

***N*-linked Glycan Release Efficiency: A Quantitative Comparison between NaOCl and PNGase F Release Protocols**

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There are several methods, both chemical and enzymatic, to release *N*-linked glycans for structural characterization. One of the most common enzymatic release methods is the use of peptide:*N*-glycosidase F (PNGase F). A less expensive and quicker alternative has been reported for the release of *N*-linked glycans chemically using sodium hypochlorite (NaOCl), which hydrolyzes the peptide-glycan bond, yielding the intact glycan with a free reducing terminus. Here, we quantitatively analyzed the efficiency of the NaOCl release protocol compared with the PNGaseF release protocol for small-scale analysis (300 µg) using liquid chromatography–single reaction monitoring–mass spectrometry. We determined that the relative glycan composition of released *N*-linked glycans from the NaOCl protocol is similar to a typical PNGase F protocol, but the absolute recovery of *N*-linked glycans is significantly lower with the chemical procedure.

KEY WORDS: glycomics, peptide:*N*-glycosidase, release efficiency, sodium hypochlorite

INTRODUCTION

Glycosylation is a post-translational modification that affects both the structure and function of glycoproteins. Characterization of these intact and released glycans, especially *N*-linked glycans, has the potential to lead to therapeutic targets and possibly treatments for many of genetic defects and their disorders. Many carbohydrate-deficient glycoprotein syndromes have been identified since the 1980s through glycomic profiling.^{1, 2} For example, a difference in one's glycan profile compared with a control subject could be indicative of a disease and possible route for therapy. The ability to completely characterize a subject's glycomic profile has become important in understanding the overall biologic makeup of a subject.

Studying *N*-linked glycan profiles relies on the ability to release the complete, intact glycans from their glycoproteins. A typical protocol for analyzing the glycan profile of a glycoprotein starts with a protein digest (usually trypsin) to digest the protein, followed by one of the glycan release methods. Following the release, the glycans are collected and usually tagged [*i.e.*, procainamide (ProA) labeling] before a mass spectroscopy (MS) technique can be utilized.

There are several release mechanisms for *N*-linked glycans that can be broken up into 2 distinct categories: chemical release and enzymatic release. Two of the oldest and most well-studied methods for releasing *N*-linked glycans are both chemical. The first is β -elimination, which takes place under alkaline conditions and hydrolyzes the glycan linkage to release the complete glycan from the peptide backbone. This is performed in the company of a reducing agent, which aids in the prevention of base peeling.^{3, 4} Although it is normally utilized for *O*-linked glycan release, this technique can be used for *N*-linked glycans, albeit under much harsher alkaline conditions.⁵ The other major chemical release technique employed is hydrazinolysis.⁶ Hydrazinolysis is the technique of using anhydrous hydrazine to cleave the complete glycan from the peptide backbone.^{6, 7} Unfortunately, both the use of hydrazine and β -elimination cause degradation of the peptide backbone and can lead to unwanted modifications of the released glycans, which need to be rectified postrelease, such as loss of *N*-acetylation and the loss of the free reducing end.^{3, 4, 6, 7}

The use of enzymes has been commonly employed to cleave *N*-linked glycans from their peptides. Many of the earliest enzymes used to cleave *N*-linked glycans were the endoglycosidases, where a glycosidic bond is cleaved. Many of these enzymes are efficient but are limited by the types of glycans they can cleave. Endoglycosidase H (Endo H), which was one of the first commercially available endoglycosidases, only releases glycans that are high mannose or hybrids.⁸ Endo F (usually sold as a combination of Endo F1, F2, and F3) is very efficient for releasing high mannose and

