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Direct Comparison of Derivatization Strategies for LC-MS/MS Analysis of N-Glycans

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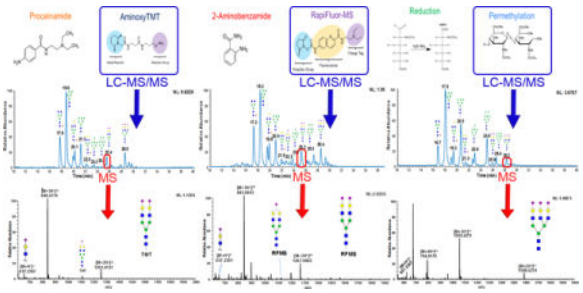
Abstract

Protein glycosylation is a common post-translational modification that has significant impacts on protein folding, lifespan, conformation, distribution and function. N-glycans, which are attached to asparagine residues of proteins, are studied most often due to their compatibility with enzymatic release. Despite the ease of N-glycan release, compositional and structural complexity coupled with poor ionization efficiency during liquid chromatography mass spectrometry (LC-MS) make quantitative glycomic studies a significant challenge. To overcome these challenges, glycans are almost always derivatized prior to LC-MS analyses to impart favorable characteristics, such as improved ionization efficiency, increased LC separation efficiency and the production of more informative fragments during tandem MS. There are a number of derivatization methods available for LC-MS analysis of glycans, each of which imparts different properties that affect both glycan retention on LC columns and MS analyses. To provide guidance for the proper selection of derivatizing reagents and LC columns, herein, we describe a comprehensive assessment of 2-aminobenzamide, procainamide, aminoxyTMT, RapiFluor-MS (RFMS) labeling, reduction and reduction with permethylation for N-glycan analysis. Of the derivatization strategies examined, RFMS provided the highest MS signal enhancement for neutral glycans, while permethylation significantly enhanced the MS intensity and structural stability of sialylated glycans.

Graphical abstract



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Author Contributions

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Conflict of interest statement

The authors declare no conflict of interest.

Keywords

2-aminobenzamide (2AB); procainamide (ProA); aminoxyTMT; RapiFluor-MS (RFMS); permethylation; N-glycan; liquid chromatography tandem mass spectrometry (LC-MS/MS); hydrophilic interaction chromatography (HILIC); reverse phase liquid chromatography (RPLC); glycomics

Introduction

Glycosylation is one of the most complex and common protein post-translational modifications (PTM) that exhibits significant roles in protein functions,[1, 2] stability, and disease development. Reliable quantitation of glycans is essential for understanding the roles of glycans in biological processes[3] and disease.[4] The compositional and structural complexity of glycans makes quantitative glycomics profiling an ongoing challenge for analytical chemists. Quantitation strategies using separation techniques coupled with optical detection or mass spectrometry (MS) are becoming the dominant approaches for quantitative glycomics. However, native glycans are not well suited for this type of analysis. Efforts have been made towards developing glycan derivatization reagents and optimizing glycan derivatization protocols over the past several decades.

Due to the unique chemical properties of the reducing end of glycans, reducing end labeling is the most common glycan derivatization method. The variety of reducing end labeling tags available to choose from makes this derivatization strategy compatible with a variety of analytical methods. The detection of native glycans by UV or fluorescence is impossible because glycans themselves do not contain chromophore or fluorophore moieties. 2-Aminobenzoic acid (2-AA),[5] 2-aminobenzamide (2-AB)[6] and 2-aminopyridine (PA)[7] are examples of labeling reagents for liquid chromatography (LC) that can be coupled with optical detection methods. The identification of glycans in this strategy relies on the comparison of glycan standards or is determined by glycan retention time libraries generated using different LC columns.[8–10] Hence, the optical spectroscopy-based glycomics quantitative methods are only applicable to pre-determined glycans and rely on efficient (baseline-resolved) separation of the glycans.

MS is one of the most powerful bioanalytical approaches available and is advancing the field of reliable glycan identification and quantitation. In addition to identification of glycan structures with a 5 ppm mass accuracy using high resolution MS (HRMS), structural elucidation can be accomplished by tandem MS. The combination of precursor and product ion transitions can be utilized with triple quadrupole MS operated in multiple reaction monitor (MRM) mode for reliable quantitative analysis.[11, 12] Although native glycans can be analyzed by MS,[13] low sensitivity in positive mode MS and the potential in-source fragmentation always impede intact glycan analysis.

Reducing end labeling derivatization is also well developed for MS-based glycomics. Tertiary amine groups are usually utilized in the tags, for example, procainamide (ProA),[14, 15] N^2, N^2, N^4, N^4-tetraethyl-6-hydrazinyl-1, 3, 5-triazine-2, 4-diamine (Meladrazine)[16] and RapiFluor-MS (RFMS)[17] all contain tertiary amines for increased

ionization efficiency in positive ion mode. Quantitative glycomics can also be achieved using multiplexing reagent derivatization. Multiplexing reagents, such as heavy/light 2-AB, aminoxyTMT,[18, 19] iARTs,[20] QUANTITY[21] and INLIGHT,[22] have been reported for quantitative glycomics based on full MS or MS/MS reporter ions. Multiplexed LC-MS strategies reduce analysis time by analyzing multiple samples at once and eliminate the variation induced by ionization efficiency fluctuation and MS response bias.

Permethylation is another glycan derivatization method that specifically works for MS-based glycomics analysis.[23–25] During the process of permethylation, all active hydrogens in glycan molecules are converted to methyl groups by iodomethane, resulting in the alteration of glycan properties. There are several benefits introduced by the permethylation of glycans, including enhanced ionization efficiency in positive mode MS analysis and the elimination of the loss of labile sialic acid and fucose moieties.[26] Moreover, permethylation facilitates structure elucidation by tandem MS, through the formation of more informative fragments.[26–28] The increased hydrophobicity of glycans after permethylation allows for their separation by reverse phase LC (RPLC). However, limited isomeric separations are achieved by RPLC separation. A previous publication has indicated the possibility of isomeric separation of permethylated glycans on a porous graphitized carbon (PGC) column.[29] Recently, we have optimized separation conditions to overcome peak broadening for acidic glycans and improve resolution for neutral glycans, thus achieving isomeric separation of permethylated glycans.[26, 28]

As mentioned above, there are multiple derivatization methods and separation techniques for LC-MS analysis of glycans. Each type of derivatization induces different properties and modifies glycan retention on different LC columns. A comprehensive assessment is necessary for providing guidance in selecting derivatization reagents and LC columns. In this study, we have investigated the derivatization reagents 2AB, ProA, aminoxyTMT, RFMS labeling, and permethylation. We compared the separation efficiency of the derivatized glycans on C18, hydrophilic interaction chromatography (HILIC), and PGC columns using both analytical LC-MS and nanoLC-MS. The quality of MS^2 spectra generated by the different strategies was also assessed for glycan structure elucidation. This investigation represents the first critical assessment of such a wide spectrum of N-glycan labeling reagents for quantitative glycomics.

