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Systematic Comparison of Reverse Phase and Hydrophilic Interaction Liquid Chromatography Platforms for the Analysis of *N*-linked Glycans

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Abstract

Due to the hydrophilic nature of glycans, reverse phase chromatography has not been widely used as a glycomic separation technique coupled to mass spectrometry. Other approaches such as hydrophilic interaction chromatography and porous graphitized carbon chromatography are often employed, though these strategies frequently suffer from decreased chromatographic resolution, long equilibration times, indefinite retention, and column bleed. Herein, it is shown that through an efficient hydrazone formation derivatization of *N*-linked glycans (~4 hr of additional sample preparation time which is carried out in parallel), numerous experimental and practical advantages are gained when analyzing the glycans by online reverse phase chromatography. These benefits include an increased number of glycans detected, increased peak capacity of the separation, and the ability to analyze glycans on the identical liquid chromatography-mass spectrometry platform commonly used for proteomic analyses. The data presented show that separation of derivatized *N*-linked glycans by reverse phase chromatography significantly out-performs traditional separation of native or derivatized glycans by hydrophilic interaction chromatography. Furthermore, the movement to a more ubiquitous separation technique will afford numerous research groups the opportunity to analyze both proteomic and glycomic samples on the same platform with minimal time and physical change between experiments, increasing the efficiency of ‘multi-omic’ biological approaches.

Introduction

Glycosylation is a post-translational protein modification that is ubiquitous in biological systems¹⁻² and plays a significant role in numerous biological processes including cell-cell interactions, cellular recognition, protein stability, and immune response.³ Therefore, it is important to develop efficient, high-throughput strategies in order to analyze and understand these modifications. Many current glycomic strategies have evolved and benefitted from existing proteomic strategies, which are comprised mainly of some fractionation method followed by mass spectrometric analysis. One of the most effective proteomic strategies is online separation by reverse phase (RP) liquid chromatography (LC) coupled online to electrospray ionization (ESI) mass spectrometry (MS). However, the translation of this technology to the field of glycomics has been difficult for numerous reasons including the lack of retention on RP columns and the suppressed ESI response of the much more hydrophilic glycans. Thus, researchers have adapted this strategy to use more compatible separation techniques⁴ (hydrophilic interaction chromatography and graphitized carbon

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chromatography) and derivatization techniques⁵⁻⁶ (permethylation, reductive amination, and hydrazone formation) in order to overcome the inherent disadvantages in the analysis of glycans.

When analyzing *N*-linked glycans cleaved from complex mixtures (such as plasma) in ESI MS, the separation of glycans prior to MS analysis is necessary due to the wide dynamic range and variable ionization efficiencies that can cause analyte suppression. Thus, numerous chromatographic techniques have been adapted for glycan separation including hydrophilic interaction (liquid) chromatography (HILIC), graphitized carbon chromatography, and less frequently RP chromatography.⁴ Chromatography is effective in increasing the peak capacity of the measurement and facilitating the detection of lower abundant glycans.⁷

HILIC has been used for the separation of both native⁸⁻¹² and derivatized¹³⁻¹⁷ glycans in online LC-MS experiments⁴ and is often used for glycomic analyses due to the strong retention of polar compounds in comparison to RP stationary phases. However, there are several disadvantages that have been reported in comparison to RP chromatography including peak fronting and tailing, column bleed, irreversible sorption, and slow equilibration times.¹⁸ These problems can be overcome by switching the HILIC stationary phase¹⁸ or increasing the buffer concentration.¹⁹ Additionally, HILIC separation efficiency is generally accepted to be inferior to RP chromatography,²⁰⁻²¹ and though this depends on the analyte and the type and amount of buffer reagent, peak widths tend to be broader in HILIC than RP chromatography. This decreases the peak capacity of the separation and increases the possibility for competition of analytes in the electrospray droplet, which can significantly hinder glycan analysis. Our group has generated data previously that show a range of separation efficiencies depending on the *N*-linked glycan composition in HILIC with the peak widths (FWHM) ranging from just under 1 minute to several minutes.^{13, 22} This is in contrast to the same chromatography instrument platform using RP C₁₈ stationary phase for peptide analysis, which consistently produces peak widths ~30 seconds.²³ This disparity leads one to the hypothesis that it is possible to significantly enhance the analysis of *N*-linked glycans by separating glycans with RP chromatography rather than HILIC.

