

# Separation of monosaccharides hydrolyzed from glycoproteins without the need for derivatization

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**Abstract** Chromatographic separation of monosaccharides hydrolyzed from glycoconjugates or complex, aggregate biomaterials, can be achieved by classic analytical methods without a need for derivatizing the monosaccharide subunits. A simple and sensitive method is presented for characterizing underivatized monosaccharides following hydrolysis from N- and O-linked glycoproteins using high-performance liquid chromatography separation with mass spectrometry detection (LC-MS). This method is adaptable for characterizing anything from purified glycoproteins to mixtures of glycoforms, for relative or absolute quantification applications, and even for the analysis of complex biomaterials. Use of an amide stationary phase with HILIC chromatography is demonstrated to retain the highly polar, underivatized monosaccharides and to resolve stereoisomers and potentially interfering contaminants. This work illustrates an original approach for characterization of N- and O-linked glycoprotein standards, mixtures, and for complex biological materials such as a total yeast extract.

**Keywords** Bioanalytical methods · HPLC · Mass spectrometry / ICP-MS · Separations/Instrumentation

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## Introduction

Characterization of carbohydrates and glycans in biological systems is of critical interest in several diverse areas including therapeutics development [1, 2], disease diagnostics (cancer, heart disease, diabetes, and numerous others) [3–5], and elucidation of biological processes [6–9] (cellular metabolism, biosynthesis, and development). Additionally, greater understanding of protein folding [10, 11], immunity, energy storage, and other areas are dependent on knowledge of glycan composition. Due to the sheer numbers of possible forms, along with their chemical similarity and structural complexity, carbohydrates are among the most difficult classes of biomolecules to characterize and/or quantify whether they exist as purely saccharide forms (mono-, oligo-, or poly-saccharide), or bound to other types of biomolecules forming glycoconjugates (glycoproteins, glycolipids, or proteoglycans). While complex in the aggregate, glycoconjugates are principally composed of a small class of similar, yet measurable, subunits of monosaccharides. Mammalian glycosylations are made up of a limited number of monosaccharide forms – principally galactose, glucose, mannose, fructose, fucose, N-acetyl glucosamine (GlcNAc), N-acetyl galactosamine (GalNAc), N-acetyl neuraminic acid (NANA), and rarely, N-glycolyl neuraminic acid (NGNA). Other minor components are seldom included such as xylose, iduronic acid, others. Each of these monomeric forms can be uniquely detected in a mass spectrometer as an underivatized compound based on a fragmentation pattern alone, or in cases of stereoisomers, with additional chromatographic retention information.

Monosaccharide characterization offers valuable complementary data to the analysis of intact glycans or glycoprotein characterization. Total monosaccharide characterization is possible for simple or complex mixtures, and can take account

of monosaccharides originating from non-targeted proteins or unexpected impurities. The characterization of biosimilars – protein therapeutics sharing similar function but with intrinsic structural variability – relies on data obtained at the glycan level, a technically difficult task often hampered by the lack of commercially available homogenous glycoprotein standards and by the complexity of known and potentially unknown glycoforms. Furthermore, biases of omission are common in glycan profiling. For instance, contributions from host-cell proteins, excipients, or glycation to the overall carbohydrate composition of a biosimilar product are often overlooked when measured at the glycan level, but are intrinsically considered by monosaccharide measurements. The qualitative approach presented here using mass spectrometry detection offers the potential to perform quantitative measurements using isotope dilution (ID) techniques, which is not currently possible using HPAEC-PAD or other detection techniques. Monosaccharide internal standards are available commercially, and may be applied generally to IS-MS measurements. Monosaccharide characterization is therefore suggested to be a valuable *complementary* tool for assessing the degree of biosimilarity.

Although there are previous literature reports that describe acid hydrolysis of glycoproteins into their constituent monosaccharide units with subsequent analysis using LC-MS [12–17] or capillary electrophoresis [18], each of these studies requires derivitization of monosaccharides for detection, likely incorporating measurement bias in proportion to the labeling inefficiency. The current industry standard for analysis of non-derivatized, hydrolyzed sugars – anion exchange chromatography with pulsed amperometric detection [19] (HPAEC-PAD) – is capable of chromatographic resolution of monosaccharides and can be a very sensitive detection technique, but is inherently incompatible with direct mass spectrometry detection, and therefore incompatible with isotope dilution techniques. In contrast, the work described here presents a novel method focusing on chromatographic resolution and mass spectral detection of monosaccharides without modification. One certain way to improve monosaccharide measurements is through simplifying the sample preparation. This effort reports a workflow for releasing monosaccharides from intact glycoproteins and characterizing them by multiple reaction monitoring (MRM) using liquid chromatography-tandem mass spectrometry (LC-MS/MS) techniques. An amide stationary chemistry was used within a normal phase (HILIC) environment to resolve ten underivatized monosaccharides prior to negative polarity electrospray ionization, and detection in a triple quadrupole mass spectrometer. This analytical approach is demonstrated in principle for N-linked glycoproteins (immunoglobulins, RNase B), O-linked glycoproteins (casein, fetuin), and a complex yeast extract. The combination of simplified sample preparation presented here with chromatographic separation and mass spectrometric detection provides

an approach for monosaccharide characterization that isn't currently available.

