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Development of a Robust and High Throughput Method for Profiling *N*-linked Glycans Derived from Plasma Glycoproteins by Nano LC FT-ICR Mass Spectrometry

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Abstract

Recent investigations continue to emphasize the importance of glycosylation in various diseases including cancer. In this work, we present a step by step protocol describing a method for *N*-linked glycan profiling of plasma glycoproteins by nano-flow liquid chromatography Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). A large experimental space was initially explored and is described herein. Three internal standards were spiked into the sample and provided normalization of plasma glycan abundance across different experimental conditions. Incubation methods, times and the effect of NP40 detergent on glycan abundance were explored. It was found that an 18-hour incubation with no detergent lead to the greatest ion abundance; however, data could be obtained in less than one day from raw plasma samples utilizing microwave irradiation or shorter incubation periods. The inter-sample precision of three different glycans was less than 5.5% (RSD) when the internal standards were added prior to the initial processing step. The high mass measurement accuracy (<3 ppm) afforded by the FT-ICR mass spectrometer provided confident identifications of several glycan species.

Introduction

The study of glycans derived from glycoproteins, commonly referred to as glycomics, is an emerging research area. Most glycans are either *O*-linked to a serine or threonine or *N*-linked to an asparagine residue and occur on an estimated 50% of all translational products.¹ Glycosylation is known to play significant roles in numerous biological processes including: protein folding, stability, cellular adhesion, signaling and disease. For biomarker investigations, glycomics offers a more targeted approach as opposed to searching for a relevant disease marker in the proteome. However, the study of an entire glycome is no simple task as the complex biological processing of glycans within the Golgi apparatus of a cell leads to a range of diverse structures. In addition, both the lack of chromophores and the hydrophilic nature, inherent of the glycan structure, make glycan analysis difficult. Due to its unparalleled molecular specificity, mass spectrometry offers the most comprehensive approach for glycan analysis allowing for both determination of glycan composition and structural elucidation which are more difficult to determine by other analytical techniques.

Wu *et al.* first implicated the significance of glycosylation in cancer when it was demonstrated that healthy fibroblasts have smaller membrane glycoproteins than their diseased counterpart.² In the late 1970's, Rostenburg *et al.* reported altered glycosylation patterns in alpha-1-antitrypsin in various types of cancer.³ Around the same time, Gehrke

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and coworkers linked glycan abundance to ovarian and lung carcinomas by gas-liquid chromatography (GLC).⁴ Recently, glycosylation has been further implicated in cancer development.^{5–11} Current investigations have taken a global glycan approach for biomarker discovery in which glycans are cleaved from glycoproteins in complex biological matrices (e.g., serum). These studies have shown promise for identifications of potential physiologically important species in several types of cancer including breast^{12, 13}, ovarian¹⁴, liver¹⁵, and prostate malignancies.¹⁶

Although there are several factors that must be considered when conducting a large scale discovery experiment (e.g., sample collection, sample type, statistical analyses, analytical variability) of two different physiological states (e.g., disease vs. control), one of the most important and initial steps is the development of a robust and reproducible method while maximizing throughput. We have recently detailed a method utilizing β -elimination chemistry¹⁷ for the analysis of *O*-linked glycans derived from glycoproteins in plasma by both MALDI-FT-ICR mass spectrometry¹⁸ and nanoLC Orbitrap mass spectrometry.¹⁹ The analysis of the same sample by both techniques revealed that the low molecular weight glycans observed by MALDI-FT-ICR mass spectrometry were potential fragments of larger species detected intact by nanoLC mass spectrometry.¹⁹ This fragmentation can be contributed to both the harsh ionization conditions of MALDI, relative to ESI, and the long detection times associated with FT-ICR mass spectrometry. Although β -elimination targets the release of *O*-linked glycans, the majority of species identified in these studies by nanoLC MS are believed to be *N*-linked (based on exact mass and tandem MS spectra). This observation may be due to two reasons: **1)** β -elimination chemistry is known to release portions of *N*-linked glycans^{20, 21} and **2)** glycosylation occurring on asparagine residues may be considerably more abundant than its *O*-linked counterpart as it has been estimated that 75% of all glycoproteins contain only *N*-linked glycans.¹ Therefore, it was decided that it would be beneficial for future discovery investigations to develop an optimized procedure that specifically targets these species.