RP chromatography is not compatible with native glycan analysis due to the lack of interaction of the glycans with the non-polar stationary phase. However, glycans that are derivatized either at the reducing terminus^{6, 24-37} or permethylated^{4, 38-39} are able to be retained on a C₁₈ column. This is due to the increase in hydrophobicity of the derivatized glycan molecule so that the derivatized glycans will interact (either by partitioning or adsorption⁴⁰) more effectively with the stationary phase and be retained on a RP column.^{4, 7} However, there have been minimal studies demonstrating the inherent advantages of using each separation technique for glycan analysis.

Glycan derivatization at the reducing terminus is common in *N*-linked glycan analysis due to the enzymatic cleavage of *N*-linked glycans from proteins using PNGase F.⁴¹ Upon cleavage, *N*-linked glycans are left intact with an accessible reducing terminus at the core HexNAc residue. Reductive amination is a prominent derivatization strategy at the reducing terminus that has been used for the incorporation of hydrophobic tags,^{32, 42-44} UV and fluorescent tags,^{27, 29, 37, 45} as well as the incorporation of stable isotope labeled tags for relative quantification.¹⁶⁻¹⁷ Depending on the derivatization reagent, studies have demonstrated that reductively aminated glycans are capable of being retained on a reverse phase column.^{32, 37} Recent reports have shown the separation of reductively aminated glycans by both HILIC^{16, 43} and RP^{32, 37} chromatography, and the development of commercialized kits (e.g. GlycoProfile for 2-AB labeling) for glycan derivatization have made glycan analysis using this derivatization strategy popular and versatile.

Pyrazolone derivatization of carbohydrates has also been used to facilitate MS and UV detection.⁶ The tag 1-phenyl-3-methyl-5-pyrazolone (PMP) is a common pyrazolone reagent that has been used frequently to derivatize glycans, and several reports have described separation of PMP-derivatized glycans using RP chromatography.^{6, 26, 30, 46-47} Perreault and coworkers²⁶ compared separation and detection efficiencies of both RP chromatography and HILIC (NH₂ column) of PMP-glycans cleaved from ovalbumin and found that HILIC provided better glycan chromatographic resolution, but RP chromatography provided more sensitive ESI. Additionally, the glycan profiles were found to be different depending on the separation method used. PMP-derivatization of glycans was also used in the profiling of urinary oligosaccharides in mucopolysaccharidosis (MPS) type II (Hunter syndrome).³⁰ The authors were able to separate the derivatized glycans and use the glycan profiles to distinguish between unaffected control samples and MPS II patients, leading to possible diagnosis strategies for Hunter syndrome.

Hydrazone formation is a similar derivatization strategy to reductive amination and pyrazolone derivatization in which hydrazine or hydrazide reagents can react with the reducing terminus of oligosaccharides. Dansylhydrazine was the first reagent used in the hydrazone derivatization of glycans,⁴⁸ and since, numerous reagents have been employed.^{6, 13-15, 24, 49-53} Hydrazone chemistry is often chosen for glycan derivatization due to the relatively simple and stable reaction that does not require a clean-up step after derivatization.^{14, 24, 49, 54} Numerous reaction conditions are presented in the literature,^{31, 51-52, 55-56} and recent studies from this group have shown consistent reaction efficiencies of hydrazide reagents with all classes of *N*-linked glycans to be 95%.¹³⁻¹⁴ Mopper and coworkers have demonstrated the RP separation of dansylhydrazine derivatized glycans,³¹ and Perreault and coworkers have demonstrated the RP separation of glycans derivatized with the phenylhydrazine reagent.^{24-25, 54} The latter authors were able to separate *N*-linked glycan standards and *N*-linked glycans cleaved from ovalbumin, both derivatized with phenylhydrazine, on C₈ and C₁₈ columns.

