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IDENTIFICATION AND CHARACTERIZATION OF ISOMERIC *N*-GLYCANS OF HUMAN ALFA-ACID-GLYCOPROTEIN BY STABLE ISOTOPE LABELLING AND ZIC-HILIC-MS IN COMBINATION WITH EXOGLYCOSIDASE DIGESTION

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Abstract

In this study, a ZIC-HILIC-MS methodology for the analysis of *N*-glycan isomers was optimized to obtain greater detection sensitivity and thus identify more glycan structures in hAGP. In a second step, this method was combined with glycan reductive isotope labelling (GRIL) through [$^{12}C_6$]/[$^{13}C_6$]-aniline and exoglycosidase digestion to characterize the different glycan isomers. The GRIL method allows the peak areas resulting from two different labelled samples to be compared, since neither retention time shifts nor variations in the ionization of glycans between these samples are obtained. First, sialic acid linkage assignations were performed for most hAGP glycan isomers with α2-3 sialidase digestion. Bi-, tri- and tetraantennary glycan isomers with different terminal sialic acid linkages to galactose (α2-3 or α2-6) were assigned, and the potential of this technique for the structural characterization of isobaric isomers was therefore demonstrated. Furthermore, fucose linkage isomers of hAGP glycans were also characterized using this isotope-labelling approach in combination with α1-3,4 fucosidase and β1-4 galactosidase digestion. α1-3 antennary fucoses and α1-6 core fucosylation were detected in hAGP fucosylated glycans. These established methodologies may be extremely useful for patho-glycomic studies to characterize glycoproteins of biomedical interest and find novel glycan isomers that could be used as biomarkers in cancer research.

1. Introduction

Glycoproteins play an important role in major biological processes, which are modulated and controlled by the oligosaccharides attached to these proteins [1–4]. These oligosaccharide structures (also called glycans) are involved in many diseases such as hereditary disorders, immune deficiencies, chronic inflammation and cancer [5, 6]. Hence, a wide range of research activities to find glycan-based biomarkers for the detection of different pathologies have been conducted [7–10]. In the case of cancer, typical *N*-glycan modifications in tumours are associated with changes in sialylation, fucosylation or branching. Increased levels of α2-6 sialic acids and overexpression of Sialyl Lewis^X (SLe^x) and Sialyl Lewis^A (SLe^a) epitopes were reported in carcinomas [11–14].

The *N*-glycans of a glycoprotein may present several isomers that mainly differ in the linkage type of the terminal sialic acids or the fucoses. N-acetylneuraminic acids (NeuAc) can be α2-3 or α2-6 linked to galactose (Gal). Moreover, fucoses can be α1-3 and α1-4 linked to N-acetylglucosamine (GlcNAc), on an external branch (Fuc Antenna), α1-2 linked to Gal or α1-6 linked to the core GlcNAc (Fuc Core) [15–17]. Some studies in recent years have shown the importance of correctly identifying and characterizing these isomers, since one type of isomer can be associated with a specific pathology. The possibility of quantitatively analysing alterations in specific sialic acid or fucose linkage isomers could therefore facilitate the discovery of diagnostic biomarkers rather than simply analysing the total sialylation or fucosylation levels of the glycans [16–19]. Some authors have recently proposed the analysis of N-linked glycans by liquid chromatography coupled to mass spectrometry detection (LC-MS) in combination with exoglycosidase digestion to elucidate and structurally characterize isomeric glycans [6, 16, 20–27]. This characterization is carried out by observing

retention time shifts and changes in the peak areas of the isomers after exoglycosidase treatment. However, the strategies reported present certain drawbacks. Some do not present sufficient retention time reproducibility to build a reliable structure/retention time library. Moreover, the differences in ionization yield between glycans and between samples can lead to errors in the assignation of the isomers, especially when there is an increase in the complexity of the glycans and the number of isomers.

Glycan reductive isotope labelling (GRIL) makes it possible to compare two samples in the same run, correct the lack of retention time reproducibility between runs and avoid variations in the ionization of glycans due to ion-suppression effects or tuning of the mass spectrometer [28–30]. The GRIL methodology allows the glycan fingerprints to be precisely compared before and after digestion with a specific exoglycosidase, and therefore provides an accurate characterization of glycan isomers. In a previous work, a GRIL strategy using [$^{12}C_6$]/[$^{13}C_6$] coded aniline was developed for the relative quantification of *N*-glycans, and the labelled glycans were analysed by μZIC-HILIC-MS [31]. This methodology rigorously separated and quantified the different isomers of a glycan and was recently evaluated for the characterization of human alpha-1-acid-glycoprotein (hAGP) glycans in serum samples from healthy volunteers and patients with pancreatic ductal adenocarcinoma (PDAC) and chronic pancreatitis (ChrP) [32]. In that study, we observed an increase in several fucosylated glycans in PDAC and demonstrated that some of them made it possible to differentiate between the two pathologies. Nevertheless, changes in the proportion of some glycan isomers were also observed in certain PDAC samples [32]. These results found in hAGP samples are a clear example of the need to establish new methodologies to thoroughly characterize isomeric *N*-glycans of glycoproteins.

To that end, an analytical method to improve the detection and characterization of isomeric *N*-glycans by µZIC-HILIC-MS was developed in this work. Studies using different exoglycosidases in combination with relative quantification by isotope labelling with [$^{12}C_6$]/[$^{13}C_6$] aniline have been performed to reliably identify the linkage type of sialic acids and fucoses in the glycan isomers. hAGP has been selected as a glycoprotein model because it appears to show great potential as a biomarker in pancreatic cancer and other diseases [12, 32–35], and because it presents a wide range of complex-type *N*-glycans and isomers, thereby allowing the proposed method to be properly evaluated. The goal of these linkage assignations is to establish a robust and reliable methodology for the characterization of isomeric glycans in patho-glycomic studies in order to identify new glycoprotein biomarkers.

