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Evaluation of oligosaccharide methods for carbohydrate analysis in a fully human monoclonal antibody and comparison of the results to the monosaccharide composition determination by a novel calculation

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ABSTRACT

Carbohydrates can change a drug's properties including solubility, affinity towards antigen, pharmacokinetics and pharmacodynamics. Due to this importance, carbohydrate composition is utilized as a parameter to evaluate a drug candidate's quality. In this study, the compositional monosaccharides of a drug candidate are measured by HPAEC-PAD, while the oligosaccharides are studied by HPAEC-PAD, CE-LIF and LC–MS. The advantages and limitations of these various approaches for oligosaccharide analysis are reviewed in this work. While the methods used for oligosaccharide analysis are well established we have devised a new and novel calculation for determining monosaccharide content using the relative percentages of the N-glycans. This calculation was used to evaluate the accuracy of the oligosaccharide determination methods by comparison of the N-glycan data to the experimental monosaccharide data. The results obtained from this novel calculation demonstrate that the relative abundance of carbohydrates as determined from these various approaches are consistent.

1. Introduction

Monoclonal antibodies are playing an increasing role as therapeutics in clinics across the world, with 21 currently approved for treatment in the United States and hundreds in the clinical pipeline of many pharmaceutical and biotechnology companies [1]. Monoclonal antibodies elicit two major effector functions: antibody-dependent-cell mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) [2–4]. It is known that recombinant proteins including monoclonal antibodies are subject to post-translational modifications such as proteolytic cleavage, modification of one or more amino acids and glycosylation during biological process or production [5,6]. Among the many different post-translational modifications, glycosylation is extensively studied to obtain structural and compositional information due to its important role in pharmacokinetics (ADCC and CDC), pharmacodynamics (antigen binding), secretion, antigenicity, stability, folding, and clearance of glycoproteins [7–12].

Immunoglobulin G (IgG) is the predominant antibody in serum and its presence is associated with immunity for many infectious diseases. The IgG protein is composed of two heavy chains and two light chains that are covalently linked by disulfide bridges. In general, IgG has one N-linked glycosylation site in the CH_2 domain of the Fc region with a second possible glycosylation site in Fab region [13,14]. The increasing interest in the development of antibodies for therapeutic purposes has created a high demand for methods to characterize the sugar moieties bound to IgG.

More than 20 years ago, high-performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD) was developed [15]. One of the advantages of this method was that it offered direct analysis of the carbohydrate species without the need for derivatization. Recently, capillary electrophoresis (CE) has emerged as a powerful tool in carbohydrate analysis with its enhanced separation efficiencies [16]. By coupling CE with laser-induced fluorescence detection a method (CE-LIF) that is highly sensitive for the analysis of carbohydrates was developed [17–19].

Although these methods are suitable for lot-to-lot testing of glycoproteins, the lack of key structural information has led to the use of off-line mass spectrometry techniques, such as matrix-assisted laser desorption/ionization time of flight (MALDI-TOF). The major drawback of MALDI-TOF is its ability to detect low abundance glycans and to quantitate sialylated glycans. Furthermore, most samples must be desalted before analysis and because of the hydrophilic behavior of carbohydrates their recovery is limited during the desalting process. With the improvement of mass spectrometric methods, on-line Liquid chromatography coupled to mass spectrometry (LC–MS) and quadrapole time-of-flight (Q-TOF)

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have been employed to determine the heterogeneity of carbohydrates and glycoproteins [20–22]. In the current work, LC–MS was employed to determine the overall relative percentage of each N-glycan after a PNGase F digestion of the antibody. The relative percentage of each N-glycan at different glycosylation sites was also determined through trypsin digestion followed by LC–MS. The carbohydrates and glycopeptides were identified by their single and/or double charge and the structures were confirmed by tandem mass spectrometry.

In this paper, the accuracy of the different methods used for the determination of carbohydrates, which included HPAEC-PAD, CE-LIF and HPLC–MS, are evaluated and compared to the method for determination of monosaccharide content. A new and novel approach to calculating the monosaccharide relative abundance from the oligosaccharide methods results is reported. By using this novel calculation, we have demonstrated that the N-glycan experimental data from the various analytical methods is comparable to the monosaccharide assay results.

2. Experimental

2.1. Apparatus

Determination of monosaccharide and oligosaccharide content in the glycoprotein were performed using a Dionex BioLC, DX-600 system (Dionex Corp, Sunnyvale, CA) consisting of an AS50 autosampler and thermal compartment, an ED50 Electrochemical Detector with a non-disposable gold working electrode and a GP40 Gradient Pump. The sample temperature was set to 4.0 °C and the column temperature was 30.0 °C. The flow rate was set to 1 mL/min using Waveform A for detection [23]. The monosaccharides were separated by isocratic elution on a Dionex CarboPac PA10 column and a Dionex AminoTrap guard column with 18 mM NaOH for 18 min. The oligosaccharides were separated by gradient elution with 5–225 mM sodium acetate in 100 mM sodium hydroxide over 50 min on a Dionex CarboPac PA100 column and CarboPac PA100 guard column [24].

Characterization of the antennary N-linked oligosaccharide profile by capillary electrophoresis was performed on a P/ACE MDQ Capillary Electrophoresis System (Beckman Coulter, Fullerton, CA) equipped with a LIF 488 Laser Module using a 50.0 cm (40.0 cm to the window) eCAP N-CHO coated capillary. Direct laser-induced fluorescence detection was employed with an excitation of 488 nm and emission at 520 nm. Separation was performed by voltage in reverse polarity mode at 500 V/cm for 20 min with a 0.17 min ramp. The capillary temperature was set at 20.0 °C. The sample storage temperature was set at 4.0 °C.

Characterization of antennary N-linked oligosaccharide profile by high-performance liquid chromatography-electrospray ionization mass spectrometry (HPLC–ESMS) was performed using a Vydac 218TP52 C18 column with a Vydac 218GD52 C18 High Performance Guard Column (Grace, Deerfield, IL) on an Agilent 1100 HPLC system on-line with a LCQ DECAXP mass spectrometer from Thermo Scientific (Waltham, MA). For oligosaccharide analysis, the mobile phases consisted of 0.15% formic acid in H_2O (buffer A) and 0.12% formic acid in ACN (buffer B). Samples were separated by gradient elution at the following conditions: 0–2 min 100% buffer A, 2–10 min gradient 100–85% buffer A, hold for 4 min, 14–15 min gradient 85–100% buffer A and 15–30 min re-equilibrate with 100% buffer A. The flow rate was 0.20 mL/min. The sheath gas flow rate used was 77 (arb) units. The spray voltage was set to 5 kV. The capillary temperature was 200 °C and the column temperature was 40 °C. The mass range setting was 400–2000 Da. The ionization mode was positive electrospray ionization. For the glycopeptide analysis, the HPLC and mass spectrometry conditions can be found elsewhere [25].

2.2. Materials

For monosaccharide analysis and characterization of antennary N-linked oligosaccharide profile by HPAEC-PAD, the monosaccharide standard for analysis was purchased from Dionex Corporation (Sunnyvale, CA). Certified sodium hydroxide 50% (w/w) was purchased from Sigma–Aldrich (Saint Louis, MO). Trifluoroacetic acid (TFA) was from Pierce Chemical (Rockford, IL). Biomax 10kD Ultrafree 0.5 mL centrifuge filters were purchased from Millipore (Billerica, MA). High purity deionized water purified by a Millipore Milli-Q system (Billerica, MA) with a resistivity of 18.2 MΩ cm was used for buffer and sample preparation. PNGase F and 10X G7 reaction buffer were from New England Biolabs (Ipswich, MA).

For characterization of antennary N-linked oligosaccharide profile by capillary electrophoresis, 1 M sodium cyanoborohydrate in THF was purchased from Sigma–Aldrich (Saint Louis, MO). 200 proof, anhydrous ethyl alcohol was obtained from Fisher Scientific (Waltham, MA). High purity deionized water purified by a Millipore Milli-Q system (Billerica, MA) with a resistivity of 18.2 MΩ cm was used for buffer and sample preparation. The eCAP Carbohydrate Labeling and Analysis Kit containing the carbohydrate separation gel buffer, APTS-labeling dye (L6), labeling dye solvent (L3), glucose ladder standard (G20) and the eCAP N-CHO coated capillary was purchased from Beckman Coulter. The PNGase F (N-glycanase) Kit was obtained from New England Bio Lab. Biomax 10kD Ultrafree 0.5 mL centrifuge filters were purchased from Millipore (Billerica, MA).

