the 20.4 nL/min flow rate. The multiple reaction monitoring (MRM) scan targeted the doubly charged species of APTS labeled

glycan masses at 15 msec, and the data were evaluated and visualized with PeakView® Software 2.2 (SCIEX).

Practicing traditional isothermal denaturation methods during the analysis of protein glycosylation usually results in precipitation when larger than several hundred micrograms of proteins are used, making downstream sample processing difficult or even impossible. The recently introduced temperature gradient denaturation protocol successfully addressed this problem and allowed precipitation-free denaturation of up to 1.5 mg protein-containing samples in a volume of 50 µL.[4]

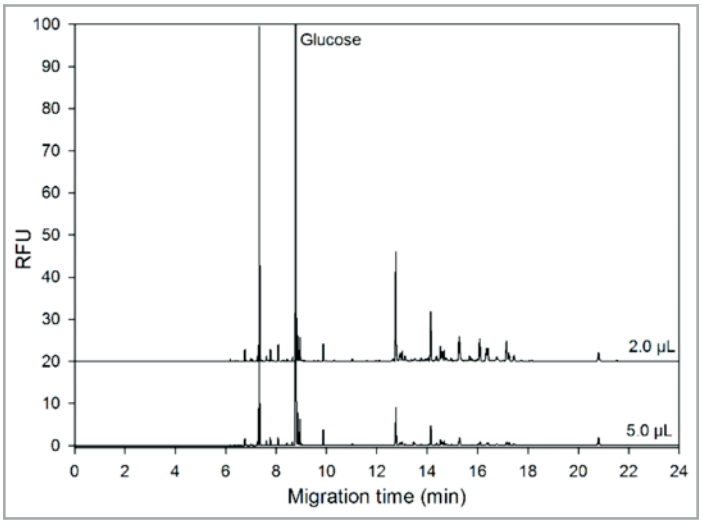


Figure 2. Comparative CE-LIF analysis of the human serum N-glycome using 2 and 5 µL starting material. Conditions: HR-NCHO gel buffer in 50 cm effective (60 cm total) and 50 um ID bare fused-silica capillary column; E=500 V/cm, t= 30 °C. Injection: water for 5 s at 5 psi, samples for 3 s at 6 kV and bracketing standard 1 s at 1 kV.

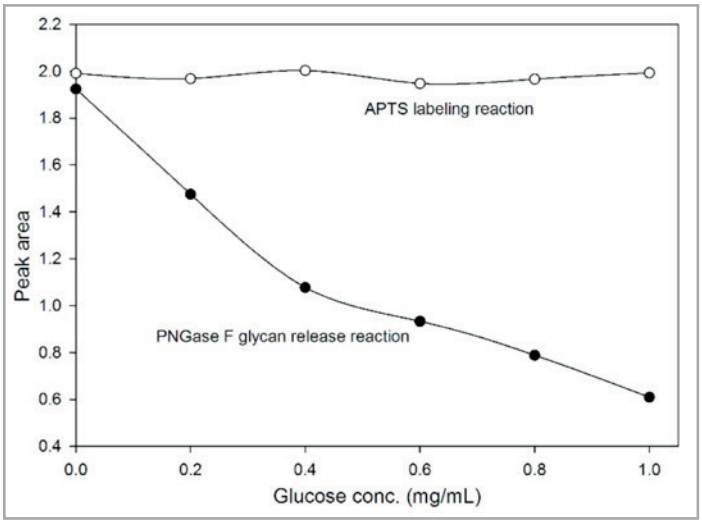


Figure 3. Effect of the extra glucose added to the reaction mixture on the APTS labeling (◦) and PNGase F digestion (•) effectivity.

Figure 2 compares the CE-LIF electropherograms of the PNGase F released, and APTS labeled human serum N-glycome using 2 and 5 µL starting material. With otherwise equal glucose peak heights, the separation trace generated by the use of 5 µL starting material unexpectedly resulted in lower peak intensities for the N-glycome peaks than those that resulted from starting with 2 µL human serum.

Figure 3 shows the resulting average peak intensity changes. To understand this counterintuitive phenomenon, both the APTS labeling and the PNGase F digestion processes were closely scrutinized by the addition of extra glucose to the reaction mixtures. Extra glucose was added prior to the APTS labeling reaction step (◦) and before the endoglycosidase digestion reaction step (•). As one can observe, the APTS reaction kinetics were not affected by the presence of the extra glucose, while the PNGase F digestion reaction was significantly influenced, losing most of its effectivity at the 1 mg/mL added glucose level, probably due to the inhibitory effect of small sugars on reaction kinetics.[4]

Based on these observations, a novel sample preparation scale-up workflow has been developed for deep N-glycomic analysis by CE-LIF and CESI-MS, comprising the following steps.