## Experimental

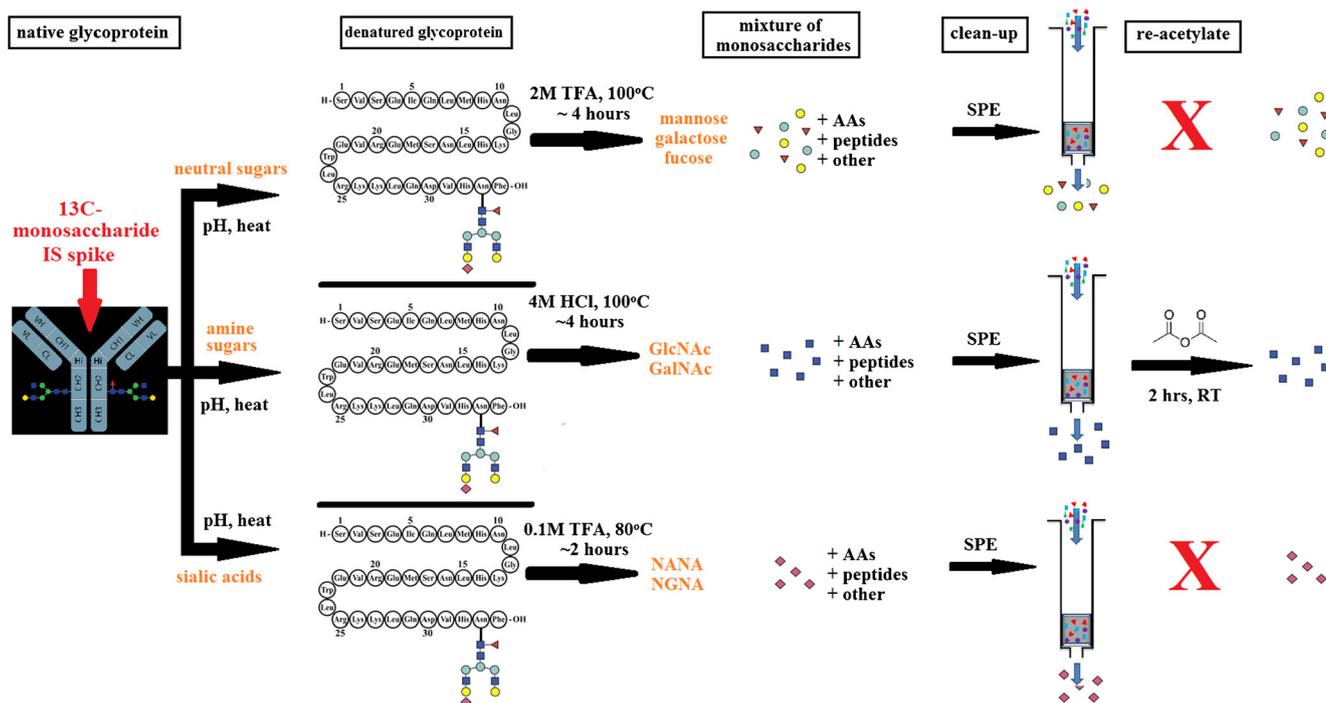
**Disclaimer** Certain commercial equipment, instruments, and materials are identified in this paper to adequately specify the experimental procedure. Such identification does not imply recommendation or endorsement by NIST nor does it imply that the equipment, instruments, or materials are necessarily the best available for the purpose.

## Materials

All chemicals and solvents were purchased through Sigma (St. Louis, MO) unless otherwise noted below. Isotope-labeled monosaccharide standards were purchased through Omicron Biochemicals (South Bend, IN). Unlabeled monosaccharide standards were purchased through Sigma (Aldrich and Fluka). Casein from bovine milk (C7078), fetuin from fetal calf serum (F2379), and yeast extract (Y4250) were also purchased through Sigma.

## Sample preparation

Stable-isotope ( $^{13}\text{C}$ ) labeled analogs of each monosaccharide were purchased from commercial sources and pre-spiked at the earliest possible step in the sample preparation (see Electronic Supplementary Material (ESM) Table SI). In the case for a total yeast extract sample, a glycoprotein extraction step with methanol/chloroform (MeOH/ $\text{CHCl}_3$ ) was necessary to first precipitate the protein sample prior to spiking the labeled analogs. Protein precipitation was performed within a 400- $\mu\text{L}$  glass autosampler vial insert (Agilent 5181-3377) that was then used for the acid hydrolysis, reducing protein loss. In all other cases, pre-spiked samples were dried in a speed vac (ThermoSavant SPD1010) and subjected separately to each of three unique acid hydrolyses. Hydrolysis was performed in a similar manner to previously reported studies by Mechref's group [16, 17]. In all cases, gas-phase hydrolysis was achieved within a capped glass vial housing the glass autosampler insert. Figure 1 provides a schematic of the sample preparation procedure for each monosaccharide class. Briefly, glycoproteins were denatured, and neutral sugars (mannose, galactose, glucose, fructose, and fucose) or amine sugars (GlcNAc and GalNAc) were liberated from their glycoprotein precursors using 2 mol/L trifluoroacetic acid (TFA, Fluka 91699) or 4 mol/L hydrochloric acid (HCl, Fluka 84410), respectively, at 100 °C for 4 h. Sialic acids (NANA, NGNA), being terminal subunits and less chemically stable, were hydrolyzed under milder conditions (0.1 mol/L TFA for



**Fig. 1** Schematic for the hydrolysis and preparation of each monosaccharide class (neutrals, amine sugars, sialic acids) prior to LC-MS analysis

2 h at 80 °C). N-acetyl groups that were observed to degrade due to the harshness of the hydrolysis conditions, as reported previously [16] for amino sugars, required a re-acetylation step. Re-acetylation was performed for one hour at room temperature by the addition of 50  $\mu$ L of  $\text{NH}_4\text{HCO}_3$  buffer, pH 7.8, with 25  $\mu$ L of neat acetic anhydride to the dried sample. Following hydrolysis, samples were cooled, dried completely, and reconstituted in water for subsequent enrichment using a solid-phase extraction (SPE) cartridge. A  $\text{C}_{18}$  SPE cartridge (Sep-Pak, Waters WAT023590) was primed according to the manufacturer's instructions and samples were slowly ( $\approx 0.2$  mL/min) vacuum-drawn through the cartridge. Flow-through fractions were pooled with the first single-bed volume wash and were retained for analysis. Any partially hydrolyzed proteins, lipids, and other contaminants that were bound to the SPE sorbent were discarded. Washed samples were dried and reconstituted in 50/50 % (v/v)  $\text{H}_2\text{O}/\text{ACN}$  for LC-MS analysis.

### LC-MS analysis

Liquid chromatographic separation was achieved using a normal phase method on an XBridge BEH amide XP analytical column (2.1 mm  $\times$  150 mm, 2.5  $\mu$ m particles; Waters) at a flow rate of 200  $\mu$ L/min. Mobile phase A and B consisted of 0.1 % (v/v) ammonium hydroxide ( $\text{NH}_4\text{OH}$ ) in water or acetonitrile, respectively (Honeywell, Burdick and Jackson, MS Grade). Monosaccharide elution was accomplished primarily by an isocratic separation for the first 20 min at 95 % (v/v) mobile

phase B, followed by a gradient elution of the sialic acids using a linear gradient over 25 min from 95 to 50 % (v/v) mobile phase B, followed by a column wash and re-equilibration. Column temperature was maintained at 80 °C; autosampler plate temperature control was set at 5 °C. An Agilent 1200 HPLC system (Santa Clara, CA) was coupled in-line with an Applied Biosystems API 5000 triple quadrupole mass spectrometer (Foster City, CA) equipped with a standard micro-flow electrospray source. Ions were detected using a multiple-reaction monitoring (MRM) method in negative polarity with a dwell time of 100 ms.