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TABLE 1

| Scheduled transitions for pro-labeled <i>N</i> -linked glycans of fetuin and the ProA-labeled 6 GU standard | | | |
|---|---------------|---------------|----------------------|
| Glycan | Q1 transition | Q3 transition | Retention time (min) |
| 6GU + ProA | 1210.7 | 399.7 | 30.7 |
| A2G2S1 + ProA | 1076.5 | 440.8 | 36.5 |
| A2G2S2 + ProA | 1222.2 | 440.8 | 42.5 |
| A3G3S2 + ProA | 1259.1 | 440.8 | 40.5 |
| A3G3S3 + ProA | 1550.0 | 440.8 | 51.0 |
| A3G3S4 + ProA | 1695.7 | 440.8 | 55.0 |

hybrids like Endo H (F1 and F2), but Endo F (F3) is also capable of releasing bi- and triantennary complex glycans.^{9–12} Besides the limitations of which glycans are cleaved, the biggest sacrifice in utilizing these enzymes is their inability to release complete, intact glycans because it requires a glycosidic bond to be cleaved.

Glycoamidases, including peptide:*N*-glycosidase F (PNGase F), have been the most widely utilized methods for the release of *N*-linked glycans for decades because of their ability to release complete and intact *N*-linked glycans. Unlike endoglycosidases, which cleaves the glycosidic bond between 2 sugars, glycoamidases hydrolyze the amide link of a glycosamide. Both glycoamidases, PNGase F, and glycopeptidase A (PNGase A), cleave the bond between the innermost *N*-acetylglucosamine (GlcNAc) and the asparagine residue. PNGase A is less commonly used but has the ability to cleave all the high mannose, hybrid, and short complex glycans, with the additional ability to cleave glycans containing a 1,3-core fucose.^{13–15} However, PNGase A is unable to release larger, more complex glycans, such as those containing a sialic acid residue.^{15–18} Similarly to PNGase A, PNGase F keeps the peptide backbones intact but is also capable of cleaving nearly all *N*-linked glycans.^{10, 19–21} The one exception is that PNGase F is unable to cleave 1,3-core fucosylated species (usually found in plants).²² Lastly, although enzymes such as PNGase F and PNGase A are expensive and time consuming to use, the simplicity and the ability to release intact glycans without causing degradation of the peptide backbone or amino acid side chains, the enzymatic release of glycans has become the preferred method.

It was recently reported that an alternative chemical release technique using the sodium hypochlorite (NaOCl) in bleach as opposed to PNGase F has been developed for large-scale processing.²³ Song *et al.* demonstrated that the NaOCl chlorinates the glycan-peptide amide bond, thus forming an *N*-chloroamide. A pericyclic reaction leads to a glycan-isocyanate intermediate, which is further hydrolyzed, yielding the intact glycan with a free reducing terminus.²³ The protocol established by Song *et al.* is much cheaper and quicker than using

PNGase F. The researchers demonstrated that the matrix-assisted laser desorption ionization–time of flight–MS profiles obtained when the NaOCl protocol was used to release *N*-linked glycans from standard glycoproteins were the same as those obtained using the traditional enzymatic release without unwanted modifications. However, would the much cheaper and faster procedure produce quantitatively similar results on a microscale experiment to a PNGase F glycan release experiment? In order to compare the NaOCl procedure with PNGase F on a microscale level using absolute quantitation, both protocols were performed in parallel and analyzed using a liquid chromatography (LC)—single reaction monitoring (SRM)-MS.

MATERIALS AND METHODS

Sample preparation

Fetuin (1.8 mg) from fetal bovine serum (MilliporeSigma, Burlington, MA, USA) and 180 μ g of a maltohexaose (6

