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Glycans, Glycosite, and Intact Glycopeptide Analysis of N-Linked Glycoproteins Using Liquid Handling Systems

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Abstract

Aberrant glycosylation has been shown to associate with disease progression, and with glycoproteins representing the major protein component of biological fluids this makes them attractive targets for disease monitoring. Leveraging glycoproteomic analysis via mass spectrometry (MS) could provide the insight into the altered glycosylation patterns that relate to disease progression. However, investigation of large sample cohorts requires rapid, efficient, and highly reproducible sample preparation. To address the limitation, we developed a high-throughput method for characterizing glycans, glycosites, and intact glycopeptides (IGPs) derived from N-linked glycoproteins. We combined disparate peptide enrichment strategies (i.e., hydrophilic and hydrophobic) and a liquid handling platform allowing for a high throughput and rapid enrichment of IGP in a 96-well plate format. The C18/MAX-Tip workflow reduced sample processing time and facilitated the selective enrichment of IGPs from complex samples. Furthermore, our approach enabled the analysis of deglycosylated peptides and glycans from enriched IGPs following PNGase F digest. Following development and optimization of the C18/MAX-Tip methodology using the standard glycoprotein, fetuin, we investigated normal urine samples to obtain N-linked glycoprotein information. Together, our method enables a high-throughput enrichment of glycan, glycosites, and IGPs from biological samples.

Glycosylation is one of the most common and diverse protein modifications,^{1–3} with N-linked glycosylation being the most prevalent and most studied, which has a consensus glycosylation motif (N-X-T or N-X-S, with X not equal to proline^{4,5}). Aberrant protein glycosylation has been found to be related to different diseases, including various cancer

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.9b03761>.

Additional supporting figures describing binding time evaluation, enrichment effect of different urine samples using C18/MAX-Tip, enrichment efficiency, and reproducibility of the C18/MAX-Tip (PDF)

Additional supporting tables describing C18/MAX binding time evaluation (Table S1), urine sample information (Table S2), identified glycan structure using different detection methods (Table S3), urine IGP identification using GPQuest (Table S4), and reproducibility evaluations (Tables S5 and S6) (XLSX)

The authors declare no competing financial interest.

types,^{3,6} rheumatoid arthritis,⁷ and heart disease,⁸ and there is evidence of altered glycosylation impacting the function of biological systems. Thus, deciphering the heterogeneity of protein glycosylation is crucial in characterizing the glycosylation of cells.

In recent years, high-throughput sample preparation has been the focus of proteomics.^{9,10} On the other hand, high-throughput glycoproteomic analysis method has been limited to glycosite analyses with glycan released from the peptides.^{11–13} Intact glycoproteomic enrichment followed by LC–MS/MS allows simultaneous characterization of glycosylation sites and glycosite-specific glycosylation.¹⁴ Traditionally, intact glycopeptides are enriched by hydrophilic chromatography or lectin.^{14–18} While sialylated glycopeptides can be specifically enriched by TiO₂, strong cationic exchange (SCX) and IMAC based on the charge selection^{3,19,20} or capturing sialylated glycopeptides using periodate-oxidation, which sialic acid information is lost after desialylation release from hydrazide beads.^{21,22} With the limits that lie ahead of high-throughput intact glycoproteomics analysis, a high-throughput method for this process is crucial for speedy and reproducible analysis of glycoproteins.

Investigation of N-linked glycosylation is a multistep process, involving proteolytic digestion of proteins, followed by enrichment of N-linked glycopeptides for subsequent downstream analysis.²³ With the rapid advancement of mass spectrometry (MS)-based analysis, there is a need to develop glycoproteomic sample preparation strategies that allow for higher sample throughput.^{13,24,25} Several challenges that need to be addressed to move toward increased sample preparation throughput include reducing the time span needed for sample preparation, limiting sample loss during sample preparation (e.g., peptide desalting, drying), and reproducibility of sample recovery. To overcome these obstacles, we developed a high-throughput methodology that would enable the analysis of glycans, glycosites, and intact glycopeptides (IGPs). Utilizing a combination of tip-based hydrophilic and hydrophobic peptide extraction chemistries and a microplate format compatible with liquid handling systems, our approach allowed for the enrichment of IGPs after 4 h of processing, and the inclusion of an additional step of PNGase F digestion, allowed for the profiling of glycosites and glycans from the same sample. This strategy offered the flexibility of analyzing various aspects of N-linked glycosylation (i.e., glycosite, glycan), while also allowing for orthogonal verification of glycosylation site identification via analysis of IGP and deglycosylated N-linked glycopeptide profiles.