Three or four fragmentation transitions were monitored for each monosaccharide for both the native (non-labeled) sugar and its labeled analog. A total of 35 fragmentation transitions were monitored in one MRM method. During data acquisition, all source and fragmentation parameters were set identically for non-labeled/labeled transition pairs. Source conditions were as follows: collision gas =  $2.7 \times 10^4$  Pa (4 psi), unit resolution in Q1 & Q3, curtain gas (CUR) =  $7.5 \times 10^4$  Pa (11 psi), intensity threshold = 0, ion source gas 1 (GS1) =  $2.1 \times 10^5$  Pa (30 psi), settling time = 3 ms, ion source gas 2 (GS2) =  $2.8 \times 10^5$  Pa (40 psi), pause between mass ranges = 3 ms, ion spray voltage (IS) = 4500 V, capillary temperature (TEM) = 400 °C. Fragmentation conditions were as follows: for all monosaccharides, the entrance potential was set to 10 V; exit cell potential was set to 15 V. Collision energies and declustering potentials were compound dependent and are detailed in ESM Table SII. Data acquisition was performed using Analyst v1.5 software (Applied Biosystems).

## Results

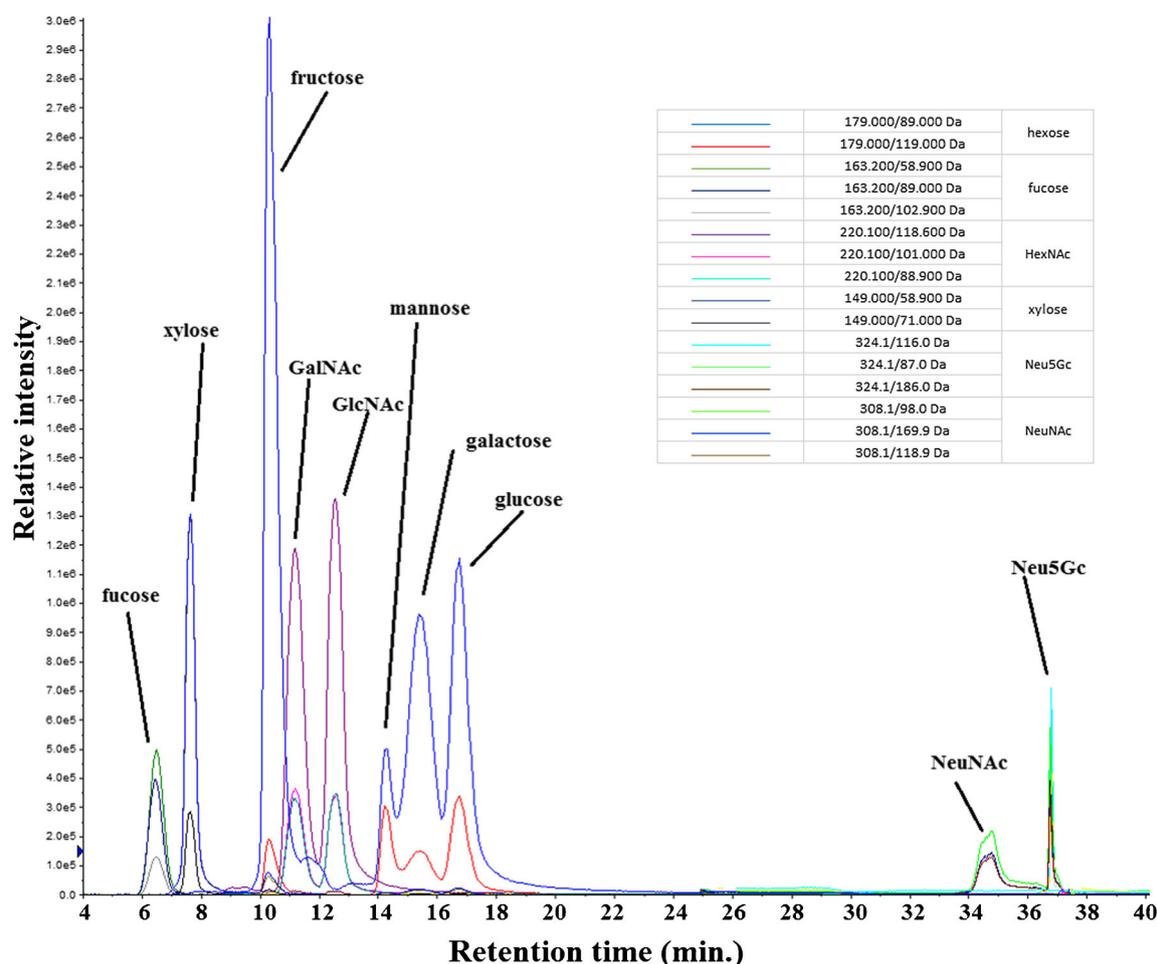
Chromatographic resolution was achieved for ten underivatized monosaccharides commonly found in natural mammalian glycoconjugates using an amide stationary chemistry under normal-phase liquid chromatography conditions. Figure 2 shows a typical MRM mass chromatographic separation of monosaccharide standards with their heavy analogs. It was important for this work to demonstrate that the separation method was capable of resolving stereoisomers that could be present within the same mammalian glycoprotein (i.e. GlcNAc/ GalNAc; mannose/ galactose), as well as resolving stereoisomers or isobars that could be present as contaminants (glucose, fructose). Non-isomeric monosaccharides were differentiated by their mass and fragmentation patterns if not also resolved chromatographically. Amine sugars, GlcNAc and GalNAc, are nearly baseline resolved under isocratic conditions, with resolution ( $R_s$ ) estimated as  $\approx 1.4$  using Eq. (1) below where  $w_{0.5,1}$  and  $w_{0.5,2}$  are the peak widths measured at

half the peak height, and assuming complete baseline resolution has  $R_s > 1.5$ .