Materials and Methods

Materials

ProA, 2-AB, iodomethane, and ammonium borane complex were purchased from Sigma-Aldrich (St. Louis, MO). The AminoxyTMT kit was provided by Thermo Scientific (Rockford, IL) and the RFMS kit was provided by Waters (Milford, MA) (Figure 1). Formic acid and all HPLC grade solvents including water, methanol, ethanol, acetonitrile, and isopropanol were obtained from Fisher Scientific (Pittsburgh, PA). Sodium hydroxide beads and high purity DMSO for permethylation were bought from Sigma-Aldrich (St. Louis, MO). Ribonuclease B (RNase B) and fetuin were obtained from Sigma-Aldrich (St. Louis, MO) and the mAb standard (Intact mouse IgG1) was provided by Waters (Milford, MA).

Dialysis membrane with MWCO 500–1000 Da was purchased from Spectrum Laboratories, Inc.

Sample Preparation

Enzymatic Release of Glycans—The N-glycans were released from a glycoprotein mixture consisting of 6 μg RNase B, 6 μg fetuin and 4 μg IgG standard using the conventional overnight PNGase F digestion protocol.[30, 31] Briefly, samples were mixed with 50 mM phosphate buffer solution, pH 7.5, in a 1:1 ratio and denatured by incubation in an 80 °C water bath for 30 min. Then, samples were allowed to cool to room temperature, an excess of PNGase F was added, and the mixture was incubated in a 37 °C water bath for 18 hours. Protein precipitation was conducted after digestion to partially purify released N-glycans. Partially purified glycans were then subjected to either 2AB labeling, ProA labeling, aminoxyTMT labeling, reduction or reduction and permethylation.

Reductive Amination Labeling—2AB labeling and ProA labeling was conducted using reductive amination conditions.[32] Dried glycans with 0.39 mg of 2AB or 0.75 mg of ProA were resuspended in 2.8 μL of 0.1M acetic acid, and 4.7 μL of 1.0 M sodium cyanoborohydride in THF was then added to the mixture. The reaction was carried out in a 60 °C water bath for 2 hours. After incubation, 100 μL of water was added to stop the reaction. 2-AB or ProA labeled samples were purified by floating dialysis for 18 hours to remove excess labeling and reducing reagents.[33–35]

Labeling Through Oxime Bond Conjugation—AminoxyTMT labeling was conducted following the protocol we recently published.[36] Dried glycans were dissolved with an excess AminoxyTMT^0-126 reagent in 200 μL of 95% methanol, 0.1% acetic acid aqueous solution, with agitation for 10 minutes using a vortex mixer. Then the solution was dried with a centrifugal vacuum concentrator. Another 200 μL of 95% methanol aqueous solution was added to the dried sample, and a vortex mixing-drying cycle was performed. Finally, 100 μL of a 10 % acetone solution was added to quench unreacted aminoxyTMT reagent.

Reduction and Permethylation—The reduction was conducted by adding 10 μL of 10 μg/μL borane ammonium complex to dried glycans and incubating at 60 °C for one hour. A methanol wash was applied to remove excess reducing reagent. A solid-phase permethylation protocol was employed to permethylated reduced glycans.[25, 37, 38] Briefly, dried glycans were resuspended in 30 μL of DMSO, 1.2 μL of water and 20 μL of iodomethane and applied to a freshly packed sodium hydroxide bead spin column. After 25 minutes of incubation at room temperature, another 20 μL of iodomethane was added to the spin column and incubation continued for 10 minutes. The mixture was then eluted from the spin column by centrifugation. A final elution of remaining permethylated glycans was achieved by passing 100 μL of acetonitrile through the column. The eluent was then dried overnight in a centrifugal vacuum concentrator due to the existence of DMSO and high concentration salts. For each derivatization method, three independent preparations were performed to estimate the variation from sample handling protocols.

Glycosylamine Derivatization—The same amount of model glycoproteins was denatured at 90 °C for 3 minutes in the presence of RapiGest and Rapid PNGase F buffer. Deglycosylation was then conducted in a 50 °C water bath, for 5 minutes, with the addition of 1.2 μL of Rapid PNGase F. The digested samples were directly subjected to RFMS labeling without purification. Briefly, 12 μL of RapiFluor-MS Reagent solution was added to the deglycosylation mixture, and the reaction was allowed to proceed at room temperature for 5 minutes. After which, 358 μL of acetonitrile was added in preparation for HILIC solid phase extraction (SPE). A GlycoWorks μElution Plate was used during the SPE procedure. After loading the sample and washing twice with 600 μL of 1:9:90 (v/v/v) formic acid/water/acetonitrile, RFMS labeled glycans were eluted with 3 30 μL volumes of GlycoWorks SPE Elution Buffer (200 mM ammonium acetate in 5% acetonitrile).

LC-MS Methods

HILIC—Derivatized samples were first subjected to analytical LC coupled to an Exactive Orbitrap MS. Mobile phase A was 50 mM ammonium formate solution, and mobile phase B was acetonitrile. Reducing end labeled and reduced native samples were separated on a Waters ACQUITY UPLC BEH amide HILIC column (1.0 mm × 150 mm i.d., particle size 1.7 μm) with the recommended gradient provided by the RFMS kit. The temperature was set to 60 °C, and the flow rate was set to 80 μL/min. Initially, the gradient ramped mobile phase A from 25 to 46%, over 35 minutes. The gradient then ramped from 46 to 100% solvent A, from 35 to 36.5 minutes. 100% solvent A was held constant from 36.5 to 39.5 minutes, after which the percentage of solvent A decreased to 25%, from 39.5 to 43 minutes. A 25% solvent A was maintained from 43 to 55 minutes, completing the run.

RPLC—Permethylated glycan samples were separated by a Kinetex C18 column (2.1 mm × 50 mm, particle size 5 μm, pore size 100 Å). The gradient was adjusted to a reverse phase LC gradient, while the mobile phase and flow rate were kept the same as the HILIC separation in order to reduce the influence of LC conditions on the quantitative results. Briefly, 20% solvent B was maintained from 0.0 to 10 minutes. Solvent B was next ramped from 20 to 38%, from 10 to 12 minutes. Solvent B was then ramped from 38 to 70%, from 12 to 41 minutes, and then solvent B was again ramped from 70 to 95%, from 41 to 42 minutes. From 42 to 48 minutes solvent B was held at 95%. After which solvent B was decreased from 95 to 20%, from 48 to 50 minutes. Finally, solvent B was held at 20% 5 additional minutes, thus completing the LC run.

Exactive Orbitrap Settings—The Exactive Orbitrap MS was set to a resolution of 50,000 and a 500–2000 *m/z* acquisition range for full MS scanning was used. A heated electrospray ionization (HESI) source was utilized with the capillary temperature set at 275 °C and the heater set at 300 °C. The spray voltage was set to 3.6 kV, and the sheath and auxiliary gas settings were 15 and 5 (arbitrary units), respectively. A mass accuracy of 10 ppm with boxcar (7-point) peak smoothing was used for plotting the extracted ion chromatograms (EIC) for all data acquired by the Exactive Orbitrap MS.

