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Facile removal of high mannose structures prior to extracting complex type N-glycans from de-N-glycosylated peptides retained by C18 solid phase to allow more efficient glycomic mapping

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Abstract

The relative amount of high mannose structures within an N-glycomic pool differs from one source to another but quite often it predominates over the larger size complex type structures carrying biologically important glyco-epitopes. An efficient method to separate these two classes of N-glycans would significantly aid in detecting the lower abundant components by mass spectrometry. Capitalizing on an initial observation that only high mannose type structures were recovered in the flow through fraction when PNGase F digested peptides were passed through a C18 cartridge in 0.1% formic acid, we demonstrated here that native complex type N-glycans can be retained by C18 cartridge and to be efficiently separated from both the smaller high mannose type structures, as well as de-N-glycosylated peptides by stepwise elution with increasing acetonitrile concentration. The weak retention of the largely hydrophilic N-glycans on C18 resin is dependent not only on size but also increased by the presence of α6-fucosylation. This was shown by comparing the resulting N-glycomic profiles of the washed and low acetonitrile eluted fractions derived from both a human cancer cell line and an insect cell line.

Keywords

N-glycan fractionation; C18 RP SPE; Glycomics; Mass spectrometry

Protein N-glycosylation proceeds from first acquiring the high mannose type structures before being further processed into hybrid and complex type N-glycans without a predefined template or genetic code. The resulting N-glycomic pool as released from a protein or any biological source is therefore a heterogeneous mixture of structures ranging from the smaller high mannose and incompletely extended hybrid or complex type to fully elaborated structures. Recent advances in mass spectrometry (MS) have prompted rapid developments in MS-based glycomics, which aims to map the full glycosylation profile of a cell or tissue and how it may be altered by genetic defects or pathophysiological stimuli. In many cases, high mannose structures dominate the N-glycomic pool and preclude other low abundant components of biological interest from being detected [1]. Although increasing sensitivity and capability in MS mapping and MS/MS sequencing coupled with online LC-separation

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has yielded impressive glycomic data rich in information content, an urgent need remains in developing a front-end glycan sample preparation step that would efficiently fractionate the diverse classes of glycans to allow independent optimization of the analytical parameters, or simply to reduce the dynamic range required for simultaneous detection of the most and least abundant components. Methods do exist to enrich for the complex type structures based on their larger size or charge contributed by sialylation [2]. Endoglycosidase H has also been used to digest and thus remove the high mannose glycans along with the hybrid types [3]. Similarly, ConA lectin has been employed to retain specifically the high mannose structures, which it binds with highest affinity [4]. Each of these approaches has its strengths and limitations but most would require subsequent clean-up steps prior to MS and/or LC mapping, with and without additional chemical derivatization.

In a typical glycomic workflow, the N-glycans are most commonly released from the glycopeptides by the enzyme PNGase F or A, followed by one of the several methods that would separate and recover the released glycans from the de-N-glycosylated peptides in bulk. Among these is the use of a C18 cartridge, to which peptides are retained while the largely hydrophilic N-glycans flow through [5]. Since native O-glycans are known to be retained on RP C18 stationary phase albeit weakly [6], it occurred to us that it may be possible to incorporate a separation during this initial step by exploring differential washing and elution conditions. Such an idea and its feasibility was first suggested by our fortuitous observation that conditioning and washing the C18 Sep-Pak (Waters) in different acidic solvents affected the distribution of the N-glycans, as monitored by MALDI-MS mapping of the respective wash and eluted fractions. A case example is provided by bovine fetuin. Using the 1-propanol/5% acetic acid solvent system widely adapted by many for this purpose based on published protocol [5], all known N-glycans can be efficiently recovered in the flow through (Fig. 1A) when the PNGase F treated tryptic digests of fetuin was loaded and passed through the C18 Sep-Pak in 5% aqueous acetic acid. As expected, the dominant peaks correspond to the sialylated complex type N-glycans (as annotated in Fig. 1A). However, if an acetonitrile/0.1% formic acid solvent system commonly used in RP C18 LC-MS/MS was applied instead, only the high mannose $Man_5GlcNAc_2$ structure was recovered in the 0.1% aqueous formic acid flow through fraction, along with a much reduced amount of the disialylated biantennary structures (Fig. 1B). The apparently missing complex type N-glycans could subsequently be eluted off the cartridge by either 5% acetic acid, or low acetonitrile concentration *e.g.* 5% acetonitrile/0.1% formic acid (Fig. 1C). In other words, under the 0.1% formic acid but not the 5% acetic acid conditions, the sialylated complex type structures can be efficiently retained on the C18 Sep-Pak cartridge used, allowing the high mannose type structures to be washed away.