$$R_s = (t_{R2} - t_{R1}) / (1.7 * 0.5 (w_{0.5,1} + w_{0.5,2})) \quad (1)$$

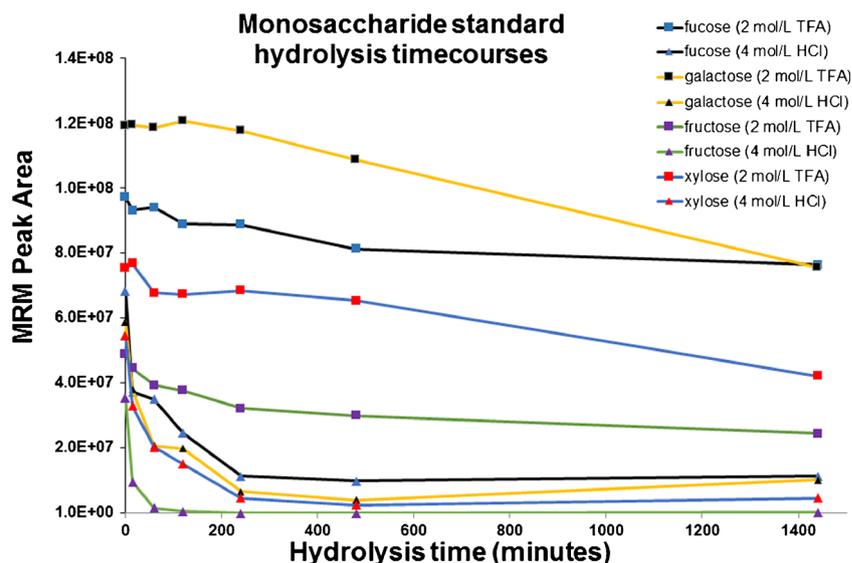
Neutral hexose monosaccharides (mannose, galactose, glucose, and fructose) that are detected in the MS using identical MRM transitions, are mostly, but not completely baseline resolved, and can be reproducibly quantified based on peak heights if necessary, rather than total peak areas. Other monosaccharides, such as fucose, xylose, and the sialic acids, are chromatographically resolved and distinguishable by their fragmentation within the mass spectrometer.

Sample preparation conditions were initially optimized on monosaccharide standards for hydrolysis time, temperature, and pH. To determine stability of the measurands during hydrolysis, monosaccharide standards were subjected to a timecourse of hydrolysis over 24 h under three optimized conditions for the amine, neutral, and sialic acid monosaccharide classes (4 mol/L HCl, 100 °C; 2 mol/L, TFA 100 °C;



**Fig. 2** Representative MRM mass chromatogram for the separation of underivatized monosaccharides using amide stationary chemistry under normal phase LC conditions. The method is capable of resolving stereoisomers likely to be present within mammalian glycoproteins

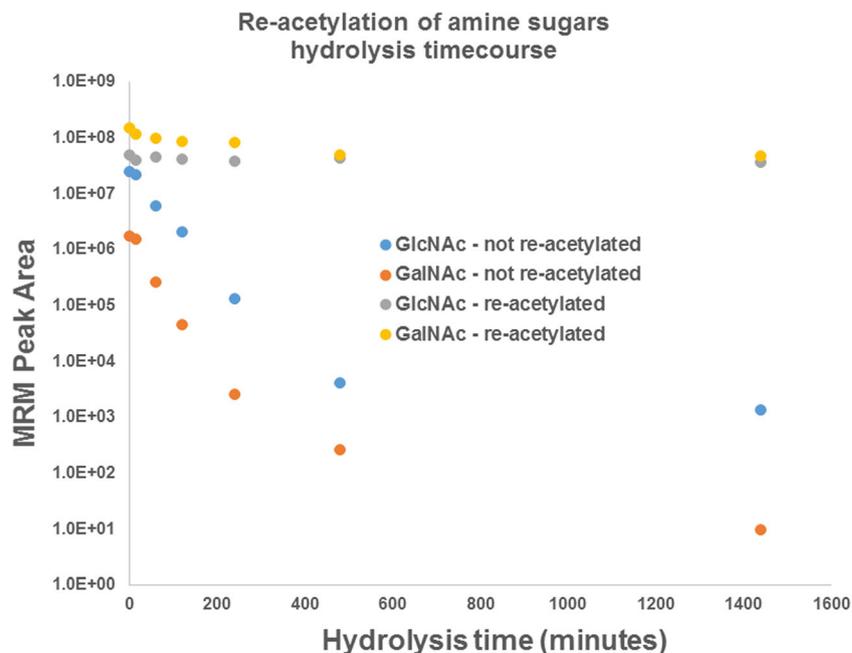
**Fig. 3** Representative time-course plot for the hydrolysis of monosaccharide standards demonstrating the relative stability of each measurands at various time, temperature, and pH conditions



0.1 mol/L TFA, 80 °C), respectively. Representative plots of normalized MRM peak area vs. time are provided in Fig. 3 for monosaccharides hydrolyzed under optimal conditions. In general, neutral monosaccharides were demonstrated to be stable under milder hydrolysis conditions (0.1 or 2 mol/L TFA at 80 °C to 100 °C) but were not stable when subjected to 4 mol/L HCl for more than one hour. Similarly, amine sugars (GlcNAc / GalNAc) were demonstrated to be completely stable under very mild conditions (0.1 mol/L TFA), but significant degradation was observed after 1 h in 2 mol/L TFA with complete degradation observed at 8 h; in 4 mol/L HCl, amine sugar degradation was complete at 4 h. This is attributable to the hydrolysis of the N-acetyl group and not degradation of the entire sugar. Further timecourse studies

of hydrolysis demonstrated that amine sugars can be re-acetylated after hydrolysis and should be considered stable for the purposes required in this work (Fig. 4). Sialic acids were completely destroyed under harsher hydrolysis conditions even after 15 min. Re-acetylation was not found to be an effective remedy. Under mild conditions (0.1 mol/L TFA, 80 °C), sialic acids were verified to be stable for up to 4 h. For all subsequent analyses presented below, 4 mol/L HCl was used for hydrolysis of the amine sugars, 2 mol/L TFA for neutral sugars, and 0.1 mol/L TFA was used for hydrolysis of the sialic acids. However, because there were only minimal differences observed in recovery of amine sugars subjected to 2 mol/L TFA or 4 mol/L HCl, either hydrolysis condition could be used.

**Fig. 4** Representative time-couse plots for standards of the amine sugars subjected to 4 mol/L HCl hydrolysis conditions and analyzed with and without re-acetylation. The plot demonstrates the need for re-acetylation of GlcNAc and GalNAc prior to LC-MS analysis