Following method development and optimization, we utilized our described method to investigate the N-linked glycoproteome of human urine samples. With the comprehensive analysis of (1) global peptides, (2) IGPs, (3) glycosite containing peptides, and (4) glycans, this method could also achieve a comprehensive and site-specific N-linked glycosylation platform that would be both rapid and high throughput when comparing with conventional methods. The tip-based format of the C18/MAX-Tip strategy offers a high degree of user flexibility and is able to process samples using a single pipet, a multichannel pipet, or liquid handling systems.

EXPERIMENTAL SECTION

Chemicals and Materials.

Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless specified otherwise. The Eppendorf 96-well plate, matrix D.A.R.T.s tips (20–300 μL), and automation reservoirs were obtained from Thermo Fisher Scientific (Hudson, NH). The C18 SepPak columns and Oasis Mix Mode Anion eXchange (MAX) column were purchased from Waters Corporation (Milford, MA). MS-grade trypsin and Lys-C were from Promega Corporation (Madison, WI). C4 reverse phase resin and C18 analytical LC column (NanoViper, 75 μm , 150 mm, 2 μm particle size) were purchased from Separation Method Technologies (Newark, DE) and Fisher Scientific (Waltham, MA), respectively. The polyethylene sheet has a median pore size of 15–45 μm (thickness = 1.57 mm or 0.062 in.; Interstate Specialty Products; Sutton, MA).

Sample Preparation.

Fetuin from bovine was obtained from Sigma-Aldrich (St. Louis, MO). Human urine samples for this study were collected and analyzed under the approval by the Johns Hopkins Institutional Review Board. First Void Urine was collected from normal donors ($n = 3$) and stored at $-20\text{ }^{\circ}\text{C}$. All the urine samples were subjected to spinning at 3000g for 10 min, followed by extraction of supernatant and disposal of sediments. Protein concentration from all the urine samples were measured via Bradford assay, while protein concentration of fetuin was measured via BCA assay.

C18/MAX-Tip Fabrication.

A polyethylene sheet (2 mm diameter) was inserted into the Thermo Scientific D.A.R.T.s tip with a volume capacity up to 300 μL . In total, 4 mg of MAX resin and 20 mg of C18 reverse phase resin was loaded sequentially into the tip, followed by a second polyethylene sheet (5 mm diameter) inserted to seal the resin material. The packed C18/MAX-Tips were then ready for use in the Versette Liquid Handler (Thermo Scientific).

C4-Tip Fabrication.

C4-Tip fabrication was previously developed in our group.¹⁰ Briefly, 30 mg of C4 reverse phase resin in 300 μL of methanol was loaded into the tip. The C4-Tips were packed and sealed and then ready for use in the Versette Liquid Handling system (Thermo Scientific).

C18-Tip Fabrication.

A polyethylene sheet (2 mm diameter) was inserted into the Thermo Scientific D.A.R.T.s tip with a volume capacity up to 300 μL . In total, 20 mg of C18 reverse phase resin was loaded into the tip, followed by a second polyethylene sheet (5 mm diameter) inserted to seal the resin material. The packed C18-Tips were then ready for use in the Versette Liquid Handler (Thermo Scientific).

Sample Preparation and IGP Enrichment Using Versette Automating Handling System.

All the samples were handled using the Thermo Fisher Scientific Versette Liquid Handling System with one cycle (aspiration/dispense) performed in approximately 2 min. C4-Tip procedures were derived from Clark et al.,¹³ with minor procedure changes. Briefly, C4-Tips were conditioned with 50% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA), followed by 0.1% TFA (10 cycles each). Proteins were acidified with formic acid (pH < 3) and bound to C4-Tips (60 cycles). C4-Tips were washed with 0.1% TFA and 50 mM triethylammonium bicarbonate (TEAB) (10 cycles each). Reduction and alkylation of proteins were performed with 10 mM TCEP and 15 mM IAA for 20 cycles each. Protein digestion was carried out with trypsin/Lys-C mix (1:50 enzyme/protein for trypsin and 20 mU enzyme/1 mg protein for Lys-C) in 50 mM TEAB and 30% ACN for 120 cycles. The digested peptides were eluted with 50% ACN in 0.1% TFA twice to ensure that all the peptides were collected (10 cycles each). The elution buffer (50% ACN in 0.1% TFA) is collected two times consecutively and then pooled together with the digestion buffer (30% ACN and 50 mM TEAB, which contains digested peptides that released from C4 beads). The temperature settings are all room temperature for C4-Tips.