Permethylation is an alternative derivatization strategy in which all hydroxyl, amino, and carboxylic acid groups are converted to their respective methyl-ether functions. This modification substantially increases the hydrophobicity of the glycans and allows for retention on a RP (C₁₈) column. This was first demonstrated by Vouros and coworkers, where a standard maltooligosaccharide ladder and standard branched complex *N*-linked glycans were permethylated and separated by RP chromatography.³⁸ A more recent study has demonstrated a chip-based RP separation of permethylated glycans.³⁹ The authors profiled the *N*-linked glycans in blood serum in search of diagnostic markers to distinguish breast cancer patients from controls. Permethylation is an advantageous technique also because permethylated glycans can yield more informative MS/MS spectra, and often, branching patterns can be determined.⁵⁷⁻⁵⁹

The derivatization steps necessary for glycan separation by RP chromatography often require a significant amount of additional sample preparation time, sample clean up procedures, and an increase in the analytical variability of the sample preparation strategy. However, often the benefits of glycan derivatization can outweigh these downsides by providing more glycan structural information,⁵⁷⁻⁵⁹ more glycome coverage,¹³ the ability to relatively quantify glycans,^{16-17, 22, 60-64} and facilitated detection either by increased ion abundance in MS^{13, 15, 43-44, 49} or by adding a fluorescent or UV tag.^{27-28, 45, 48, 65-66}

Herein, we demonstrate that the separation of hydrazone derivatized *N*-linked glycans by RP chromatography has several significant advantages in comparison to HILIC including an increased number of detectable glycans, increased peak capacity due to better chromatographic resolution, reduced complexity of the spectra due to the lack of ammonium

adduction, reduced equilibration times, an increase in laboratory efficiency, and increased repeatability across laboratories currently equipped with instrumentation for proteomic analyses. Each of these advantages will be discussed with specific examples and data, which were collected on the same LC platform for both chromatographic techniques (the platforms only differed in the packing material of the trap and analytical chromatography columns). RP and HILIC separation will be discussed with respect to two samples: 1) maltodextrin oligosaccharide ladder and 2) *N*-linked glycans cleaved from pooled human plasma. In both samples, the number of glycans detected was greater when separated by RP chromatography rather than HILIC. Thus, hydrophobic derivatization of *N*-linked glycans and separation by RP chromatography is a significant advantage over separation by HILIC and a significant advancement in the development of a high-throughput strategy for the analysis of *N*-linked glycans.

Experimental

Materials

The hydrazide reagent, 4-phenethylbenzohydrazide, used for *N*-linked glycan derivatization was synthesized previously²² in the Department of Chemistry at North Carolina State University. Peptide: *N*-glycosidase F (PNGase F) was purchased from New England BioLabs (Ipswich, MA). The maltodextrin oligosaccharide ladder was purchased from V-Labs, Inc. (Covington, LA). TSK-Gel Amide-80 and Magic C₁₈AQ stationary phases were purchased from TOSOH Bioscience (San Jose, CA) and Michrom BioResources (Auburn, CA), respectively. Pooled human plasma, acetic acid, ammonium acetate, ammonium bicarbonate, dithiothreitol, and formic acid were all purchased from Sigma Aldrich (St. Louis, MO). HPLC grade acetonitrile, water, and methanol were purchased from Burdick & Jackson (Muskegon, MI).

N-linked Glycan Derivatization Procedure

N-linked glycans were derivatized with 4-phenethylbenzohydrazide (P2PGN) via hydrazone formation, as described previously.¹³ Briefly, immediately prior to reaction, a 1 mg/mL solution of P2GPN reagent was made in 75:25 (v/v) MeOH:acetic acid solution. One hundred microliters of the reagent solution were then added to the lyophilized glycan sample, and the sample was vortexed, centrifuged, and allowed to incubate at 56° C for 3 hr. The samples were then immediately lyophilized to dryness and stored at -20° C.

Maltodextrin Sample Preparation

Aliquots were made such that upon lyophilization, each contained 50 µg of maltodextrin ladder. No further sample preparation was performed until derivatization with either the light or heavy P2GPN reagent. After derivatization, the glycans were reconstituted in 200 µL of the LC initial conditions.