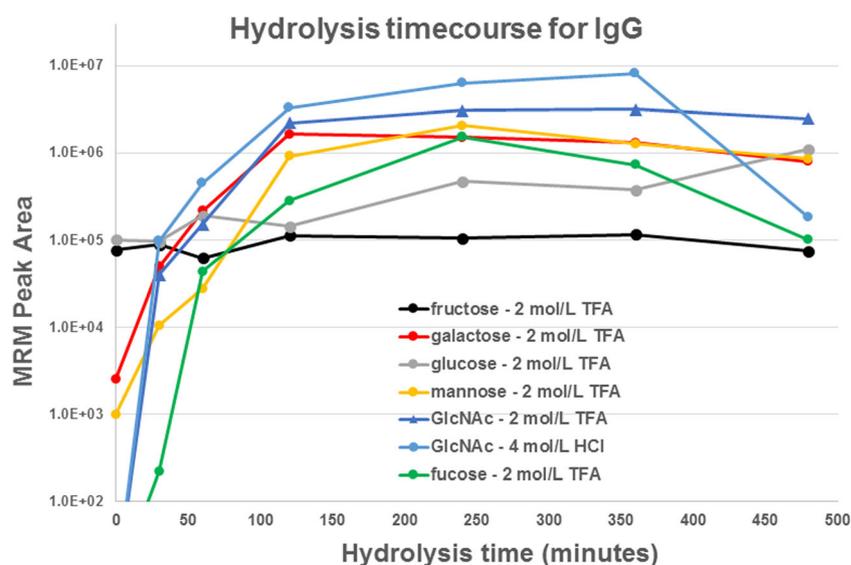
This separation scheme was subsequently applied to simple and complex biological samples to demonstrate applicability of the separation scheme, and to validate the robustness of the sample preparation. Different classes of glycoproteins (N-linked, O-linked, mixtures) were subjected to acid hydrolysis and LC-MS/MS analysis as proof-of-principle that monosaccharide characterization can be achieved without the need for derivatization steps. As is the case with any biochemical reaction, parameters such as duration of hydrolysis should be optimized individually for a given glycoprotein to ensure comprehensive monosaccharide release. A hydrolysis timecourse was performed for a representative glycoprotein – immunoglobulin G – and the individual monosaccharide concentrations were monitored by MRM. As expected, timecourse data (Fig. 5) was incomplete for several monosaccharides (fructose, glucose, GalNAc, sialic acids) that were not expected to be part of the glycosylation pattern for this N-linked glycoprotein. Fructose was observed to reach a maximum concentration at 4 h hydrolyzed with 2 mol/L TFA and then degraded thereafter; galactose and mannose were saturated between 4 and 6 h of hydrolysis with 2 mol/L TFA and remained relatively stable; GlcNAc was equally hydrolyzed by 4 h using either 2 mol/L TFA or 4 mol/L HCl, and degraded somewhat by 8 h.

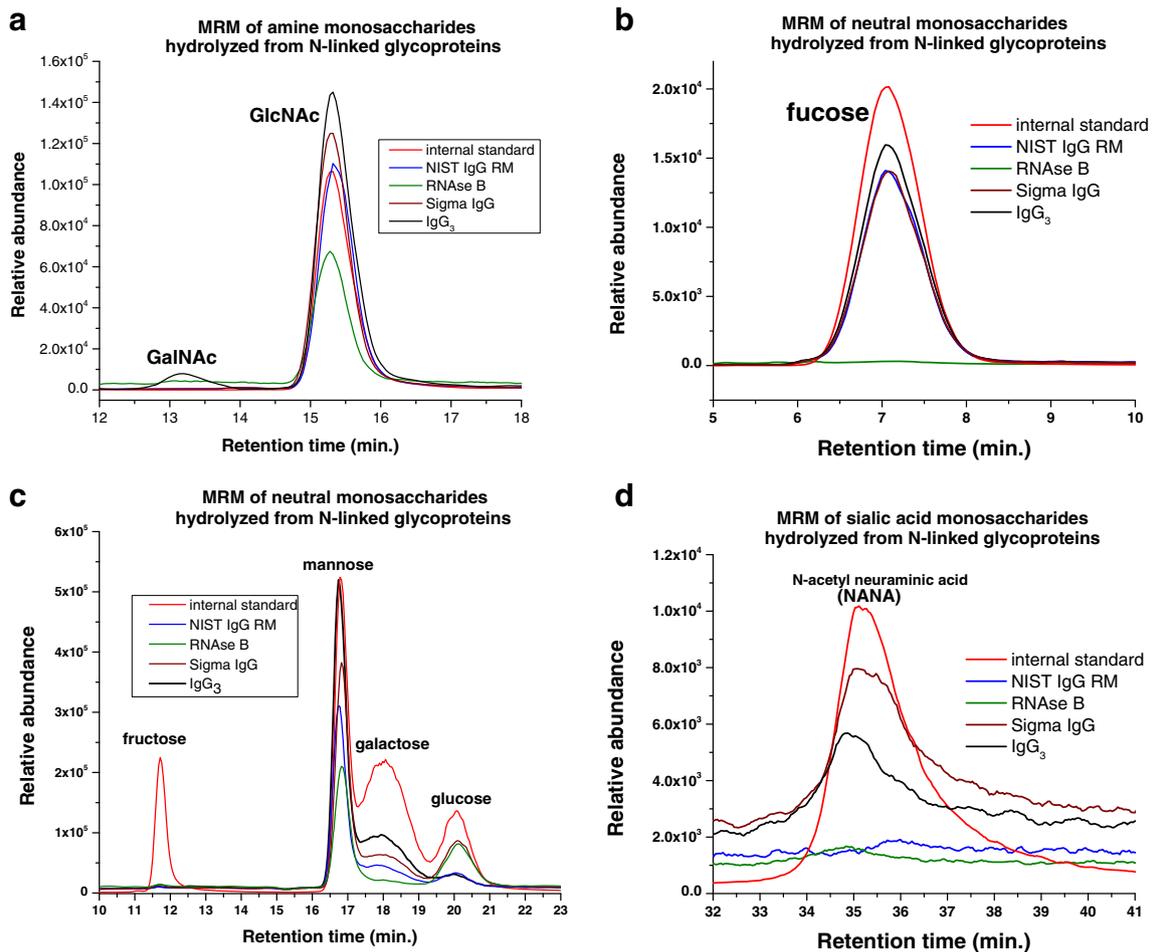
Optimized hydrolysis conditions were applied in parallel to N-linked glycoprotein standards. Three different immunoglobulin standards and ribonuclease B (RNase B) were separately subjected to acid hydrolysis followed by LC-MS analysis (no derivatization) to demonstrate the capability of this workflow. Total ion extracted mass chromatograms for IgG from human serum (Sigma standard I4506), a purified IgG<sub>3</sub> material (prepared in-house), an IgG monoclonal antibody reference material which is currently under development (NIST RM 8670), and an RNase B standard (Sigma R7884)