Samples were dried and reconstituted in 0.1% TFA while C18/MAX-Tip conditioning was performed using (1) ACN, (2) 100 mM triethylammonium acetate (TAAB), (3) 95% ACN + 1% TFA, and (4) 0.1% TFA (sequentially, 10 cycles each) as described in the previous publication.¹⁶ Reconstituted samples were bound onto the C18/MAX-Tip (20 cycles) and washed with 0.1% TFA for 10 cycles. The global peptides were eluted from the C18/MAX-Tip with 95% ACN in 0.1% TFA (3 × 10 cycles). The IGP were sequentially enriched with 50% ACN (3 × 6 cycles). BCA assays could be performed to estimate the yield of urinary IGP after enrichment, however, since glycosylated peptides are usually scarce compared to global peptides, an estimation of yield using BCA assays could be unfeasible if the starting material is limited.

After IGP were enriched, half of the samples were aliquoted for direct IGP analyses. Another half will undergo PNGase F digestion. Briefly, IGP was dried and reconstituted in 25 mM ammonium bicarbonate, PNGase F were added as an estimated ratio of 1 mg of peptide/1 mL of PNGaseF. IGP were mixed and reacted thoroughly at 37 °C for 3 h. After glycans were cleaved off the IGP, the deglycosylated peptides and glycans were acidified while an additional C18-Tip were applied as a desalting step for 20 cycles. The flow through would be glycans, and deglycosylated peptides were eluted with 50% ACN + 0.1% FA.

NanoLC–MS/MS Analysis.

For urinary glycoproteomic analysis, individual urine samples were enriched for IGP and 10% of enriched IGP were analyzed by MS regardless of their initial urine protein concentration, unless specified otherwise.

Samples were analyzed by a Thermo Orbitrap Fusion Lumos mass spectrometer (Thermo Scientific) unless specified otherwise. Peptides were separated with a 28 cm × 75 μ m C18 column on an Easy-nLC 1200 at a flow rate of 300 nL/min with a 110 min linear gradient (from 6 to 25% B over 85 min; A = 0.1% formic acid, 3% ACN in water, B = 0.1% formic

acid in 90% ACN). Full MS scans were acquired over the mass range of 350–2000 m/z with a mass resolution of 60 000. The automated gain control (AGC) target value was set at 400 000. The fifteen most intense peaks were fragmented with higher energy collisional dissociation (HCD) with a collision energy of 35. MS/MS was acquired with a resolution of 15 000 with an AGC target of 100 000 and a max injection time of 150 ms. Dynamic exclusion was set for 45 s.

MALDI-MS.

MALDI-MS was conducted on a Shimadzu MAXIMA Resonance with a mass range from 50 to 7000 Da. The MALDI matrix consisted of 20 mg/mL DHB and *N,N*-dimethylaniline (DMA) was added at a ratio of 0.02 μL per μL of matrix solution. A volume of 2 μL of samples were spotted on a MALDI target plate and 1 mL of 2,5 dihydroxybenzoic acid (DHB) was added as a matrix and dried under room temperature.

Data Analysis of IGP, Deglycosylated Peptides, Global Peptides, and Glycans.—IGP data were searched with our inhouse developed glycopeptide analysis software, GPQuest 2.0^{3,26}. Any peptide-spectrum match (PSM) without glycan annotation and a glycan intensity ratio less than 20% were filtered out with consideration of a false discovery rate (FDR) less than 1%.

Deglycosylated peptides and global peptides were searched with SEQUEST in Proteome Discover 2.2 (Thermo Fisher Scientific). With consideration of static modification of carbamidomethylation (C) and variable modifications of oxidation (O) and deamidation (N), the results were filtered with peptide rank and peptide confidence (maximum rank, 1; minimum confidence, high) and protein filters as minimal of 2 peptides per protein. The FDR rate was set to 1%. MS/MS spectra were extracted with a tolerance of 10 ppm from raw files.