Michalski and coworkers have described in great detail a method for *N*-linked glycan cleavage and purification derived from protein quantities greater than 50 μ g.²² In summary, this step by step procedure is time consuming as it involves reduction (4 hours), alkylation (12 hours), proteolytic digestion (24 hours), PNGase F digestion (18 hours), solid phase extraction (SPE), permethylation, followed by purification of permethylated glycans using another SPE step.²² In addition, four 12-hour dialysis steps are recommended to purify the sample during preparation. Other similar procedures utilizing reduction and alkylation^{15, 16} or a combination of reduction, alkylation, and proteolytic digestion^{13, 23, 24} prior to *N*-glycan release, sample cleanup, derivatization and mass spectral analysis, have been briefly described throughout the literature. Wührer and coworkers recently reported a method utilizing SDS and heat for plasma protein denaturation followed by *N*-glycan release and derivatization; however, the method utilized two SPE steps for sample purification and both MALDI-MS and CE-ESI-MS for glycan analysis.²⁵

In this work, we describe a method for profiling of underivatized *N*-linked glycans from plasma by nanoLC FT-ICR mass spectrometry. Data can be collected from a plasma sample in less than one day using microwave irradiation or shorter incubation times during PNGase F digestion. The procedure involves plasma denaturation with SDS and β -mercaptoethanol, digestion by PNGase F and one SPE step. The effect of NP40 detergent and different PNGase F incubation methods on ion abundance are reported. In addition, three internal standards were spiked into the samples and allow for normalization of glycan abundance amongst samples across different experimental conditions. Finally, several glycan species were identified using the high mass measurement accuracy afforded by FT-ICR mass spectrometry.

Experimental

Materials

Peptide-N-Glycosidase F (2.5 mU/ μ L), the denaturing mixture consisting of 1 M β -mercaptoethanol and 2% (w/w) sodium dodecyl sulfate, and the detergent solution consisting of 15% nonidet P40 (NP40) in water were all purchased from Prozyme (San Leandro, CA). Formic acid, trifluoroacetic acid (TFA), ammonium acetate, lacto-*N*-difucohexaose I (LND), lacto-*N*-fucopentose (LNF), maltoheptaose, pronase E (6 U/mL) were purchased from Sigma Aldrich (St Louis, MO). HPLC grade acetonitrile and water were obtained from Burdick & Jackson (Muskegon, MI). Pooled human plasma was purchased from Innovative Research (Novi, MI). Graphitized solid phase extraction cartridges (Part Number 210101) were from Alltech (Deerfield, IL) and drop dialysis membranes were from Millipore (Burlington, MA). Slide-A-Lyzer dialysis cassettes, molecular weight cut-off of 7 kDa, were obtained from Pierce (Rockford, IL).

Methods

A large experimental space was initially explored in order to optimize this procedure and these parameters are summarized in Table 1. Herein, the final optimized protocol is described. Certain parameters from Table 1 are further discussed (*vide infra*). A conservative estimate of the total time for each step is given and is based on a sample size of 15–20. This estimate is broken up into “personnel hours” and hours that require no attention (e.g., incubation).