To examine if the observed effects can be extended to a full range of N-glycomic complexity including non-sialylated glycans, we tested the differential wash and elute conditions on N-glycans released from a colonic adenocarcinoma cell line, *colo205*. As shown in Fig. 2A, the MALDI-MS profile on the released, permethylated c*olo205* N-glycans comprises a highly heterogeneous mixture of multifucosylated complex type structures, ranging from biantennary to those carrying more than 6 LacNAc units at m/z > 5000. The signals afforded were, however, less than 20% of the base peak intensities, corresponding to $Man_{8-9}GlcNAc_2$, with slightly lesser amount of $Man_{5-7}GlcNAc_2$. Such a dominance of high mannose type structures would generally prevent more of the low abundant complex type structures from being readily detected particularly if the total sample amount that can be loaded is limiting, such as in the case of nanoLC-MS/MS applications. We showed that by loading and washing instead in 0.1% formic acid, only the high mannose structures would flow through (Fig. 2B), whereas the bulk of the complex type structures were retained and could then be enriched by 5% acetonitrile/0.1% formic acid elution (Fig. 2C). Removal of a majority of the high man-nose structures in this instance apparently did not alter the relative

intensities of the complex type N-glycans retained. More extensive washing with increasing volume of 0.1% formic acid to get rid of the residual amount of high mannose structures would, however, affect the retention of smaller non-core fucosylated complex type structures (Fig. 1B, C) and therefore unwarranted. It should be noted that our findings also imply that if the PNGase F digestion of N-glycopeptides were subjected to direct RP C18 LC-MS/MS analysis using the acetonitrile/0.1% formic acid solvent system, the majority of the released complex type N-glycans would survive pre-column washing in 0.1% formic acid and then be carried through to analytical column and concentrated in near void peak, as soon as the percentage of acetonitrile is elevated. This may allow a rapid mapping of the N-glycans, in this case as non-permethylated structures, with simultaneous proteomic identification of the peptides and de-N-glycosylated peptides.

At present, the mechanistic basis underlying differential effects shown by 0.1% formic acid (pH 3) versus 5% acetic acid (pH 2.5) is not clear, particularly since it affects not only sialic acid containing but also neutral N-glycans. It may be argued that the differences in pH strength is a contributing factor. However, if 1% acetic acid, which gives a similar pH 3 aqueous solvent, is used instead for the initial loading and wash, substantial amount of the high mannose structures was still found as base peaks in the subsequent 5% acetic acid eluates (Fig. 2D). In fact, a significant proportion of both the high mannose and complex type structures were already washed off by the 1% acetic acid conditions (not shown). Thus the chromatographic behavior of the N-glycans in 1% acetic acid is more similar to that in 5% acetic acid rather than in 0.1% formic acid but lowering the acetic acid strength did allow more to be retained on the C18 Sep-Pak cartridge. Finally, if non-acidified milliQ water is used for the initial conditioning, loading and wash, an even higher amount of the high man-nose structures was retained but some would likewise flow through. We thus recommend to use 5% acetic acid solvent system if it is intended that all N-glycans to be collected in one pool, separated from the de-N-glycosylated peptides. On the other hand, 0.1% formic acid solvent system appears to be better suited to remove most of the high mannose structures from the larger complex type N-glycans. These can then be separately eluted off, free from the more tightly retained de-N-glycosylated peptides by increasing the acetonitrile concentration to 5% level.