Nano RPLC for reducing end labeled glycans—All samples were also subjected to nanoLC-MS analysis. On this platform, the LC flow rate was set to 0.35 μL/min. C18 nano

and PGC nano columns were employed for the separations. For nano C18 separations a Thermo Scientific Acclaim PepMap RSLC column (75 μm × 150 mm i.d., particle size 2 μm, pore size 100 Å) was used, solvent A consisted of 98% acetonitrile with 2% water and 0.1% formic acid and solvent B was 100% acetonitrile with 0.1% formic acid. The gradient initially ramped from 2% to 5% solvent B, from 0.0 to 36 minutes. Then, from 36 to 68 minutes the solvent ramped from 5 to 40% solvent B. The gradient next ramped from 40 to 90% solvent B, from 68 to 69 minutes. From 69 to 72 minutes the gradient was held at 90% solvent B. Finally, from 72 to 75 minutes the solvent ramped from 90 to 5% solvent B.

Nano RPLC for permethylated glycans—The mobile phases, flow rate and RPLC column were the same as the ones used for analyzing reducing end labeled glycans in order to make a relative fair comparison. The gradient started at 20% mobile phase B for 10 min, and increased to 38% at 11 min. The organic phase gradually developed to 60% in the next 32 min. After that, it increased to 90% within 3 min and maintained for the next 4 min. Finally, the percentage of mobile phase B decreased to 20% in 1 min and kept at 20% for 9 min to pre-equilibrate the system.

Nano PGC Chromatography—For nano PGC separations a Thermo Scientific HyperCarb PGC column (75 μm × 100 mm i.d., particle size 3 μm, pore size 250 Å) was used, and the flow rate was 0.35 μL/min. The solvents utilized were the same as the solvents used for C18 nano separations. The gradient began at 2% solvent B and ramped to 15% solvent B over 36 minutes. It then ramped from 15 to 60% solvent B, from 36 to 47 minutes. After which the gradient ramped from 60 to 90% solvent B from 47 to 60 minutes. A 90% solvent B was maintained from 60 to 72 minutes, then the gradient was ramped from 90 to 5% solvent B, from 72 to 75 minutes.

LTQ Orbitrap Velos Settings—nanoLC was coupled to an LTQ Orbitrap Velos tandem MS through nano-electrospray ionization (ESI). The capillary temperature was set to 300 °C, and the spray voltage was 1.6 (kV). In full scan mode, the resolution was set to 15,000. Each MS scan preceded 4 MS/MS events using ion trap collision-induced dissociation (CID) and higher-energy collisional dissociation (HCD) operated in data dependent acquisition (DDA) mode. CID collision energy was set to 30% of the normalized energy value with a 15 ms activation time, and activation Q was set to 0.250. A mass accuracy of 10 ppm with boxcar (3-point) peak smoothing was used for EICs for data acquired by the LTQ Orbitrap Velos MS.

Result and Discussion

The time spent for sample preparation using each derivatization method are summarized in Table 1. Due to the Rapid PNGase F release method provided by the RFMS kit, the RFMS derivatization method exhibited the fastest sample preparation time. Derivatization protocols for reducing end labeling by 2-AB, ProA, aminoxyTMT and reduction and permethylation all contain 20 hours for glycan PNGase F digestion and purification. The sample handling time for these derivatization methods varies from one to two days. The advantage of the RFMS labeling method is obvious when sample preparation time is considered.

The assessment of MS intensity enhancement of the different labeling methods was conducted using analytical LC-MS because standard flow HESI is more stable and reliable compared to nano-ESI. All derivatized glycans were separated on a HILIC column (Figure 2a–e) except for the permethylated glycans, whose hydrophobicity was significantly increased by permethylation. Permethylated glycans were instead separated on a C18 column (Figure 2f). Both columns were operated using the same mobile phase and flow rate to make sure the MS intensity comparison was not influenced by LC conditions. From the EIC traces of the different methods, we observed good separation of the different glycans on both HILIC and C18 columns. HILIC-LC provided isomeric separation of structural or linkage isomers; however, C18-LC was not able to separate all isomers present in the permethylated glycan samples.

Table S-1 depicts the distributions of differently derivatized high mannose glycans derived from RNase B. For all derivatization methods, the standard deviation was less than 10% for all, indicating a good reproducibility of each derivatization method. Since the intensities of unlabeled glycans we observed in full ms were lower than 1%, taking ionization efficiency into account, it is safe to claim that labeling efficiency of higher than 95% can be acquired from all derivatization methods. RFMS labeled glycans exhibited the highest intensity of all derivatization strategies investigated, for glycans released from IgG (Figure 3a), where permethylation resulted in intensities 1.4, 2.2, 6.0, 3.1 times higher than ProA, aminoxyTMT, reduced native and 2-AB labeled glycans; meanwhile, the intensities of RFMS glycans were approximately 1.3 times higher than the permethylated glycans. These results indicated that RFMS provided the highest signal enhancement for neutral glycans. However, it must be stated that because the RFMS release protocol involves the use of the surfactant RapiGest SR, it is possible that increased glycan accessibility played a role in the signal enhancement observed. We tried to investigate the possibility of adding RapiGest to the other protocols, but the addition of RapiGest before carrying out the other derivatization procedures (reductive amination, permethylation etc.) will result in substantial byproducts. To address this issue, additional purification steps were needed to remove RapiGest, which would influence the quantitative comparisons negatively.

For the sialylated glycans released from fetuin, the advantage of permethylation in enhancing signals from acidic glycans was apparent, since permethylation is the only derivatization method, among the six methods, examined, that modifies the carboxylic acid groups of glycans. As shown in Figure 3b, the intensities of permethylated sialylated glycans are much higher than the reducing end labeled or native glycans. Take the tri-antennary tri-sialylated glycan as an example, the intensity from permethylation is about 5 times higher than RFMS labeling, about 10 times higher than ProA and aminoxyTMT labeling, and more than 100 times higher than 2-AB derivatization and the reduced native form. Also, the distribution of sialylated glycans from fetuin differ between the different derivatization methods. For example, the intensity ratio of the tri-antennary tri-sialylated glycan over the bi-antennary bi-sialylated glycan in the analysis of permethylated glycans is 3.5, while that ratio for RFMS, ProA, aminoxyTMT, 2-AB labeling, and reduced native form are 1.2, 1.2, 1.1, 1.3 and 1.5, respectively. While according to NMR data, in the scientific literature, the ratio is 3.5[39]. This indicates that the intensity observed from the permethylation method for

quantitative analysis of sialylated glycans is a more accurate representation of the molar abundance of the different glycans.

This can be explained by the different ionization efficiency improvement mechanisms utilized by permethylation and reducing end labeling strategies and that fact that sialic acid can be lost in the ESI source for non-permethylated glycans. Sialic acid in a glycan structure functions to decrease the overall proton affinity of the molecule. Although the labeling tags in reducing-end derivatization methods increase the probability of positive charges attaching, the overall ionization efficiency is still significantly influenced by the number of sialic acids present. The ionization efficiency of tri-sialylated glycans is lower than bi-sialylated glycans, resulting in the offset of the MS intensity ratio from the molar ratio. While in the case of permethylation, sialic acid is also modified in order to eliminate the heterogeneity of ionization efficiency between the different types of monosaccharides. In this strategy, all glycans have the same or similar proton affinity despite the different chemical properties of individual monosaccharides. The MS intensity distributions of permethylated glycans are comparable to their molar distributions suggested by NMR data.[39] All data are summarized in Tables S-1 and S-2.