The cleaved glycans were collected and desalted by NuTip Carbon (Hypercarb) (Glygen, MD). Glycans were analyzed by a Shimadzu MALDI (matrix-assisted laser desorption/ionization) mass spectrometer. Glycan composition was determined by GlycoWorkbench data analysis software²⁷ and verified by MS/MS if necessary.

RESULTS AND DISCUSSION

Workflow of High-Throughput Analysis of Glycans, Glycosites, Site-Specific Glycosylation of N-Linked Glycoproteins.

As seen in Figure 1, the overall workflow comprises three different tip-based procedures: C4 digestion, C18/MAX enrichment, and C18 desalting. The C4-Tip binds proteins from biological samples, followed by protease digestion.¹⁰ The C18/MAX-Tips are used to rebinding the tryptic peptides and enrich IGP from nonglycopeptides (i.e., global peptides), with (1) nonglycopeptides observed in the flow-through and IGP remaining bound to the MAX resin. Following elution with 50% ACN to release (2) IGP, an aliquot (~50%) was subjected to in-solution PNGase F digestion to cleave N-glycans. Peptide/glycan mixtures will then be reloaded on a C18-Tip to separate (3) deglycosylated peptides and (4) glycans. The (1) nonglycosylated peptides (global), (2) IGP, and (3) deglycosylated peptides are

analyzed by LC–MS/MS using the Orbitrap Fusion Lumos (Thermo). The (4) glycans are then analyzed with matrixassisted laser desorption/ionization-time-of-flight (MALDITOF) using a Shimadzu Axima Resonance. The entire procedure can be performed in 8 h using a liquid handling system. Of note, this entire procedure can also be performed manually, using a pipet and constructed C4- and C18/MAX-Tips.

Evaluating the Peptide Binding Time for C18/MAX-Tip.

To determine the optimal binding time for C18/MAX-Tip, we used a glycoprotein standard, fetuin. Total protein was digested with trypsin and the resulting peptides bound to the C18/MAX-Tip. A total of 100 μg of digested fetuin peptides in 250 μL 0.1% TFA was aspirated/dispensed through the C18/MAX-Tips, and the remaining unbound peptides in the solution was measured at 10 min (5 cycles), 20 min (10 cycles), 30 min (15 cycles), 60 min (30 cycles), and 90 min (45 cycles). The unbound peptides were measured via BCA assay to determine the peptides that were not bound at each time point. We observed over 90% of peptides bound after 20 min as shown in Figure S1. Within 30 min, 96% of peptides were bound to the C18/MAX-Tip. The peptide binding plateaued after 30 min. These results prompted our decision to perform the peptide binding step for 40 min, which corresponded to 20 aspiration/dispense cycle iterations.

Rapid Enrichment of Fetuin Global Peptides, IGPs, Glycans, and Glycosites.

In order to validate the entire enrichment pipeline, fetuin was used to test the entire workflow in triplicate. Global peptides, IGPs, deglycosylated peptides, and glycans were sequentially acquired as described in the Experimental Section and outlined in Figure 1. The (1) global peptides, (2) IGPs could be rapidly obtained by the C18/MAX-Tip in 4 h postdigestion, while the (3) deglycosylated peptides and (4) glycans could be obtained sequentially afterward with additional 4 h. Compared to nonglycosylated peptides that were identified in the flow through and IGPs obtained in the C18/MAX-Tip eluate, deglycosylated peptides displayed a distinct amino acid composition modification following PNGase F digestion, resulting in an Asn to Asp transition on the glycosylation site. This was observed in the deglycosylated peptides on all the three N-linked glycosylation sites from fetuin. One of the three identified fetuin Nglycosites with and without glycosylation at the glycosylation site was presented in Figure 2. Integrating these results allows for verification of a site-specific identification of N-linked glycosites from the same biological sample.