2. Materials and methods

2.1. Chemicals

All chemicals used to prepare buffers and solutions were of analytical reagent grade. Acetic acid (HAc, glacial), formic acid (FA 98%-100%), dimethyl sulfoxide (DMSO) and acetone were supplied by Merck (Darmstadt, Germany). Ammonium hydrogen carbonate (NH_4HCO_3), sodium phosphate dodecahydrate ($Na_3PO_4 \cdot 12H_2O$), sodium cyanoborohydride ($NaBH_3CN$), [$^{12}C_6$]-aniline ([$^{12}C_6$]AN), [$^{13}C_6$]-aniline ([$^{13}C_6$]AN), 2-mercaptoethanol (β-ME) and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen chloride (HCl, 37%) was supplied by Panreac (Barcelona, Spain) and "NP-40 alternative" by Calbiochem (Darmstadt, Germany). Ammonium acetate (NH_4Ac) and acetonitrile (ACN), from Merck (Madrid, Spain), and LC-MS grade water from Fluka (Madrid, Spain), were used for μZIC-HILIC-TOF-MS analysis. Peptide-N-glycosidase F (PNGase F) and neuraminidase (α2-3,6,8 sialidase) were obtained from Roche Diagnostics (Basel, Switzerland), and α2-3 neuraminidase S (α2-3 sialidase), α1-3,4 fucosidase and β1-4 galactosidase came from New England Biolabs (Ipswich, MA, USA). ESI low concentration (ESI-L) tuning mix was supplied by Agilent Technologies (Waldbronn, Germany) for the tuning and calibration of the oa-TOF mass spectrometer.

2.2. Release, purification and labelling of hAGP *N*-glycans

Standard of human α1-acid-glycoprotein (hAGP, 99%) was purchased from Sigma-Aldrich. Twenty-five micrograms of hAGP was reduced with 0.5% β-ME in the presence of 0.5% SDS in 50 mM Na_3PO_4 (pH 7.5) and boiled for 30 min in a water bath. When the sample was at room temperature, a volume of 50 mM Na_3PO_4 (pH 7.5)

with 1% (v/v) of NP-40 alternative was added to achieve a final concentration of 0.1% SDS and β-ME in the sample. To release the *N*-glycans, 1μL of PNGase F (1 U) solution was added and the mixture was carefully vortexed and incubated at 37ºC for 18 h. Digestion was stopped by heating the sample in a thermoblock at 100ºC for 15 min and the sample was stored at -20ºC until use. The *N*-glycans released were purified by solid phase extraction (SPE) using Hypercarb cartridges (25 mg, 1 mL volume, Thermo Fisher Scientific). First, SPE cartridges were conditioned and equilibrated with 1 mL of 60% ACN, 0.1% FA and 2 mL of water, respectively. After dissolving the digested glycoprotein sample in ~500 μL of water, it was loaded and the SPE cartridge was rinsed with 1 mL of water. Retained *N*-glycans were eluted with 600 μL of 60% ACN and 0.1% FA, and the eluate was evaporated to dryness in a SpeedVac. The dried *N*-glycans were stored at -20ºC until use. The labelling was carried out using a reaction mixture of 0.35 M aniline and 1 M $NaCNBH_3$ in DMSO with 30% HAc. The dried hAGP glycans were mixed with 10 μL of the reaction mixture and incubated at 70ºC for 2 h in a water bath. Samples were cooled to room temperature and the labelled glycans were precipitated with acetone as described in [32]. Finally, samples were evaporated to dryness in a SpeedVac to remove the excess acetone, and the dried *N*-glycans were stored at -20ºC. Centrifugations were performed in a Mikro 220R centrifuge (Hettich-Zentrifugen, Tuttlingen, Germany).

2.3. Exoglycosidase digestion of hAGP *N*-glycans

hAGP *N*-glycans were treated with different exoglycosidases. *Sialidase digestion*: Sialic acids were released by enzymatic digestion with sialidase (α2-3,6,8 sialidase or α2-3 sialidase) using two different digestion methods. Method 1: Once the hAGP sample was subjected to PNGase F digestion, the sample was evaporated to dryness in a SpeedVac

and reconstituted to the initial volume with 50 mM NH_4Ac (pH 5.0). Subsequently, 1 µL of α2-3,6,8 sialidase (50 mU) or 6.25 µL of α2-3 sialidase (50 U) was added, and the solution was incubated at 37ºC for 18 h (α2-3,6,8 sialidase) or 1h (α2-3 sialidase). Method 2: In this case, sialidase digestion was performed after PNGase F treatment in the same buffer solution of 50 mM Na_3PO_4 (pH 7.5) and incubated at the same conditions of temperature and digestion time as Method 1. In both procedures, digestion was stopped by heating the samples in a thermoblock for 10 min at 100ºC, and the samples were purified and labelled with aniline in accordance with the protocol described in Section 2.2. *Fucosidase digestion*: α1-3,4 fucosidase was used to release specific fucose residues of hAGP *N*-glycans. Three variants of the fucosidase digestion protocol that differed in the procedure or the number of enzymes used were tested. Method 1: After digestion of hAGP with PNGase F, the sample was evaporated to dryness in a SpeedVac and fucosidase digestion was carried out. Method 2: Before fucosidase digestion, the glycans released with PNGase F were purified and labelled as described in Section 2.2. Method 3: Once the hAGP *N*-glycans were purified and labelled, digestion with fucosidase was carried out in combination with other enzymes (1 µL of α2-3,6,8 sialidase (50 mU) and/or 1µL of β1-4 galactosidase (8 U)). In all these methods, the dried glycans were dissolved in 8 µL of water in order to perform fucosidase digestion. Subsequently, 1 µL of 50 mM NaAc:5 mM $CaCl_2$ (pH 5.5) and 1 µL of BSA at a concentration of 1000 µg/mL, provided by the manufacturer, were added [36]. Afterwards, 1 µL of fucosidase (4 U) was added and the samples were incubated at 37ºC overnight. Digestion was stopped by heating the samples in a thermoblock at 100ºC for 10 min, and samples were stored at -20ºC until analysis.