N-linked Glycan Extraction from Pooled Plasma

Pooled plasma aliquots were prepared by pipetting 50 µL into individual vials, and 1 µg of internal standard (maltoheptaose) was added to each sample. The samples were then lyophilized to dryness. The glycan cleavage and extraction methodology has been developed and described in detail previously,^{10, 67} and the following briefly outlines the procedure used in this experiment with the deviations elaborated on from previous publications. The lyophilized pooled human plasma and internal standard were reconstituted in 191 µL of 100 mM ammonium bicarbonate buffer, vortexed, and centrifuged. In order to denature the proteins, 2 µL of 1 M dithiothreitol (DTT) were added to make a final concentration of 10 mM DTT. The plasma sample was then incubated in 95° C for 15 s followed by incubation at 25° C for 15 s. The denaturation heat cycle was repeated 3 additional times. Following

denaturation, 2 μ L (1000 units) of PNGase F (glycerol free) were added to the sample for *N*-linked glycan cleavage. The mixture was vortexed, centrifuged, and allowed to incubate at 37° C for 18 hr. Following incubation, ethanol precipitation was performed to crudely separate the glycans from the remaining proteins and plasma matrix.⁶⁷ This was accomplished by adding 800 μ L of chilled ethanol to the sample and letting it incubate for 1 hr at –80° C. The sample was then centrifuged at 13,200 rpm, and the supernatant was extracted and lyophilized. Solid phase extraction (SPE) was then performed on the sample to remove any other contamination, remaining plasma matrix, or protein and has been described previously.¹⁰ The SPE fractions were then combined, lyophilized, and stored at –20° C. After derivatization, the glycans were reconstituted in 200 μ L of the LC initial conditions.

nano-Flow Hydrophilic Interaction Chromatography

Both derivatized and native *N*-linked glycans were separated by online HILIC prior to mass analysis, as described previously.^{9-10, 13} An Eksigent nanoLC-1D PLUS system (Eksigent, Dublin, CA) equipped with an HTS PAL autosampler (LEAP Technologies, Carrboro, NC) was set up to run in the vented column configuration.²³ The trap and analytical columns were packed in-house with TSK-Gel Amide-80 stationary phase (TOSOH Bioscience, San Jose, CA). A 100 μ m inner diameter IntegraFrit capillary (New Objective, Woburn, MA) was packed to 5 cm and used as the trap column, and a 75 μ m inner diameter PicoFrit (New Objective, Woburn, MA) was packed to 15 cm and used as the analytical column. Mobile phase A consisted of 50 mM ammonium acetate in water (pH 4.5), and mobile phase B was 100% acetonitrile. *N*-linked glycan samples were reconstituted in a 20/80% (v/v) ratio of mobile phase A/B and loaded onto the trap column at 2 μ L/min in the same solvent conditions. The sample was washed on the trap column for approximately 10 column washes, and then, a gradient elution was performed where mobile phase B was varied from 80% to 45% over 35 min at a flow rate of 500 nL/min. Column equilibration in 20% mobile phase A and column washing in 15% mobile phase B were performed at the beginning of the gradient, and a final column re-equilibration was performed in the final 10 min of the run in 20% mobile phase A for a total run time of 70 min.

nano-Flow Reverse Phase Chromatography

The same equipment and vented-column setup was used for reverse phase chromatography as stated above for HILIC. The columns were the same dimensions but were packed with Magic C₁₈AQ stationary phase (Michrom Bioresources, Auburn, CA). The stationary phase consisted of 5 μ m particles with 200 Å pore size. Mobile phase A consisted of 98/2/0.2% water/acetonitrile/formic acid, and mobile phase B was 2/98/0.2% water/acetonitrile/formic acid. Sample was reconstituted in a solution of 95/5/0.2% water/acetonitrile/formic acid solution and loaded onto the trap column at 2 μ L/min in mobile phase A. Approximately 10 column washes were performed with mobile phase A, and then the sample was eluted from the trap column at 500 nL/min using a gradient elution. The column was allowed to equilibrate for 5 min at 98% mobile phase A, and the gradient was ramped from 20% to 35% B over 33 min. The column was washed in 98% B for 5 min and allowed to re-equilibrate at the initial gradient conditions for 10 min for a total run time of 55 min.