Denaturing Plasma Proteins (0.5 Hours + 1 Hour of Lyophilization)—50 μ L of pooled human plasma was lyophilized to dryness at 35° C. 250 μ L of 50 mM tris-HCl buffer (pH=7.5) was added to the dried proteins and samples were vortexed vigorously for 5 minutes. 28 μ L of stock denaturing solution (2% SDS/1 molar β -mercaptoethanol) was added to the sample, vortexed (30 s), centrifuged and heated in a 95° C water bath for 5 minutes.²⁶ NP40 detergent was *not* used in the final protocol; however, in the developmental stages 30 μ L of stock detergent solution was added after protein denaturation. It is important to note that higher concentrations of plasma protein, higher temperatures, and/or longer incubation times resulted in an aggregate formation. This formation led to extremely viscous samples and both was irreversible and detrimental to analysis in our studies. If desirable, the volume of plasma starting material could be decreased; however, 50 μ L of plasma resulted in 840 μ L of sample yielding approximately 200 LC/MS runs.

Enzymatic Digestion (0.5 Hours Sample Preparation + 18 Hours Incubation)—After allowing the mixture to cool to room temperature (20 mins), 5 μ L of enzyme solution (12.5 mU) was added to the sample. The sample was vortexed (10 s), centrifuged, and incubated at 37° C for 18 hours. After 18 hours the enzyme was quenched by adding 500 μ L of aqueous 0.1% TFA to lower the pH (~3.5). It is important to note shorter enzyme incubation times or the use of a microwave reactor could be used to decrease the length of this step and still resulted in acceptable data (*vide infra*).

Solid Phase Extraction (SPE) using Nonporous Graphitized Carbon (3 Hours for SPE + 4 Hours for Lyophilization)—Graphitized carbon is well suited for these applications as the material binds strongly to molecules with a range of hydrophobicities. Small molecules and salts were removed in **step A** and peptides and proteins were separated from glycans in **step B** (elution step) by strong interactions with the stationary phase.

Three solutions were used for SPE.

1. Wash solution (100% 18 M Ω nanopure H₂O containing 0.1% TFA)

2. Conditioning solution (80% ACN with 0.05% TFA)
3. Elution solution (25% ACN in 0.1% TFA)

A. Conditioning the Extraction Cartridges—The extraction cartridges were conditioned with two column volumes of solution 1, one column volume of solution 2, and two more column volumes of solution 1. External pressure could be applied during the conditioning phase to achieve a flow rate of approximately 1 mL/min.

B. Loading and Wash Phases—The sample was loaded onto the SPE cartridge and allowed to pass through the cartridge with no external pressure. The ependorf tube containing the sample was rinsed twice with 500 μ L of aqueous 0.1% TFA and both rinses were added to the column. The sample was then washed with 50 mL of solution 1. This step removed salts and other small molecules from the sample. If necessary, external pressure could be applied during the wash phase to achieve a flow rate of $\sim$ 0.5 mL/min.

C. Elution—1 mL of the elution solution (solution 3) was added to the column and the contents were collected in a 1.5 mL ependorf tube. This step was repeated 3 additional times for a total of four 1 mL aliquots. A greater number of elution aliquots was explored with no additional benefits in glycan abundance (Table 1). External pressure could be applied in the elution step to achieve a flow rate of 0.5–1 mL/min. However, it should be noted that too much external pressure could result in protein eluting from the SPE cartridge. In addition, at higher flow rates an unidentified black material was observed in the elution, presumably carbon from the SPE packing material. It also should be noted that higher concentrations of organic modifier ($>25\%$) in the elution solution could result in peptide contamination. The four elutions were then lyophilized to dryness at 35° C for 4 hours.

D. Reconstitution—Each aliquot was reconstituted in 10 μ L of HPLC grade water and fractions were combined for a total of 40 μ L of sample. It was occasionally observed that in the first elution aliquot, after lyophilization and reconstitution, there was insoluble protein material. In these cases the aliquot was centrifuged for 30 seconds and only the supernatant was used for analysis. This was not found to be detrimental to glycan analysis by LC/MS. All samples were stored at -20° C prior to analysis.