2.4. μZIC-HILIC-ESI-MS

The μLC-TOF-MS experiments were performed in a 1200 Series capillary liquid chromatography system coupled to a 6220 oa-TOF LC/MS mass spectrometer with an orthogonal G1385-44300 interface (Agilent Technologies). To perform the chromatographic separation, a ZIC-HILIC column packed with 3.5 mm particles, 150 x 0.3 mm L_T x ID (SeQuant, Umeå, Sweden) was used; the stationary phase consisted of a surface with immobilized zwitterionic sulfobetaine moieties. Experiments were performed at room temperature with gradient elution at a flow rate of 4 μL/min and injections of between 0.15-0.25 μL of glycan sample labelled with aniline (glycan concentration: ~100-50 pmol/μL). Eluting solvents were A: 1 mM NH_4Ac solution and B: acetonitrile. The following gradient conditions were used: solvent B from 90% to 80% (within 5 min) and from 80% to 65% (within 20 min) as the linear gradient, followed by cleaning and equilibration steps of B: 65%→50% (within 5 min), 50%→0% (within 5 min), 0% (over 15 min), 0%→90% (within 5 min) and 90% (over 10 min). The mass spectrometer was equipped with a dual-nebulizer ESI source, and the orthogonal nebulizer was used for the μLC-TOF-MS experiments; the second nebulizer, which is generally used to introduce the internal reference mass standard solution in conventional LC–MS experiments, was disabled to avoid any interference with the μLC-TOF-MS experiments [37]. Tuning and calibration of the mass spectrometer were carried out in accordance with the manufacturer's instructions. The measurement parameters were subsequently fine-tuned by direct infusion of maltohexaose labelled with [$^{12}C_6$]-aniline to maximize the signal for the singly charged molecular ion [31]. The best ("optimum") operational conditions in negative mode were: capillary voltage -3500 V, drying gas (N_2) temperature 200ºC, drying gas flow rate 4 L/min, nebulizer gas (N_2) 15 psi, fragmentor voltage 190 V, skimmer voltage 70 V and OCT 1 RF Vpp

voltage 300 V. Data were collected in profile (continuum) at 1 spectrum/s (approx. 10,000 transients/spectrum) between m/z 100 and 3200, at the highest resolution mode (4 GHz). μLC-TOF-MS control, data acquisition and analysis were performed using MassHunter Workstation software (Agilent Technologies).

3. Results and discussion

3.1 Optimization of chromatographic conditions by µZIC-HILIC-TOF-MS

In a previous work, a µZIC-HILIC-TOF-MS method was developed to separate hAGP *N*-glycan isomers labelled with aniline [32]. Although this method made it possible to separate the different glycan isomers, it was not sensitive enough to detect minor glycans when the glycoprotein of interest had a very low concentration in biological samples or when it presented a low percentage of glycosylation. In order to improve sensitivity, the chromatographic conditions used for this work were optimized by evaluating the influence of the pH and ionic strength of the mobile phase. Different pH conditions (pH values of 3, 5, 7 and 8) were tested. Although changes in pH were expected to produce selectivity changes for glycans with the stationary phase due to the deprotonation degree of sialic acids, there was no improvement in separation and the MS signal of *N*-glycans decreased considerably compared to the initial conditions (10 mM NH_4Ac, pH=6.4). Afterwards, the effect of the salt content was also evaluated, with concentrations of 10, 5 and 1 mM of NH_4Ac in the mobile phase. Figures 1A and 1B show the extracted ion chromatograms (EICs) of the major *N*-glycans released from hAGP and labelled with [$^{12}C_6$]-aniline ([$^{12}C_6$]AN), using 10 mM NH_4Ac and 1 mM NH_4Ac mobile phases, respectively. In both cases, the gradient elution used was that reported in [31, 32]. The retention of glycans was affected by the ionic strength and the elution order of some was altered (e.g. 2Ant2NeuAc), but separation generally did not significantly improve with respect to 10 mM NH_4Ac. By contrast, glycan signals considerably increased at a low salt concentration. Hence, 1 mM of NH_4Ac at pH 6.4 was chosen as the optimum aqueous mobile phase. Once the mobile phase composition was established, the gradient elution was optimized to improve separation of glycan isomers and obtain an appropriate analysis time.

In line with our previous works [32, 33], several bi-, tri- and tetraantennary fucosylated and non-fucosylated *N*-glycans were detected using this approach (see Table 1). Nevertheless, the improvement in sensitivity made it possible to detect more glycan structures of hAGP standard in this study. The biantennary glycan with one NeuAc and the tetraantennary mono- and disialylated glycans were also identified. In addition, it was possible to detect the glycans 4Ant3NeuAc and 4Ant4NeuAc with one and two extra units of N-acetyllactosamine (HexHexNAc or LacNAc), some of them also fucosylated. Isobaric isomers were detected for most hAGP glycans using the ZIC-HILIC method reported in [32]. Moreover, the gradient elution optimized in this work provided a better resolution of isomers, especially for less abundant glycans (data not shown).

3.2 Characterization of sialic acid linkage isomers

Once the method for separating and detecting hAGP glycans and their corresponding isomers was established, these structural isomers were characterized using α2-3 sialidase, since it was expected that the main differences between them would be the linkage type of the terminal NeuAc (α2-3 or α2-6). Prior to the characterization of glycan isomers, α2-3,6,8 sialidase (total sialidase) was used to establish the optimum digestion conditions. Two digestion procedures were tested to completely remove the sialic acids from the hAGP glycans. Method 1 used two different buffer solutions for digestion with PNGase F and sialidase, and maintained the optimum working pH for each enzyme (50 mM Na_3PO_4, pH 7.5 for PNGase F and 50 mM NH_4Ac, pH 5.0 for sialidase). By contrast, digestion of the two enzymes in method 2 was carried out using the same buffer solution (50 mM Na_3PO_4, pH 7.5). Higher MS

signals for the desialylated glycans were obtained by digesting both enzymes in the PNGase F optimum working pH (Method 2), even though the optimum pH for sialidase digestion is 5-6. This method was therefore selected as the most suitable for performing sialidase digestion, as sialic acids with the highest yield were removed and less time was required to carry out the enzymatic digestion.