It would seem that size, rather than charge is the most critical factor in achieving the separation and, in general, the $Man_{5-9}GlcNAc_2$ structures are smaller than most of the complex type N-glycans. However, the non-sialylated, core fucosylated biantennary structure (m/z = 2244), which is smaller than the $Man_9GlcNAc_2$ structure (m/z 2396), was clearly retained (Fig. 2C) and not washed down by 0.1% formic acid along with the high mannose structures (Fig. 2B). Likewise for the non-sialylated, core fucosylated biantennary structures with one additional HexNAc (m/z 2489), or one and two additional Fuc residues (m/z 2418, 2592), which were retained but not the $Man_9GlcNAc_2$ structure with an additional Hex (m/z 2600). These deviations from a simple size factor may be attributed to the presence of core α6-Fuc, which is known to increase the retention of reducing end 2-amino pyridine tagged N-glycans on an RP HPLC column [7]. To test this possibility, insect N-glycans from *Sf9* cells known to carry a range of truncated mannose type structures with and without core α6-fucosylation [8] were released by PNGase F after tryptic digestion and passed through the C18 Sep-Pak cartridge conditioned in either the 0.1% formic acid or 5% acetic acid solvent system (Fig. 3).

Interestingly, the 0.1% formic acid washed through fraction (Fig. 3B) not only contained the $Man_3GlcNAc_2$ and high mannose ($Man_{5-9}GlcNAc_2$) structures but also the non-core fucosylated trimannosyl core structures with one or two additional HexNAcs (m/z 1416 and 1662), whereas the core α6-fucosylated counterparts (m/z 1590, 1836) were retained (Fig. 3C). This is fully consistent with our observations that core α6-fucosylation increases the

binding affinity of native N-glycans to C18 cartridge and would require a stronger eluting condition afforded by 5% acetic acid or 5% acetonitrile/0.1% formic acid. However, as exemplified by the case of core α6-fucosylated trimannosyl core (m/z 1345), the mere presence of a core α6-Fuc is insufficient to allow the full retention of this smallest N-glycan. It could thus be concluded that the primary determining factor is size but binding is also strengthened by core α6-fucosylation. In contrast, as would be expected from previous findings on the elution order of 2-amino pyridine tagged N-glycans on RP C18 column, core α3-fucosylation commonly found on plant and other lower animal N-glycans would weaken the retention [7]. We indeed found that the single major N-glycan from horseradish peroxidase, a xylosylated and α3-fucosylated trimannsoyl core structure [9], was almost completely washed through by 0.1% formic acid (data not shown).

In conclusion, we have developed a facile separation method for removing the high mannose type structures from the larger complex type N-glycans. The most attractive feature of this approach is its simplicity as it involves no additional enzymes or chromatography media other than the same RP C18 cartridge used to collect released N-glycans from PNGase-digested peptides. It can therefore be readily incorporated also into current glycoproteomic workflow in which removal of N-glycans for glycomic mapping is often coupled with identification of de-N-glycosylated peptides. In glycomic samples dominated by high mannose structures, this simple two-step elution will allow separate and thus better detection of the less abundant species including the smaller core α6-fucosylated structures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviation

ConA	Concanavalin A
PNGase F/A	Peptide-N-glycosidase F/A
GlcNAc	N-acetylglucosamine
Man	Mannose
Fuc	Fucose
LacNAc	N-acetyllactosamine
HexNAc	N-acetylhexosamine
Hex	Hexose