For characterization of antennary N-linked oligosaccharide profile by HPLC–ESMS, myoglobin, guanidine HCl, tris base, sodium iodoacetate, formic acid and ammonium bicarbonate were purchased from Sigma–Aldrich (Saint Louis, MO). TFA was purchased from Pierce Chemical (Rockford, IL). Dithiothreitol (DTT) was obtained from Bio-Rad (Hercules, CA). Sequencing grade modified trypsin was from Promega (Madison, WI). HPLC grade water was purchased from Honeywell Burdick & Jackson (Morristown, NJ). 1N hydrochloric acid, 1N NaOH, acetonitrile and 6.0–8.0 pH paper were obtained from Fisher Scientific (Waltham, MA).

2.3. Methods

2.3.1. Standard and sample preparation for monosaccharide analysis by HPAEC-PAD

The Dionex monosaccharide standard was diluted with Milli-Q water to yield a 400 μM stock solution. The 400 μM stock solution was then further diluted to 150, 75, 60, 25, 12.5 and 3.5 μM for construction of the standard curve. A system suitability sample was prepared from an aliquot of the 25 μM standard which was hydrolyzed following the same procedure as the antibody sample. Six replicate injections of the system suitability sample were performed.

The fully human monoclonal antibody (IgG_1) sample was desalted using a pre-rinsed centrifuge filter unit. The protein concentration of the washed antibody sample was determined at a wavelength of 280 nm and a volume equal to 1.125 mg of protein was transferred into a 2-ml glass vial. The sample was placed into a speedvac and dried at 45 °C. The sample was then hydrolyzed in 2 M TFA for 2 h at 100 °C in a standard heatblock. After hydrolysis the sample was placed into a speedvac and dried at 45 °C. The sample was reconstituted with Milli-Q water. Triplicate injections were performed for each sample and standard.

2.3.2. Antibody sample preparation for characterization of antennary N-linked oligosaccharide profile by HPAEC-PAD

The fully human monoclonal antibody (IgG_1) sample was desalted using a pre-rinsed centrifuge filter unit. The sample was transferred to an Eppendorf tube and 10× reaction buffer, PNGase F and Milli-Q water were added. The sample was incubated overnight at 37 °C. Following incubation the antibody sample was filtered by a centrifuge filter unit. The filtrate was collected for analysis. Triplicate injections were performed for each sample.

2.3.3. Glucose ladder and sample preparation for characterization of antennary N-linked oligosaccharide profile by capillary electrophoresis

The sample preparation procedure was adapted from the Beckman Coulter ProteomeLab Carbohydrate Labeling and Analysis Guide. The fully human monoclonal antibody (IgG_1) sample was desalted using a pre-rinsed centrifuge filter unit. The washed antibody sample remaining in the upper chamber of the filter unit was transferred to an Eppendorf tube. 10× releasing buffer (G7) and PNGase F were then added. The sample was incubated overnight in a 37 °C incubator. At the end of the incubation cold ethanol (−20 °C) was added to the sample and the sample was put on ice for 10 min. The sample was centrifuged and the supernatant was transferred to another Eppendorf tube which was dried down in an Eppendorf vacufuge at 45 °C.

To prepare the glucose ladder, 5 mg of the G20 glucose ladder standard was diluted with 80 μL of HPLC grade water. The sample was divided into 2 μL aliquots and dried down in an Eppendorf vacufuge at 45 °C.

Labeling dye (L6) was added to the dried test sample and glucose ladder standard. 1 M sodium cyanoborohydride in THF was then added to all samples in a fume hood. All samples were incubated overnight in a 37 °C incubator. After incubation HPLC grade water was added to all samples to stop the reaction.

2.3.4. Trypsin enzyme digestion of test samples for characterization of antennary N-linked oligosaccharide profile by HPLC-ESMS

As previously described [25], the antibody samples were transferred into 15 mL polypropylene centrifuge tubes. 8 M guanidine HCl and 2.5 M Tris base were added to each tube. All samples were incubated at 37 °C for 30 min. After incubation 1 N HCl was added to each tube and the pH was checked to ensure it was in the 8–9 range. DTT was added to each tube and the tubes were then saturated with nitrogen, mixed and incubated at 37 °C for 2 h. After incubation sodium iodoacetate was added to each tube and the sample pH was measured again to confirm it was between 8 and 9. The samples were saturated with nitrogen and incubated in the dark at ambient temperature for 15 min. The samples were rinsed with 0.1 M ammonium bicarbonate by centrifugation in a centrifuge filter unit. The supernatant from the samples was transferred to separate glass vial with screw caps and reconstituted in 0.1 M ammonium bicarbonate. The samples were treated with 1 μg/μL trypsin at a ratio of 50:1 (w/w) of protein to trypsin and incubated for 3 h at 37 °C. LC–MS analysis was performed on the samples.

2.4. Assay performance

The accuracy of the monosaccharide analysis by HPAEC-PAD method was assessed by comparing the relative abundance of each criterion monosaccharide in two antibody samples to the relative abundance of the corresponding monosaccharide in an antibody reference material. The accuracy is expressed as a % of determined replicate mean value of each criterion monosaccharide in the sample to the mean value found in the reference sample. Linearity of the standard curve for each monosaccharide from 4 to 150 μM by triplicate injections was determined by analysis of the correlation coefficient (r^2). Recovery of each monosaccharide was evaluated using the ratio of the observed concentration to the expected concentration by repeat analysis of the system suitability sample ($n = 6$). Precision was evaluated using the %R.S.D. of the observed monosaccharide concentration from triplicate injections of three concentrations of the reference sample.

Intermediate precision of the oligosaccharide analysis by HPAEC-PAD assay was determined using six independent preparations of a reference sample on three separate days. Intermediate precision of the oligosaccharide analysis by CE-LIF assay was determined using three independent preparations of a reference sample on three separate days. Intermediate precision of the characterization of antennary N-linked oligosaccharide profile by HPLC-ESMS assay was determined using two independent preparations of a reference sample on four separate days.

3. Results and discussion

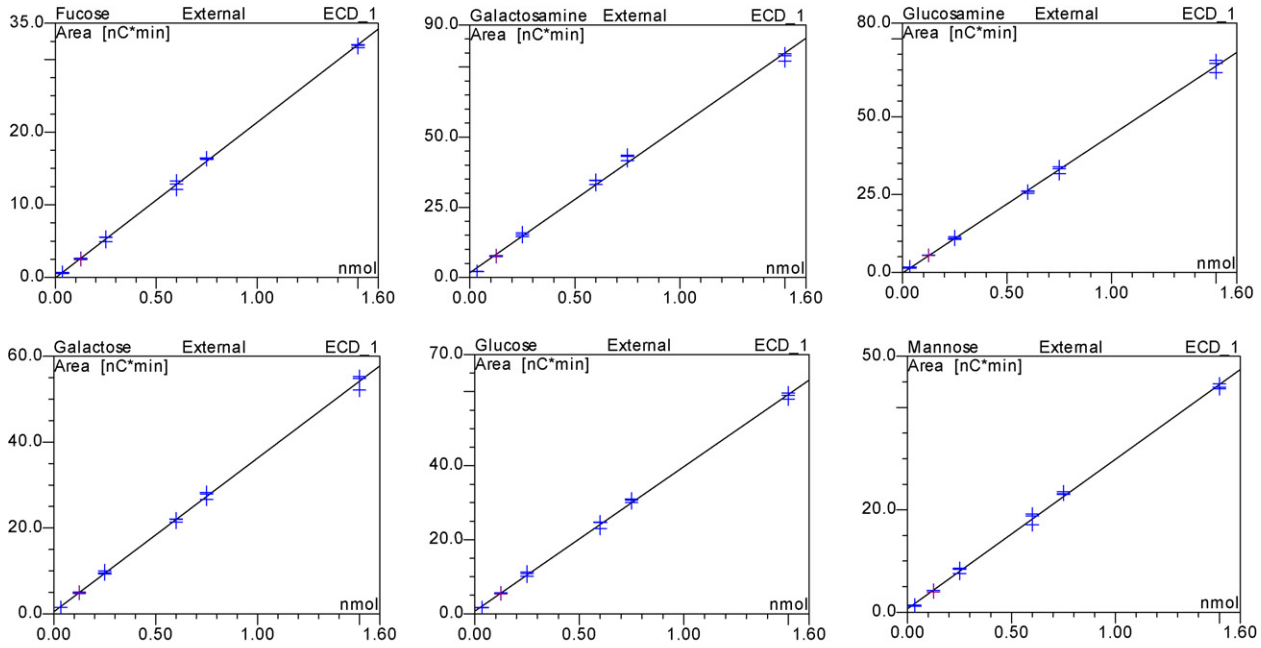
3.1. Monosaccharide analysis by HPAEC-PAD