LTQ-FT-ICR Mass Spectrometry

Mass analysis was performed on a hybrid linear ion trap Fourier transform ion cyclotron resonance (LTQ-FT-ICR) mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a 7 Tesla superconducting magnet. The instrument was calibrated per the manufacturer specifications. ESI was achieved by attaching 2.25 kV to the trap column union. The capillary was heated to 225° C with the capillary and tube lens voltages set at 42 and 120 V, respectively. Data dependant acquisition was performed such that for every

precursor scan in the ICR cell, a maximum of 5 ms/ms fragmentation scans were collected in the ion trap determined by the most intense precursor ions. However, the second time a specific m/z was chosen for ms/ms, it was placed on an exclude list for 3 min, allowing for lower abundant analytes to be chosen for ms/ms as well. The ions were dissociated in the ion trap using collision induced dissociation with a normalized collision energy (NCE) of 28. An AGC of 8×10^3 with a maximum injection time of 100 ms was used in the ion trap scans, and an AGC of 1×10^6 with a maximum injection time of 500 ms was used in the FT scans. The glycans were identified manually by accurate mass (composition identification) and analyzed using Xcalibur software (v.2.0.7).

Results and Discussion

The separation of *N*-linked glycans by RP chromatography affords several advantages including an increase in separation efficiency, an increase in the number of glycans detected, and practical advantages such as the ability to use a more widely employed LC-MS technique (e.g. proteomic analyses). In order to demonstrate these advantages, two samples, maltodextrin and *N*-linked glycans cleaved from pooled human plasma, were used to compare the effectiveness of HILIC and RP chromatography on the separation of both native and hydrazide derivatized glycans. As HILIC has been shown to effectively retain and separate native and derivatized glycans,^{10, 13} this was used as the control separation method. The *N*-linked glycans cleaved from plasma and the maltodextrin samples were first analyzed by HILIC coupled online with LTQ FT-ICR MS in order to insure sample integrity. Aliquots of the same samples (stored frozen at -20°C) were then analyzed by online RP chromatography coupled to FT-ICR MS using the same LC instrument platform.

Figure 1 contains the base peak chromatograms for both native and derivatized maltodextrin samples on HILIC (**1a** and **1b**, respectively) and RP chromatography (**1c** and **1d**, respectively). As shown previously, both native and derivatized glycans are retained and separated by HILIC.^{10, 13-15, 22} In contrast, Figure 1c shows that there is no retention of native maltodextrin on the RP column, as is expected due to the polar nature of the glycans and the non-polar nature of the C_{18} stationary phase. Upon derivatization of maltodextrin, Figure 1d shows that maltodextrin is capable of being separated and retained on a RP C_{18} column. It must be mentioned that these are the optimized HILIC and RP gradients for derivatized glycans. Though the gradient for RP analysis of native glycans begins at $>20\%$ B, studies were also performed in RP starting at 2% B in order to optimize the native glycan retention. However, there were no conditions in which native glycans were retained on RP chromatography.

Several additional important observations are made from the base peak chromatograms in Figure 1. The HILIC base peak chromatograms show that the separation efficiency for both native and derivatized glycans varies as a function of the glycan retention time. This is in contrast to the separation by RP chromatography, where the peak widths are uniform throughout the chromatogram. It is important in the analysis of *N*-linked glycans to have as narrow peak widths as possible in order to reduce the amount of possible co-elution and peak suppression due to the more easily ionized or more abundant glycans. In the HILIC chromatograms, the glycans that elute over a large time window are the more hydrophobic and more abundant glycans, meaning that these glycans will likely elute at the same time as, and out-compete, the more hydrophilic, less abundant glycans. Figure S1 in the supplemental material contains example extracted ion chromatograms (EIC) for both HILIC and RP chromatography to further demonstrate the difference in chromatographic efficiency between the two approaches.