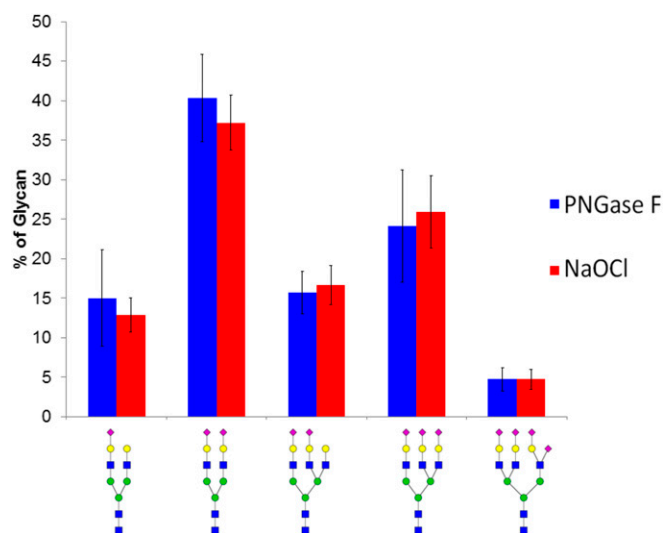


FIGURE 1

Glycan composition analysis: PNGaseF vs. NaOCl. The relative glycan profile for each replicate was taken for the 5 most abundant glycans of fetuin. The technical replicates for each were averaged, and the averages for the biologic replicates were averaged.

GU) standard (MilliporeSigma) were resuspended in 600 μl of H_2O . Six equal aliquot fractions (3 replicates for each preparation) were dried down. The glycans were then released following the assigned protocols.

PNGase F samples: Each was resuspended with 15 μg [20:1 (w/w)] of tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (MilliporeSigma) in 60 μl of 100 mM ammonium bicarbonate buffer. The samples were incubated overnight at 37°C. The samples were then heated for 5 min at 100°C to deactivate the trypsin. One microliter (500 U) of PNGase F (New England Biolabs, Ipswich, MA,

USA) was then added to the samples, and the samples were incubated for another 12 h at 37°C. The PNGase F samples were then cleaned using a C18 extraction column (Avantor Performance Materials, Radnor, PA, USA) and dried down.

NaOCl release²³: Samples were resuspended in 15 μl of H_2O (20 mg/ml), and 2.18 μl of Clorox bleach (6.85% NaOCl) was added while stirring. The samples were stirred for 15 min at room temperature. Formic acid (0.5 μl) was then added to quench the reaction, and the samples were stirred for an additional 5 min. The samples were then spun down on a benchtop microcentrifuge. The supernatant was