After assessment of the MS quantitation performance of the different derivatization methods, all samples were subjected to analysis using a nanoLC-MS platform to investigate their performance in a nano-flow system. The first test was conducted using a C18 nano column for nanoLC separation. The resolution of permethylated glycans was determined to be similar to the resolution observed on the analytical scale column, described above. Due to the fact that native glycans cannot be retained on a C18 column, there was no separation for reduced native glycans on C18. The separation of reducing end labeled glycans was quite different from the separation observed on the HILIC column. Here we take the RFMS labeled glycans as a representative example to discuss their retention on C18. Figure S-1a represents the separation of high mannose glycans. With the addition of mannose, the retention time of glycans decreases. A similar trend was also observed for the IgG glycans, which also exhibited reduced retention time with increased galactose residues (Figure S-1b). Hence, we can draw the conclusion that the hydrophobicity of a hexose (Hex) is less than the glycan core structure plus the tag. This property directly resulted in the weak retention of high mannose glycans observed. There was unexpected peak splitting for Man8 and Man9 due to the weak interaction between glycan and stationary phase. For the fucosylated bi-antennary mono-galactosylated glycan, the isomeric separation was also observed during nano C18 separation with a slightly lower resolution compared with the HILIC separation. The CID MS/MS spectrum demonstrated different fragment ion ratios that may be utilized to elucidate structure. However, as previously reported,[40] fragments resulting from fucose migration were also observed in MS^2, indicating instability of the reducing end-labeled glycan structures in the gas phase (Figure S-2).

Sialic acid, under the acidic separation conditions used here, is the monosaccharide that contributes most to glycan retention on a C18 column. For example, the RFMS labeled bi-antennary mono-sialylated glycan has a retention time similar to the tri-antennary mono-sialylated glycan, and the bi-antennary bi-sialylated glycan has a retention time comparable to the tri-antennary bi-sialylated glycan. However, the bi-antennary neutral glycan (Figure

4a), which was not reported in a previous NMR study,[39] was found to have two clusters of peaks with retention times matching the mono and bi-sialylated structures. Also, in the EIC of the bi-antennary mono-sialylated glycan, there were two peak clusters in addition to the cluster that appeared at the correct retention time (Figure 4b). The two unexpected peak clusters match the retention times of the bi-sialylated glycans (Figure 4c and d) and the tri-antennary tri-sialylated glycan (Figure 4e), indicating that these additional peaks for the bi-antennary mono-sialylated glycan are from sialic acid loss or sialic acid branch (GlcNAc-Gal-Neu5Ac) loss during the ionization process. Under the full MS spectra at the retention time of the tri-antennary tri-sialylated glycan, we also identified the *m/z* of the tri-antennary bi-sialylated glycan and GlcNAc-Gal-Neu5Ac (Figures 4f and 5). Figures 5 and 6 both display EICs of a GlcNAc-Gal-Neu5Ac glycan fragment, at *m/z* 657.2, that is derived from in-source fragmentation of reducing end labeled sialylated structures. In Figure 5a–c the mass spectra averaged over the peak width of RFMS labeled biantennary monosialylated, bi-antennary disialylated and triantennary tri-sialylated structures are shown, respectively. While in Figure 6a–d the mass spectra averaged over the peak width of aminoxyTMT labeled biantennary monosialylated, biantennary disialylated, triantennary tri-sialylated and tri-antennary tetra-sialylated structures are shown, respectively. In all of the mass spectra depicted in Figures 5 and 6 the ion at *m/z* 657.2 representing the GlcNAc-Gal-Neu5Ac glycan fragment can be observed accompanying the ions corresponding to the intact structures. The structural analyses were done using nano ESI with capillary voltage set to 1.6 kV, which was lower than the voltage added to ESI source (3.7 kV) that are commonly used for reducing end labeled glycans.[41, 42] Thus, the loss of sialic acid or the GlcNAc-Gal-Neu5Ac fragment is a commonly existed phenomenon in MS analysis of reducing end labeled glycans.

During nano-ESI approximately 10% of the sialylated glycans endured in-source fragmentation, while the fragmentation percentage for normal flow HESI was around 2%. In this case, optimized ionization conditions for the analysis of all N-glycans, including both neutral and acidic structures, were utilized. Optimized conditions for the reduction of sialic acid fragmentation of labeled glycans have been reported for glycans using the individuality normalization strategy;[43] however, in our study, a more widely applicable method was deemed appropriate for our comparisons. Additionally, despite reduced sialic acid fragmentation being observed with optimized ionization conditions used in conjunction with the INLIGHT strategy, higher sensitivity is still achieved through the sialic acid stabilizing process of permethylation. Sialic acid loss is always an issue with native or reducing end labeled glycans, in nanoLC-MS analysis the problem becomes more serious. While this problem can be overcome by esterification of sialylated structures,[44–46] this introduces additional derivatization and clean up steps which create the potential for sample loss. From our observations, we can draw the conclusion that a C18 column can be used for the separation of reducing end labeled and permethylated glycans. More isomeric separation was observed for reducing end labeled glycans, whereas permethylation masked the structural differences resulting from linkage isomers. However, the instability of reducing end labeled glycan structures presents a significant problem and is the main drawback to their analysis by nano-LC-MS.

During nano scale, PGC-LC-MS analysis, a separation similar to what was achieved on C18 was observed for reducing end labeled neutral glycans (Table 1).[32] However, it was reported that the elution of highly sialylated glycans from PGC is difficult for reducing end labeled and reduced native glycans.[47–49] This issue can be overcome by adjusting the mobile phase to increase ion pairing interactions.[47, 48] However, ion suppression has been reported towards different ion paring reagents,[50] yet On the other hand, PGC separation of permethylated glycans was observed to be efficient, in agreement with our previous publications,[26–28] and all isomers were baseline resolved.

The adduct formations and charge state distributions were found to be different for the different derivatization methods under the same LC-MS conditions. Figure S-2 displays the spectra for differently derivatized Man5 separated on a C18 column, in the case of permethylated samples, or a HILIC column and analyzed using an Exactive Orbitrap MS. The permethylated Man5 mainly existed as a doubly charged ammonium adduct. The fully protonated form was present at less than 1% of the total abundance (Figure S-2a). Reduced native Man5 was found as singly charged protonated, ammoniated and sodiated forms (Figure S-2c). ProA and AminoxyTMT had similar patterns (Figure S-2b and e, respectively), which were doubly charged protonated and sodiated forms. 2-AB and RFMS labeled glycans exhibited the simplest adduct forms and charge states (Figure S-2d and f, respectively). 2-AB labeled glycans were mainly monoprotonated and RFMS labeled glycans were doubly protonated. This can be an advantage, because the simplified adduct forms and charge states may contribute to quantitative reliability; and a disadvantage, because sometimes formation of different adducts is required to obtain different fragmentation patterns in MS^2. For example, the aminoxyTMT labeled glycans require sodiated forms to ensure a high yield of reporter ions.