Sample Preparation for NanoLC MS Analysis (< 0.5 hours)

Preparation of LC/MS sample

LND, LNF and maltoheptaose were chosen for internal standards because these carbohydrates are not found attached to plasma glycoproteins, have different LC retention times and are commercially available. A 10 μ M mixture of each internal standard was prepared. For experiments with ISDs added prior to sample processing, 40 μ L of this mixture (400 pmols) were added directly to raw plasma. For other experiments the 10 μ M mixture was diluted 25-fold in ACN/H₂O (80:20) and mixed with 5 μ L sample, resulting from reconstitution of the SPE elutions, prior to analysis. The sample was then placed in a low volume LC vial (part number CTV-0910P, Chromtech, Apple Valley, MN) for subsequent analysis by nanoLC mass spectrometry. The glycan ratio factor was calculated by summing the integrated areas of the extracted ion chromatograms of 6 plasma glycans and dividing this number by the areas of the internal standards. Due to significant ammonium exchange with hydroxyl hydrogens, which produced satellite peaks 17.03 Da apart, both the integrated areas from these species and the protonated species were used in the calculation.

Microwave Experiments

Plasma samples (~50 μ L), that were prepared and denatured according to the described procedure, were placed in a microwave reaction vial and subjected to digestion with 5 μ L of enzyme solution (12.5 mU) under the following microwave conditions using a CEM Discover system: 1) standard mode (37°C, 1 hour, ~10 W), 2) power mode (~55°C, 5 mins, 100 W), or 3) under simultaneous cooling using a jacketed reaction vessel (CEM Coolmate) that enabled temperature control via continuous flow of coolant (–40°C), allowing a maximal power input at a lower temperature in standard mode (25° C, 20 mins, ~250 W). Temperatures were measured using a fiber optics probe. Condition 3 was found to be optimal and was further investigated with and without detergent. Irradiated samples were subsequently subjected to the described solid phase extraction (*vide supra*).

Time Course Experiments

We investigated the effect of different incubation times on glycan abundance using the described non-microwave protocol. Five different incubation periods (37° C) were explored in triplicate: 1) T=0, 2) T=20 mins, 3) T=6 hours, 4) T=12 hours, 5) T=18 hours. Immediately after adding the enzyme to the denatured plasma sample and vortexing for 10 seconds, the reaction was quenched and this set of samples (n=3) corresponded to T=0. After each incubation period the set of corresponding samples (n=3) was quenched by adding 500 μ L aqueous 0.1% TFA solution and stored at –20°C. After 18 hours, solid phase extraction was performed on all samples following the described protocol. These samples were analyzed by nanoLC mass spectrometry in random order to avoid any measurement biases.

Nano LC FT-ICR Mass Spectrometry

Liquid chromatography was performed using an Eksigent nanoLC-2D system (Dublin, CA) operating under hydrophilic interaction chromatography (HILIC) conditions.²⁷ Solvents A and B were 50 mM ammonium acetate (pH=4.5) and acetonitrile, respectively. A vented column configuration was used in these studies and was recently found in our laboratory to provide superior chromatography of peptides when compared to the discontinuous configuration.²⁸ Four μ L of sample were injected onto a 10 μ L loop and flushed out of the loop onto a ~5 cm self packed IntegraFrit (New Objective, Woburn, MA) trap at 2 μ L/min (80% ACN). During the wash phase the nano-flow pumps were running over a 15 cm self-packed IntegraFrit “dummy” column to provide sufficient backpressure prior to the valve switching. After approximately 10 trap washes, the 10-port valve (VICI, Houston, TX) switched in-line with the gradient and data collection commenced. Glycans were eluted at 500 nL/min from a 75 μ m I.D. PicoFrit capillary column with a 15 μ m I.D. tip (New Objective, Woburn, MA) packed in-house (10 cm) with TSK-Gel Amide80 stationary phase (Tosoh Biosciences, San Francisco, CA). After 3 minutes, the gradient ramped to 60% solvent A over 37 minutes, held constant for 5 minutes and was then brought back to initial conditions (80% B) to re-equilibrate the column for an additional 10 minutes. The total run time was 60 minutes.