1. Protein capture:

- Add 50 µL of complex biological or bioprocessing sample into a 1.5 mL Eppendorf tube.
- Add 50 µL of 1 M NaBH3CN (in THF) to the sample.
- Vortex the sample at maximum speed for 5 s.
- Wait for 1 min.
- Add 1.0 mL of 0.1x PBS solution.
- Add 20 µL of amine-functionalized magnetic beads (50 mg/mL, 1.0 µm diameter).
- Wait for 30 min at room temperature (RT).

2. Removal of the unbound proteins:

- Remove the supernatant after pulling down the magnetic beads by a magnetic stand.
- Reconstitute the beads in 500 µL of HPLC grade water.
- Vortex the sample at maximum speed for 10 s.
- Apply a quick (2–3 s) spin-down in a benchtop microfuge.
- Wait for 5 min at RT.
- Remove the supernatant while the vial is on the magnetic stand.

3. Temperature gradient denaturation:

- Prepare the denaturation solution by supplementing 70 µL of the Fast Glycan Labeling and Analysis Kit denaturation solution with 10 µL of glycerol. (The Fast Glycan Labeling and Analysis Kit denaturation solution consists of: 50 µL of D4 + 10 µLD1 + 10 µL of D3. Other similar purpose denaturation solutions from other vendors may be used with the glycerol supplement.)
- Add 20 µL of HPLC grade water to the beads.
- Apply a quick (2–3 s) spin-down in a benchtop microfuge to remove any sample from the wall of the Eppendorf vial.
- Add 10 µL of denaturing solution to the sample (do not touch the beads with the pipette tip).
- Vortex the sample at maximum speed for 10 s.
- Apply a quick (2–3 s) spin-down in a benchtop microfuge.
- Denature the sample for 15 min using the following temperature gradient: 30 °C to 80 °C using a 5 °C/min heating program, followed by 5.0 min incubation at 80 °C.

4. PNGase F digestion on the bead:

- Apply a quick (2–3 s) spin-down in a benchtop microfuge.
- Add 50 µL of HPLC grade water after the denaturation step.
- Add 2.0 µL of PNGase F enzyme.
- Vortex the sample at maximum speed for 5 s.
- Apply a quick (2–3 s) spin-down in a benchtop microfuge.
- Incubate the sample at 50 °C for 1.0 h.

5. Evaporative APTS labeling

- Apply a quick (2–3 s) spin-down in a benchtop microfuge.
- Add 120 µL of ice-cold acetonitrile.
- Keep the sample at -20 °C for 15 min.
- Centrifuge the sample at 10,700 x g for 5 min.
- Transfer the sample supernatant (200 µL) into a new 0.5 mL Eppendorf vial.
- Dry the sample in a SpeedVac for 60 min at 60 °C.
- Prepare the labeling stock solution of 9.0 µL of 20 mM APTS (in 20% acetic acid), 1.0 µL of 1 M NaBH3CN (in THF) and 10 µL of additional THF per sample.
- Add 20 µL of labeling solution to the dry sample (wash down the sample from the Eppendorf wall with the labeling solution).
- Vortex the sample at maximum speed for 10 s.
- Apply spin-down for 10 s in a benchtop microfuge.
- Incubate the sample for 1 h at 50 °C with the lid closed.
- Incubate the sample for 1 h at 55 °C with the lid open (or until the sample is completely dry).

6. Purification and elution of the APTS labeled glycans:

- Reconstitute the dry sample with 20 µL of Fast Glycan Labeling and Analysis Kit magnetic beads in water (concentrated from 400 µL of M1 process solvent by storage solution replacement with water on a magnetic stand).
- Vortex the sample at maximum speed for 10 s (wait for 3 min for complete reconstitution after addition of the beads, then vortex again).
- Add 170 µL of acetonitrile to the sample (do not vortex after this step).
- Wait for 1 min at RT.
- Remove the supernatant after placing the vial on a magnetic stand.
- Repeat the last 3 steps 3 more times using 20 µL of HPLC grade water and 170 µL of acetonitrile per the Fast Glycan Labeling and Analysis Kit user manual.
- Add 50 µL of HPLC grade water after the last supernatant removal step.
- Vortex the sample at maximum speed for 10 s.
- Apply a quick (2–3 s) spin-down in a benchtop microfuge.
- Place the sample on the magnetic stand for at least 1 min.
- Transfer 45 µL of sample (avoid magnetic bead transfer) into a new 200 µL PCR tube.
- Store the sample at 4 °C until analyzed.
- Use 5.0 µL of sample for up to 5 consecutive injections in a CE nanovial for CESI-MS measurements.