The HPAEC-PAD method is a classic procedure used for the analysis of monosaccharides released from a glycoprotein. With high selectivity and specificity it permits direct quantitation of non-derivatized monosaccharides. Currently, the concentration (in μM) of each monosaccharide present in the antibody samples is determined by extrapolation off a standard curve for each individual monosaccharide. The standard curves and their respective correlation coefficients are depicted in Fig. 1. Monosaccharides in the samples are identified by comparison to the retention times of the monosaccharide peaks in the standard. A representative HPAEC-PAD chromatogram of the monosaccharides of an IgG_1 sample is shown in Fig. 2. The percent relative abundance of each monosaccharide was determined by dividing the amount of the individual monosaccharide by the total amount of all the monosaccharides as shown in Table 1. Galactosamine and glucose were excluded since galactosamine was not present in the sample and the determined glucose concentration in the sample was below the quantitation limits of the assay. This method has proven to be successful for regular monosaccharide analysis. However, the assay is limited to specialized equipment since the monosaccharides are detected by pulsed amperometric detection and the sodium hydroxide should not be run through a typical HPLC with stainless steel lines.

3.2. Oligosaccharide analysis

3.2.1. Oligosaccharide analysis by HPAEC-PAD

The HPAEC-PAD method for determination of carbohydrate populations is successful for glycoproteins that do not contain a complicated glycan profile. Fig. 3 contains the HPAEC-PAD results for oligosaccharides from an antibody with one glycosylation site (Fig. 3b) and an antibody with two glycosylation sites (Fig. 3a). The multi-component peak in Fig. 3a contains more than one type of N-glycan, which could not be resolved by this method. Peaks were identified by comparison of their retention time to injections of glycan standards. The limited separation power of this ion exchange method makes accurate quantitation for those carbohydrates difficult for complex products. Further analysis of the antibody sample with two glycosylation sites by CE-LIF showed six different oligosaccharides and two unknown peaks (Fig. 4). Another difficulty is that the PNGase digestion step employed in the sample preparation for the HPAEC-PAD analysis is not specific for individual glycosylation sites. Therefore, if two different glycosylation sites



Peak Name	Slope	Offset	Corr. Coeff. %
Fucose	21.401	-0.053	99.959
Galactosamine	52.196	1.702	99.851
Glucosamine	44.258	-0.170	99.926
Galactose	35.836	0.449	99.923
Glucose	38.998	0.717	99.949
Mannose	29.210	0.667	99.916

Fig. 1. Results for individual monosaccharide standard curves from HPAEC-PAD analysis of the IgG_1 antibody sample. Upper row from left to right the standard curves are fucose, galactosamine and glucosamine. Bottom row from left to right the standard curves are galactose, glucose and mannose. Analysis was done in triplicate at six concentration levels.

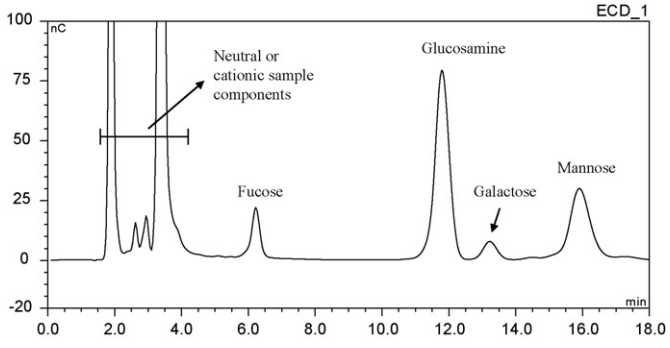


Fig. 2. A typical HPAEC-PAD chromatographic profile of the monosaccharides from acid hydrolysis of the IgG_1 antibody sample. Monosaccharides were released by incubation of the sample with 2M TFA for 2 h.

exist, only the total glycan profile can be obtained and a different method must be used to determine the carbohydrate population at each possible site.

3.2.2. Capillary electrophoresis method

Although the HPAEC-PAD method is successful, more information about the carbohydrate populations can be obtained by using capillary electrophoresis with laser-induced fluorescence detection. CE-LIF can resolve certain oligosaccharide species better than HPAEC-PAD. For example, by using CE-LIF, two peaks are obtained for G1F due to the linkage of the galactose on the 1–6 or 1–3 arm. Fig. 4 shows the electropherogram of an antibody sample with two glycosylation sites (same sample analyzed by HPAEC-PAD in Fig. 3a). The glycans were identified by comparison of their migration time to injections of glycan standards. The

Table 1
HPAEC-PAD monosaccharide experimental data in six IgG_1 samples.

Sample ID	Determined average concentration in μM ($n = 3$)[a]				Calculated percent relative abundance			
	Fucose	Glucosamine	Galactose	Mannose	Fucose	Glucosamine	Galactose	Mannose
Sample 1	69.37 ± 0.02	292.39 ± 0.01	30.03 ± 0.01	144.03 ± 0.01	12.95	54.57	5.60	26.88
Sample 2	62.23 ± 0.01	298.99 ± 0.00	24.79 ± 0.01	167.15 ± 0.01	11.25	54.05	4.48	30.22
Sample 3	51.83 ± 0.02	280.26 ± 0.03	21.17 ± 0.01	148.73 ± 0.02	10.32	55.83	4.22	29.63
Sample 4	65.27 ± 0.01	282.24 ± 0.07	28.34 ± 0.00	155.45 ± 0.05	12.28	53.12	5.33	29.26
Sample 5	45.68 ± 0.02	180.70 ± 0.04	14.52 ± 0.01	168.63 ± 0.03	11.15	44.12	3.55	41.18
Sample 6	48.72 ± 0.03	185.59 ± 0.10	21.29 ± 0.02	159.15 ± 0.12	11.75	44.75	5.13	38.37

[a] Average values from three injections ± S.D.

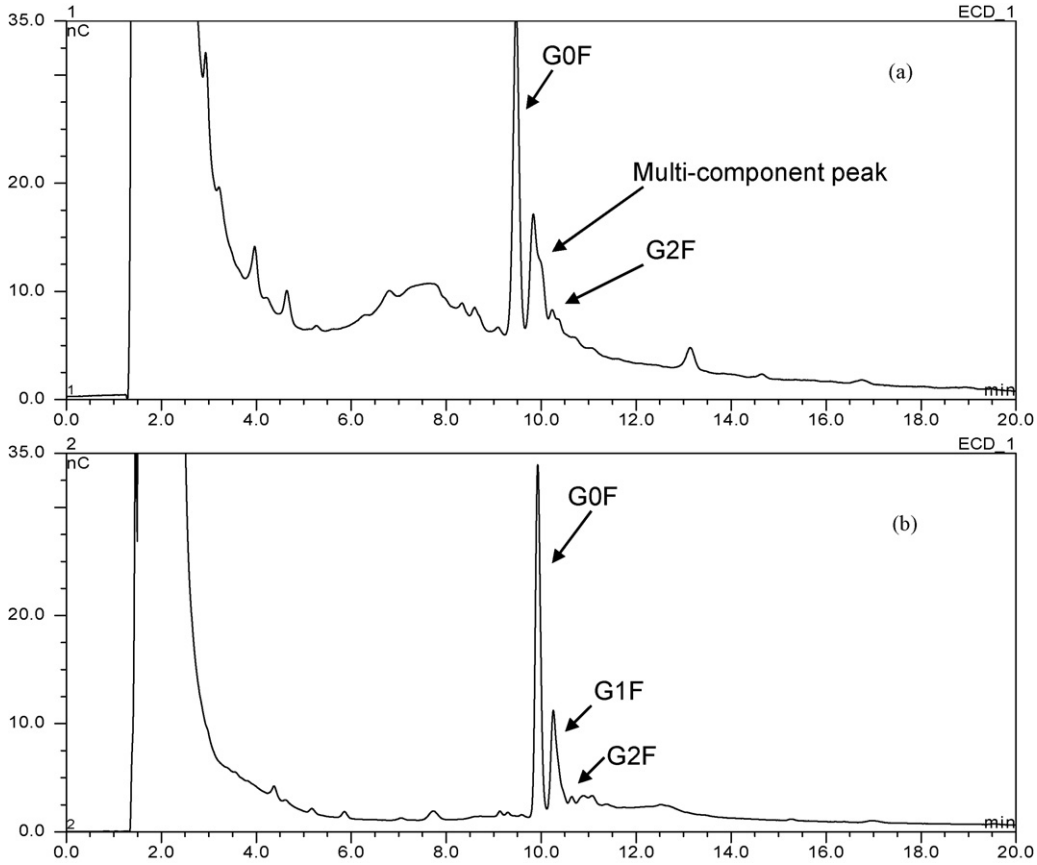


Fig. 3. (a) HPAEC-PAD oligosaccharide profile from analysis of the IgG_1 antibody sample with multiple glycosylation sites and (b) HPAEC-PAD oligosaccharide profile from analysis of the IgG_1 antibody sample with a single glycosylation site.

oligosaccharide profile from CE-LIF analysis exhibits eight resolved peaks. The drawbacks of this CE-LIF method are the time consuming labeling, the lack of direct structural confirmation, and the lack of commercial standards for all possible glycans. Furthermore, the PNGase digestion step employed in the sample preparation for CE-LIF analysis cannot determine the oligosaccharide population at individual glycosylation sites within the same recombinant product.