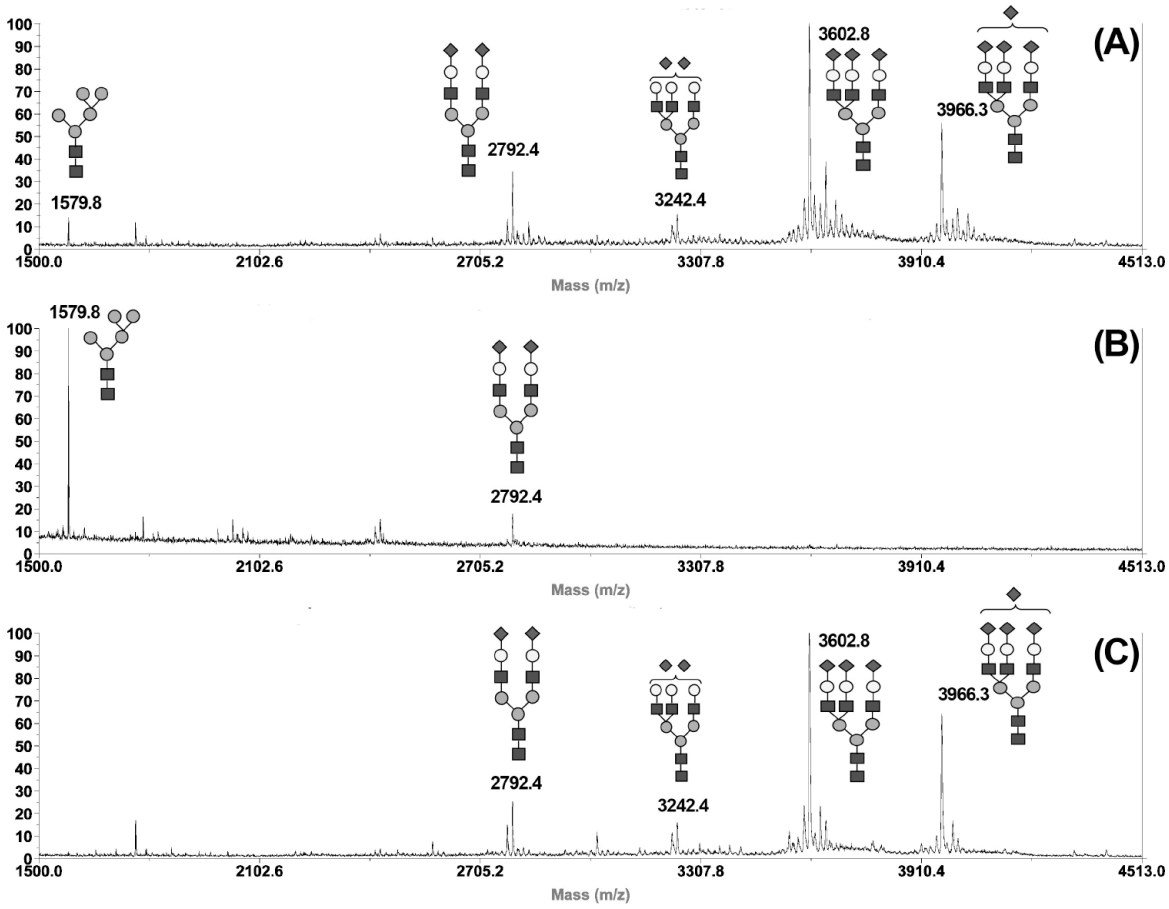

Figure 1. Sialylated complex type N-glycans from fetuin were retained on C18 cartridge conditioned in 0.1% formic acid

The de-N-glycosylated peptides along with the released N-glycans from fetuin were loaded onto C18 Sep-Pak conditioned in 5% acetic acid (A) or 0.1% formic acid (B) and washed through with the same acidic aqueous solvent. The collected N-glycans in these flow through fractions (A, B) or those subsequently eluted by 5% acetonitrile/0.1% formic acid (C) after the initial 0.1% formic acid wash were permethylated and analyzed by MALDI-MS. The N-glycans were assigned and annotated based on the molecular compositions defined by the detected m/z for the $[M+Na]^+$ ions and prior knowledge of the fetuin N-glycan structures.