Mass spectrometric analyses were performed using a hybrid linear ion trap Fourier transform ion cyclotron resonance mass spectrometer (Thermo Fischer Scientific, San Jose, CA) equipped with a 7 Tesla superconducting magnet. The instrument was calibrated by following the manufacturer’s standard procedure. Electrospray ionization was achieved by applying a potential of 2 kV to a liquid junction pre-column. The capillary and tube lens voltage were set to 42 V and 120 V, respectively. The capillary temperature was 225° C. Full scans were performed in the ICR cell at a resolving power of 100,000_{FWHM} @ m/z 400, AGC of 1×10^6 , and a maximum injection time (IT) of 1 second. Five MS/MS scans were

performed in the ion trap per full scan at a normalized collisional energy of 22. For MS/MS spectra the AGC was set to 1×10^4 with a maximum IT of 400 ms. A dynamic exclusion of 120 s was used to avoid repeated interrogation of abundant peaks. Glycans were identified by using the OSCAL software provided by the Lebrilla laboratory (Personal Communication) and Glycoworkbench.²⁹ Xcalibur software version 2.0.5 was used for data analysis and peak integration.

Results and Discussion

Figure 1 displays a typical base-peak ion chromatogram of glycans derived from plasma using the described protocol. Glycans eluted between 20 and 35 minutes and any interfering peptides eluted prior to 20 minutes. It is worth noting that peptide contamination observed in these studies was minimal, in contrast to our previously published procedure using β -elimination for *O*-linked glycan analysis.^{19, 30} In order to compare the abundances of glycans under various experimental conditions, and for future studies across different physiological states, 3 standard glycans LND, LNF and maltoheptaose were spiked into the sample. Figure 1 shows the elution order and relative abundances of these internal standards. The internal standards were chosen based on availability and the fact they are not found linked to glycoproteins. This gave samples a point of reference at which plasma glycan abundances could be quantitatively compared across different experimental conditions. As described in the method sections, a ratio of abundances between the plasma glycans and the three internal standards was used to evaluate the various experimental conditions discussed herein.

One of the major problems discovered in the early stages of method development was the strong polymer signal that was present throughout the LC-MS analysis as shown in Figure 1. This polymer signal was determined experimentally to be the result of NP40, the detergent used in the early stages of method development. It has been reported that non-ionic detergents such as NP40 are used to maximize PNGase F activity in the presence of SDS²² and thus, increasing the efficiency of glycan cleavage.²⁵ In our studies the addition of NP40 was found to be detrimental as polymer was observed in these samples and slowly bled off the nanoLC column as shown in Figure 1. After running a few samples, even with blanks in between, the polymer signal began to significantly suppress glycan intensity.

Two main options existed in order to remedy the polymer contamination: **1)** Eliminate the use NP40 detergent during the sample preparation process or **2)** To investigate another method of sample cleanup (e.g., dialysis, organic solvent precipitation, or a second solid phase extraction). Due to the fact that adding another sample preparation step would add a significant amount of time to the sample preparation process and increase both the possibility for sample loss and contamination, we decided to explore the first option. We were initially concerned about the enzymatic activity of PNGase F in the presence of SDS without the NP40 detergent. As a result, microwave radiation was utilized in an effort to increase enzymatic activity, thus both counteracting the inhibitory effects of SDS and decreasing the overall time needed for incubation allowing for higher sample throughput. Microwave technology has been utilized previously to increase the enzymatic activity of PNGase F; however, those studies focused on model proteins.^{31, 32} Microwaving the sample for 20 minutes @ 20°C and 250 watts was found to provide the most favorable results in terms of absolute glycan abundance (data not shown) for the three conditions investigated (Table 1).