In this study, isotope-coded labelling with [$^{12}C_6$]-aniline ([$^{12}C_6$]AN) and [$^{13}C_6$]-aniline ([$^{13}C_6$]AN) was performed to reliably evaluate the changes in hAGP glycans caused by digestion of the protein with a certain exoglycosidase. This GRIL strategy made it possible to unequivocally assign the sialic acid linkage types present in each isomer by direct comparison of the peak areas of the intact glycans (hAGP only digested with PNGase F) with those digested with a specific sialidase (hAGP digested with PNGase F and α2-3 sialidase), since it avoids experimental variability in retention times and peak areas between runs. For this purpose, three independent samples of 25 µg of standard hAGP were digested with PNGase F and labelled with [$^{13}C_6$]AN, while another three were digested with PNGase F and α2-3 sialidase in accordance with method 2, and labelled with [$^{12}C_6$]AN. In this case, the time allowed for PNGase F digestion was 18 h to ensure the complete de-glycosylation of hAGP, but α2-3 sialidase digestion was limited to one hour, as recommended by the manufacturer, to avoid any possible loss of α2-6 sialic acids in overnight digestion. After derivatization, equimolar mixtures of hAGP glycan-[$^{13}C_6$]AN and hAGP α2-3-desialylated glycan-[$^{12}C_6$]AN were prepared and analysed by µZIC-HILIC-TOF-MS. Figure 2 illustrates the results obtained for the bi-, tri- and tetraantennary non-fucosylated glycans. The EICs of the glycans detected in the hAGP sample digested only with PNGase F are shown in red, and the EICs of the glycans detected in the hAGP sample additionally digested with α2-3 sialidase are shown in black. With regard to biantennary non-fucosylated glycans

(Figure 2A), hAGP digested with PNGase F showed the presence of 2Ant2NeuAc with two isomers and 2Ant1NeuAc. After α2-3 sialidase digestion, isomer 1 of 2Ant2NeuAc disappeared and the peak area of the glycan 2Ant1NeuAc increased, while isomer 2 of 2Ant2NeuAc was maintained at the same retention time and presented practically the same peak area (see Figure 2A and Table 2). These facts reveal that isomer 2 of 2Ant2NeuAc only presents α2-6 linked sialic acids and isomer 1 contains one α2-6 linked sialic acid and one α2-3 linked sialic acid. Moreover, the sialic acid of 2Ant1NeuAc is only α2-6 linked, as the 2Ant0NeuAc was not detected after α2-3 sialidase digestion (see Table 2). It is worth mentioning that the peak area of 2Ant1NeuAc after α2-3 sialidase digestion was not exactly equal to the sum of the peak areas corresponding to isomer 1 of 2Ant2NeuAc and the glycan 2Ant1NeuAc. This is because a glycan with two sialic acids shows a higher ionization yield in negative mode compared to the same glycan with only one sialic acid residue.

With respect to the triantennary non-fucosylated glycans, isomers 1 and 2 of the glycan 3Ant3NeuAc completely disappeared after α2-3 sialidase treatment (EICs in black), and only the third isomer remained. This presented a similar peak area, which suggests that this isomer presents three α2-6 NeuAc linkages. To correctly assign the remaining isomers of glycan 3Ant3NeuAc, it was first necessary to study the behaviour of glycan 3Ant2NeuAc. In this case, after specific sialidase digestion, the first isomer disappeared, the peak area of the second increased considerably and a third peak appeared at a different retention time (Isomer 3). Additionally, it was assumed that hAGP does not present any triantennary glycan with all α2-3 NeuAc linkages, since the glycan 3Ant0NeuAc was not detected in the hAGP sample digested with α2-3 sialidase. Isomer 1 of the glycan 3Ant2NeuAc shows one α2-3 and one α2-6 linked sialic acid and, after sialidase digestion, it turned into the glycan 3Ant1NeuAc, which appeared at

a t_R = 19.6 min, with a similar peak area (292825 versus 314765, see Table 2). This fact revealed that no other isomer contributed to the peak area of the glycan 3Ant1NeuAc. Hence, isomers 1 and 2 of the glycan 3Ant3NeuAc contain two α2-6 linked sialic acids and one α2-3 linked sialic acid, and the second isomer of 3Ant2NeuAc also showed sialic acids with α2-6 linkages. As with the biantennary glycans, the differences in ionization between the intact and desialylated glycans made it difficult to accurately compare the areas obtained (see Table 2). After evaluation of the different possibilities, however, we propose that isomers 1 and 2 of the glycan 3Ant3NeuAc turn into isomers 3 and 2 of the glycan 3Ant2NeuAc, respectively, after α2-3 sialidase digestion.

hAGP tetraantennary glycans were more difficult to characterize, since multiple combinations of the terminal sialic acid linkages were possible. Nevertheless, most linkages in each glycan isomer were characterized by evaluating all of the possible assignations and taking account of the peak areas obtained in the two samples (intact hAGP glycans and desialylated α2-3 hAGP glycans, Figure 2C). The tentative assignation obtained for the tetraantennary glycans is also presented in Table 2. The sialic acid linkage assignation of the remaining glycans, including fucosylated glycans (illustrated in Table 3), was carried out with the same strategy by direct comparison of the peak areas. Less abundant glycans could not be characterized, however, since the MS signal was too low to perform a reliable assignation (e.g. 4Ant4NeuAc3Fuc, 4Ant2LacNAc4NeuAc, 4Ant1LacNAc4NeuAc1Fuc and 4Ant2LacNAc4NeuAc1Fuc). It is important to note that, although certain ionization differences between the intact and desialylated glycans could not be avoided with the GRIL approach, this complex characterization of isomeric glycans would not have been possible without this strategy. To our knowledge, very few studies in the literature have characterized triantennary and tetraantennary glycans with such a large number of isomers for each glycan [27]. This is

mainly due to the limitations presented by other methodologies [6, 16, 20–26]. In our previous work [32], certain isobaric glycans (e.g. isomer 1 and 2 of 3Ant3NeuAc) were found to be underexpressed in pancreatic cancer with respect to control (ratio values were between 0.6 and 0.4, depending on the stage of the PDAC), while the abundance of isomer 3 of 3Ant3NeuAc was similar in the control and PDAC samples (ratio values close to 1). These findings could be related to our sialic acid linkage assignations, since isomer 3 only presented α2-6 linked sialic acids, while isomers 1 and 2 showed α2-3 linked-type sialic acids. These results demonstrate the importance of characterizing these isomers, because changes in the expression of α2-3 and α2-6 linked sialic acids have also previously been described in cancer [13].

