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Enhanced Protocol for Quantitative *N*-linked Glycomics Analysis Using Individuality Normalization when Labeling with Isotopic Glycan Hydrazide Tags (INLIGHT)TM

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Abstract

The analysis of *N*-linked glycans using liquid chromatography and mass spectrometry (LC-MS) presents significant challenges, particularly owing to their hydrophilic nature. To address these difficulties, a variety of derivatization methods has been developed to facilitate improved ionization and detection sensitivity. One such method, the Individuality Normalization when Labeling with Isotopic Glycan Hydrazide Tags (INLIGHT)TM strategy for labeling glycans, has previously been utilized in the analysis of *N*- and *O*-linked glycans in biological samples. To assess the maximum sensitivity and separability of the INLIGHTTM preparation and analysis pipeline, several critical steps were investigated. First, recombinant and nonrecombinant sources of PNGase F were compared to assess variations in the released glycans. Second, modifications in the INLIGHTTM derivatization step were evaluated including temperature optimization, solvent composition changes, and reaction condition length and tag concentration. Optimization of the modified method resulted in 20–100 times greater peak areas for the detected *N*-linked glycans in fetuin and horseradish peroxidase compared to the standard method. Furthermore, the identification of low abundance glycans, such as (Fuc)₁(Gal)₂(GlcNAc)₄(Man)₃(NeuAc)₁ and (Gal)₃(GlcNAc)₅(Man)₃(NeuAc)₃, was possible. Finally, the optimal LC setup for the INLIGHTTM

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COMPLIANCE WITH ETHICAL STANDARDS

The pooled male and female human plasma samples used in these studies were sourced from a licensed entity, Golden West Biologicals (Temecula, CA). The samples were de-identified by Golden West Biologicals as to which participants were used to make up the male and the female pool. Upon arrival and for this study, we further pooled the male and female plasma 1:1 (v/v) prior to analysis. No data in our study can be linked to any human subject.

CONFLICTS OF INTEREST

None of the authors report any conflicts of interest.

derivatized *N*-linked glycan analyses was found to be a C18 reverse-phase (RP) column with mobile phases typical of RPLC.

Keywords

Glycomics; Orbitrap; Sample preparation; INLIGHT™; *N*-linked glycans; HILIC; PGC; Reversed-phase; Chromatography

INTRODUCTION

Glycosylation of proteins is a crucial post-translational modification associated with intra- and extracellular communication [1], protein folding and stability [2], and protein trafficking [3]. Since glycan properties, including protein binding locations, monomer linkages, and synthesis site and time, greatly dictate their functional roles further investigations are greatly needed. *N*-linked glycan studies are of great interest for assessment of disease onset and progression as their disruption have been associated with neurodegenerative disorders [4, 5], inflammation-based diseases [6], and numerous types of cancer [3, 7], among many others. In proteins containing the motif asparagine-X-serine/threonine, *N*-linked glycans are covalently bound to the asparagine (Asn) residue through a glycosidic bond, where X is any amino acid except proline [8]. More than 70% of proteins found in eukaryotic systems have the sequon, asparagine-X-serine/threonine, necessary for *N*-glycosylation and as a consequence [9], more than half of proteins are decorated with *N*-linked glycans[10]. While many methods exist for the removal and purification of *N*-linked glycans from glycoproteins, one of the most common methods utilizes the enzyme peptide-*N*-glycosidase F (PNGase F) for glycan release [11]. PNGase F cleaves *N*-linked glycans from glycoproteins at the nitrogen located in Asn, converting Asn to aspartic acid (Asp) and leaving the glycan structure intact [8]. This cleavage and conversion results in a 0.084 Da mass shift, allowing the assessment of potential glycosylation sites on the peptide and complete characterization of the released glycan. The released glycans can then be analyzed either directly or following chemical derivatization with chromatography with either optical detectors or mass spectrometry (MS).