Figure 2 compares the absolute glycan abundance using the glycan ratio factor (y-axis) for four different experimental conditions. Two technical replicates were investigated for each set of conditions. Microwave-assisted cleavage with and without NP40 was compared to conventional 18 hour digestion with and without NP40. Surprisingly, there were minimal

differences in the ratio factors (glycan abundance) between the 18-hour digestion with and without the detergent. The microwave experiments without NP40 were comparable to the 18 hour digestion; however, they were completed in only 20 minutes at 25° C – a 50-fold reduction in digestion time. Interestingly, in the presence of detergent, glycan abundance was significantly lower (Figure 2).

Due to the capability to perform parallel processing under conventional enzymatic procedures as opposed to serial processing with the microwave procedure, it was clear that for a large sample set it would be more beneficial to optimize the 18 hour digestion protocol. To further increase throughput, the length of incubation and its effect on glycan abundance was investigated. Figure 3 shows the ratio factor as a function of incubation time. Five different time periods were investigated: 1) T=0; 2) T=20 mins; 3) T=6 hours; 4) T=12 hours; 5) T=18 hours. For each period there were 3 technical replicates for a total of 15 samples. All samples were digested without the presence of NP40. As displayed in Figure 3, the ratio factor significantly increases with only 20 minutes of incubation at 37°C. As the length of incubation increases, glycan abundance also increases. The ratio factor begins to level off at 12 to 18 hours; however, the highest reproducibility was achieved by the 18-hour incubation, indicated by the small range in the confidence interval. Although comparable ratio factors were obtained with these shorter incubation times (T=20 mins and T=6 hours) some of the less abundant glycans in plasma were not always detected in these samples (data not shown).

The final step of development was to evaluate the reproducibility of the 18 hour non microwave digestion procedure. The comparison of the average ratio in the triplicate injection of three technical replicates was used to determine the inter-sample variability of three glycans. We observed relatively large RSDs by adding the internal standards just prior to LC/MS. As a result, we explored introducing the ISDs at the initial stages of sample processing and observed significantly higher reproducibility. These data are summarized in Figure 4.

Glycans identified in plasma, using the described procedure, are shown in Table 2. The high mass measurement accuracy afforded by the FT-ICR mass spectrometer provides a confident glycan composition as the upper limit of the 95 % CI in the average MMA was below 3 ppm. The absolute MMAs originating from 5 different runs were used to perform this calculation. It is important to note that several glycans containing sialic acid residues were also identified. These glycans are thought to mediate various critical physiological processes.³³ Changes in the amount, type, and bonding of sialic acids to neighboring molecules have also been reported in human cancers.^{34–36} The ability to identify and determine differential expression of these species across a large sample set will prove critical for future biomarkers studies.

Conclusions

A fast and robust method for profiling *N*-linked glycans from glycoproteins in plasma is reported. A large experimental space was initially explored and is summarized. The final procedure is described in detail and requires no protease digestion, one solid phase extraction step, and no glycan derivatization. Internal standards were spiked into the samples and provided a point of reference to normalize plasma glycans across numerous experimental conditions. Incubation times, effects of NP40 detergent, and the use of microwave irradiation were investigated. Although microwave irradiation and lower incubation times could potentially be used to increase throughput, these processes yielded slightly lower glycan abundances as determined through the use of internal standards. Future studies will utilize the described optimized procedure for initial screening of glycan profiles

derived from plasma taken from benign gynecologic tumor controls and epithelial ovarian cancer patients in efforts to discover a diagnostic marker regardless of disease progression. Structures of glycans identified to be significantly different between EOC and control groups will then be determined from tandem MS spectra.

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Figure 1.

A base peak ion chromatogram of glycans resulting from the cleavage and purification of 50 μ L of plasma. Glycans eluted between 20 and 35 minutes. One of the major problems discovered in the early stages of the method development was a strong polymer signal, resulting from the NP40 detergent used in the initial stages, which slowly bled off the column. After LC-MS analysis of a few samples the polymer signal began to suppress glycan intensity. The extracted ion chromatograms for three internal standards, used to normalize glycan abundance, are overlaid on the base peak ion chromatogram.