Total sialidase digestion of hAGP glycans was also performed in this work. The EICs of the resulting total desialylated glycans (non-fucosylated and fucosylated) are shown in Figure 3. It is important to highlight that, while the non-fucosylated glycans exhibited only one chromatographic peak for each glycan, different isomers were observed in the case of fucosylated desialylated glycans. The fact that they were only present in fucosylated glycans may reveal the presence of different fucose types (α1-6 core Fuc, α1-3 antennary Fuc or α1-2 linked Fuc to Gal). Although only α1-3 antennary fucosylation was previously reported in hAGP and α1-6 core fucosylation was only detected in the glycan 2Ant2NeuAc1Fuc [12], these results confirm that fucose linkage assignment in all hAGP fucosylated glycans is still required in order to identify possible alterations of these isomers in pathological samples.

3.3 Characterization of fucose linkage isomers

We ruled out the presence of α1-4 fucoses, since only type-II structures (Galβ1-4GlcNAc) were previously described in hAGP [38]. To establish the optimum release of α1-3 antennary fucoses from hAGP glycans using α1-3,4 fucosidase, three different digestion methods were evaluated. In method 1, fucosidase digestion was carried out after PNGase F digestion by changing the digestion buffer, while in method 2, it was performed after PNGase F digestion and glycan purification and labelling. Both procedures were rejected, since any fucose residue was removed from the hAGP glycans, probably due to the steric impediment of the terminal antennary monosaccharides such as sialic acids and galactoses, which hindered fucosidase accessibility. α1-3,4 fucosidase digestion only worked successfully when method 3 was used (method 2 in combination with total sialidase and/or β1-4 galactosidase digestion). The need for sialidase or galactosidase during α1-3,4 fucosidase digestion demonstrated the failure of this enzyme to release antennary fucoses in highly decorated glycans, contrary to the manufacturer's guidelines.

Once method 3 was selected as the optimum procedure, fucose linkage-type isomer assignation was carried out using the GRIL strategy. Three independent samples of 10 µg of standard hAGP were digested with PNGase F and total sialidase and labelled with [$^{13}C_6$]AN, while a further three samples were digested with PNGase F, total sialidase and α1-3,4 fucosidase and labelled with [$^{12}C_6$]AN. A 1:1 molar mixture of these samples was then prepared and analysed by µZIC-HILIC-TOF-MS. Glycan fingerprints obtained for bi-, tri- and tetraantennary desialylated glycans with one fucose are illustrated in Figure 4. The EICs, shown in red, are the hAGP glycans digested with total sialidase and labelled with [$^{13}C_6$]AN, whereas the EICs shown in black are the desialylated glycans detected in hAGP, but also digested with α1-3,4 fucosidase and

labelled with [$^{12}C_6$]AN. With respect to the desialylated biantennary glycan with one fucose (Figure 4A), isomer 3 was not detected after α1-3,4 fucosidase digestion, while isomer 1 and 2 were maintained at the same retention time and with similar peak areas (see also Table 4). This confirmed the notion that the first two isomers may present the α1-6 linked fucose to the core GlcNAc, as previously reported in [12], or α1-2 linked to Gal, while isomer 3 may show α1-3 antennary fucosylation. In the case of the glycan 3Ant0NeuAc1Fuc (Figure 4B), digestion with α1-3,4 fucosidase also caused the third isomer to disappear (compare red and black EICs), which suggests α1-3 antennary fucosylation for isomer 3 and, contrary to our expectations and inconsistent with reported studies [12, 33], an α1-6 core or α1-2 linked to Gal fucosylation for the first two isomers. Finally, the tetraantennary structures with one fucose unit (4Ant0NeuAc1Fuc, 4Ant1LacNAc0NeuAc1Fuc and 4Ant2LacNAc0NeuAc1Fuc) seemed to present one isomer with an α1-3 linked fucose and the other with an α1-6 core fucose or α1-2 fucose linked to Gal, since it was maintained after fucosidase digestion (Figure 4B and Table 4).

As occurred in the characterization of sialic acids, fucose type assignation of the highly fucosylated glycans was more difficult to perform, since their abundance was very low in hAGP. In this case, it can be only deduced that all highly fucosylated glycan isomers show at least one α1-3 antennary fucose, with the exception of isomer 1 of the glycan 4Ant0NeuAc2Fuc (see Table 4).

Finally, in order to differentiate between α1-2 fucosylation linked to Gal and core fucosylation, total sialidase and α1-3,4 fucosidase digestion in combination with β1-4 galactosidase was carried out. Three independent samples of 10 µg of standard hAGP were digested with PNGase F, total sialidase and α1-3,4 fucosidase and labelled with [$^{13}C_6$]AN, while a further three were digested additionally with β1-4 galactosidase and

labelled with [$^{12}C_6$]AN. Afterwards, equimolar mixtures of these samples were prepared and analysed by μZIC-HILIC-TOF-MS. Isomer profiles obtained after β1-4 galactosidase digestion confirmed that none of the glycans with one fucose showed α1-2 fucoses linked to Gal, as the glycan isomers which were detected after α1-3,4 fucosidase digestion were also detected after β1-4 galactosidase treatment, thereby conserving the fucose unit in their structure after removal of the galactoses (e.g. see Figure 5 for 3Ant0NeuAc1Fuc). In the case of the highly fucosylated glycans, the absence of α1-2 fucoses could not be reliably confirmed, since the MS signals were rather low. Nonetheless, the results obtained with the glycans with one fucose revealed that the majority of hAGP glycans showed α1-3 fucosylation (Fuc Antenna), but also α1-6 fucosylation (Fuc Core). The presence of two isomers with core fucosylation in glycans 2Ant0NeuAc1Fuc and 3Ant0NeuAc01Fuc (Table 4) could not be fully explained in terms of structure. One possible explanation is the presence of α1-6 core fucoses linked to either GlcNAc of the chitobiose core, although this atypical core structure has not been described previously in hAGP. Despite the fact that core fucosylation in hAGP was reported only in the glycan 2Ant2NeuAc1Fuc [12], the presence of the other core fucosylated structures detected in this work opens up an opportunity to evaluate the fucosylation type that is truly involved in the overexpression of hAGP fucosylated glycans in pathological samples, in order to explore the potential of hAGP as a pancreatic cancer biomarker.