The use of MS-based approaches for the analysis of glycans has become highly preferable as it allows for MS and MS/MS structural elucidation [12, 13]. To aid in their evaluations, the MS analysis is commonly preceded by liquid chromatography (LC) separation techniques such as hydrophilic interaction liquid chromatography (HILIC) [14] or porous graphitic carbon (PGC) chromatography[15], followed by electrospray ionization (ESI). Glycans are difficult to ionize due to their innate hydrophilicity and hydrophobic bias of the electrospray droplets [16]. However, a variety of derivatization methods have been employed to facilitate glycan analysis including protection of the alcohol functional groups, as seen with permethylation [17], or adding chemical labels to the reducing end of the sugar after cleavage from the protein. Permethylation is commonly used to derivatize *O*-linked glycans, as they do not contain a reducing end after cleavage without further chemical modification. The most popular reagents used for the derivatization of *N*-linked glycans are 2-AA and 2-AB [18] which rely on reductive amination and include the ability to fluoresce. Other

popular reducing end tags rely on other chemical techniques such as a Michael addition with 1-phenyl-3-methyl-5-pyrazolone [19] or oxime chemistry using aminooxy Tandem Mass Tags [20]. The addition of derivatization reagents can increase the hydrophobicity of glycans by expanding their non-polar surface area (NPSA), significantly enhancing their surface activity for better separations and increasing their likelihood of ionization while also providing diagnostic peaks in the mass spectrum for deep structural elucidation. Additionally, the increased hydrophobicity allows for the use of reversed-phase liquid chromatography (RPLC). Though effective, these derivatization methods have challenges including variable reaction efficiencies and additional cleanup steps that can result in long sample preparation times and increased analytical irregularities [21].

The Individuality Normalization when Labeling with Isotopic Glycan Hydrazide Tags (INLIGHT)TM strategy has previously been utilized to increase the hydrophobicity of glycans by adding 4-phenethyl-benzohydrazide (P2PGN) tags with large NPSA for improved ionization efficiency and analysis with RP-LC-MS [16, 21–23]. Furthermore, the reaction efficiency is >95%, leading to almost complete labeling of glycans present in a sample [21]. The INLIGHTTM strategy also facilitates relative quantitative analysis of glycans in biological samples as it employs the use of both a natural (NAT – ¹²C₆) or stable-isotope label (SIL – ¹³C₆) phenyl ring [23]. The co-elution of the NAT and SIL derivatized glycans is crucial for their identification and relative quantification. As the labeled reagents are added in an equal abundance and have the same reaction efficiency, relative quantification can be readily performed, even in complex matrices. This method has been used in conjunction with filter aided *N*-glycan separation (FANGS) [24, 25] and applied to the analysis of *N*-linked glycans in many types of complex biological samples, ranging from fetuin to a large-scale ovarian cancer study in human plasma [23, 26]. The INLIGHTTM strategy has also been applied to the analysis of *O*-linked glycans [27]. Evaluating *O*-linked glycans is very challenging since only one enzyme is known for the specific cleavage of mucin-type core 1 glycans [28]. Thus, their sample preparation is quite different to that used for *N*-linked glycans and chemical cleavage from the protein occurs via hydrazinolysis [29] or β -elimination [30]. Subsequent chemical derivatization steps are then needed to form the aldehyde required for labelling with the P2PGN reagent [31]. The *O*-linked derivatization method employs more acidic reaction conditions, requires a lower concentration of the P2PGN reagent (0.10 μ g/mL reagent as compared to 200 μ g/mL in previous procedures), and utilizes a shorter reaction time and lower reaction temperature which helps prevent the loss of sialic acid or fucose compared to the current *N*-linked derivatization method [27]. Herein, we report on improvements to the sample preparation and detection of *N*-linked glycans using the INLIGHTTM strategy. We also provide mechanistic insights as to why this approach out-performs previous work.

EXPERIMENTAL

Materials

Peptide N-glycosidase F (PNGase F) were purchased from New England Biolabs (Ipswich, MA) and Bulldog Bio (Portsmouth, NH). The natural (NAT) and stable-isotope labelled (SIL) P2PGN hydrazide reagents were purchased from Cambridge Isotope Labs (Andover, MA).