Figure 2.

The ratio factor (glycan abundance) is plotted as a function of different experimental conditions. Two samples were carried through each experimental condition. The NP40 detergent did not have a significant impact on 18 hour incubations performed at 37°C while the detergent significantly suppressed glycan abundance using the microwave. Microwave conditions: 250 W, 25°C, 20 mins.

Figure 3.

The 95% CI in the average ratio factor ($n=3$) is plotted as a function of 5 different incubation times at 37°C. Glycan abundance increased significantly after just 20 minutes of incubation and began to level off after 12–18 hours. It is important to note that some of the less abundant glycans were not always present at shorter incubation lengths (e.g., 20 mins and 6 hours). The 18 hour digestion was the most reproducible.

Figure 4.

Displays the RSDs of three plasma glycans as a function of when the internal standards were added during the sample preparation process. The integrated areas of the EIC of three glycans were individually normalized to the areas of the ISDs. Three technical replicates were used for each internal standard method to produce these data. Higher reproducibility was observed when the ISDs were added directly to unprocessed plasma as opposed to adding them just prior to LC/MS.

Table 1

The experimental conditions explored throughout the stages of method development.

Experiment	Hypothesis	Conclusion
Dialysis of plasma against 500 mL of nanopure H ₂ O for 24 h. MWCO 7 kDa. This was done prior to drying down and reconstituting in 50 mM Tris-HCl buffer.	Removal of low molecular weight contaminants (e.g., metabolites, salts, small peptides) would increase glycan spectra quality.	No real advantage was observed. Step significantly increased sample preparation time.
Varying the number of elutions from the SPE cartridge from 4 to 8.	Possible that elution of glycans from the SPE cartridge was incomplete using only 4 elutions.	The second set of 4 elutions showed no detectable glycans. 4 elutions is sufficient.
The use of a microwave during enzymatic digestion. Different conditions were explored.	Reduce incubation times by increasing enzymatic activity through the use of a microwave.	Condition number 3 worked the best in terms of overall glycan abundance.
<ol style="list-style-type: none"> 1 60 mins @ 37° C ~10 Watts 2 5 mins @ 55° C ~100 Watts 3 20 mins @ 25° C ~250 Watts 		
Comparing glycan abundance by normalizing glycans to internal standards between the microwave and conventional incubation methods with and without NP-40.	Is the microwave comparable with 18 hour digestion? What is the effect of NP-40 detergent.	See Figure 2 and text.
Optimizing enzymatic incubation times for total glycan abundance using internal standards.	Can decreased incubations times still yield similar results?	See Figure 3 and text.
After SPE, pronase digestion for 4 hours followed by drop dialysis for 2 hours.	Help purify the sample by removing interfering peptide peaks that eluted during SPE.	Proved detrimental as glycan abundance decreased and peptide contamination increased.

Table 2

Glycan compositions identified using the described protocol. All compositions were in the aldehyde form. Majority of species were detected in $[M+2H]^{2+}$ form.