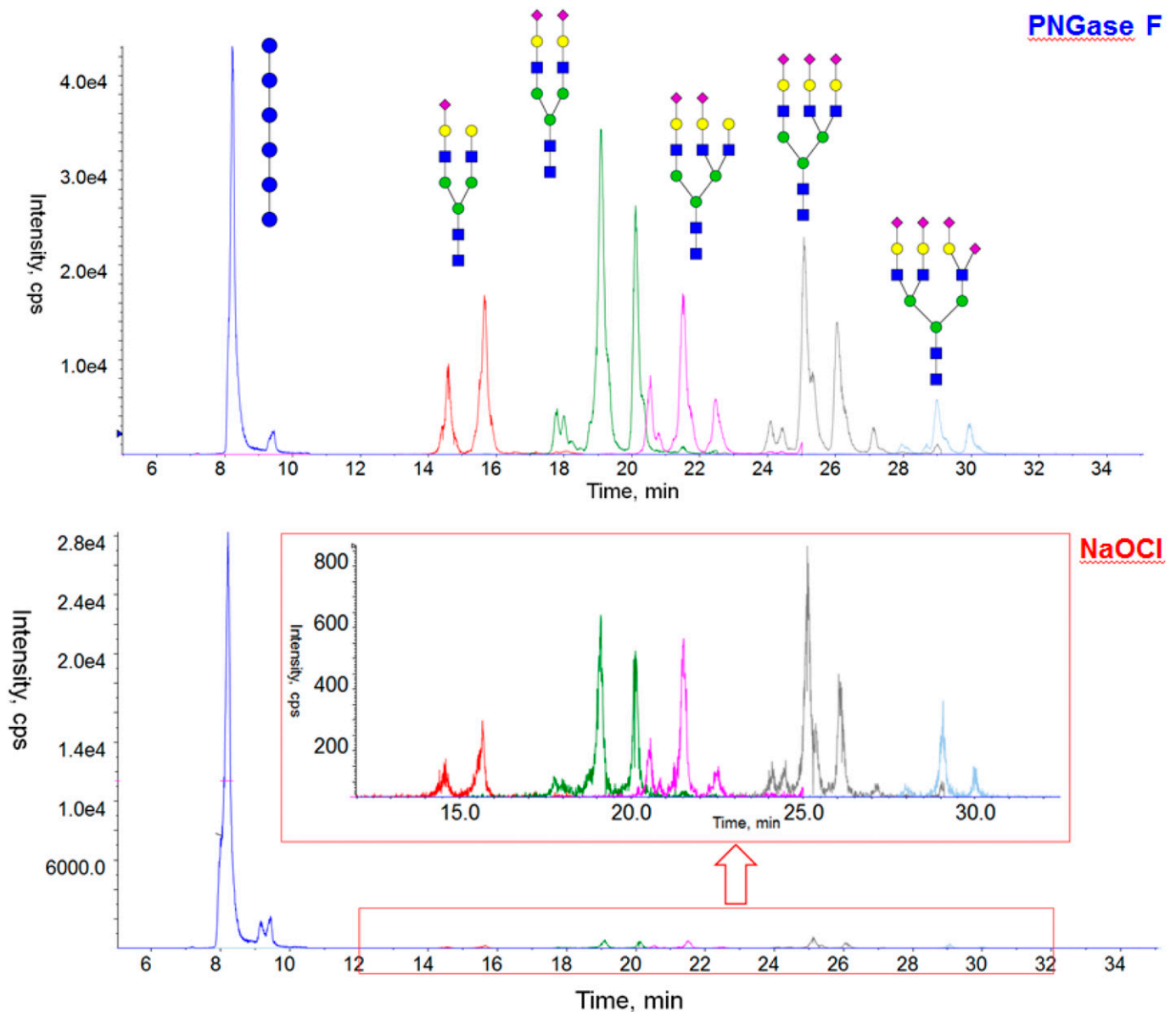


FIGURE 2

SRM LC traces of the ProA-labeled 6-GU standard and the ProA-labeled *N*-linked glycans from fetuin released using the PNGase F and NaOCl protocols. LC traces of the 5 most abundant glycans of fetuin [A2G2S1 (dark blue), A2G2S2 (red), A3G3S2 (green), A3G3S3 (gray), and A3G3S4 (light blue)] from SRM analysis following the PNGase F release (top) and NaOCl release (bottom) protocols.

collected for each sample and dried down. The samples were then cleaned on a PD MiniTrap G10 desalting column (GE Healthcare, Chicago, IL, USA) and a C18 extraction column (Avantor Performance Materials) before it was dried down.

The samples from both the chemical and enzymatic release protocols, which contained the released *N*-linked glycans of fetuin, were then resuspended in 20 μ l of H₂O, and 30 μ l of ProA-labeling solution [400 mM ProA HCL and 1 M sodium cyanoborohydride in 70/30 DMSO/acetic acid (v/v)] was added to each sample. The samples were incubated overnight at 37°C and then diluted with 250 μ l of H₂O before being cleaned on a PD MiniTrap G10 desalting column (GE Healthcare). All samples containing the ProA-labeled *N*-linked glycans were dried down.

LC–tandem MS settings and instrumentation

Data were acquired on a LC-30AC LC System (Shimadzu, Kyoto, Japan) that was coupled to a 4000 Q Trap LC/MS/MS System (Applied Biosciences, Beverly Hill, CA, USA). The mobile phase consisted of solvents A [99.9% H₂O, 5% acetonitrile (ACN), 0.1% formic acid, and 50 mM ammonium formate] and B (100% ACN). All solvents were HPLC grade (MilliporeSigma). A gradient elution was run from 68% B to 52% B over 16 min on a Halo Penta-HILIC, 2.1 \times 150 mm with 2.7- μ m pore size (Advance Material Tech, Wilmington, DE, USA) at a flow rate of 0.4 ml/min with the column being heated to 60°C. An SRM method was set up where retention times were scheduled based on previous data. For the SRM, a total of 6 transitions were scheduled (**Table 1**): 6GU Standard+ProA, A2G2S1+ProA, A2G2S2+ProA, A3G3S2+ProA, A3G3S3+ProA, and A3G3S4+ProA.²⁴ All samples were resuspended in 25 μ l of H₂O. The sample (12 μ l) was mixed with 48 μ l of ACN, and 15 μ l was injected for each run. Each replicate was run in triplicate.