LC-MS grade solvents (water, acetonitrile, and methanol), ammonium bicarbonate, ammonium acetate, and formic acid were purchased from Fisher Scientific (Hampton, NH). Human plasma (K2 EDTA, male and female) were purchased from Golden West Biologicals (Temecula, CA). All other reagents, including the biological glycoprotein standards, fetuin, and horseradish peroxidase, were purchased from Sigma Aldrich (St. Louis, MO).

Modified N-linked Glycan Preparation Protocol

A detailed, step-by-step protocol covering the cleavage and derivatization of *N*-linked glycans adapted from Hecht et al. [32] can be found in the Electronic Supplementary Material (ESM). Biological glycoprotein samples were prepared using a modified filter aided *N*-glycan separation procedure outlined previously [25, 32]. Briefly, 250 µg of protein were loaded onto the 10 kDa molecular weight cutoff filters. Proteins were denatured by adding 2 µL of dithiothreitol (DTT) to the filter then diluted in 200 µL of digest buffer (100 mM ammonium bicarbonate, pH 7.0). The centrifuge tubes were capped, lightly vortexed, and incubated at 56 °C for 30 minutes. Following denaturation, the samples were alkylated by adding 50 µL of 1 M iodoacetamide and incubated at 37 °C for 60 minutes. The alkylated, denatured proteins were then concentrated onto the filters via centrifugation at $14,000 \times g$ for 40 minutes. Next, the samples were washed with 100 µL digestion buffer and centrifuged at $14,000 \times g$ for 20 minutes. This step was repeated twice more for a total of three washes. The filters were transferred to a new, clean collection centrifuge tube prior to enzymatic removal of the glycans. This was accomplished by addition of 2 µL of PNGase F (1,000 units), from either New England Biolabs (NEB) or Bulldog Bio (BB), to the filter. An additional 98 µL of digestion buffer was added to the filters, bringing to total solution volume to 100 µL. The samples were mixed by pipetting up and down on the filter and then allowed to incubate at 37 °C for 18 hours. The glycans were eluted from the filters by centrifugation at $14,000 \times g$ for 20 minutes. The filter was then washed with 100 µL of 100 mM ammonium bicarbonate buffer and centrifuged again at $14,000 \times g$ for 20 minutes. This washing step was performed twice more for a total of three washes. The samples were then stored at -80 °C until fully frozen (~30 min), then dried to completion in a vacuum concentrator.

INLIGHT™ Derivatization of N-Linked Glycans

A standard and modified method were investigated for the derivatization of *N*-linked glycans as shown in Table 1. The standard method has been described extensively [23, 25, 32] for analysis of *N*-linked glycans released from biological samples. Briefly, the NAT and SIL INLIGHT™ reagents were suspended in 1 mL of 3:1 (v:v) methanol: acetic acid solution for a final concentration of 1 µg/µL and then, 200 µL (200 µg) of NAT and SIL reagents were added to their respective samples. The samples were lightly vortexed, briefly centrifuged (~5 sec), and incubated for 3 hours at 56 °C to allow the glycans to react with the labeling reagent. The modified method has previously been described as the optimal INLIGHT™ derivatization conditions for labeling *O*-linked glycans [27]. Briefly, the NAT and SIL INLIGHT™ reagents were suspended in 1 mL of methanol. 10 µL of NAT and SIL tag were then added to their respective samples and diluted with 45 µL of methanol and 45 µL of acetic acid, yielding a final concentration of 0.1 µg/µL of P2PGN reagent in a 55:45 (v:v) methanol: acetic acid solution. These samples were then incubated at 37 °C for 1.75 hrs.

Both methods were quenched by drying to completion in a vacuum concentrator at 55 °C. The dried, derivatized glycans were stored at –20 °C until LC-MS/MS analysis. Samples were prepared using increasing amounts of P2PGN reagent while maintaining the solution composition, incubation temperature and incubation length. The P2PGN concentrations investigated were 0.001 µg/µL, 0.01 µg/µL, 0.05 µg/µL, 0.1 µg/µL, 0.25 µg/µL, 0.50 µg/µL, 0.75 µg/µL, and 1 µg/µL.