are provided in Fig. 6a–d. Internal standard monosaccharides were spiked into the samples prior to hydrolysis, and were observed co-eluting with the native analog monosaccharides. Mass chromatograms are displayed separately and reflect their separate hydrolysis and detection. Figure 7 shows the average MRM response from three to four fragmentation transitions for each monosaccharide. When considering the amine sugars, GlcNAc was detected in each N-linked glycoprotein whereas GalNAc was only observed from the IgG<sub>3</sub> material. GalNAc is rarely observed in the N-linked glycosylation biosynthesis pathway in mammalian cells [10], and when it is observed is only found to be expressed in very small amounts [20]. Therefore it is likely that this signal was the result of contamination of the IgG<sub>3</sub> material during protein expression, or potentially due to an unknown O-linked glycosylation in IgG<sub>3</sub>. Fucose was observed in all N-linked glycoproteins with the exception of RNase B, which is a glycoprotein made of only mannose and GlcNAc, therefore the absence of fucose in this case was expected. The neutral monosaccharides, mannose and galactose, were found in each N-linked glycoprotein, as expected, with the exception that galactose was not observed in RNase B. Fructose was not observed in any sample, as expected. It was surprising to find glucose at some level in each IgG material and in RNase B, likely a result of glycation or a contamination from the purification of the proteins. NGNA is not a mammalian compound, however, NANA was observed in small amounts in both the Sigma IgG and the IgG<sub>3</sub> material.

Hydrolyses were subsequently performed for the O-linked glycoprotein  $\kappa$ -casein, a glycosylated phosphoprotein found in milk, and fetuin ( $\alpha$ 2-HS-glycoprotein), a major plasma carrier protein secreted by the liver that contains both N-linked and O-linked glycoforms. The commercial casein product was purified from milk and can likely be considered a mixture of

**Fig. 5** Hydrolysis time-course plot of a representative glycoprotein, immunoglobulin G, under optimized temperature and pH conditions. Relative monosaccharide concentrations were monitored by MRM (LC-MS)





**Fig. 6** **a** MRM of amine monosaccharides hydrolyzed from N-linked glycoproteins. **b** MRM of neutral (fucose) monosaccharides hydrolyzed from N-linked glycoproteins. **c** MRM of amine monosaccharides

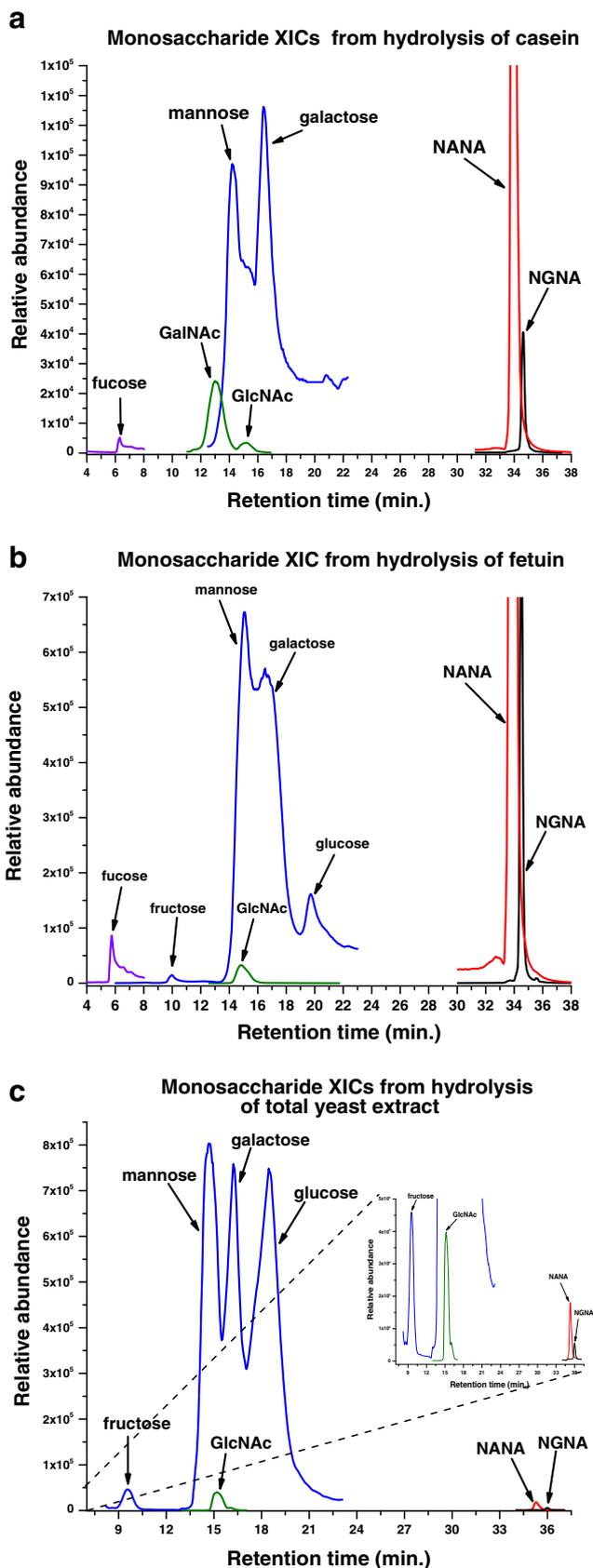
hydrolyzed from N-linked glycoproteins. **d** MRM of sialic acid monosaccharides hydrolyzed from N-linked glycoproteins

the related family of  $\alpha$ S1,  $\alpha$ S2,  $\beta$ , and  $\kappa$ -caseins. Figure 7a shows a mass chromatogram of overlaid MRM XICs for the hydrolysis of casein. The sialic acids ionize very efficiently in negative polarity ESI, and are observed with a robust signal. The precursor and product ions from NANA and NGNA are non-isobaric, consequently, these monosaccharides are distinguishable in the mass spectrometer. Sialic acids and galactose have been reported in the literature [21–24] as major monosaccharide components in O-linked glycoforms of casein, and were observed in this work. A peak corresponding to mannose was observed but was determined to be due to contamination. Glucose and fructose were not detectable. Both amine sugars, GlcNAc and GalNAc [25], were chromatographically resolved and detected from  $\kappa$ -casein. Surprisingly, a small amount of fucose was detected from this assay. There is some precedence in the literature [25, 26] for the presence of fucose on O-linked glycoforms of casein, which is supported by this observation.

The analysis of fetuin was qualitatively similar when compared to casein except that no GalNAc was detected in fetuin.

Furthermore, fructose and glucose were clearly detected in the fetuin material, and fucose was observed with a signal comparable to casein (Fig. 7b). The absence of GalNAc is not unexpected. However, the presence of fructose is most likely evidence of the purity of the glycoprotein mixture itself. Glucose is most likely explained by some degree of glycation, which is typical for serum proteins, and was evidenced for fetuin previously [27]. The difference in fucose signal can be attributable to the diversity of both O-linked and N-linked glycosylations found on fetuin. As noted in the literature [28], both sialic acids, NANA and NGNA have been previously identified in fetuin glycosylations. NGNA levels have been reported to be up to 7 % (m/m %) in fetuin [29], a level high enough to elicit an immune response.