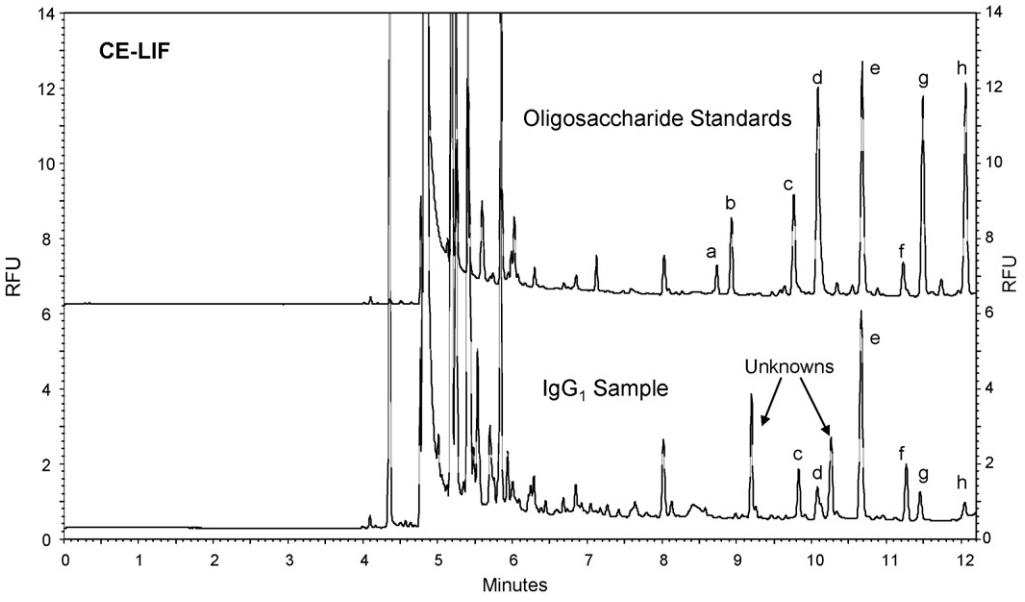


Fig. 4. CE-LIF electropherogram of the oligosaccharides released from digestion of the IgG_1 antibody sample with PNGase F and oligosaccharide standards. The following glycans were identified: (a) S2G2; (b) S2G2F; (c) S1G2; (d) S1G2F; (e) G0F; (f) G1F(1,6); (g) G1F(1,3); and (h) G2F.

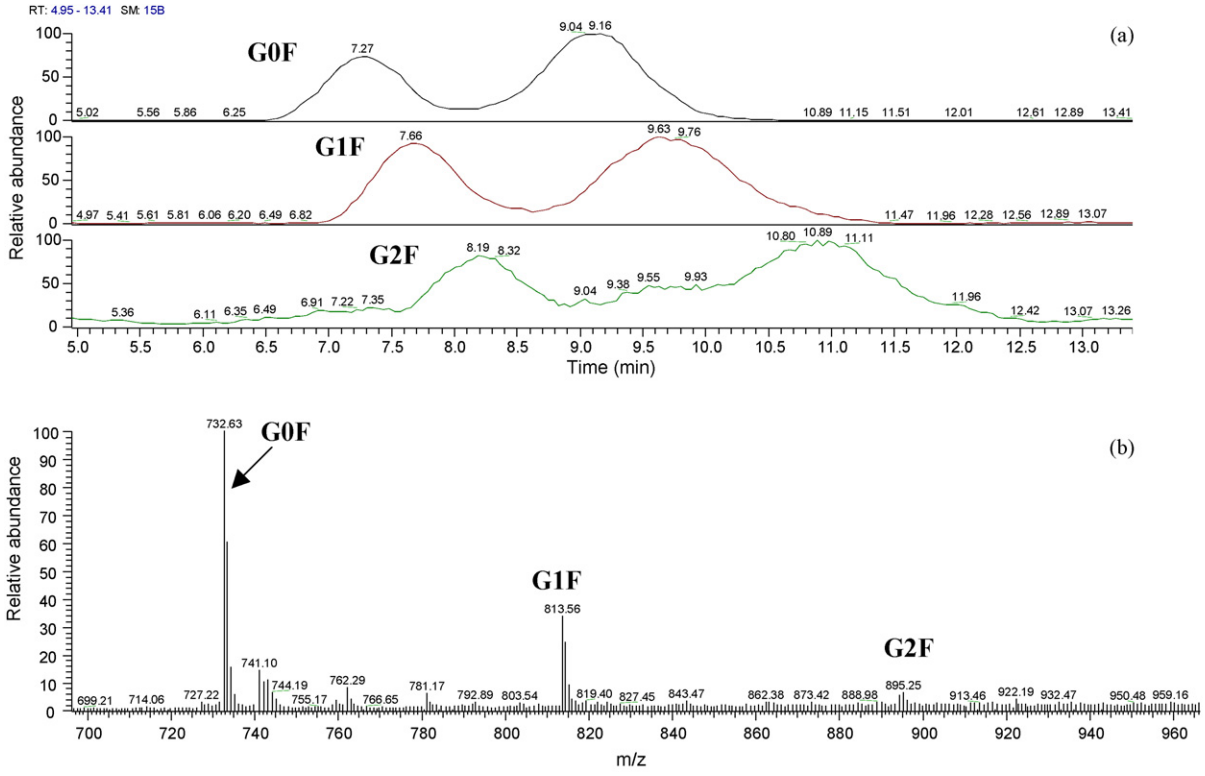


Fig. 5. (a) The extracted ion chromatogram (XIC) of the glycans released by PNGase F digestion of the IgG_1 antibody sample and (b) the mass spectrum data of the glycans released by PNGase F digestion of the IgG_1 antibody sample.

3.2.3. Liquid chromatography coupled to mass spectrometry

HPAEC-PAD and CE are good tools to analyze oligosaccharides and to determine the relative percentages of each individual glycoform. However, it is difficult to determine when new peaks should be included or excluded in the calculation because their identification may not be possible due to the lack of commercially available standards. Therefore, mass spectrometry is a great tool to provide direct structural information about the glycan population. However, the mobile phase from HPAEC-PAD cannot be directly introduced into the mass spectrometer because the high salt concentration of the eluent will interfere with the sample identification and overall sensitivity. Ideally, off-line mass spectrometry, such as MALDI-TOF, could be used to identify peaks of interest in HPAEC-PAD after collecting the fractions and desalting the sample. Although, hydrophilic oligosaccharides will not be retained by the column if hydrophobic filters are used. Additionally, due to the low recovery of glycans from size exclusion filters; minor glycan components are hard to identify. One way to directly analyze the HPAEC-PAD eluent by mass spectrometry would be with the use of an in-line membrane-desalting device. This would eliminate the need for collecting the fractions and desalting the sample. In this study, a reverse phase HPLC coupled to a mass spectrometer was used to identify carbohydrates by two different schemes. Fig. 5 depicts the chromatograms and spectrum of the glycans released by PNGase F and separated on a C18 column. The three primary N-glycans G0F, G1F and G2F exhibited two isomer peaks with each of the species being identified by mass spectrometry. The nature of these two peaks in G0F, G1F and G2F is not clear and further investigation is needed. Extracted ion chromatograms (XIC) were used for the relative abundance determination.