Nano-LC MS/MS Analysis

Prior to nanoLC-MS/MS analysis, dried down and derivatized maltoheptaose, bovine fetuin, and horseradish peroxidase were reconstituted in 50 µL of LC-MS grade water and pipetted up and down to ensure suspension of glycans in the solution. The re-suspended glycans were briefly vortexed, centrifuged at $14,000 \times g$ for 5 minutes, and the supernatant was carefully removed and placed into new centrifuge tubes to avoid the presence of excess tag in the samples during analysis. Equimolar NAT and SIL derivatized samples were combined 1:1 (v:v).

Analysis of combined NAT and SIL labeled glycan samples was performed using RP nanoLC-MS/MS by means of an Easy nLC 1200 (Thermo Fisher Scientific, Waltham, MA) coupled in-line to a high field quadrupole Orbitrap mass spectrometer (Q Exactive HF-X, Thermo Fisher Scientific, Bremen, Germany). Five microliters of glycan sample were loaded onto a C18 Acclaim™ PepMap™ trap column (2 cm $\times$ 75 µm $\times$ 3 µm) (Thermo Fisher Scientific, West Palm Beach, FL) connected to an EASY-Spray Column (25 cm $\times$ 75 µm $\times$ 2 µm) (Thermo Fisher Scientific, West Palm Beach, FL). Mobile phase A (MPA) consisted of 98% water, 2% acetonitrile, and 0.1% formic acid, while mobile phase B (MPB) was 80% acetonitrile, 20% water, and 0.1% formic acid. The glycans were eluted using a gradient that ramped from 5% to 35% MPB in 2 minutes, then 35% to 70% MPB over 40 minutes, and finally ramped to 95% MPB in 1 minute. It was then held at 95% MPB for 6 min before returning to 5% MPB in 1 minute with another hold of 10 minutes for re-equilibration. The flow rate for the gradient was 300 nL/minute and a spray voltage of 1.8 kV was utilized.

MS measurements were performed using the Top12 data dependent acquisition (DDA) mode and analyzed using an m/z range of 300 to 2000 m/z . The resolving power was set to 60,000 FWHM at 200 m/z for full MS1 acquisition and 15,000 at 200 m/z for MS/MS acquisition. AGC targets for MS and MS/MS were set to 5×10^5 and 5×10^4 with maximum ionization times of 64 ms and 100 ms for MS1 and MS/MS, respectively. Higher-energy collisional dissociation was performed using 27 as the normalized collision energy. Precursor ions were collected with an isolation window of 1.4 m/z and the dynamic exclusion window was set to 15 seconds. Each sample was injected in triplicate to ensure run-to-run reproducibility.

UHPLC MS/MS Analysis

Preceding LC-MS/MS analysis, 25 µL of LC-MS grade water were added to the dried, derivatized maltoheptaose, bovine fetuin, and human pooled plasma glycan samples and pipetted up and down to ensure the glycans were suspended. The re-suspended glycans were briefly vortexed then centrifuged at $14,000 \times g$ for 5 minutes. To reduce the amount of

unreacted tag in the samples, the supernatant was vigilantly removed and equal volumes were combined (1:1 NAT:SIL) in a new centrifuge tube.

Analysis of combined NAT and SIL labeled glycan samples was performed with 5 distinct stationary phase and mobile phase combinations by means of a Vanquish UHPLC (Thermo Fisher Scientific, Waltham, MA) coupled in-line to an ultra-high field Orbitrap tribrid mass spectrometer (Orbitrap ID-X Tribrid, Thermo Fisher Scientific, Bremen, Germany). RP-LC separations were carried out using a Waters (Milford, MA) UPLC BEH C18 (1.7 μ m, 2.1 $\times$ 100mm) column. HILIC separations utilized an Agilent (Santa Clara, CA) Zorbax HILIC RRHD (1.8 μ m, 2.1 $\times$ 100 mm), and PGC separations were carried out on a Thermo Scientific (Waltham, MA) Hypercarb™ Porous Graphitic Carbon (3 μ m, 1 $\times$ 100 mm) column. Ten μ L of each glycan sample were loaded onto the column and subsequently eluted using optimized gradient conditions appropriate for the stationary phase/mobile phase being investigated. These optimized conditions can be found in ESM Tables S1–S3.