Conclusions

In the first part of this paper, the conditions for separation and detection of glycan isomers using the μZIC-HILIC-TOF-MS methodology were optimized, which resulted in a significant improvement in sensitivity that could prove useful for the future analysis of biological samples or glycoproteins with low glycosylation percentages. In addition, this approach identified more glycan structures in hAGP than ever before with this methodology (2Ant1NeuAc, 4Ant1NeuAc and 4Ant2NeuAc, as well as some tetraantennary structures with one and two extra units of N-acetyllactosamine). In the second part, we established a methodology using a GRIL strategy with [$^{12}C_6$]/ [$^{13}C_6$]-aniline and specific exoglycosidase digestions to assign sialic acid and fucose linkage-type isomers. In contrast to other reported methodologies, this GRIL strategy made it possible to reliably characterize glycan isomers by comparing peak areas and retention times, thereby avoiding the lack of reproducibility between runs and variations in the ionization of the glycans due to ion-suppression effects or tuning of the mass spectrometer. The assignation of sialic acids linked-type in hAGP glycan isomers revealed differences in the α2-3 and α2-6 sialic acid content that can be correlated with changes in the abundance of certain isomers in pathological samples. Furthermore, characterization of fucose linkage-type isomers of the most abundant hAGP glycans confirmed that, in addition to α1-3 antennary fucosylation, core fucosylation also appears to be present in several hAGP glycans. The established methodology provides a reliable way of characterizing glycan isomers in order to identify specific biomarkers in many diseases involving protein glycosylation, and in the specific case of hAGP, it provides an insight into the role of this protein in pancreatic cancer.

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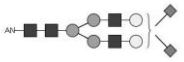
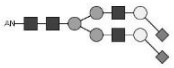
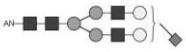
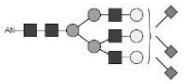
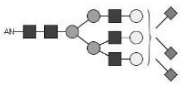
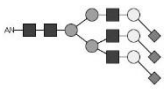
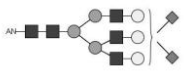
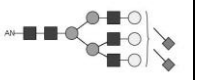
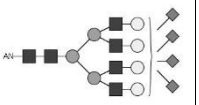
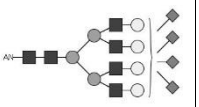
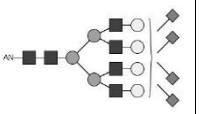
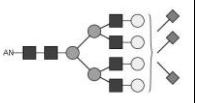
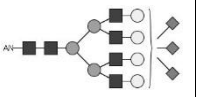
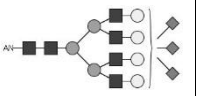
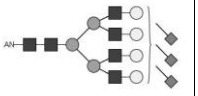
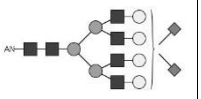
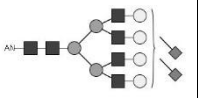
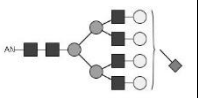
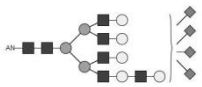
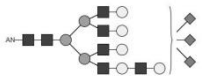
Table 1. hAGP *N*-glycans labeled with [$^{12}C_6$]AN detected by μZIC-HILIC-MS in negative ion mode (*N*-glycan concentration: 50pmol/μL)

hAGP glycan-[$^{12}C_6$]AN		t_R (min)	M_{theo}	M_{exp}	Error (ppm)
2Ant1NeuAc	Isomer 1	17.3	2008.7267	2008.7298	1.5
2Ant2NeuAc	Isomer 1	12.9	2299.8232	2299.8288	2.4
	Isomer 2	13.5	2299.8232	2299.8218	0.6
2Ant2NeuAc1Fuc	Isomer 1	14.0	2445.8810	2445.8844	1.4
	Isomer 2	14.2	2445.8810	2445.8884	3.0
3Ant2NeuAc	Isomer 1	14.8	2664.9554	2664.9590	1.4
	Isomer 2	15.4	2664.9554	2664.9539	0.6
3Ant2NeuAc1Fuc	Isomer 1	15.5	2811.0132	2810.9946	6.6
	Isomer 2	16.0	2811.0132	2810.9850	10.0
3Ant3NeuAc	Isomer 1	12.6	2956.0513	2956.0484	1.0
	Isomer 2	13.0	2956.0513	2956.0463	1.7
	Isomer 3	13.5	2956.0513	2956.0469	1.5
3Ant3NeuAc1Fuc	Isomer 1	13.1	3102.1093	3102.0830	8.5
	Isomer 2	13.5	3102.1093	3102.1025	2.2
3Ant3NeuAc2Fuc	Isomer 1	13.9	3248.1656	3248.1578	2.4
4Ant1NeuAc	Isomer 1	22.1	2738.9911	2738.9606	11.1
4Ant2NeuAc	Isomer 1	17.0	3030.0865	3030.0824	1.3
	Isomer 2	17.5	3030.0865	3030.0818	1.5
4Ant2NeuAc1Fuc	Isomer 1	17.6	3176.1444	3176.1245	6.3
	Isomer 2	18.1	3176.1444	3176.1386	1.8
4Ant3NeuAc	Isomer 1	13.5	3321.1837	3321.1616	6.6
	Isomer 2	14.1	3321.1837	3321.1778	1.8
	Isomer 3	14.4	3321.1837	3321.1772	1.9
	Isomer 4	14.9	3321.1837	3321.1664	5.2
4Ant3NeuAc1Fuc	Isomer 1	13.9	3467.2414	3467.2259	4.5
	Isomer 2	14.6	3467.2414	3467.2289	3.6
	Isomer 3	14.9	3467.2414	3467.2181	6.7
	Isomer 4	15.3	3467.2414	3467.2226	5.4
4Ant3NeuAc2Fuc	Isomer 1	15.0	3613.2978	3613.2707	7.5
	Isomer 2	15.4	3613.2978	3613.2509	13.0
4Ant4NeuAc	Isomer 1	12.8	3612.2791	3612.2714	2.1
	Isomer 2	13.1	3612.2791	3612.2582	5.8
	Isomer 3	13.5	3612.2791	3612.2681	3.0
4Ant4NeuAc1Fuc	Isomer 1	13.1	3758.3368	3758.3177	5.1
	Isomer 2	13.5	3758.3368	3758.3147	5.9
4Ant4NeuAc2Fuc	Isomer 1	13.7	3904.3932	3904.3727	5.2
4Ant4SiA3Fuc	Isomer 1	14.2	4050.4511	4050.3887	15.4
4Ant1LacNAc3NeuAc	Isomer 1	16.2	3686.3141	3686.3096	1.2
4Ant1LacNAc4NeuAc	Isomer 1	14.6	3977.4096	3977.4023	1.8
4Ant1LacNAc3NeuAc1Fuc	Isomer 1	16.5	3832.3720	3832.3859	3.6
4Ant1LacNAc4NeuAc1Fuc	Isomer 1	14.9	4123.4675	4123.4318	8.7
4Ant1LacNAc4NeuAc2Fuc	Isomer 1	15.3	4269.5254	4269.5423	4.0
4Ant1LacNAc4NeuAc3Fuc	Isomer 1	15.5	4415.5833	4415.5567	6.0
4Ant2LacNAc3NeuAc	Isomer 1	17.3	4051.4463	4051.4378	2.1
4Ant2LacNAc4NeuAc	Isomer 1	15.8	4342.5417	4342.5251	3.8
4Ant2LacNAc4NeuAc1Fuc	Isomer 1	16.2	4488.5997	4488.6103	2.4