This LC–MS method was used to directly analyze the glycans released from the same antibody product, expressed in two different cell types, B cell hybridoma and CHO cell transfectoma. Fig. 6 contains the mass spectrometric results obtained from the antibody produced by B cell hybridoma (Fig. 6a) and CHO cell transfectoma (Fig. 6b). The carbohydrate species from the antibody produced by the B cell hybridoma line are more complicated. The three major glycans, G0F, G1F, and G2F were found along with more complex structures. A reasonable hypothesis for the glycosylation differences between the cell lines is that the different cell lines contain different types and amounts of glycanases which lead to the generation of more or less carbohydrate species.

LC–MS of tryptic glycopeptides provides an alternative way for identifying N-glycan structures and measuring their relative percentages. Fig. 7 shows the extracted ion chromatogram with integrated peak areas corresponding to G0F, G1F and G2F containing peptides and the mass spectrum for these glycopeptides. The carbohydrate structures can be confirmed by their respective mass spectra and were also further verified by their tandem mass spectra as shown in Fig. 8a–c. In addition to all fragments identified in Fig. 8a, unique fragments at m/z 1223.9, 1297.1 and 1325.6 corresponding the G1F glycopeptide were identified in Fig. 8b. Two unique fragments at m/z 1399.2 and 1406.7 relating to the G2F glycopeptide were also identified in Fig. 8c. From these tandem mass spectra one can clearly identify and confirm the structure of each of the distinct glycoforms for this particular glycopeptide. Furthermore, because trypsin digestion does not break the carbohydrate to protein linkage, a calculation of the relative population of the different carbohydrates at different glycosylation sites in the same method is possible.

Utilizing theoretical glycosylation site prediction software (Bioworks, Thermo Fisher) it was determined that two proprietary antibodies may contain two possible glycosylation sites. The LC–MS method to measure and sequence glycans based on the glycopeptides released by trypsin was employed and allowed for the conclusion that one of the antibodies contained two glycosylation

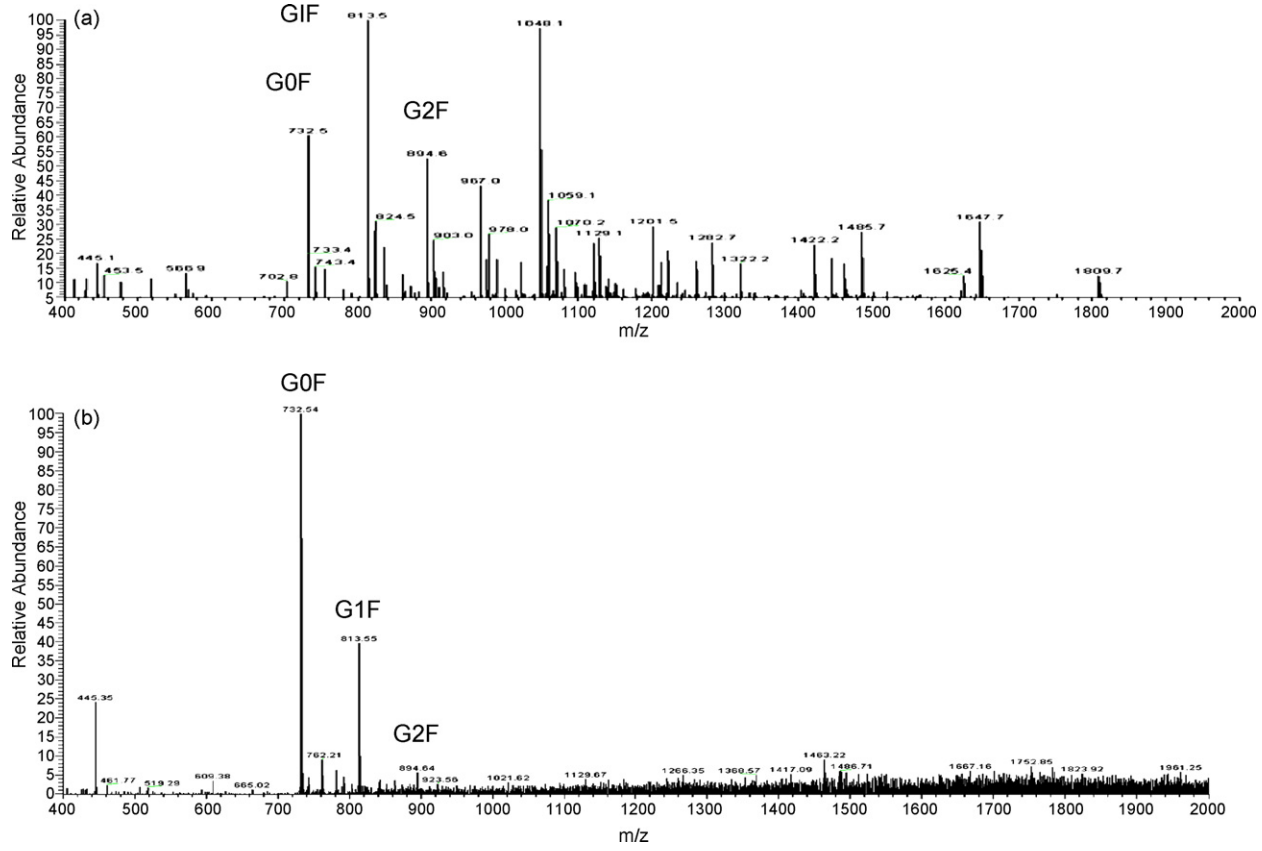


Fig. 6. (a) The mass spectrum results of the glycans released by PNGase F digestion of an IgG_1 antibody sample produced by B cell hybridoma and (b) the mass spectrum results of the glycans released by PNGase F digestion of an IgG_1 antibody sample produced by CHO cell transfectoma.

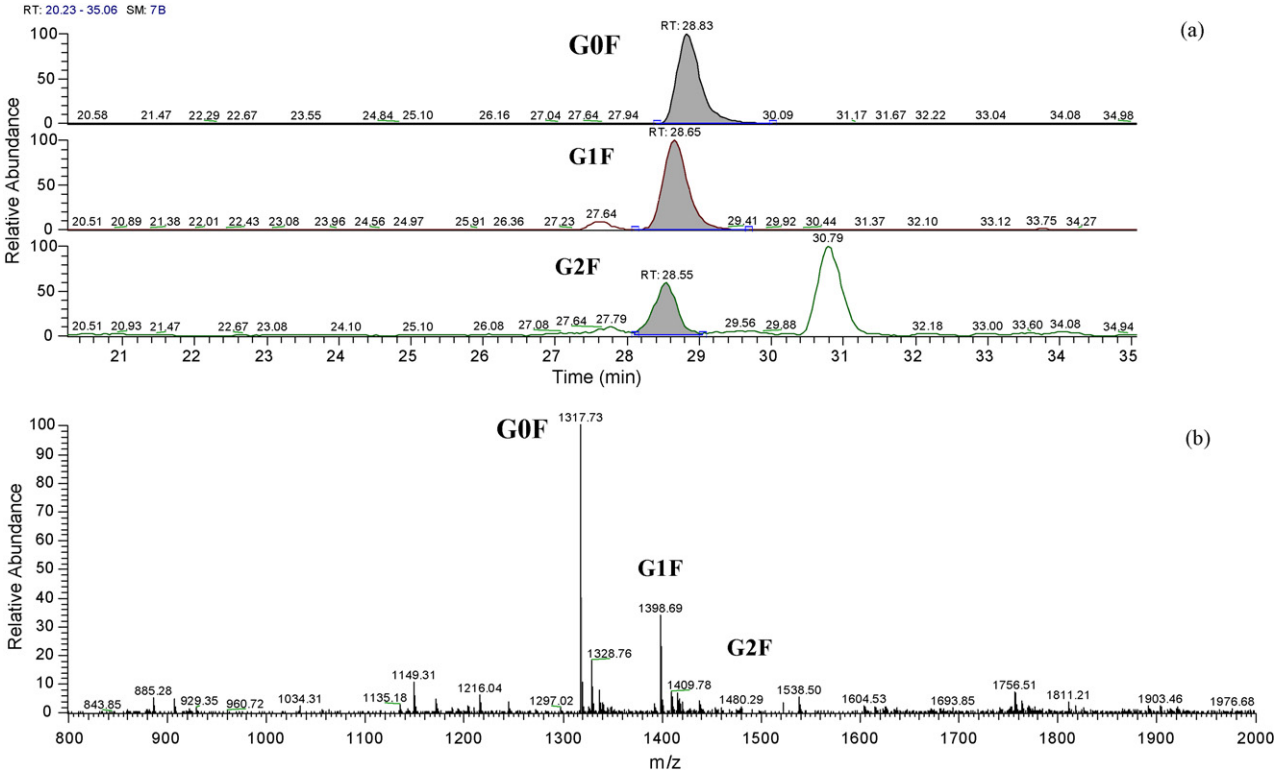


Fig. 7. (a) The extracted ion chromatogram (XIC) of the tryptic glycopeptides released by trypsin digestion of the IgG_1 antibody sample and (b) the mass spectrum data of the tryptic glycopeptides released by trypsin digestion of the IgG_1 antibody sample.