In a similar fashion, glycan analysis can be achieved either by glycomic analysis of released glycans or by identified glycans attached on IGPs. To determine whether these two glycan profiles matched, we compared the glycan identified from fetuin IGPs with the PNGase F released glycans analyzed directly by MALDI-TOF (Figure 3B). To prevent the loss of sialic acids, glycans were permethylated before analyzing on the MALDI-TOF.²⁸ A total of 56 glycan compositions are identified using two methods, with 15 glycan structures commonly identified by both methods (Table S3). The low rate of identification overlap might stem from the disparate sample preparation chemistries: released glycans are permethylated while IGPs are not chemically modified. This could result in glycans on IGPs more prone to neutral loss of sialic acids, which could cause a single glycan structure identified as multiple glycan composition (as shown in Table S3, N5H5F1S0, N5H5F1S1, and N5H5F1S2).^{29,30}

Of note, no high mannose glycans were observed in either the IGP or permethylated glycan data, which was consistent with previous literature values.³¹ This data showed that both glycomic analysis and IGP analysis provides glycan branching information; however, the enrichment of IGP offers more information, including both the glycan branching composition and site of glycosylation.

Evaluation of IGP Enrichment Efficiency and Reproducibility on Complex Samples Using Human Urine.

After establishing the optimal binding time and assessing the whole glycoproteomic pipeline, we evaluated the efficacy of enriching IGPs from complex samples via the C18/MAX-Tip. The glycoproteomic pipeline was applied to the analysis of three human urine samples (denoted F1, M2, and 27F) with three replicates for each sample. Urine samples were normalized to a total volume (500 μ L) and not by total protein amount. As seen in Figure 4A, the results indicated that for sample F1, peptide spectral matches (PSMs) increased from peptides in flow through (47 PSMs) to after the enrichment (881 PSMs, Table S4) for isolation replicates, which was in an average of 18.6-fold. As the control, we also analyzed the enriched IGPs and peptides in flow through from a single isolation in triplicates (injection replicates), which on average PSMs increased by 15.42-fold. Lower enrichment results were observed for samples M2 and 27F, which might be due to lower starting concentration (Figure S2 and Table S2). Next, we assessed the variability of identification IGPs between C18/MAX-Tips (enrichment replicate) and serial mass spectrometry analyses (injection replicate) for sample F1. We observed >27% overlap of identified IGP across the three tips, whereas we observed >30% overlap of identified IGPs across three sample injections (Figure 4B,C). These results mirror previous reports examining reproducibility of datadependent acquisition (DDA)-based mass spectrometry, wherein the differences among the replicates are more likely related to low abundant glycopeptides and stochastic nature of DDA fragmentation selection rather than variation of IGP enrichment using our described methodology.^{13,32} Furthermore, IGPs that were mutually identified across all C18/MAX-Tip and injection replicates were selected, with the intensity between runs plotted. We observed similar MS1 intensity of IGP precursor ions between tip to tip replicates and injection replicates (Figures S4 and S5). Overall, these results show our described enrichment method is reproducible and can be applied to investigate the glycoproteomic of complex biological samples.

Enrichment Effectiveness and Specificity of C18/MAX-Tip.

Finally, we want to evaluate both the performance and specificity of enrichment of IGPs by C18/MAX-Tip on complex biological samples. We selected one of the human urine samples (F1), performed tryptic digestion, and then split the peptide sample into two conditions: one was subjected to desalting using a C18 stage tip without MAX enrichment; the second was subjected to IGP enrichment using a C18/MAX-Tip (0.5 μ g). They were subjected to ESI-LC-MS/MS analysis and the resulting MS spectra searched using GPQuest 2.0. As seen in Figure 5A, the N-linked IGP spectral count increased >7-fold after IGP enrichment (115 vs 15 PSM). This indicates the C18/MAX-Tip is effective in retaining the IGP on the MAX resin relative to C18. To assess the specificity of IGP enrichment of the C18/MAX-Tip, the two eluates were further analyzed for the presence of nonglycosylated peptides. The MS

spectra were searched using Thermo Proteome Discoverer, and the results showed the number of nonglycosylated PSMs decreased by 2.3-fold with IGP enrichment (Figure 5B). We further evaluated the MS1 intensities of those nine commonly identified IGPs. Within the identified nine IGPs, eight out of nine IGPs have an increased ratio of IGP intensities after enrichment. The maximum fold increase is 516.8-fold, and the minimum increase is 5.37-fold (Figure S3). As for the one IGP with a fold decrease, it was most likely stemmed from that it was eluted early (with a retention time a lot earlier than others identified IGPs), which could suggest that it was more hydrophilic than other IGPs, therefore its binding on C18 could be weaker than other IGPs. Together, these results indicate that the C18/MAX-Tip enriches glycopeptides specifically and reduces the number of nonglycosylated peptides in the final IGP eluate.