Though the fact that HILIC is capable of retaining both native and derivatized *N*-linked glycans is often viewed as an advantage, Figure 1b shows that these two species often have overlapping analytes, and this is a disadvantage when one is only interested in analyzing the derivatized *N*-linked glycans. During derivatization, 5% of each glycan remains untagged. Though this seems like a small number, when the dynamic range of the sample comprises orders of magnitude, 5% of an abundant analyte is capable of out-competing lower abundant analytes in the electrospray droplet (e.g. Hex₇ and *Hex₁₄ in Figure 1b). In contrast, the RP chromatography of derivatized glycans has no contamination by the un-reacted glycan. This is a significant advantage in comparison to HILIC and decreases the competition in the electrospray droplet and AGC ion trap capacity for the low abundant glycans.

The final important piece of data from Figure 1 is the total number of unique glycan compositions detected in each chromatographic system. There are 12 glycan compositions detected in Figure 1a, 16 in Figure 1b, 0 in Figure 1c, and 23 in Figure 1d. This shows that independent of the chromatographic technique, it is always better to derivatize the *N*-linked glycans with hydrophobic hydrazide reagents than to analyze their native counterparts. However, Figure 1 also shows that it is significantly more productive (~50% more compositions detected) to separate derivatized glycans by RP chromatography than HILIC. There are several differences in the two chromatography techniques that are possible reasons for this outcome. First, as mentioned above (Figure S1), the efficiency of RP chromatography is more uniform throughout the glycan retention window, and in general, the peak widths are narrower. The oligosaccharide peak widths are compared in the supplemental material for the same 15 glycans (only oligosaccharides that were detected in both HILIC and RP techniques). The HILIC peak widths have a range of 0.34-10.37 min with a mean of 2.23 min and standard deviation of 2.87 (Table S1). In contrast, the RP peak widths have a range of 0.34-1.1 min with a mean of 0.49 min and a standard deviation of 0.18 (Table S1). Because peak broadening spreads the analytes out over a longer retention time, the detection limit will be lower for chromatographic techniques with narrower peak widths (*i.e.*, higher concentration). Additionally, the elution overlap of un-reacted glycans with lower abundant, derivatized glycans in HILIC causes competition in the electrospray droplet. This hinders the ionization of the lower abundant glycans, causing fewer glycans to be detected in HILIC.

Furthermore, the efficiency of HILIC is often increased and the peak tailing minimized by addition of an acidic buffer such as ammonium acetate or ammonium formate.^{19, 68} However, the addition of ammonium salts into the buffer often has an adverse affect on detection due to the ammonium adduction in ESI. Figure 2 shows the difference in mass spectra between HILIC (top/blue) and RP chromatography (bottom/red) for three different glycan compositions (Figure 2a from maltodextrin and Figure 2b and Figure 2c cleaved from human plasma – *vide infra*). It must be noted that all the glycans are detected in light and heavy pairs due to the derivatization with both the native and stable-isotope labeled glycan reagents (as published previously for the relative quantification of *N*-linked glycans²²). This was necessary to confirm that the light and heavy tagged glycans are affected identically in RP chromatography with no chromatographic shift due to the incorporation of ¹³C₆.

The absence of ammonium acetate in the RP solvent system allows for only the detection of the [M+nH]⁺ peak, whereas ammonium adduction is detected in the HILIC spectra due to the buffer system containing ammonium acetate. This negatively impacts the profiling of *N*-linked glycans because the ammonium adduction spreads the signal of one glycan composition into multiple *m/z* channels. Additionally, Figure 2a and Figure 2c show that for certain types of glycans in HILIC, the [M+nH]⁺ peak is not detected in the mass spectrum, making detection and identification more difficult.

The observations discussed above have led to several conclusions on the mechanism of *N*-linked glycan separation by HILIC and RP chromatography. Figure 3 summarizes the observations made in the separation of *N*-linked glycans, both native and derivatized, in HILIC (Figures 3a and 3b) and RP chromatography (Figures 3c and 3d). The mechanisms of both HILIC^{4, 21, 69-72} and RP chromatography^{40, 73} are frequently debated, and often, the compromise is that the separation techniques both involve a combination of partitioning and adsorption depending on the molecular properties of the analytes. These properties permit the native glycans to interact significantly with the HILIC stationary phase and allow them to be retained on the HILIC column (Figure 3a). However when derivatized, the glycan molecules become more hydrophobic, and the hydrophobic tag causes the glycan to partition more into the organic mobile phase, which decreases the retention time (as seen in Figure 1b). In the case of RP chromatography, the native glycan interacts very little with the hydrophobic C₁₈ stationary phase (Figure 3c), but when derivatized (Figure 3d), the glycan molecule becomes hydrophobic enough to interact with the C₁₈ stationary phase, and the derivatized glycans are retained and separated (as seen in Figure 1d).