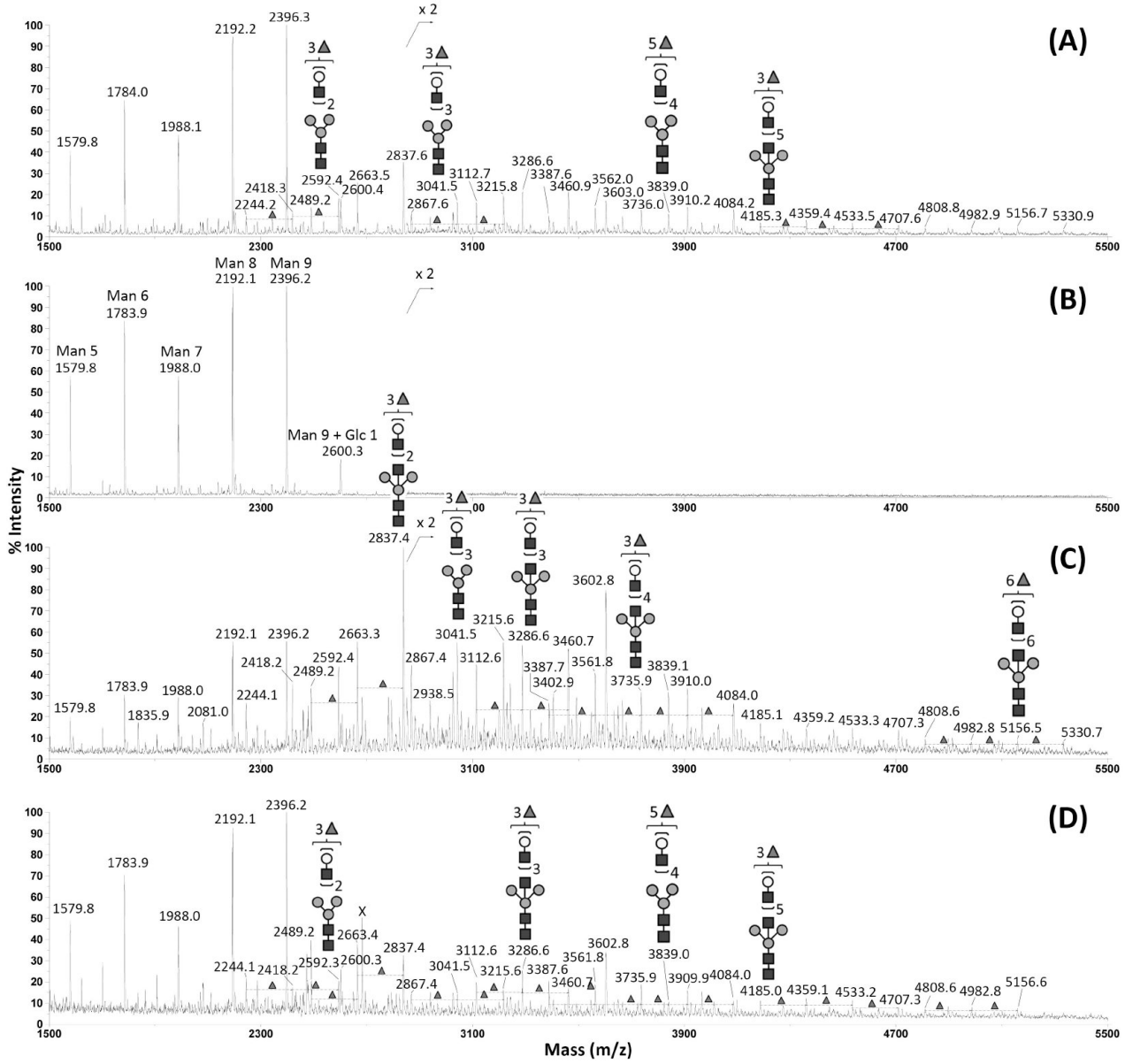

Figure 2. Enrichment of complex type N-glycans from total N-glycomic pool of colo205

The total N-glycans released from *colo205* were passed through C18 Sep-Pak cartridge conditioned in either 5% acetic acid (A); or 0.1% formic acid (B) and then further eluted with 5% acetonitrile/0.1% formic acid (C); or 1% acetic acid and then eluted with 5% acetic acid (D). The collected N-glycans in each of these flow through and eluted fractions were permethylated and analyzed by MALDI-MS. A two fold magnification was applied to *m/z* range above 2850 (except D). Major $[M+Na]^+$ signals were compositionally assigned and annotated by plausible cartoon structures or mass differences of fucose (triangle). No attempt was made to define structural details for current work. X indicates peak which could not be compositionally assigned as glycan.