	Theoretical [M+H]	Composition	95% CI MMA (ppm) n=5
1	911.3350	Hex ₃ HexNAc ₂	0.75 ±0.52
2	1073.3878	Hex ₄ HexNAc ₂	0.76 ±0.93
3	1114.4150	Hex ₃ HexNAc ₃	0.88 ±0.53
4	1235.4410	Hex ₅ HexNAc ₂	2.12 ±0.37
5	1260.4730	Hex ₃ HexNAc ₃ Fuc ₁	0.90 ±0.42
6	1276.4670	Hex ₄ HexNAc ₃	1.03 ±0.70
7	1317.4940	Hex ₃ HexNAc ₄	1.31 ±0.32
8	1397.4940	Hex ₆ HexNAc ₂	1.34 ±0.44
9	1405.5100	Hex ₃ HexNAc ₃ NeuAc ₁	1.01 ±0.77
10	1438.5176	Hex ₅ HexNAc ₃	0.49 ±0.48
11	1463.5520	Hex ₃ HexNAc ₄ Fuc ₁	1.83 ±0.30
12	1479.5470	Hex ₄ HexNAc ₄	0.95 ±0.21
13	1520.5732	Hex ₃ HexNAc ₅	1.18 ±0.16
14	1600.5728	Hex ₆ HexNAc ₃	1.77 ±0.46
15	1625.6050	Hex ₄ HexNAc ₄ Fuc ₁	1.84 ±0.14
16	1641.5994	Hex ₅ HexNAc ₄	1.49 ±0.32
17	1666.6310	Hex ₃ HexNAc ₅ Fuc ₁	2.13 ±0.06
18	1682.6260	Hex ₄ HexNAc ₅	1.43 ±0.23
19	1713.6205	Hex ₄ HexNAc ₃ Fuc ₁ NeuAc ₁	1.19 ±0.30
20	1729.6154	Hex ₅ HexNAc ₃ NeuAc ₁	0.81 ±0.61
21	1770.6520	Hex ₄ HexNAc ₄ NeuAc ₁	1.38 ±0.32
22	1787.6573	Hex ₅ HexNAc ₄ Fuc ₁	1.12 ±0.24
22	1828.6839	Hex ₄ HexNAc ₅ Fuc ₁	1.68 ±0.14
23	1844.6788	Hex ₅ HexNAc ₅	1.00 ±0.31
24	1891.6682	Hex ₆ HexNAc ₃ NeuAc ₁	1.52 ±0.86
25	1916.6999	Hex ₄ HexNAc ₄ Fuc ₁ NeuAc ₁	1.29 ±0.22
26	1932.6848	Hex ₅ HexNAc ₄ NeuAc ₁	2.09 ±0.25
27	1973.7214	Hex ₄ HexNAc ₅ NeuAc ₁	0.85 ±0.52
28	1990.7367	Hex ₅ HexNAc ₅ Fuc ₁	0.58 ±0.49
29	2078.7527	Hex ₅ HexNAc ₄ Fuc ₁ NeuAc ₁	1.81 ±0.12
30	2119.7793	Hex ₄ HexNAc ₅ Fuc ₁ NeuAc ₁	1.13 ±0.83
31	2135.7742	Hex ₅ HexNAc ₅ NeuAc ₁	1.31 ±0.46
32	2223.7902	Hex ₅ HexNAc ₄ NeuAc ₂	1.20 ±0.49
33	2281.8321	Hex ₅ HexNAc ₅ Fuc ₁ NeuAc ₁	1.23 ±0.31
34	2297.8270	Hex ₆ HexNAc ₅ NeuAc ₁	0.56 ±0.58

	Theoretical [M+H]	Composition	95% CI MMA (ppm) n=5
35	2369.8481	Hex ₅ HexNAc ₄ Fuc ₁ NeuAc ₂	1.32 ±0.52
36	2426.8696	Hex ₅ HexNAc ₅ NeuAc ₂	0.74 ±0.91
37	2572.9275	Hex ₅ HexNAc ₅ Fuc ₁ NeuAc ₂	1.60 ±0.62
38	2588.9224	Hex ₆ HexNAc ₅ NeuAc ₂	0.83 ±0.97
39	2734.9803	Hex ₆ HexNAc ₅ NeuAc ₂ Fuc ₁	1.21 ±1.02
40	2880.0178	Hex ₆ HexNAc ₅ NeuAc ₃	1.07 ±0.68
41	3026.0757	Hex ₆ HexNAc ₅ Fuc ₁ NeuAc ₃	1.14 ±0.38
42	3536.2454	Hex ₇ HexNAc ₆ NeuAc ₄	1.31 ±1.09