MS measurements were performed using DDA mode and analyzed using an m/z range of 400 to 2000 m/z . For full MS1 acquisition, the resolving power was set to 120,000 FWHM at 200 m/z . The automatic gain control (AGC) target was set to 100% and the maximum isolation time (IT) was set to 50 ms. The MS/MS acquisition parameters include resolving power of 30,000 at 200 m/z , AGC target set to predicted, and maximum IT set to 100 ms. Higher-energy collisional dissociation was performed at a setting of 35%. Precursor ions were collected with an isolation window of 1.5 m/z and the dynamic exclusion window was set to 15 seconds. Each sample was injected in triplicate to ensure run-to-run reproducibility.

Data Analysis

Glycans were identified utilizing accurate mass (mass measurement accuracy 3 ppm) and manually evaluated using Qual Browser in Thermo Scientific's XCalibur software and Skyline. The observed potential glycans were compared to a list of accurate glycan masses in literature [23, 33–37]. A transition list comprised of the exact masses of tagged glycans was curated in Skyline and used to verify co-elution of the NAT and SIL-labeled glycans. Quantification of the glycans was performed using the peak integration function in Skyline[38, 39]. Normalization was then performed to account for variability within each experiment. The quantified data was normalized to the maximum base peak (BP) ion abundance of each injection within the same experiment. Normalization coefficients were generated by dividing the maximum base peak ion abundance by each individual injection base peak abundance, and data was multiplied by the resulting coefficient.

RESULTS AND DISCUSSION

In this study, we investigated several facets of the analysis of INLIGHT™ derivatized glycans by LC-MS/MS. The first step assessed the enzymatic cleavage of *N*-linked glycans from known glycoproteins using two sources of PNGase F. The first source was the nonrecombinant, in-solution PNGase F enzyme (New England Biolabs), which has facilitated numerous glycan identifications in complex biological samples [23, 25, 26, 32, 40, 41]. In contrast, the use of the lyophilized, mutant recombinant form of PNGase F (Bulldog Bio) has primarily been implemented in Matrix Assisted Laser Desorption

Ionization (MALDI) MS imaging protocols for analyzing glycans [42–47]. To assess run-to-run reproducibility and determine if discrepancies existed between the two enzymes, fetuin and horseradish peroxidase were evaluated with both PNGase F sources in triplicate. While minor background changes were observed within each run, no differences in overall ion abundances or LC peak numbers were observed for either glycoprotein. Figure 2 shows representative base peak chromatograms (BPCs) for the glycans enzymatically released from fetuin (Figure 2A). The insets in Figure 2A show the mass spectra summed over the retention window for INLIGHT™ derivatized glycans enzymatically released using either the recombinant (BB, top) and nonrecombinant (NEB, bottom) PNGase F. The mass spectra were similar in both the number of NAT/SIL paired species present, as well as the relative peak abundances in the mass spectra as shown in Figure 2B for the INLIGHT™ derivatized glycan, GlcNAc₂Man₅. Comparisons between the absolute spectral abundances for the NAT and SIL peaks showed similar ion abundances regardless of the type of enzyme used. For both glycoprotein samples, the same derivatized glycans were identified regardless of the type of PNGase F used (ESM Tables S4 and S5). This confirms that there were no biases between the nonrecombinant, in-solution, enzyme from New England Biolabs and the mutant recombinant, lyophilized enzyme from Bulldog Bio. Thus, either PNGase F source can be utilized in the FANGS-INLIGHT™ strategy of sample preparation with comparable results.