Table 2. Retention time and peak areas of native non-fucosylated hAGP-glycans (derivatized with [$^{13}C_6$]AN) and non-fucosylated hAGP-glycans desialylated with α2-3 sialidase (derivatized with [$^{12}C_6$]AN) by μZIC-HILIC-TOF-MS. Sialic acid linkage assignation for each isomer.

		t_R (min)	glycan-[$^{13}C_6$]AN area	α2-3 desialylated glycan-[$^{12}C_6$]AN area	NeuAc linkage assignation	
2Ant2NeuAc	Isomer 1	13.6	169825	-	1 NeuAc α2-6 1 NeuAc α2-3	
	Isomer 2	14.0	2010769	1783084	2 NeuAc α2-6	
2Ant1NeuAc	Isomer 1	17.3	68941	135571	1 NeuAc α2-6	
2Ant0NeuAc*	Isomer 1	26.5	-	-	-	-
3Ant3NeuAc	Isomer 1	12.6	396885	-	2 NeuAc α2-6 1 NeuAc α2-3	
	Isomer 2	13.1	4156023	-	2 NeuAc α2-6 1 NeuAc α2-3	
	Isomer 3	13.7	1536871	1412485	3 NeuAc α2-6	
3Ant2NeuAc	Isomer 1	14.9	292825	-	1 NeuAc α2-6 1 NeuAc α2-3	

	Isomer 2	15.4	386282	2277812	2 NeuAc α2-6	
	Isomer 3[&]	15.9	-	225692	-	-
3Ant1NeuAc	Isomer 1[&]	19.6	-	314765	-	-
3Ant0NeuAc[*]	Isomer 1	29.8	-	-	-	-
4Ant4NeuAc	Isomer 1	12.9	252257	-	1 NeuAc α2-6 2 NeuAc α2-3 1 NeuAc unknown	
	Isomer 2	13.3	317537	-	1 NeuAc α2-6 2 NeuAc α2-3 1 NeuAc unknown	
	Isomer 3	13.5	126903	-	2 NeuAc α2-6 2 NeuAc α2-3	
4Ant3NeuAc	Isomer 1	13.6	101293	-	1 NeuAc α2-6 2 NeuAc α2-3	
	Isomer 2	14.2	883588	-	1 NeuAc α2-6 1 NeuAc α2-3 1 NeuAc unknown	
	Isomer 3	14.6	670502	-	1 NeuAc α2-6 1 NeuAc α2-3 1 NeuAc unknown	
	Isomer 4	15.1	214102	397994	3 NeuAc α2-6	

4Ant2NeuAc	Isomer 1	16.9	195765	-	1 NeuAc α2-6 1 NeuAc α2-3	
	Isomer 2	17.4	66762	1004143	2 NeuAc α2-6	
4Ant1NeuAc	Isomer 1	22.1	43075	648373	1 NeuAc α2-6	
	Isomer 2[&]	22.7	-	76451	-	
4Ant0NeuAc[*]	Isomer 1	32.4	-	-	-	
4Ant1LacNAc4NeuAc	Isomer 1	14.6	425915	-	1 NeuAc α2-6 2 NeuAc α2-3 1 NeuAc unknown	
4Ant1LacNAc3NeuAc	Isomer 1	16.2	248701	77459	1 NeuAc α2-6 1 NeuAc α2-3 1 NeuAc unknown	
4Ant1LacNAc2NeuAc	Isomer 1[&]	19.6	-	112626	-	
4Ant1LacNAc1NeuAc	Isomer 1[&]	24.7	-	32151	-	
4Ant1LacNAc0NeuAc[*]	Isomer 1	33.9	-	-	-	

[*] Glycan not present in the native protein. Detected only after total sialidase digestion.

[&] Glycan isomer not present in the native protein. Detected only after α2-3 sialidase digestion.