CONCLUSIONS

In this study, we have developed a high-throughput method for comprehensive characterization of N-linked glycoproteins, including the identification of glycan, glycosite, and site-specific glycosylation IGP profiles. With the combination of reverse phase C18 resin and a hydrophilic resin, the C18/MAX-Tip method enabled us to rapidly obtain glycans, glycosites, and IGPs of N-linked glycoproteins in a sequential process. The described workflow decreases the amount of time needed for sample processing significantly¹⁴ and can be applied to investigate the N-linked glycoproteome in complex biological samples with high reproducibility. To the best of our knowledge, our study described here reported the first study of high-throughput analysis of IGPs with glycosite, glycan, and glycosite-specific glycosylation information. Compared to previously reported urinary glycoproteomic studies,^{15,18,22} we observed an increased number of intact glycopeptide identifications and comprehensive coverage of glycopeptides carrying different glycans, including sialylated glycans, oligomannose glycans, and fucosylated glycans as well as other complex glycans. Our method, when used with an automated liquid handling system facilitates high-throughput sample preparation of N-linked glycans, glycosites, and IGPs and has applications for complex biological sample analysis of both large and small patient cohorts.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Workflow for glycans, glycosite, and intact glycopeptide analysis. Following on-tip proteolytic digestion of protein samples using a C4Tip, peptides are loaded onto a C18/MAX-Tip for IGP enrichment. The peptides in flow-through include (1) global peptides, while peptides in the eluate are (2) IGP. Subsequently, an aliquot of the IGPs fraction can be subjected to PNGaseF digest; (3) deglycosylated peptides and (4) glycans are separated by C18-Tips. The glycan fraction is analyzed via MALDI-MS while the (1) global peptides, (2) IGP, and (3) deglycosylated peptide are analyzed by LC-MS/MS.

Figure 2.

Nonglycosylated peptide and deglycosylated peptide spectra from the same glycosylation site of fetuin indicating site specific information and enrichment specificity. Nonglycosylated peptide (global peptide) collected in the C18/MAX-Tip flow through (A), deglycosylated peptide collected after PNGase F enzymatic digestion (B), and IGP collected after the C18/MAX-Tip elution (C). All spectra are of fetuin-derived peptide: VVHAVEVALATFNAES#GSLQLVEISR. The site of glycosylation and Asn to Asp transition are indicated by #.

A

Composition	Released Glycan Information		IGP Information			
	Possible Structure	Permethylated Glycan Mass	Retention time (min)	Oxonium Ion IR	Peptide	Morpheus Score
N3H4F2		1969.1609	81.605	43.9%	RPTGEVYDIEIDTLETT CHVLDPTLANCSVR	14.24
N4H5F2		2418.6909	80.932	51.7%	RPTGEVYDIEIDTLETT CHVLDPTLANCSVR	17.21
N4H5S2		2792.3826	14.529	63.2%	KLCPDCPLLAPLNSDR	10.22
N5H6S2		3245.6578	57.239	68.9%	KLCPDCPLLAPLNSDR	11.22
N5H6S3		3606.0506	64.805	60.2%	KLCPDCPLLAPLNSDR	10.20
N5H6S4		3963.9560	88.019	64.3%	LCPCPLLAPLNSDR	11.13

B

Figure 3.

Fetuin glycan analysis using released glycans and IGPs. (A) Six representative glycan compositions from released glycans detected in MALDI-TOF and the information detected from the IGP eluate for the same glycans. (B) Venn diagram for glycan information that was detected from released glycans and IGPs.

Figure 4.

IGP enrichment effect and reproducibility of C18/MAX-Tip. A human urine sample (F1) was enriched with three replicates using C18/MAX-Tip (isolation replicates), which also underwent three LC-MS/MS analyses for one of the replicates (injection replicates), enrichment efficiency was evaluated based on number of intact glycopeptide PSMs with error bars representing standard deviations (A). Venn diagram of unique IGPs overlap for sample F1 isolation (B) and injection (C), respectively.

Figure 5. Enrichment effectiveness and specificity of C18/MAX-Tip. Samples (F1) were prepared either without IGP enrichment (-), (green) or with IGP enrichment by C18/MAX-Tip (+), (orange). IGP spectral count increase for >7-fold following enrichment (A). Nonglycopeptide spectral count decreased >2-fold following enrichment (B).