To demonstrate the advantages of this new and efficient workflow, deep N-glycomic analysis of a human serum sample was attempted. Figure 4 depicts the separation of the N-linked human serum oligosaccharides by CE-LIF (upper panel) and CESI-MS (lower panel) using the amine-functionalized magnetic bead-based protein capture with temperature gradient denaturation-based sample preparation protocol. The use of this approach enabled a “precipitation-free” preparation of 50 µL human serum sample, 25 times larger than that of the amount usually used to avoid precipitation (2 µL). Application of this special protocol also removed the majority of the blood glucose content from the sample, alleviating endoglycosidase enzyme inhibition. All small peaks, otherwise buried in the baseline,

were clearly visible and quantifiable in the electropherogram in Figure 4 (upper panel). The inset shows the full-scale image of the separation. The reproducibility of this sample preparation protocol is depicted in Table 1. Figure 4 (lower panel) delineates the evaluation of the workflow for CESI-MS analysis featuring the total ion electropherogram of the PNGase F released and APTS labeled N-linked human serum oligosaccharides. All glycan structures identified by CE-LIF and CESI-MS methods are shown in Table 2 with the corresponding structural information and their integrated peak area values.

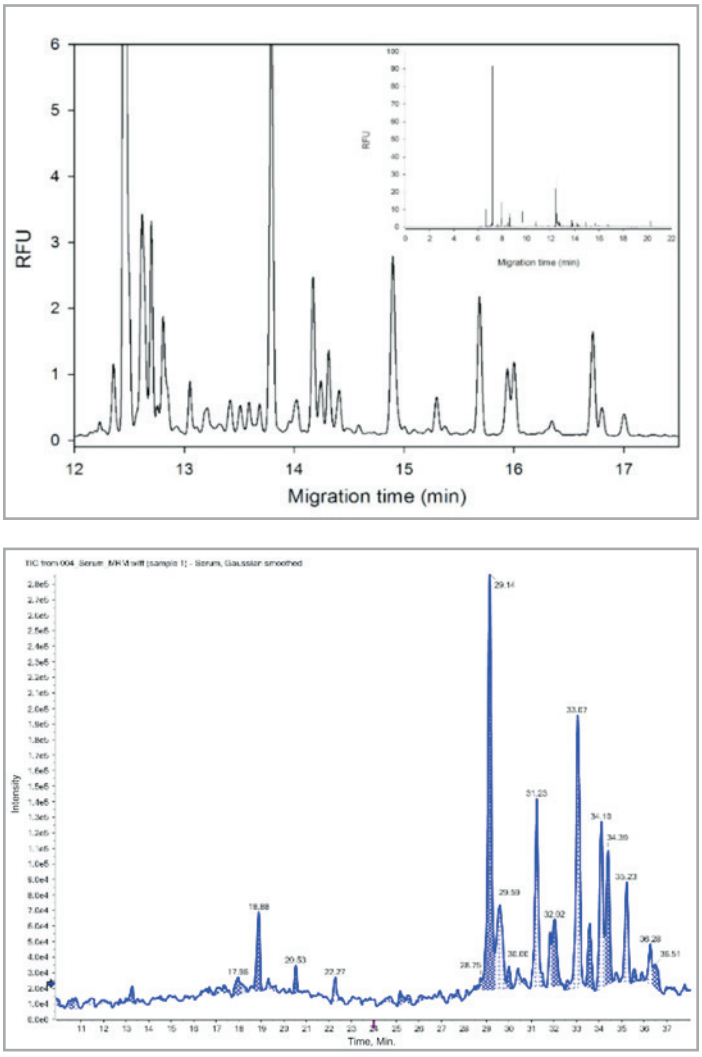


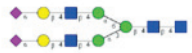
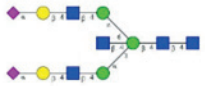
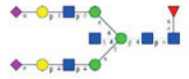
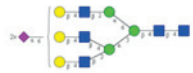
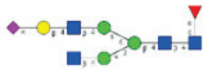
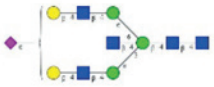
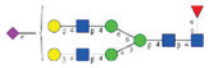
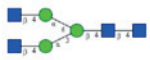
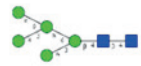
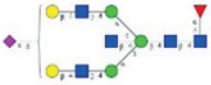
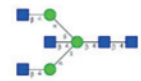
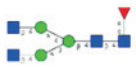
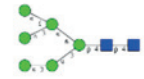
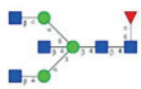
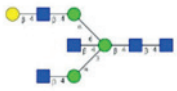
Figure 4. Capillary electrophoresis separation of PNGase F released and APTS labeled human serum N-glycans by CE-LIF (upper panel) and CESI-MS (lower panel). Separation conditions: CE-LIF, same as in Figure 2; injection, 1 kV/1 s; CESI-MS, OptiMS cartridge (91 cm total length, 30 µm ID/150 µm OD); background electrolyte, 10 mM ammonium acetate, 0.1% acetic acid (pH 4.5) and 20% isopropanol; applied voltage: E=220 V/cm in reversed polarity mode at 20 °C with additional 2 psi forward pressure at both the separation and the conductive lines; injection, 1 psi/5 s water pre-injection followed by 10 kV/20 s sample injection.

Measurement		%RSD	
		Intra-day	Inter-day