Conclusion

In this study, we comprehensively assessed six different derivatization methods and their analysis on LC-MS and nanoLC-MS systems. Different LC columns were also investigated to determine their compatibility with different derivatization methods. All methods were compared under the optimum conditions previously reported and no additional optimization was performed sine this was not the scope of this study. As summarized in Table 1 each method of analysis has pros and cons. RFMS method is enabling the shortest sample preparation while permethylation is the only method that eliminated sialic acid loss and rearrangement. However, sialic acid loss in the case of the other methods can be overcome through an additional chemical treatment step. For neutral glycans, RFMS provided the highest MS signal while permethylation exhibited a significant advantage in increasing MS intensity and structural stability for sialylated glycans. The strong RFMS signal is partially reulting from the enzymatic release method. Althouh signal for all other methods might have increased if the enzymatic release method of RFMS was uses, the focuse of this study is to compare existing methods as described in the literture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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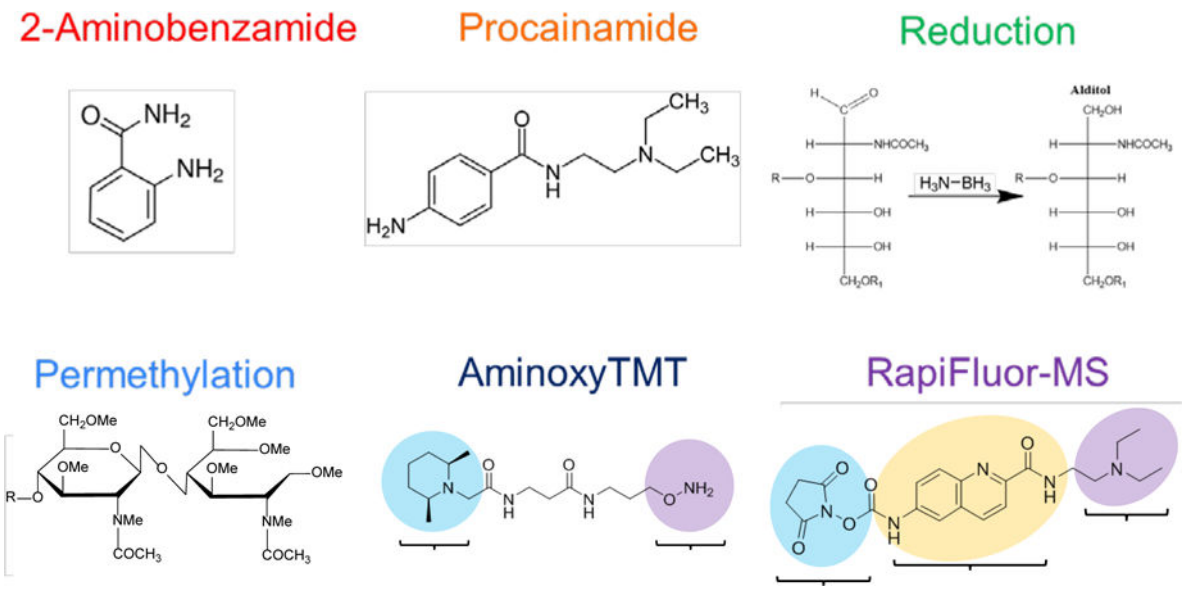
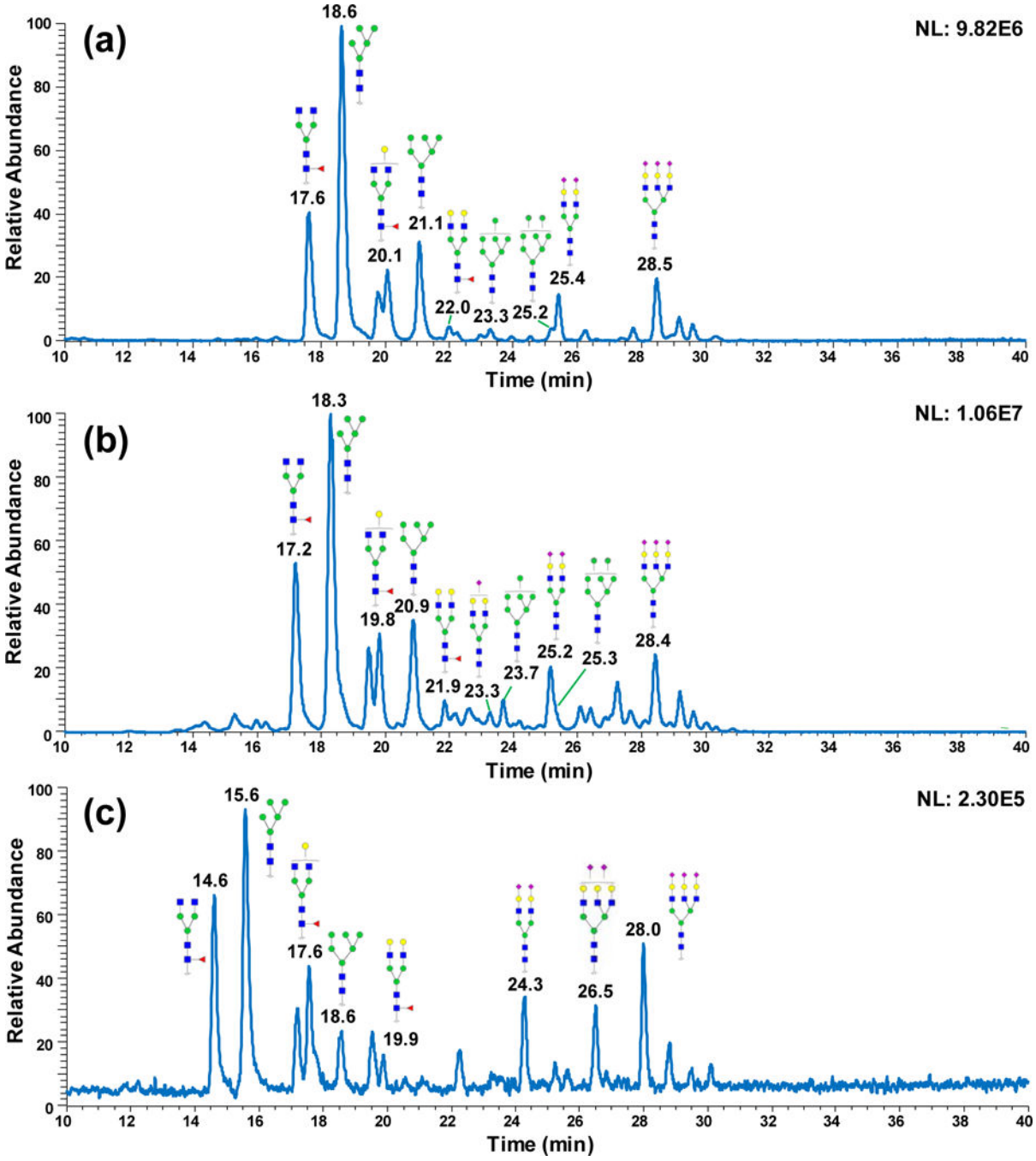


Figure 1.

An illustration of the glycan derivatization methods described herein. Regarding the reduction illustration, R, 3-linked saccharide; R1, 6-linked saccharide.