Modification of the INLIGHT™ derivatization method used for labeling the glycans was investigated next. Here, the standard method for the derivatization of *N*-linked glycans, considered to be the optimal conditions for their analysis, was compared to several modifications found by King et al., in which less INLIGHT™ reagent, more acidic conditions, a shorter reaction time and lower temperature during derivatization decreased sialic acid peeling and the loss of fucose for *O*-linked glycans. Since they used different sample preparation steps prior to the derivatization using the INLIGHT™ strategy, further investigation was necessary. Using the same optimized temperature, solvent composition, and reaction condition length as King et al, the concentration of P2PGN was varied from 1 ng/μL to 1 μg/μL and reacted with maltoheptaose (Figure 3) and *N*-linked glycans cleaved from bovine fetuin. Triplicate injections were performed to assess inter-run variability and comparisons were made for the NAT and SIL peak areas. The data from the maltoheptaose experiments is shown in Figure 3 since it only requires derivatization prior to RPLC-MS/MS analysis; therefore, the potential for sample loss from FANGS is minimal. In the analyses, the highest signal was observed for the reaction utilizing 0.1 μg/μL INLIGHT™ reagent for glycan derivatization. Derivatization reagent concentrations lower than 0.1 μg/μL was observed to have a decrease in observed abundance, which was attributed to a significant decrease in reaction efficiency. Conversely, samples labeled with concentrations of >0.1 μg/μL of the derivatization reagent had lower abundance, which was attributed to ionization suppression due to excess tag remaining and competing with the labeled glycans for charge in the ESI process. The unreacted tag was filtered out by the quadrupole to avoid overfilling the C-trap thereby excluding the ions of interest from MS detection. *N*-linked glycans from bovine fetuin were also analyzed and provided similar results.

Upon comparing samples prepared using the standard protocol to those using the modified protocol and 0.1 μg/μL P2PGN (Figure 4), a significant increase in the relative abundances

of glycans was observed. Figure 4A depicts an extracted ion chromatogram for the NAT and SIL derivatized glycan (Man)₅(GlcNAc)₂ in bovine fetuin using both methods, while Figure 4B depicts (Xyl)₁(GlcNAc)₂(Man)₃ from horseradish peroxidase. These data were normalized to the maximum base peak ion abundance within this experiment. Normalization was done to identify the true difference in signal increase between the two methods by normalizing the signal to the highest base peak abundance from each system in the experiment so all of the data has the same relative abundances. The lower ion abundances observed when using the standard protocol is attributed to ion suppression from the excess (unreacted) tag as the tag bleeds from the column taking away a significant amount of charge from the electrospray droplets. As previously mentioned, the unreacted tag is not observed in the mass spectrum because it is purposefully filtered out by the quadrupole to allow filling of the C-trap with the labeled glycans.

The average peak area for (Man)₅(GlcNAc)₂ increased ~96 times when compared to the standard method and (Xyl)₁(GlcNAc)₂(Man)₃ increased by ~23 times. This difference demonstrated the innate ionization differences between the two representative *N*-linked glycans while also portraying a total ion signal increase of over a magnitude instead of four times as originally stated when the P2PGN tag was first characterized[21]. It was also apparent across sample types that excess of the derivatization reagent, even when carefully avoided during sample preparation, results in issues with ionization and detection of low abundance glycans. For instance, the glycans (Fuc)₁(Gal)₂(GlcNAc)₄(Man)₃(NeuAc)₁ and (Gal)₃(GlcNAc)₅(Man)₃(NeuAc)₃ found in fetuin were only identified in the samples prepared using the modified method (ESM Table S4).

The final optimization performed was evaluating the ideal LC separation parameters for the INLIGHT™ *N*-linked glycans. Numerous LC separation techniques exist for derivatized *N*-linked glycans, although the stationary phases, mobile phases and separation conditions vary greatly. The most commonly employed stationary phases are PGC, HILIC, and RPLC, so each was evaluated. Mobile phase compositions were chosen based on those commonly employed when separating glycans with each specific stationary phase. To investigate which LC setup best separated the INLIGHT™ derivatized *N*-linked glycans, derivatized maltoheptaose, bovine fetuin, and human pooled plasma were analyzed with multiple columns and mobile phases (Figure 5).