The magnitude of the arrows in Figure 3 are used to portray the distribution coefficient (C_S/C_M) of the analyte and its dependence on the physical properties of the molecule. Molecules that are hydrophobic will tend to interact more with the C₁₈ stationary phase, whereas hydrophilic molecules will interact more with the mobile phase. A molecule that has both hydrophobic and hydrophilic character (such as the derivatized glycans) will have a competing interaction between the mobile phase and stationary phase. Figure 3c implies that there is little to no interaction between the native glycans and the RP stationary phase because no retention is observed. However, because separation of derivatized *N*-linked glycans is observed on a RP column, the glycans must have some interaction with the stationary phase in order to have different retention times. If the glycan molecules are assumed to have no interaction with the stationary phase, then all possible retention would be due to the hydrophobic tag interaction, and since all the glycans are derivatized with the identical tag, one would assume that there would be no separation of the glycans. Since this was not observed, the partitioning of derivatized glycans was estimated by calculating the ratio of the hydrophobic surface area (NPSA) and the hydrophilic surface area (MW) (equation development in supplemental Equation S1). A plot of both retention time and $\frac{NPSA}{MW}$

vs. the number of hexose monosaccharide units is shown in Figure 4. It is seen that the estimated hydrophobicity:hydrophilicity ratio predicts a similar trend as experimentally observed for the retention of derivatized glycans. This supports a partitioning mechanism for derivatized glycans based on both the hydrophobic and hydrophilic character of the molecule. However, eventually (based on the extrapolation of Figure 4), the glycans will be so large, adding an additional hexose monosaccharide (Hex_{n+1}) will not afford any chromatographic separation from the previous oligosaccharide (Hex_n). Thus, a limitation of the *N*-linked glycan RP separation will be at the most hydrophilic (largest) glycan molecules, where separation of the analytes will be limited, and competition in the electrospray droplet could occur. HILIC could be a possible alternative to RP for these large glycans; however, due to the competition in the ESI droplet with underivatized glycans (*vide supra*), HILIC does not perform better than RP for glycans derivatized by hydrazone formation.

In order to apply and study the advantages of RP separation over HILIC to complex mixtures, *N*-linked glycans cleaved from pooled human plasma were used to further compare the two chromatographic techniques. The maltodextrin oligosaccharide ladder is composed only of hexose monosaccharide residues; thus, it was necessary to test and compare more complex glycans (containing fucose and sialic acid residues) and glycan mixtures on the RP system. The base peak HILIC and RP chromatograms for derivatized *N*-

linked glycans are shown in Figure 5a and Figure 5b, respectively (the native glycan chromatograms were not shown due to the results presented above – *vide supra*). These chromatograms are a complex mixture of glycans. However, Figure 5a shows that several glycans in HILIC are dominating the glycan retention window with large abundances and retention times on the order of minutes. This is not seen in the RP chromatogram, implying less spectral competition due to the most abundant analytes. In Figure 5c, it is seen that all glycans that were detected in HILIC (42 unique compositions) were also detected in RP. Moreover, 18 additional *N*-linked glycans were detected using RP separation rather than HILIC. The majority of these glycans were of the large, highly sialylated, complex glycan type that are typically low abundant and hydrophilic, two properties that make detection by ESI-MS difficult. By increasing the chromatographic resolution, reducing ammonium adduction, and reducing the competition in the ESI droplet, the effective detection limit of the instrument was decreased so that one is able to delve deeper into the *N*-glycome of human plasma. In a few cases (mostly sialylated glycans), it was observed that the glycan compositional mass was detected at two distinct retention times. This could possibly be due to isomeric separation, which would allow for quantification of glycan isomers. However, this needs to be investigated more thoroughly in order to determine the cause and utility of this peak splitting.