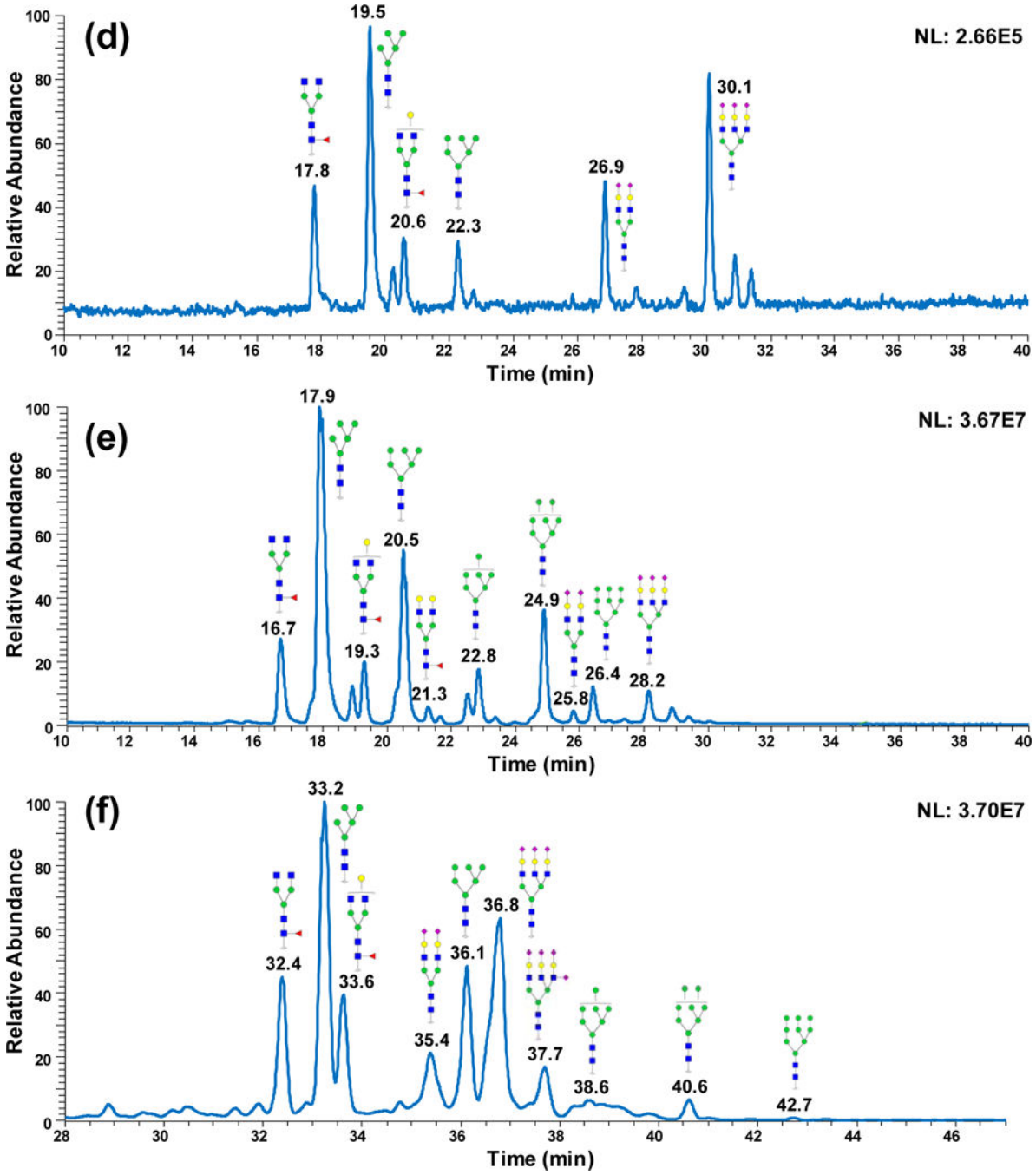


Figure 2.

EIC of differently derivatized glycans, **a**-AminoxyTMT labeled, **b**-ProA labeled, **c**-2AB labeled, **d**-Reduced native, **e**-RFMS labeled and **f**-Permethylated. Reducing end labeled glycans and reduced native glycans were separated using HILIC-MS. Permethylated glycans were separated using RPLC-MS.

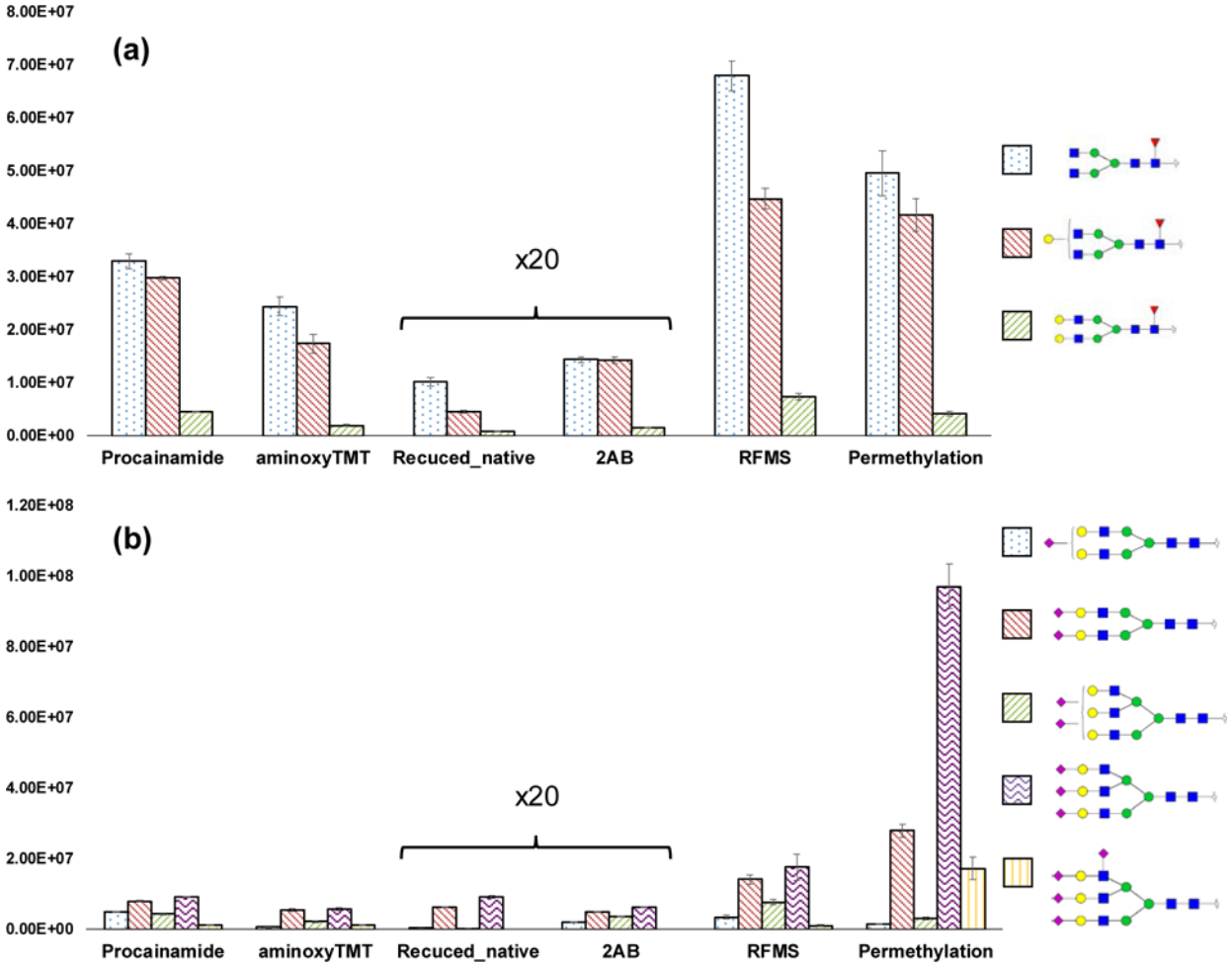


Figure 3.