In the LC evaluations for all stationary phases, the composition of MPA was varied while maintaining a consistent MPB composition (98% acetonitrile, 2% water, and 0.1% formic acid). For PGC, MPA normally consists of 10 mM ammonium bicarbonate, 10 mM ammonium acetate, or water with small amounts of acetonitrile and formic acid[50–56], therefore, two separate MPA compositions were tested. The first MPA consisted of 10 mM ammonium acetate with 0.1% formic acid, while the second was comprised of 98% water, 2% acetonitrile, and 0.1% formic acid (Figure 5A). It was noted that a washing step using 95% methanol was ideal for maintaining the integrity of the PGC stationary phase so a blank sample consisting of each MPA was run with MPC of 95% methanol after every run to regenerate the analytical column[56]. HILIC mobile phase compositions for derivatized glycans typically have various concentrations of ammonium formate or acetonitrile for MPA, while MPB consists of primarily acetonitrile[34, 57–60]. The Optamax NG ionization

source on the Orbitrap ID-X and utilized in this study, does not function optimally with ion pairing reagents or other additives with concentrations that exceed 10 mM. Thus, two different MPA compositions selected for the HILIC analysis were: 10 mM ammonium formate in water with 0.1% formic acid and 98% water, 2% acetonitrile and 0.1% formic acid (Figure 5B). Finally, RPLC uses a MPA primarily comprised of water and MPB mainly consisting of acetonitrile[23, 25, 27, 61–63], therefore, 98% water, 2% acetonitrile, and 0.1% formic acid was used for MPA (Figure 5C).

To assess the retention of INLIGHT™ derivatized samples, derivatized maltoheptaose was analyzed for each setup. INLIGHT™ derivatized maltoheptaose, maltohexaose, maltopentaose and maltotetraose were retained in all cases except for the HILIC column using the water and acetonitrile mobile phases. *N*-linked glycans enzymatically removed from bovine fetuin were analyzed next using each LC setup to observe the retention of the derivatized glycans. Glycans were identified only when both NAT and SIL versions co-eluted and the mass spectrum contained both isotopic distributions. No INLIGHT™ derivatized *N*-linked glycans were retained on the PGC column with either mobile phase systems. Four INLIGHT™ derivatized *N*-linked glycans from bovine fetuin were retained on the HILIC column when using the water and acetonitrile mobile phase system, while five INLIGHT™ derivatized *N*-linked glycans were retained on the HILIC column with the ammonium formate and acetonitrile mobile phases. The highest number of INLIGHT™ derivatized *N*-linked glycans, 18, were retained on the C18. For those chromatographic conditions where glycans were retained, the gradient and method were optimized to further improve the separation (ESM Tables S1–S3).

Finally, *N*-linked glycans enzymatically removed from human pooled plasma proteins were analyzed using the optimized gradient for each LC system. INLIGHT™ derivatized *N*-linked glycans were not retained on the PGC column using either mobile phase systems, nor were any detected following separation via the HILIC column with the water and acetonitrile mobile phases. However, 21 INLIGHT™ derivatized *N*-linked glycans were retained on the HILIC column when using ammonium formate and acetonitrile and 57 were retained and subsequently detected using the RPLC system. Thus, the RPLC setup provided the best separation for INLIGHT™ derivatized *N*-linked glycans with the majority of detected glycans eluting between 6 and 9 minutes out of the 30-minute gradient (ESM Table S3). While the majority of the glycans eluted early within this gradient, it was necessary to keep the length of this gradient at 30 minutes as lessening the time resulted in the inability to efficiently separate derivatized glycans.

CONCLUSIONS

Three steps of the INLIGHT™ derivatized *N*-linked glycan pipeline were investigated and optimized. First, evaluation of recombinant and nonrecombinant PNGase F illustrated no differences in the number of glycan observed. Second, modifications in the INLIGHT™ derivatization step including temperature optimization, solvent composition changes, reaction condition length and tag concentration resulted in over an order of magnitude increase in signal for the detected *N*-linked glycans in fetuin and horseradish peroxidase compared to the standard method. Not only did the modified method significantly increase