A mass chromatogram of a total yeast extract is provided in Fig. 7c. The results reflect the glycoprotein complexity expected from a natural biomaterial, and the capacity of this analytical method for characterizing this complexity. Yeast, in contrast to bacteria, are capable of higher forms of post-translational modifications including glycosylation although



**Fig. 7** **a** Monosaccharide XICs from the hydrolysis of casein. **b** Monosaccharide XICs from the hydrolysis of fetuin. **c** Monosaccharide XICs from the hydrolysis of a total yeast extract

there are considerable differences observed between the glycan structures of yeast and mammalian origin. Yeast glycan structures are typically represented as high-mannose types, or as non-enzymatic glycation (glucose) products [30, 31]. Accordingly, mannose, along with galactose and glucose, were observed as the major monosaccharides from this extract. Other minor monosaccharides were detected in this complex yeast extract and include GlcNAc [32] and fructose, as well as very minor contributions from the sialic acids, NANA and NGNA, which are not typically expected to be present in yeast, but which have been recently suggested in several reports for yeast, and other fungi [33–36]. Although it is very uncommon to see sialic acids in fungi, the very low levels detected in this work could also potentially be a result of contamination or carry-over. As expected, neither fucose nor GalNAc were observed in the yeast extract hydrolysis.

## Discussion

The use of a silica-based amide stationary phase in a bridged ethylene hybrid column was shown to eliminate a major contributing factor of quantitative bias – the need to derivatize monosaccharides prior to LC-MS analysis. Several chemistries and column types were tested unsuccessfully for the ability to bind and resolve all relevant monosaccharides without derivatization. These included  $C_{18}$ , amine, amino, pentafluorophenyl (PFP), ion-exclusion, organic acid, HILI C-sugar, PGC (porous graphitic carbon), and polymer-based amide columns. Although several of these phases were shown to bind and resolve some or most monosaccharides, the silica-based amide chemistry provided the best resolution and most importantly provided the most robust column. The separation conditions were necessarily optimized at high temperatures ( $80\text{ }^{\circ}\text{C}$ ) and high pH ( $>9$ ) often leading to poor reproducibility and often to column degradation. Although this is not an exhaustive list of column chemistries, the results of this work demonstrate that the goal of reproducible underivatized monosaccharide separation is attainable.

Monosaccharides, like other classes of metabolites, often share isomeric or structural similarity making MS characterization challenging. When considering which monosaccharides to include in the current LC separation, it is necessary to consider only those of biological significance. Of the eight possible D-isomers of hexose, for example, altrose is considered an unnatural monosaccharide and idose is not commonly found in nature as a monosaccharide. Allose, talose, and gulose are not found in mammalian glycoproteins and are very rare otherwise. The remaining hexoses – galactose, glucose,

and mannose – are common to glycoproteins in mammalian cells and were targeted in this separation. A similar approach was considered for the other monosaccharide classes. Exoglycoside digestions were also considered for monosaccharide release from glycoconjugates, but this approach was rejected due to the inherent quantitative biases associated with using multiple enzymes and buffers for hydrolysis of numerous monosaccharide linkages.

Characterization of monosaccharides is directly applicable to the relative comparison of biomaterials. Future and ongoing work is focused on considering a comparison of biosimilars (follow-on therapeutic biologics) by relating the relative quantity of all monosaccharides between a biosimilar and its innovator product. Monosaccharide characterization should be viewed as a valuable complementary, orthogonal technique to current validation approaches for glycosylation of biosimilars. In addition to providing indirect measure of comparability between total glycan concentrations on biosimilars, monosaccharide characterization accounts for biases ignored by other assays such as, 1) contribution of excipients (specifically binders or coatings) and/or host-cell proteins to the biosimilar's total glycan content, 2) the incomplete knowledge of the glycan heterogeneity resulting in glycoforms which may not be targeted by other assays, or which may be present at concentrations lower than arbitrarily established thresholds, 3) the lack of suitable commercial glycoprotein standards, and 4) detection bias due to the large dynamic range of potential glycoforms when compared to that of monosaccharides. Additionally, this technique can be important in the direct detection of immunogenic saccharides which may be present in some of the different culture conditions such as bacterial, yeast, or mammalian (non-human and human) expression systems.

In limited cases such as purified glycoforms or simple mixtures of glycoforms, monosaccharide quantification can also be used to indirectly measure glycan concentrations. Consider a homogenous glycoprotein with a single glycosylation site (i.e. A<sub>1</sub>F) containing one fucose, four GlcNAc, three mannose, two galactose, and one NANA residue. One can equate glycan concentration to monosaccharide concentrations as:

$$\begin{aligned} [\text{glycan}] &= [\text{fucose}] = 1/4 * [\text{GlcNAc}] = 1/3 * [\text{mannose}] \\ &= 1/2 * [\text{galactose}] = [\text{NANA}] \end{aligned}$$

In such a case, agreement among monosaccharide concentrations further validates the measurement accuracy. In the case where total protein concentration can be determined concurrently, such as with the use of amino acid analysis, an occupancy rate for the glycosylation can be estimated. Consider the example of a mixture of two common glycoforms, A<sub>1</sub>F and G<sub>1</sub> where

[A<sub>1</sub>F]=[x], and [G<sub>1</sub>]=[y]. In this simple mixture, one set up a system of linear equations which can be solved simultaneously:

$$4[x] + 4[y] = [\text{GlcNAc}]_x + [\text{GlcNAc}]_y = [\text{GlcNAc}]_{\text{total}}$$

$$3[x] + 3[y] = [\text{mannose}]_x + [\text{mannose}]_y = [\text{mannose}]_{\text{total}}$$

$$2[x] + [y] = [\text{galactose}]_x + [\text{galactose}]_y = [\text{galactose}]_{\text{total}}$$

$$[x] = [\text{fucose}]_x = [\text{fucose}]_{\text{total}}$$

$$[x] = [\text{NANA}]_x = [\text{NANA}]_{\text{total}}$$

This approach is limited by the complexity of the glycoproteins in any given mixture, and prior knowledge of what is being measured.