Bar graphs representing the intensities of differently derivatized glycans released from **a**-IgG and **b**-fetuin analyzed by LC-MS. The intensities of reduced native and 2-AB labeled glycans were increased by 20-fold.

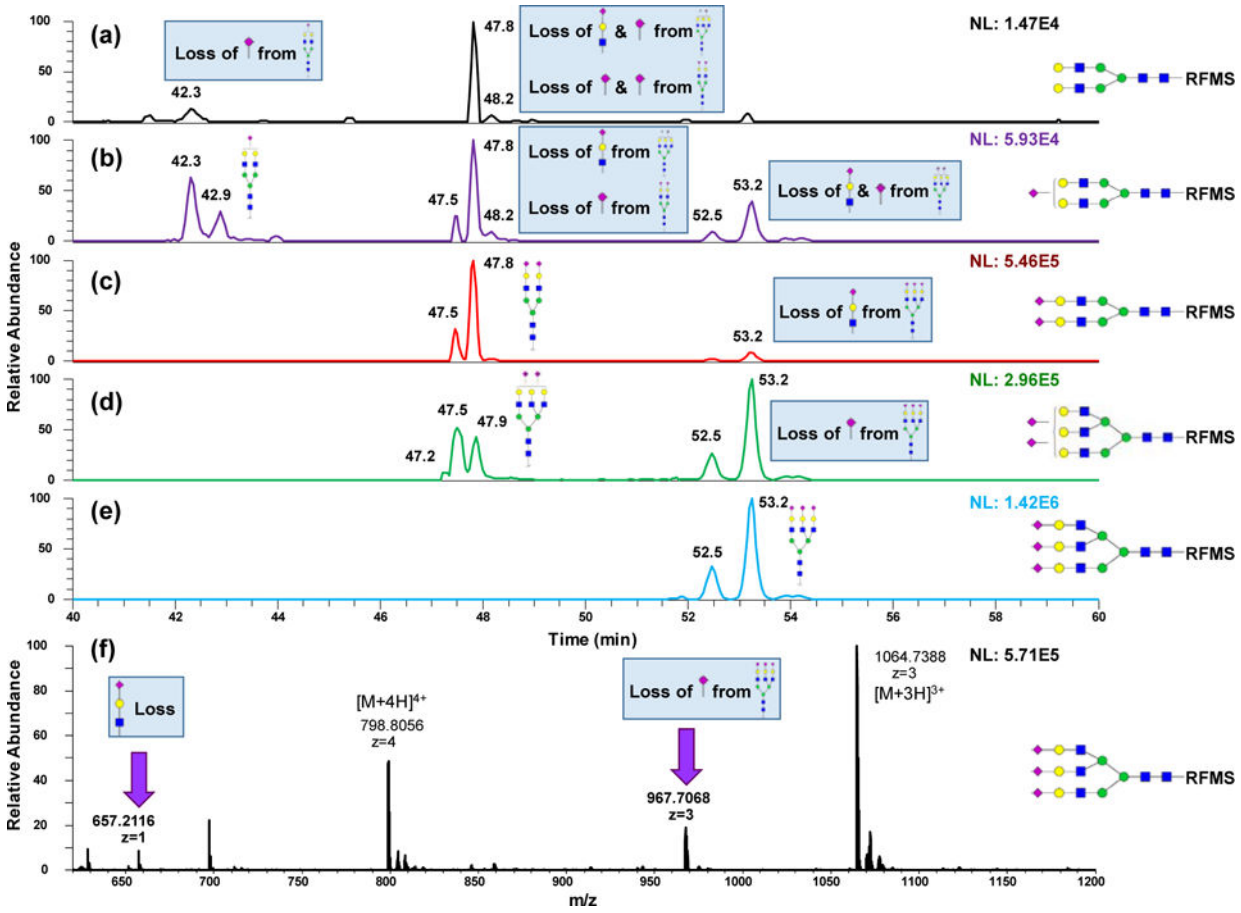


Figure 4.

EICs of RFMS labeled glycans released from fetuin separated on a C18 nano column (**a-e**). Panel **f** depicts the full MS spectrum corresponding to the peak at 53.2 min, the retention time of the tri-sialylated glycan shown in panel **e**.