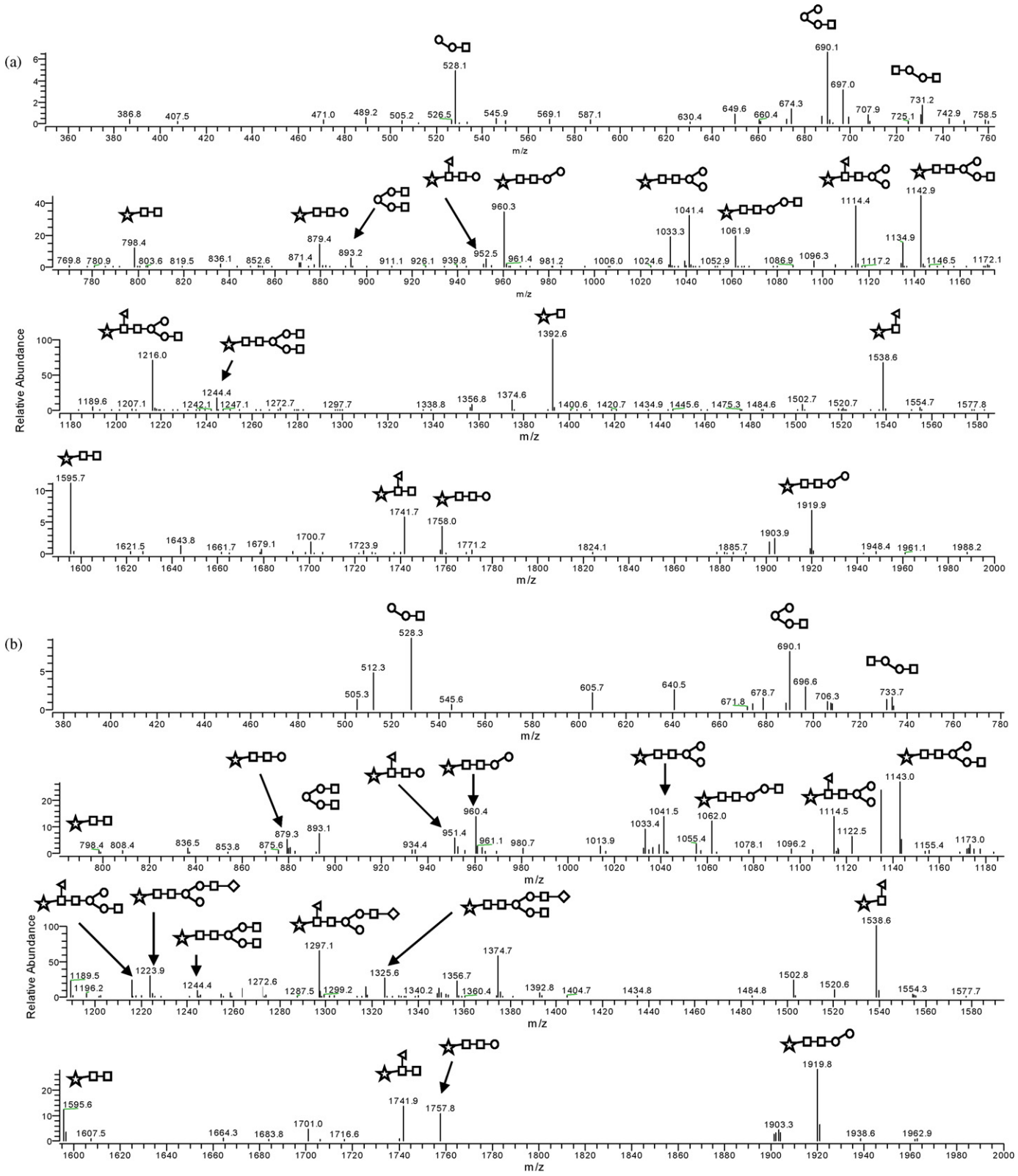


Fig. 8. (a) The tandem mass spectra of the G0F tryptic glycopeptide (☆ = EEQYNSTYR) released by trypsin digestion of the IgG_1 antibody sample, (b) the tandem mass spectra of the G1F tryptic glycopeptide (☆ = EEQYNSTYR) released by trypsin digestion of the IgG_1 antibody sample, and (c) the tandem mass spectra of the G2F tryptic glycopeptide (☆ = EEQYNSTYR) released by trypsin digestion of the IgG_1 antibody sample. Monosaccharide symbols as follows: ◊ = galactose, □ = N-acetylglucosamine, ○ = mannose and ◁ = fucose.

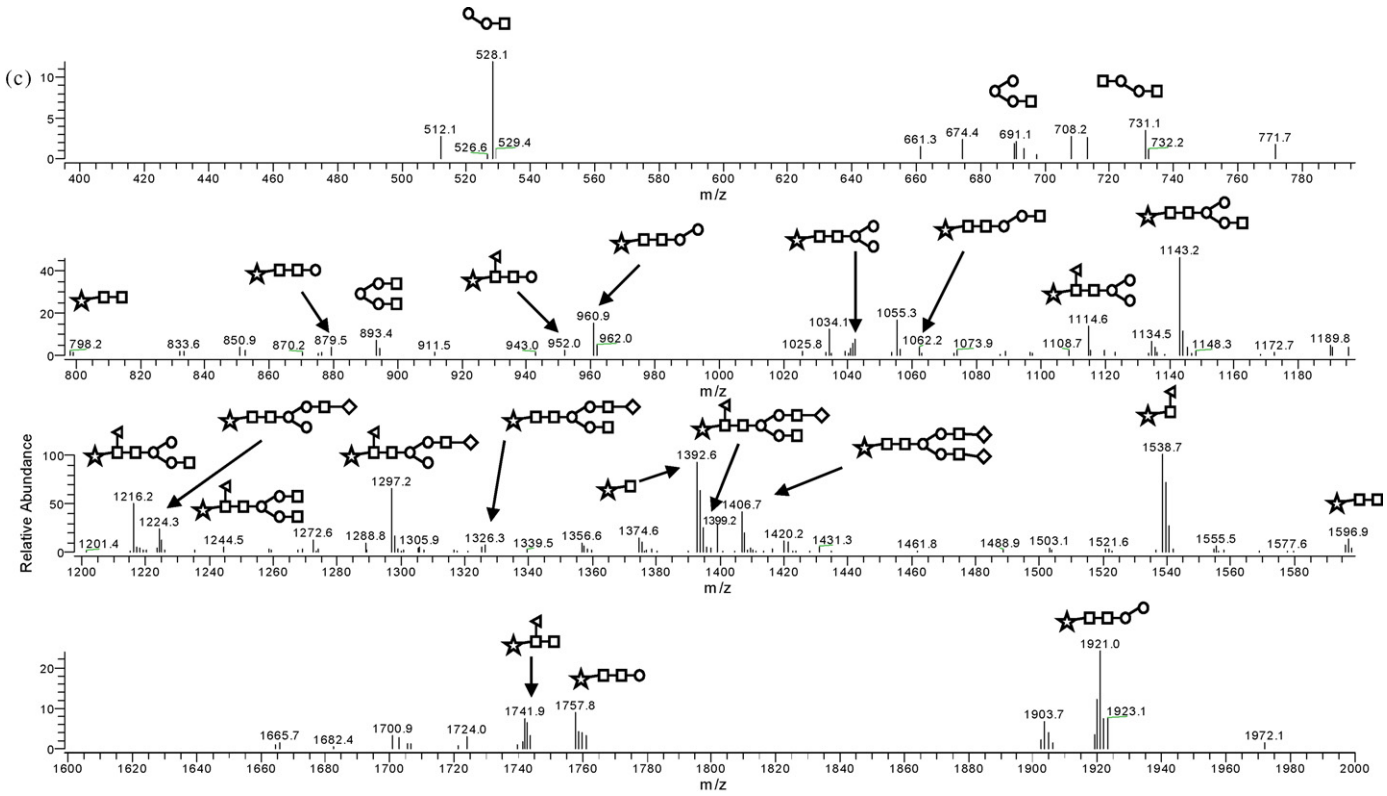


Fig. 8. (*Continued*).

sites while the other antibody only contained one despite the prediction (Table 2). The other methods for oligosaccharide analysis would not have been able to determine if one possible site was devoid of carbohydrate. Furthermore, with one trypsin digestion of the same sample, N-terminal sequencing of the heavy and light chains, peptide mapping, and determination of relative percentage the carbohydrate populations can be obtained in one LC–MS run. This makes the LC–MS method for determination of glycosylation pattern and structure a more attractive option due to the time savings and amount of information gained.