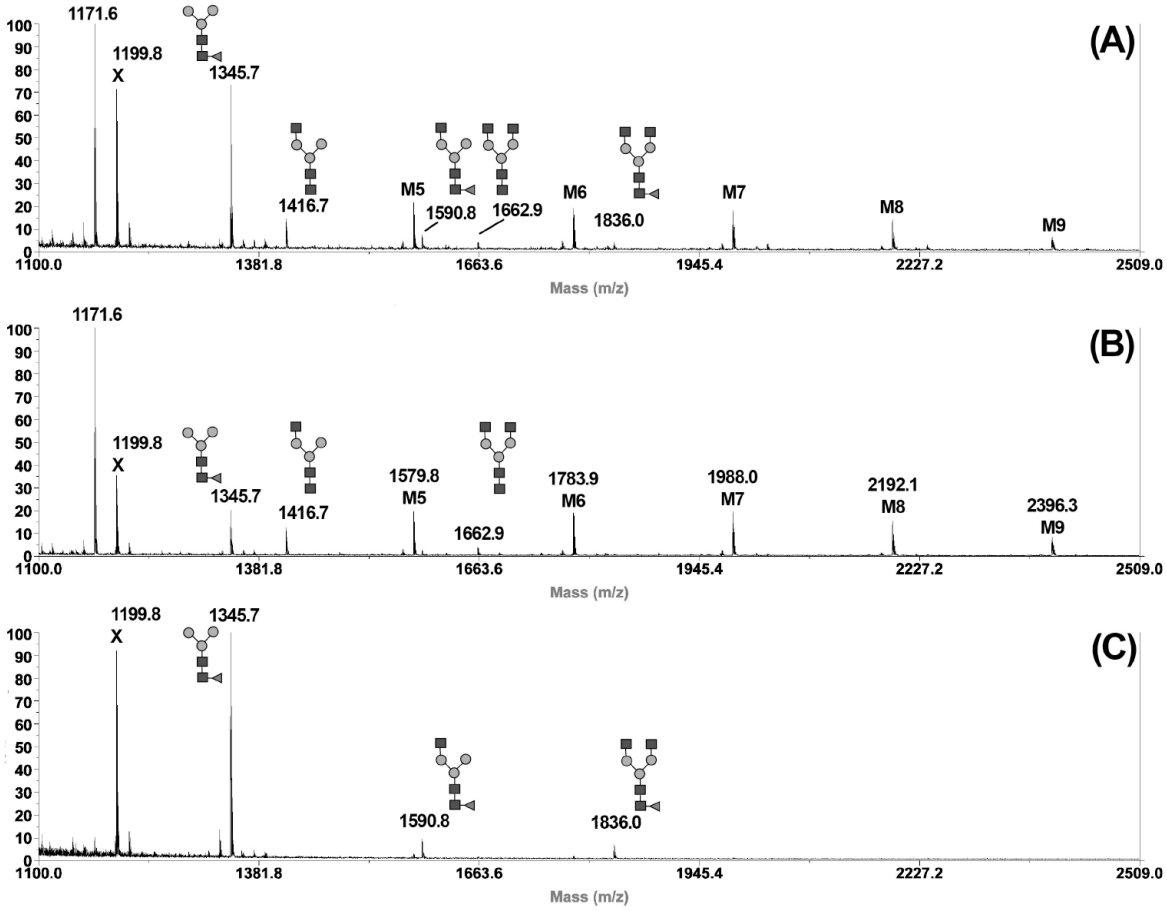

Figure 3. Core α1-6 fucosylation increases binding of N-glycans to C18 cartridge and affects size fractionation

The total N-glycans released from *Sf9* cells were passed through C18 Sep-Pak cartridge in either 5% acetic acid (A); or 0.1% formic acid (B) and then further eluted with 5% acetonitrile/0.1% formic acid (C). The collected N-glycans in each of these fractions were permethylated and analyzed by MALDI-MS. Major $[M+Na]^+$ signals were compositionally assigned and annotated by plausible cartoon structures based on prior knowledge of the Sf9 N-glycomic profile [8]. X indicates unknown peak which could not be compositional assigned as glycan.