For the glycan degradation analysis, an SRM was also performed, and the q3 transitions were either GlcNAc+ProA or Mannose + ProA, depending on which glycan residues we were analyzing.

Data analysis of SRM

All of the peaks representing the 5 scheduled glycans along with the peak representing the scheduled internal standard were integrated using Analyst software (Applied Biosciences) to determine the total ion abundance of each peak.

RESULTS

Cost comparison

The comparative costs in labor and dollars of these 2 procedures were striking. The PNGase F procedure costs

between 10 and 15 dollars more as well as taking 2 d in prep time compared with 15 min.

Relative glycan composition

The sum of all individual peaks representing a specific glycan was divided by the sum of all integrated peaks representing a glycan for each run and multiplied by 100 to determine the relative percentage of each glycan for each run. The technical replicates of each sample were averaged, and the averages for each of the 3 biologic replicates were then averaged. There was no detectable difference in the relative abundance of the 5 major fetuin glycans between the 2 different release protocols (**Fig. 1**).

Recovery of all released *N*-linked glycans

The absolute recovery of glycans released by the 2 methods was significantly different as shown by the LC-SRM traces obtained by glycans released from the 2 release protocols (**Fig. 2**). To ensure that this difference was not related to instrument performance, the sum of all the peaks of the corresponding glycans were then divided by the abundance of the 6 GU internal standard for that replicate. The ratio of glycans compared with the standard was averaged for all technical replicates from the PNGase F protocol, and then all replicates representing the NaOCl protocol. The average ratios were 3.12 and 0.14 for the PNGase F protocol and NaOCl release protocol, respectively (**Fig. 3**).

Glycan degradation analysis

A possible reason for the reduction in *N*-linked glycan recovery in the NaOCl protocol would be degradation,

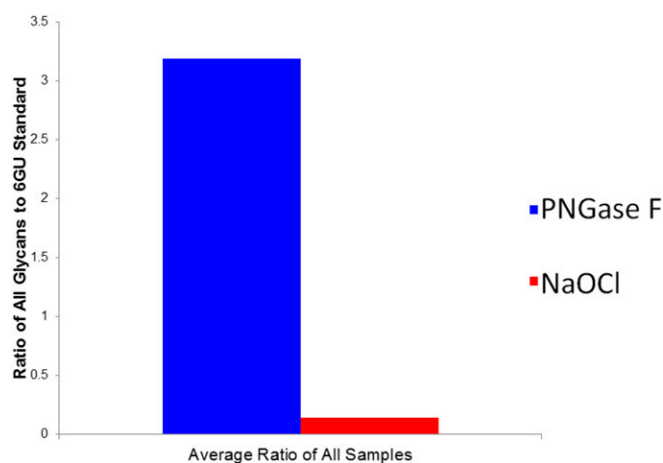


FIGURE 3

Recovery of glycans compared with the 6-GU standard. The total abundance of the 5 analyzed glycans in a single run were combined and divided by the abundance of the internal standard for that run. All results of the PNGase F replicates were then averaged (blue), and all of the results for the NaOCl replicates were averaged (red).