In summary, this study focuses on one approach for limiting biases in monosaccharide quantification. Quantitative biases arise from many places in the sample preparation workflow, including incomplete hydrolysis, improper internal standard selection, sample losses during preparation and cleanup, derivatization/labeling inefficiencies, and detection bias. It is essential to demonstrate that glycoprotein hydrolysis has reached completion, and that minimal degradation of monosaccharides biases the results through timecourse analysis. Internal standards selection requires that an exact-matched, stable-isotope labeled analog with known purity and labeling amount be employed for each measurand. Sample losses should be minimized and internal standards should be spiked in at the earliest possible time to account for any losses. Monosaccharide derivatization should be avoided in most cases due to the variability of labeling efficiency between measurands and variability between preparations. Furthermore, although it has been reported in other publications, it is not recommended to derivatize a measurand and quantify/detect that derivatized measurand by monitoring (MRM-MS) the loss of the derivative in a triple quadrupole instrument (i.e. the fragmentation transition of M+(derivative) → M+). Lastly, it is suggested that mass spectrometry should be considered the gold standard for quantitative measurement of monosaccharides due to its unmatched selectivity and specificity, wide detection dynamic range, sensitivity, and the ability to perform experiments using an isotope dilution approach. The novel method described in the current study demonstrates the potential use of LC-MS for characterizing underivatized monosaccharides from typical mammalian glycans. Future efforts will be required to obtain comprehensive coverage of all possible sugars including rare abundance and non-mammalian types and towards applying a quantitative approach for reference material certification.

## References

1. Doerner T, Strand V, Castaneda-Hernandez G, Ferraccioli G, Isaacs JD, Kvien TK, Martin-Mola E, Mittendorf T, Smolen JS, Burmester GR (2013) *Ann Rheum Dis* 72:322–328
2. Hristodorov D, Fischer R, Linden L (2013) *Mol Biotechnol* 54:1056–1068
3. Badr HA, AlSadek DMM, Darwish AA, ElSayed AI, Bekmanov BO, Khussainova EM, Zhang X, Cho WCS, Djansugurova LB, Li C-Z (2014) *Expert Rev Proteomics* 11:227–236
4. Christiansen MN, Chik J, Lee L, Anugraham M, Abrahams JL, Packer NH (2014) *Proteomics* 14:525–546
5. McCarthy C, Saldova R, Wormald MR, Rudd PM, McElvaney NG, Reeves EP (2014) *J Proteome Res* 13:3131–3143
6. Wang L-X, Amin MN (2014) *Chem Biol* 21:51–66
7. Defaus S, Gupta P, Andreu D, Gutierrez-Gallego R (2014) *Analyst* 139:2944–2967
8. Doyle HA, Mamula MJ (2012) *Curr Opin Immunol* 24:112–118
9. Nagae M, Yamaguchi Y (2012) *Int J Mol Sci* 13:8398–8429
10. Taylor ME, Drickamer K (2006) *Introduction to Glycobiology*. Oxford University Press, New York
11. Lepenies B, Yin JA, Seeberger PH (2010) *Curr Opin Chem Biol* 14:404–411
12. Yasuno S, Kokubo K, Kamei M (1999) *Biosci Biotechnol Biochem* 63:1353–1359
13. Fu DT, Oneill RA (1995) *Anal Biochem* 227:377–384
14. Harazono A, Kobayashi T, Kawasaki N, Itoh S, Tada M, Hashii N, Ishii A, Arato T, Yanagihara S, Yagi Y, Koga A, Tsuda Y, Kimura M, Sakita M, Kitamura S, Yamaguchi H, Mimura H, Murata Y, Hamazume Y, Sato T, Natsuka S, Kakehi K, Kinoshita M, Watanabe S, Yamaguchi T (2011) *Biologicals* 39:171–180
15. Marino K, Lane JA, Abrahams JL, Struwe WB, Harvey DJ, Marotta M, Hickey RM, Rudd PM (2011) *Glycobiology* 21:1317–1330
16. Hammad LA, Saleh MM, Novotny MV, Mechref Y (2009) *J Am Soc Mass Spectrom* 20:1224–1234
17. Hammad LA, Derryberry DZ, Jmeian YR, Mechref Y (2010) *Rapid Commun Mass Spectrom* 24:1565–1574
18. Guttman A (1997) *J Chromatogr A* 763:271–277
19. Davies M, Hounsell E (1998) In: Hounsell E (ed) *HPLC and HPAEC of Oligosaccharides and Glycopeptides*. Humana Press, Totowa, NJ, pp 79–100
20. Sakiyama T, Kabayama M, Tomita M, Nakamura J, Mukai H, Tomita Y, Furukawa K (1998) *Biochim Biophys Acta* 10:268–274
21. Holland JW, Deeth HC, Alewood PF (2005) *Proteomics* 5:990–1002
22. O’Riordan N, Kane M, Joshi L, Hickey RM (2014) *Glycobiology* 24:220–236
23. Madsen JA, Ko BJ, Xu H, Iwashkiw JA, Robotham SA, Shaw JB, Feldman MF, Brodbelt JS (2013) *Anal Chem* 85:9253–9261
24. Choi BK, Jimenez-Flores R (2001) *J Agric Food Chem* 49:1761–1766
25. Dev BC, Sood SM, Dewind S, Slattery CW (1993) *Prep Biochem* 23:389–407
26. Stromqvist M, Falk P, Bergstrom S, Hansson L, Lonnerdal B, Normark S, Hernell O (1995) *J Pediatr Gastroenterol Nutr* 21:288–296
27. Kennedy DM, Skillen AW, Self CH (1993) *Clin Chem* 39:2309–2311
28. Gimenez E, Sanz-Nebot V, Rizzi A (2013) *Anal Bioanal Chem* 405:7307–7319
29. Noguchi A, Mukuria CJ, Suzuki E, Naiki M (1995) *J Biochem* 117:59–62
30. Gomes RA, Miranda HV, Silva MS, Graca G, Coelho AV, Ferreira AE, Cordeiro C, Freire AP (2006) *Febs J* 273:5273–5287
31. Gomes RA, Sousa Silva M, Vicente Miranda H, Ferreira AEN, Cordeiro CAA, Freire AP (2005) *Febs J* 272:4521–4531
32. Kukuruzinska MA, Bergh MLE, Jackson BJ (1987) *Annu Rev Biochem* 56:915–944
33. Soares RMA, Alviano CS, Soares RMD (1998) *Revista De Microbiologia* 29:31–35
34. Soares RMA, Soares RMD, Alviano DS, Angluster J, Alviano CS, Travassos LR (2000) *Biochim Biophys Acta Gen Subj* 1474:262–268
35. Rodrigues ML, Dobroff ASS, Couceiro E, Alviano CS, Schauer R, Travassos LR (2002) *Glycoconj J* 19:165–173
36. Alviano CS, Travassos LR, Schauer R (1999) *Glycoconj J* 16:545–554