CE-LIF	Hardware performance (peak area)	0.92	1.35
	hIgG1 sample preparation (peak area)	3.40	5.42
	Human serum sample preparation (peak area)	7.83	9.63
CESI-MS	Peak area (total)	6.11	8.53
	Migration time	1.42	2.31

Table 1. Reproducibility of the sample preparation scale-up and analysis workflow.

Conclusions

Based on the significant sample preparation improvements of the individual optimized steps described in this technical note, a novel workflow was established to analyze the N-linked carbohydrates of complex biological samples readily applicable for bioprocessing specimens. The sample preparation scale-up protocol was applied for N-linked glycosylation analysis of human serum samples without any precipitation-related problems up to 1.5 mg protein content by using the combination of amine-functionalized magnetic bead-based protein capture and temperature gradient denaturation with our earlier reported evaporative labeling protocol. In addition, this approach efficiently removed the free sugar content of complex biological samples (e.g., serum glucose) that would otherwise inhibit the endoglycosidase digestion reaction during N-glycan release. Deep N-glycomic analysis of human serum samples was demonstrated using CE-LIF and CESI-MS methods, and 28 individual structures were identified.

No.	Abbreviated structural interpretation	Glycan composition	Neutral mass	Doubly charged APTS labeled mass	Glycan structure
1	A2G2S2	H5N4A2	2222.78	1330.87	
2	A2BG2S2	H3N5	1519.57	979.26	
3	FA2G2S2	H5N4A2F1	2368.84	1403.89	
4	FA2BG2S2	H5N5A2F1	2571.92	1505.43	
5	A2G1S1	H4N4A1	1769.63	1104.29	
6	A3G3S2	H6N5A2	2587.92	1513.43	
7	FA2G1S1	H4N4A1F1	1915.69	1177.32	
8	A2G2S1	H5N4A1	1931.69	1185.32	
9	A2BG2S1	H5N5A1	2134.77	1286.86	
10	FA2G2S1	H5N4A1F1	2077.75	1258.35	
11	A2	H3N4	1316.49	877.72	
12	M5	H5N2	1234.43	836.69	
13	FA2BG2S1	H5N5A1F1	2280.82	1359.89	
14	A2B	H3N5	1519.57	979.26	
15	FA2	H3N4F1	1462.54	950.75	
16	M6	H6N2	1396.49	917.72	
17	A2G1	H4N4	1478.54	958.74	
18	FA2B	H3N5F1	1665.62	1052.29	
19	A2BG1	H4N5	1681.62	1060.28	

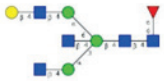
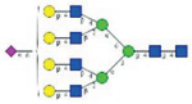
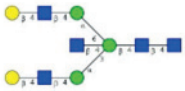
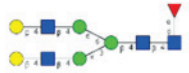
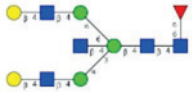
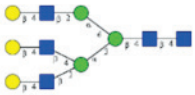
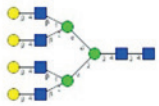
20	FA2G1	H4N4F1	1624.60	1031.77	
21	FA2BG1	H4N5F1	1827.68	1133.31	
22	A4G4S1	H7N6A1	2661.95	1550.45	
23	A2G2	H5N4	1640.59	1039.77	
24	A2BG2	H5N5	1843.67	1141.31	
25	FA2G2	H5N4F1	1786.65	1112.80	
26	FA2BG2	H5N5F1	1989.73	1214.34	
27	A3G3	H6N5	2005.72	1222.34	
28	A4G4	H7N6	2370.86	1404.90	

Table 2. Identified N-glycan structures by CE-LIF and CESI-MS.[5]

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