Table S2 in the supplemental material details the glycan compositions that were detected and their respective molecular weights that were detected using both HILIC and RP chromatographic techniques. It is seen that numerous large complex glycans are only detected with RP separation. The charge state has also been listed in Table S2 for each glycan, and it was observed that glycans separated and analyzed in the RP solvent system are detected in higher charge states. This could be due to the elution of the glycans in different organic:aqueous solvent ratios and the difference in acidic buffer (formic acid or ammonium acetate). In comparison to other studies, the 60 unique *N*-linked glycan compositions detected using this method is on par with current literature. Reinhold and coworkers were able to detect 53 unique compositions,⁷⁴ and this was increased to 106 by using plasma depletion methods. Lebrilla and coworkers report up to 64 unique compositions,⁶⁷ and Ruhaak and coworkers report 47 unique compositions.⁷⁵ However, utilizing the hydrazide quantification strategy presented previously,²² each sample consisted of a 1:1 combination of a light and heavy derivatized sample, and only the glycans that were detected with a light and heavy pair in the spectra were determined to be identified. Thus, each of the glycans that was detected is also capable of being relatively quantified using stable-isotope labeled derivatization.

Conclusion

RP separation of *N*-linked glycans, both simple and cleaved from pooled human plasma, was more efficient than HILIC with respect to both separation and detection by MS. The discussed benefits of separating *N*-linked glycans by RP chromatography are in addition to the practical advantages that will increase the efficiency of ‘multi-omics’ laboratories. The opportunity to use the same chromatographic instrumentation, solvents, columns, and set-up as are used with proteomic analyses is a practical advantage that cannot be understated. Since many mass spectrometry groups who study glycosylation often study proteomics as well, the use of RP (C₁₈) chromatography for both analyses nearly eliminates the time it takes to switch between techniques (which takes at least one day to fully switch out solvents and equilibrate the columns when transitioning to HILIC from RP chromatography and vice versa), greatly increasing the efficiency of instrument time.

Furthermore, the ability to use a derivatization strategy that adds only 4 hr to the glycan sample preparation time while being able to analyze the samples on a RP-MS platform

allows researchers to apply this derivatization strategy to glycans from any protein sample. This compatibility with numerous research labs combined with the previously demonstrated advantages including relative glycan quantification²² and increased ESI efficiency¹³ make this a versatile, high-throughput strategy for the enhanced profiling and quantification of *N*-linked glycans cleaved from proteins. Additionally, the ability to acquire data in nearly any laboratory studying proteomics or glycomics using the exact same LC-MS strategy will allow for more accurate comparisons across laboratories, reproducibility studies, and ultimately allow the field of glycomics to stride toward a more in-depth biological understanding of glycosylation including aberrant glycosylation in disease, cellular interactions, and biomarker discovery efforts.

Supplementary Material

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Figure 1.

The base peak chromatograms of both native and derivatized (**bold with ***) maltodextrin (Hex_n) oligosaccharides analyzed by HILIC (**a** and **b**, respectively) and RP chromatography (**c** and **d**, respectively).

Figure 2.

Example mass spectra for (a) a hexose oligosaccharide in the maltodextrin sample and (b) and (c) two glycans cleaved from pooled human plasma. The top spectra (blue) were analyzed using HILIC-MS and the bottom spectra (red) were analyzed with RP-MS. The difference in ammonium adduction is observed for several different types of glycans.

Figure 3.

The observed retention behavior of native and derivatized *N*-linked glycans in HILIC and RP chromatography. The magnitude of the arrows indicates the observed relative amount of interaction of the glycan with the stationary phase. The *N*-linked glycan shown is a general hexose structure to demonstrate the collective behavior of the maltodextrin glycans.

Figure 4.

The retention times (left y-axis) of the maltodextrin ladder with respect to the size of the maltooligosaccharide. The calculated 'interaction' of derivatized glycans with the stationary phase is modeled by the ratio of the NPSA/MW and is overlaid (right y-axis).

Figure 5.

The (a) HILIC and (b) RP chromatography base peak chromatograms for derivatized *N*-linked glycans. (c) Venn diagram of the total number of unique *N*-linked glycan compositions cleaved from pooled human plasma and analysed by online HILIC- and RP-MS.