the signal for *N*-linked glycans, it also improved sample throughput by decreasing the time necessary for sample preparation and minimized chances of sialic acid peeling and fucose losses by using a lower temperature. Finally, LC setup optimization illustrated that a C18 RPLC column, and MPA: 98% water, 2% acetonitrile, and 0.1% formic acid and MPB: 98% acetonitrile, 2% water, and 0.1% formic acid, enabled the best retention and separation of the INLIGHT™ derivatized *N*-linked glycans compared to two PGC and two HILIC setups. These method optimizations were demonstrated on a complex sample (human plasma) and enabled the best selectivity and sensitivity for INLIGHT™ derivatized *N*-linked glycan analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Modified FANGS workflow for glycan sample preparation. **A)** Fetuin and horseradish peroxidase samples were added to molecular weight cut-off filters. **B)** These protein samples were denatured using DTT, then alkylated with iodoacetamide. **C)** The glycans were cleaved by addition of PNGase F purchased from either New England Biolabs or Bulldog Bio. **D)** The glycans were eluted and **E)** derivatized using either natural (NAT) or stable isotope label (SIL) INLIGHT™ tags using one of two derivatization methods. **F)** The NAT and SIL pairs were then combined 1:1 before analysis via LC-MS/MS.

Figure 2.

PNGase F enzyme comparisons. **A)** Representative base peak chromatograms (BPC) for glycans cleaved from fetuin using both Bulldog Bio (BB) PNGase F (top) and New England Biolabs (NEB) PNGase F (bottom). The mass spectra from the LC window in which most glycans elute (8.0 min–12.0 min) are displayed. **B)** The ion abundances in each of the representative mass spectra for the observed glycan (GlcNAc)₂(Man)₅ were comparable for both sources of PNGase F.

Figure 3.

P2PGN concentration optimization for maximum peak area and equivalent NAT/SIL ratios by derivatizing 50 μg of maltoheptaose in each sample. Average peak areas normalized to the maximum base peak ion abundance of INLIGHT™ derivatized maltoheptaose. The error bars represent the standard error from the individual peak areas in the triplicate analyses.

Figure 4. Sensitivity enhancement utilizing the modified method. Extracted ion chromatograms and average peak areas, normalized to the maximum base peak abundance for **A)** (Man)₅(GlcNAc)₂ in fetuin and **B)** (Xyl)₁(GlcNAc)₂(Man)₃ in horseradish peroxidase. The observed glycans were derivatized using the modified and standard methods described in Table 1. Deviations from 1:1 ratio are likely due systematic sample preparation errors and a slight bias of increased signal from the heavy labelled glycan previously noted by Walker et al [48]. Error bars were derived from the peak areas in the triplicate analyses.

Figure 5.

LC separation and sensitivity optimization for INLIGHT™ derivatized glycans. Three stationary phases: **A)** porous graphitic carbon (PGC); **B)** hydrophilic interaction chromatography (HILIC); and **C)** reversed-phase liquid chromatography (RPLC), and their most common mobile phases all containing 0.1% formic acid. Maltoheptaose and *N*-linked glycans from bovine fetuin and pooled human plasma were used for their evaluation due to the increasing complexity and known glycans from literature. The chromatograms represent the separation of the INLIGHT™ labeled *N*-linked glycans derived from fetuin. Representative theoretical isotopic distributions for the NAT (●) and SIL (●), calculated using enviPat[49], are overlaid with the experimental data demonstrating how spectral accuracy can aid in the identification of the glycans. RPLC using a C18 stationary phase with mobile phase A comprising of 98% water, 2% acetonitrile (ACN) and 0.1% formic acid and mobile phase B comprising of 98% ACN, 2% water and 0.1% formic acid was

determined to be the best LC system for separating and identifying INLIGHT™ derivatized *N*-linked glycans.

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Table 1.

Differences between 2 derivatization conditions used for labeling of released biological *N*-linked glycans.

Derivatization Conditions	Standard Method	Modified Method
Methanol: Acetic acid	75:25	55:45
Concentration of INLIGHT™	1 µg/µL	0.1 µg/µL
Temperature	56° C	37° C
Reaction Length	3.00 hours	1.75 hours