3.3. Calculations of monosaccharides from oligosaccharide methods

Monosaccharide and oligosaccharide analyses are usually run at the same time by different methods, yet the results are usually not directly compared. A new and novel approach to calculating the monosaccharide relative % abundance from the intact N-glycan experimental data was derived. By using the calculated monosaccharide values it was demonstrated that the oligosaccharide methods results are accurate and comparable to the monosaccharide assay. As shown in Fig. 9 and Table 3, there are four identified N-glycans in the IgG_1 sample as determined by the oligosaccharide methods. These are asialo-, agalacto-, biantennary, core-substituted with fucose (G0F), asialo, monogalactosylated, biantennary core-substituted with fucose (G1F), asialo-, galactosylated biantennary, core-substituted with fucose (G2F) and mono-sialylated-, galactosylated biantennary, core-substituted with fucose (S1G2F). The oligosaccharide relative abundance for the LC–MS and HPAEC-PAD analysis is based on peak areas and does not take into account any response differences between the oligosaccharides.

A calculation was performed to obtain the monosaccharide relative abundance by first computing the relative abundance of each monosaccharide in one molecule of a specific N-glycan. This value was calculated by dividing the number of residues of the individual monosaccharide by the total number of monosaccharide residues present in the specific N-glycan. The relative abundance of each

Table 2
The relative percentages of tryptic glycopeptides released by trypsin digestion of two IgG_1 antibody samples.

Antibody 1		Antibody 2	
Fab site		Fab site	
Glycan	Relative %	Glycan	Relative %
Not glycosylated	S2G2F	59	
	S1G2F	33	
	S1G1F	8	
Fc site		Fc site	
Glycan	Relative %	Glycan	Relative %
G0F	66	G0F	75
G1F	30	G1F	22
G2F	4	G2F	3

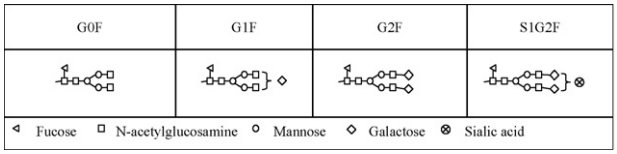


Fig. 9. The major oligosaccharides identified in the IgG_1 sample. These are asialo-, agalacto-, biantennary, core-substituted with fucose (G0F), asialo, monogalactosylated, biantennary core-substituted with fucose (G1F), asialo-, galactosylated biantennary, core-substituted with fucose (G2F) and mono-sialylated-, galactosylated biantennary, core-substituted with fucose (S1G2F).

Table 3
HPAEC-PAD, LC–MS and CE-LIF oligosaccharide percent relative abundance in six IgG_1 samples.

Sample ID	Oligosaccharide percent relative abundance			
	G0F	G1F	G2F	S1G2F
Sample 1 by HPAEC-PAD[a]	63.80 ± 0.10	32.30 ± 0.08	2.30 ± 0.05	1.60 ± 0.05
Sample 1 by LC–MS[b]	64.40	29.30	3.50	2.80
Sample 1 by CE-LIF[c]	57.90 ± 0.24	32.60 ± 0.20	4.00 ± 0.02	5.50 ± 0.03
Sample 2 by HPAEC-PAD	66.10 ± 0.09	30.50 ± 0.13	1.80 ± 0.04	1.60 ± 0.15
Sample 2 by LC–MS	63.40	29.90	4.70	2.00
Sample 2 by CE-LIF	60.70 ± 0.14	31.20 ± 0.16	3.30 ± 0.03	4.80 ± 0.06
Sample 3 by HPAEC-PAD	66.00 ± 0.16	30.80 ± 0.08	1.90 ± 0.07	1.30 ± 0.01
Sample 3 by LC–MS	61.30	33.60	3.70	1.60
Sample 3 by CE-LIF	60.50 ± 0.13	31.20 ± 0.14	3.40 ± 0.01	4.90 ± 0.03
Sample 4 by HPAEC-PAD	64.30 ± 0.09	32.00 ± 0.05	2.10 ± 0.01	1.60 ± 0.10
Sample 4 by LC–MS	58.90	35.20	4.10	1.90
Sample 4 by CE-LIF	57.00 ± 0.03	33.50 ± 0.13	3.90 ± 0.06	5.60 ± 0.14
Sample 5 by HPAEC-PAD	66.67 ± 0.33	29.12 ± 0.18	2.87 ± 0.34	1.34 ± 0.46
Sample 5 by LC–MS	66.79	30.20	3.01	0.00
Sample 5 by CE-LIF	67.01 ± 0.06	30.08 ± 0.10	2.91 ± 0.04	0.00
Sample 6 by HPAEC-PAD	62.25 ± 0.52	32.85 ± 0.19	2.96 ± 0.56	1.95 ± 0.34
Sample 6 by LC–MS	60.08	35.42	4.50	0.00
Sample 6 by CE-LIF	60.66 ± 0.13	34.59 ± 0.13	4.74 ± 0.00	0.00

[a] Average values from three injections ± S.D.
[b] Values obtained from a single injection.
[c] Average values from three injections ± S.D.

monosaccharide was then multiplied by the relative percent abundance of the specific N-glycan in the IgG_1 sample as determined from each set of data. This gives a monosaccharide relative percent abundance based on the relative abundance of the specific N-glycan in the IgG_1 sample as measured by a specific method. In the N-glycan species, one molecule of N-acetylglucosamine is considered to be equivalent to one molecule of glucosamine because during the TFA hydrolysis of the sample N-acetylglucosamine is hydrolyzed to glucosamine. These values were summed up for each individual monosaccharide for all N-glycans to yield the theoretical monosaccharide relative % abundance in the IgG_1 sample. An example of the calculation used to determine the theoretical monosaccharide relative percent abundance is shown in Table 4.

As shown in Table 5, comparisons of the experimentally determined monosaccharide relative percent abundances to the theoretical values determined from LC–MS, HPAEC-PAD and CE-LIF N-glycan analysis proved to be consistent by analysis of the Δ range between the experimental and theoretical data. The Δ range is calculated by taking the highest and lowest absolute value for the theoretical relative % abundance of each monosaccharide from the

Table 4
Example of the theoretical monosaccharide relative percent abundance calculation from N-glycan experimental data.

N-glycan species	Monosaccharide content of the N-glycan species	Monosaccharide residue/N-glycan molecule	Monosaccharide relative abundance per N-glycan molecule	Relative % abundance N-glycan in IgG_1 sample from experimental data	Monosaccharide relative % abundance for N-glycan species adjusted for relative % abundance N-glycan in IgG_1 sample
G0F	Galactose	0	0.0000	64.4	0.00
	Mannose	3	0.3750		24.15
	Fucose	1	0.1250		8.05
	Glucosamine	4	0.5000		32.20
	Total	8			
G1F	Galactose	1	0.1111	29.3	3.26
	Mannose	3	0.3333		9.77
	Fucose	1	0.1111		3.26
	Glucosamine	4	0.4444		13.02
	Total	9			
G2F	Galactose	2	0.2000	3.5	0.70
	Mannose	3	0.3000		1.05
	Fucose	1	0.1000		0.35
	Glucosamine	4	0.4000		1.40
	Total	10			
S1G2F	Galactose	2	0.2000	2.8	0.56
	Mannose	3	0.3000		0.84
	Fucose	1	0.1000		0.28
	Glucosamine	4	0.4000		1.12
	Total	10			
Theoretical monosaccharide relative percent abundance in IgG_1 sample				Galactose	4.52
				Mannose	35.81
				Fucose	11.94
				Glucosamine	47.74

Table 5
LC–MS, HPAEC-PAD and CE-LIF theoretical calculation for monosaccharide relative percent abundance versus HPAEC-PAD experimental results for monosaccharide relative percent abundance.