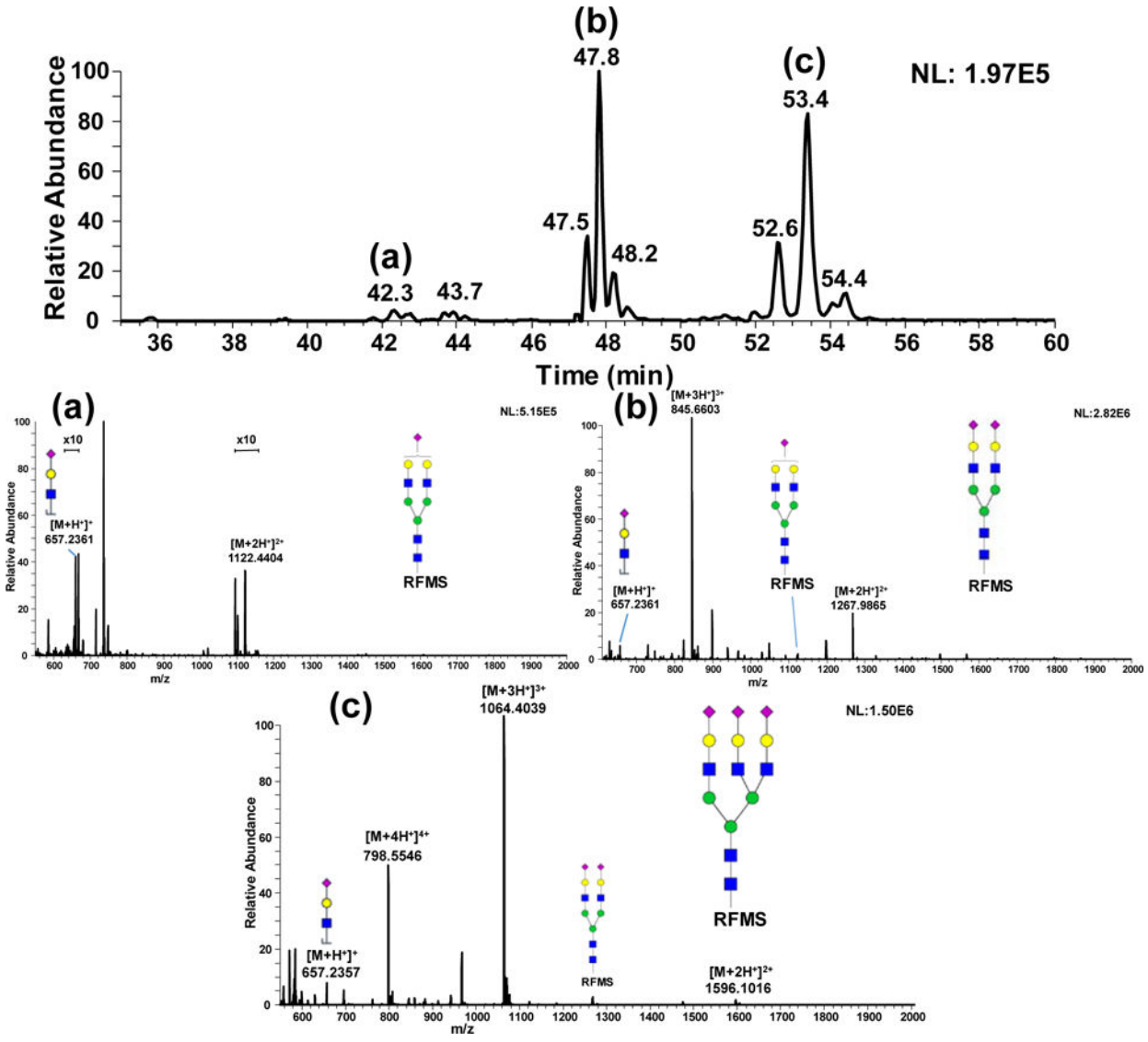


Figure 5.

EIC at *m/z* 657.2 of a glycan fragment of GlcNAc-Gal-Neu5Ac arising from in-source fragmentation of RFMS labeled glycans. Panels **a**, **b** and **c** depict the mass spectra averaged over the peak width of biantennary monosialylated, biantennary disialylated and triantennary trisialylated structures, respectively. The separation was performed using a nano C18 column and a LTQ Orbitrap Velos MS was used for detection.

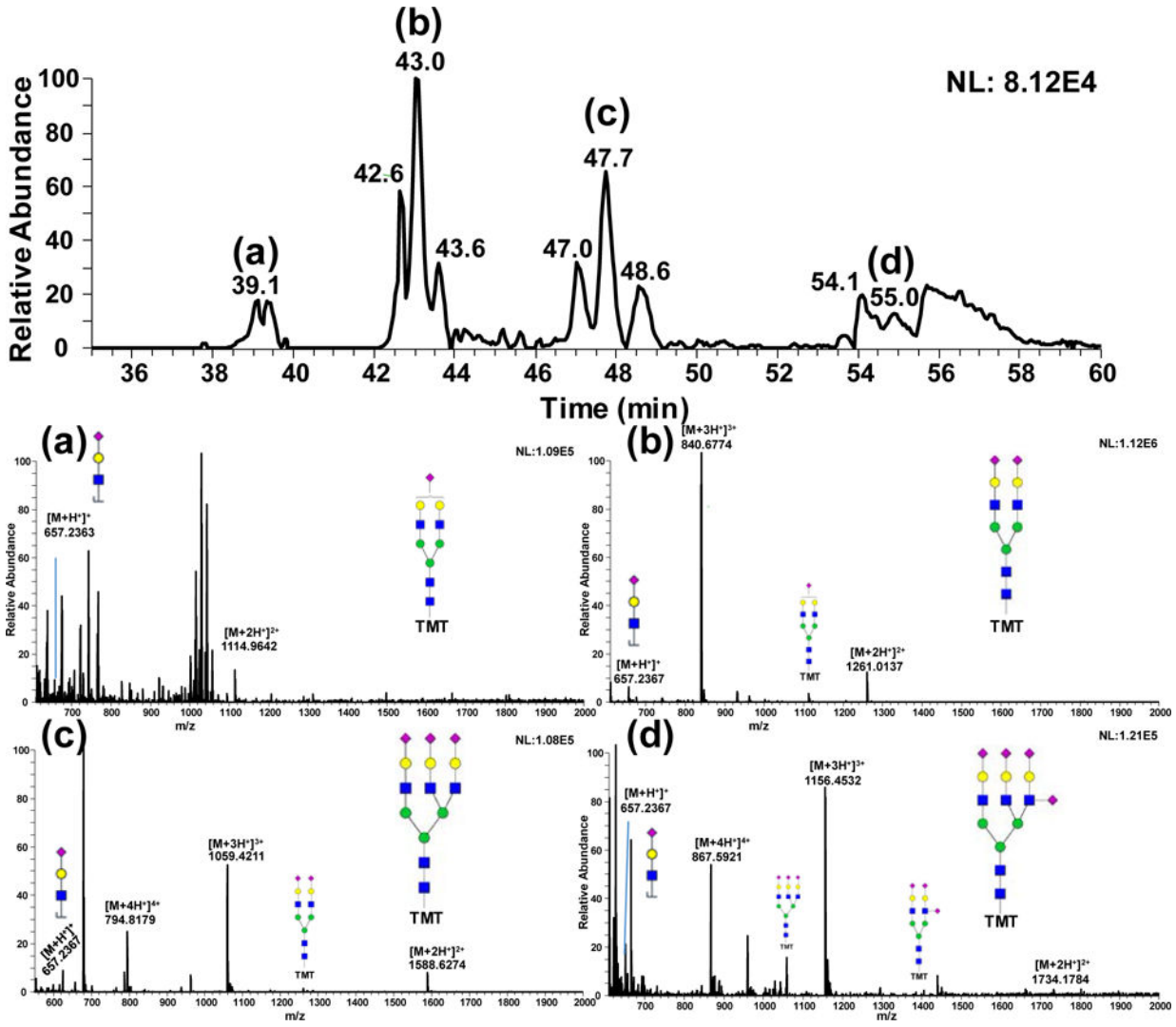


Figure 6.

EIC at *m/z* 657.2 of a glycan fragment of GlcNAc-Gal-Neu5Ac arising from in-source fragmentation of aminoxyTMT labeled glycans. Panels **a**, **b**, **c** and **d** depict the mass spectra averaged over the peak width of biantennary monosialylated, biantennary disialylated, triantennary trisialylated and triantennary tetrasialylated structures, respectively. The separation was performed using a nano C18 column and a LTQ Orbitrap Velos MS was used for detection.

Table 1

Summary of different derivatization methods sample preparation time, susceptibility to sialic acid loss and rearrangement and separation performance on different columns.

	Procainamide	**aminoxyTMT**	**Reduced native**	**2AB**	**RapiFluor-MS**	**Permethylation**
Sample prep duration	~ 40 hrs	~ 22 hrs	~ 24 hrs	~ 40 hrs	~ 1 hr	~ 48 hrs
Rearrangement/ Sialic acid loss	Yes	Yes	Yes	Yes	Yes	No
Separation on HILIC column	isomeric separation	isomeric separation	isomeric separation	isomeric separation	isomeric separation	N/A
Separation on C18 column	isomeric separation except for high mannose	isomeric separation except for high mannose	N/A	isomeric separation except for high mannose	isomeric separation except for high mannose	partial isomeric separation
Separation on PGC column	isomeric separation for neutral glycan	isomeric separation for neutral glycan	isomeric separation	isomeric separation for neutral glycan	isomeric separation for neutral glycan	isomeric separation