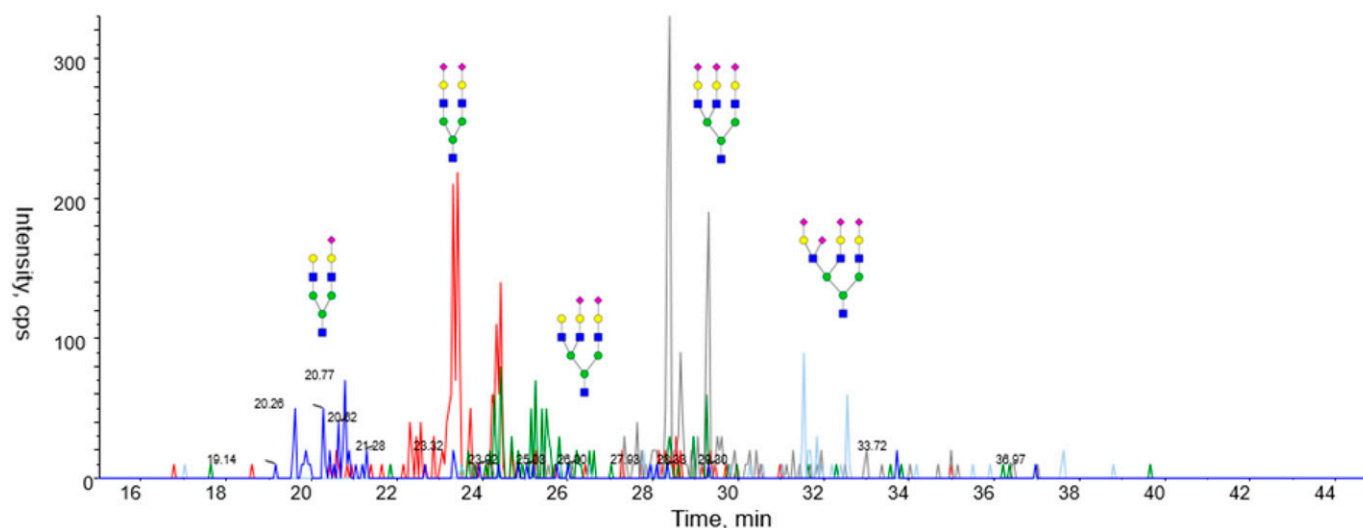


FIGURE 4

Loss of GlcNAc residue on the reducing ends following NaOCl release of *N*-linked glycans. An SRM was run on the NaOCl release sample. Along with the 5 most abundant intact glycans [A2G2S1 (dark blue), A2G2S2 (red), A3G3S2 (green), A3G3S3 (gray), and A3G3S4 (light blue)], the glycans with losses of sialic acids, GlcNAc residues, and mannose residues were analyzed. An extracted-ion chromatogram of the 5 most abundant glycans minus a GlcNAc residue is shown.

potentially *via* a base peeling reaction due to the high pH used for this release. Losses of a GlcNAc residue on the reducing terminus were observed by LC-SRM analysis of the NaOCl-released glycans (Fig. 4). Similar LC-SRM analysis of a PNGase F-released sample did not contain peaks corresponding to these degradation products. Losses of sialic acids or more than the loss of one GlcNAc were not observed by LC-SRM analysis. Although the degradations will lead to a sample reduction, the level of these fragments does not correspond to the large difference determined in the absolute recovery discussed in the preceding paragraph, and thus we conclude that the NaOCl process also releases glycans less efficiently than PNGase F.

DISCUSSION

It was previously reported that the NaOCl release protocol was just as effective as the more commonly applied PNGase F release protocol.²³ We have determined quantitatively that the glycan profile of fetuin using the NaOCl protocol is experimentally indistinguishable from the glycan profile resulting from PNGase F release on a microscale. However, although the overall glycan composition of released *N*-linked glycans for the 5 most abundant *N*-linked glycans of fetuin using the NaOCl protocol was experimentally indistinguishable from the PNGase F release protocol, the absolute recovery of all glycans from a 300 μ g sample of fetuin is significantly different. The results demonstrate quantitatively that the absolute recovery from the NaOCl protocol was <20 times lower than that obtained with enzymatic release of these *N*-linked glycans. It appears that the NaOCl release protocol was not as efficient as the PNGase F release protocol and led to

glycan degradation from the base peeling reaction. Therefore, we cannot be certain if the lower recovery of *N*-linked glycans is due to the efficiency of the technique or complete degradation of the glycans. Although the NaOCl is a much cheaper and quicker option, the low recovery of glycans using the NaOCl procedure implies that this approach is not suitable with small amounts (<500 μ g) of sample.

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