Sample ID	Assay	Relative % abundance			
		Galactose	Mannose	Fucose	Glucosamine
Sample 1	Monosaccharide[a]	5.60 ± 0.15	26.88 ± 0.43	12.95 ± 0.31	54.57 ± 0.75
	LC–MS N-glycan[b]	4.52	35.81	11.94	47.74
	HPAEC-PAD N-glycan[c]	4.37 ± 0.01	35.86 ± 0.00	11.95 ± 0.00	47.82 ± 0.01
	CE-LIF N-glycan[d]	5.52 ± 0.03	35.43 ± 0.01	11.81 ± 0.00	47.24 ± 0.02
	Δ range[e]	0.08–1.23	8.55–8.98	1.00–1.14	6.75–7.33
Sample 2	Monosaccharide	4.48 ± 0.13	30.22 ± 0.39	11.25 ± 0.13	54.05 ± 0.30
	LC–MS N-glycan	4.66	35.75	11.92	47.67
	HPAEC-PAD N-glycan	4.07 ± 0.02	35.97 ± 0.01	11.99 ± 0.00	47.97 ± 0.01
	CE-LIF N-glycan	5.09 ± 0.01	35.59 ± 0.00	11.86 ± 0.00	47.46 ± 0.01
	Δ range	0.18–0.61	5.37–5.75	0.61–0.74	6.08–6.59
Sample 3	Monosaccharide	4.22 ± 0.22	29.63 ± 0.67	10.32 ± 0.48	55.83 ± 0.22
	LC–MS N-glycan	4.79	35.78	11.93	47.70
	HPAEC-PAD N-glycan	4.06 ± 0.02	35.98 ± 0.01	11.99 ± 0.00	47.97 ± 0.01
	CE-LIF N-glycan	5.13 ± 0.01	35.58 ± 0.01	11.86 ± 0.00	47.44 ± 0.01
	Δ range	0.16–0.91	5.95–6.35	1.54–1.67	7.86–8.39
Sample 4	Monosaccharide	5.33 ± 0.53	29.26 ± 0.97	12.28 ± 0.99	53.12 ± 0.59
	LC–MS N-glycan	5.11	35.62	11.87	47.49
	HPAEC-PAD N-glycan	4.30 ± 0.02	35.89 ± 0.01	11.96 ± 0.00	47.85 ± 0.01
	CE-LIF N-glycan	5.62 ± 0.01	35.39 ± 0.00	11.80 ± 0.00	47.19 ± 0.00
	Δ range	0.22–1.03	6.13–6.63	0.32–0.48	5.27–5.93
Sample 5	Monosaccharide	3.55 ± 0.21	41.18 ± 0.34	11.15 ± 0.28	44.12 ± 0.27
	LC–MS N-glycan	3.96	36.02	12.01	48.02
	HPAEC-PAD N-glycan	4.08 ± 0.06	35.97 ± 0.02	11.99 ± 0.01	47.96 ± 0.03
	CE-LIF N-glycan	3.92 ± 0.01	36.03 ± 0.00	12.01 ± 0.00	48.04 ± 0.00
	Δ range	0.37–0.53	5.10–5.21	0.84–0.86	3.84–3.92
Sample 6	Monosaccharide	5.13 ± 0.19	38.37 ± 0.70	11.75 ± 0.36	44.75 ± 0.50
	LC–MS N-glycan	4.84	35.69	11.90	47.58
	HPAEC-PAD N-glycan	4.63 ± 0.10	35.77 ± 0.04	11.92 ± 0.01	47.69 ± 0.05
	CE-LIF N-glycan	4.79 ± 0.01	35.70 ± 0.01	11.90 ± 0.00	47.60 ± 0.01
	Δ range	0.29–0.50	2.60–2.68	0.15–0.17	2.83–2.94
Overall Δ range		0.08–1.23	2.60–8.98	0.15–1.67	2.83–3.89

[a] Average values from three injections ± S.D.
[b] Values from a single injection.
[c] Average values from three injections ± S.D.
[d] Average values from three injections ± S.D.
[e] The Δ range is calculated by taking the highest and lowest absolute value for the theoretical relative % abundance of each monosaccharide from the three assays less the experimental results from the monosaccharide analysis.

three assays less the experimental results from the monosaccharide analysis. The theoretical values for galactose and fucose were close to the experimental value while the values for mannose and glucosamine were more varied. In Table 6, the monosaccharide data was also compared to the glycoprotein as a secondary confirmation of the results using methods previously described by Mechref et al. [26]. The results of the monosaccharide analysis in nmoles were compared to the amount of glycoprotein in nmoles. These values were then normalized to three mannose residues by dividing the ratio of monosaccharide to antibody values by a factor of 4.12. The values were normalized to three mannose residues since there are three mannose in the core glycan structures. As expected, for a core-fucosylated biantennary structure, there is approximately a 3:1 ratio of mannose to fucose and a 3:4 ratio of mannose to N-acetylglucosamine. A 3:0.5 ratio of mannose to galactose supports the findings that the glycoprotein is comprised of mainly G0F. These results further confirm the accuracy of the monosaccharide analysis.

Table 6
Comparison of the average monosaccharide content in six IgG_1 samples as analyzed by HPAEC-PAD.

Monosaccharide	Monosaccharide amount experimentally determined (nmole)[a]	Glycoprotein amount (nmole)	Ratio of monosaccharide to antibody (nmole/nmole)[b]	Ratio normalized to three mannose residues[c]	Monosaccharide Amount (ng)[d]
Fucose	0.57 ± 0.1	0.127	4.50	1.10	93.90 ± 16.0
Glucosamine	2.53 ± 0.5		19.95	4.88	546.35 ± 118.2
Galactose	0.23 ± 0.1		1.84	0.45	42.08 ± 10.1
Mannose	1.57 ± 0.1		12.38	3.00	283.19 ± 17.7
Total mass of monosaccharides (ng)					965.52 ± 132.0
% glycans in glycoprotein					5.15 ± 0.7

[a] Average values ± S.D. determined from six samples in Table 1.
[b] The molecular weight of the glycoprotein is 147,992 Da.
[c] The ratios of monosaccharide to antibody were divided by a factor of 4.12 to normalize the ratios to three mannose residues.
[d] The amount ± S.D. determined from six samples was calculated by multiplying the experimentally determined amount (nmole) by the formula weight for each monosaccharide.

3.4. Assay performance

3.4.1. Monosaccharide analysis by HPAEC-PAD

Precision (repeatability) as determined by the percent relative standard deviation (%R.S.D.) for the concentration of each criteria monosaccharide in the reference sample by repeat analysis was ≤4.4% ($n = 9$). Accuracy expressed as a % of determined replicate mean value of each criterion monosaccharide in the samples to the mean value found in the reference sample ranged from 93.3 to 107.6%. Linearity of the standard curve based on the correlation coefficient (r^2) was greater than 0.992. Recovery of each monosaccharide as evaluated using the observed concentration to the expected concentration by repeat analysis of the system suitability sample ($n = 6$) was 89.8% for fucose, 95.9% for galactosamine, 93.0% for glucosamine, 90.1% for galactose, 93.4% for glucose and 96.8% for mannose.

3.4.2. Oligosaccharide analysis by HPAEC-PAD

Intermediate precision was determined using six independent preparations of a reference sample on three separate days. Intermediate precision expressed as % relative standard deviation of the relative retention times and relative abundance of the criteria peaks was less than 1% R.S.D. for relative retention times for all N-glycans, less than 3% R.S.D. for relative abundance of high abundance N-glycans and less than 16% R.S.D. for relative abundance of low abundance N-glycans.

3.4.3. Oligosaccharide analysis by CE-LIF

Intermediate precision was determined using three independent preparations of a reference sample on three separate days. Intermediate precision expressed as % relative standard deviation of the relative retention times and relative abundance of the criteria peaks was less than 0.2% R.S.D. for relative retention times for all N-glycans, less than 2% R.S.D. for relative abundance of high abundance N-glycans and less than 11% R.S.D. for relative abundance of low abundance N-glycans.

3.4.4. Characterization of antennary N-linked oligosaccharide profile by HPLC-ESMS

Intermediate precision was determined using two independent preparations of a reference sample on four separate days. Intermediate precision expressed as % relative standard deviation of the relative abundance of the criteria peaks was less than 7% R.S.D. for high abundance N-glycans and less than 16% R.S.D. for low abundance N-glycans.

4. Conclusion

In this work, three different analytical methods for oligosaccharide detection and their relative percentage determination in an IgG_1 antibody were discussed and compared. It was demonstrated that all methods lead to comparable results but LC–MS after trypsin digestion is the only method that can offer glycosylation site-dependent information. The calculation presented here provided a new and novel way to assess the accuracy of the oligosaccharide analytical methods. By utilizing the calculation, cross-confirmation of the accuracy of the oligosaccharide composition determination in an IgG_1 antibody by the various methods has been established.

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