Table 3. Retention time and peak areas of native fucosylated hAGP-glycans (derivatized with [$^{13}C_6$]AN) and fucosylated hAGP-glycans desialylated with α2-3 sialidase (derivatized with [$^{12}C_6$]AN) by μZIC-HILIC-TOF-MS. Sialic acid linkage assignation for each isomer.

		t_R (min)	glycan-[$^{13}C_6$]AN area	α2-3 desialylated glycan-[$^{12}C_6$]AN area	NeuAc linkage assignation
2Ant2NeuAc1Fuc	Isomer 1	13.9	40007	-	1 NeuAc α2-6 1 NeuAc α2-3
	Isomer 2	14.2	43446	38801	2 NeuAc α2-6
2Ant1NeuAc1Fuc	Isomer 1[&]	18.6	-	31219	-
2Ant0NeuAc1Fuc[*]	Isomer 1	26.4	-	-	-
	Isomer 2	27.2	-	-	-
	Isomer 3	28.2	-	-	-
3Ant3NeuAc1Fuc	Isomer 1	13.2	182067	-	2 NeuAc α2-6 1 NeuAc α2-3
	Isomer 2	13.7	2237472	-	2 NeuAc α2-6 1 NeuAc α2-3
3Ant2NeuAc1Fuc	Isomer 1	15.6	145101	-	1 NeuAc α2-6 1 NeuAc α2-3
	Isomer 2	16.2	106913	974367	2 NeuAc α2-6
	Isomer 3[&]	16.7	-	116369	-
3Ant1NeuAc1Fuc	Isomer 1[&]	20.8	-	106131	-
3Ant0NeuAc1Fuc[*]	Isomer 1	29.6	-	-	-
	Isomer 2	30.3	-	-	-
	Isomer 3	30.9	-	-	-
3Ant3NeuAc2Fuc	Isomer 1	14.0	56253	-	2 NeuAc α2-6 1 NeuAc α2-3
3Ant2NeuAc2Fuc	Isomer 1[&]	16.6	-	30442	-
4Ant4NeuAc1Fuc	Isomer 1	13.3	140718	-	1 NeuAc α2-6 2 NeuAc α2-3 1 NeuAc unknown
	Isomer 2	13.6	180386	-	1 NeuAc α2-6 2 NeuAc α2-3 1 NeuAc unknown
4Ant3NeuAc1Fuc	Isomer 1	14.1	35632	-	1 NeuAc α2-6 1 NeuAc α2-3 1 NeuAc unknown
	Isomer 2	14.7	269095	-	1 NeuAc α2-6 1 NeuAc α2-3 1 NeuAc unknown
	Isomer 3	15.0	162968	-	1 NeuAc α2-6 1 NeuAc α2-3 1 NeuAc unknown
	Isomer 4	15.4	51521	49623	3 NeuAc α2-6
4Ant2NeuAc1Fuc	Isomer 1	17.5	47920	48603	2 NeuAc α2-6

	Isomer 2	18.1	17739	152919	2 NeuAc α2-6
	Isomer 3[&]	18.6	-	30699	-
4Ant1NeuAc1Fuc	Isomer 1[&]	22.1	-	23485	-
	Isomer 2[&]	23.0	-	161345	-
	Isomer 3[&]	23.6	-	21748	-
4Ant0NeuAc1Fuc[*]	Isomer 1	32.2	-	-	-
	Isomer 2	33.0	-	-	-
4Ant4NeuAc2Fuc	Isomer 1	13.7	86151	-	1 NeuAc α2-6 2 NeuAc α2-3 1 NeuAc unknown
4Ant3NeuAc2Fuc	Isomer 1	15.0	41296	-	1 NeuAc α2-6 1 NeuAc α2-3 1 NeuAc unknown
	Isomer 2	15.4	42600	38949	3 NeuAc α2-6
4Ant2NeuAc2Fuc	Isomer 1[&]	18.7	-	29613	-
4Ant1NeuAc2Fuc	Isomer 1[&]	23.7	-	33914	-
4Ant0NeuAc2Fuc[*]	Isomer 1	33.5	-	-	-

[*] Glycan not present in the native protein. Detected only after total sialidase digestion.
[&] Glycan isomer not present in the native protein. Detected only after α2-3 sialidase digestion.

Table 4. Retention time and peak areas of desialylated hAGP-glycans (derivatized with [$^{13}C_6$]AN) and desialylated hAGP-glycans digested with α1-3,4 fucosidase (derivatized with [$^{12}C_6$]AN) by μZIC-HILIC-TOF-MS. Fucose linkage assignation for each fucosylated glycan isomer.

		t_R (min)	Desialylated glycan-[$^{13}C_6$]AN area	Desialylated + α1-3,4 defucosylated glycan-[$^{12}C_6$]AN area	Fucose linkage assignation
2Ant0NeuAc0Fuc	Isomer 1	28.2	392811	348791	-
2Ant0NeuAc1Fuc	Isomer 1	28.3	31920	29711	1 Fuc core
	Isomer 2	29.0	20008	17474	1 Fuc core
	Isomer 3	30.2	12472	-	1 Fuc Antenna α1-3 linked
3Ant0NeuAc0Fuc	Isomer 1	31.5	644160	666457	-
3Ant0NeuAc1Fuc	Isomer 1	31.5	92394	95158	1 Fuc core
	Isomer 2	32.1	19997	25852	1 Fuc core
	Isomer 3	32.5	183321	-	1 Fuc Antenna α1-3 linked
3Ant0NeuAc2Fuc	Isomer 1	32.6	48452	-	At least 1 Fuc Antenna α1-3 linked
4Ant0NeuAc0Fuc	Isomer 1	33.5	298959	345043	-
4Ant0NeuAc1Fuc	Isomer 1	33.5	64200	76963	1 Fuc core
	Isomer 2	34.0	78593	-	1 Fuc Antenna α1-3 linked
4Ant0NeuAc2Fuc	Isomer 1	33.5	24799	24713	2 Fuc core or α1-2 linked to Gal
	Isomer 2	34.1	20435	-	At least 1 Fuc Antenna α1-3 linked
	Isomer 3	34.4	22952	-	At least 1 Fuc Antenna α1-3 linked
4Ant0NeuAc3Fuc	Isomer 1	34.1	7247	-	At least 1 Fuc Antenna α1-3 linked
	Isomer 2	34.4	8236	-	At least 1 Fuc Antenna α1-3 linked

Figure legends

Figure 1: Extracted ion chromatograms (EICs) of the major *N*-glycans released from hAGP standard sample and labelled with [$^{12}C_6$]-aniline using the chromatographic gradient reported in [31], with an aqueous mobile phase of 10 mM NH_4Ac (a) and 1 mM NH_4Ac (b).

Figure 2: Equimolar mixture of aniline-labelled *N*-glycans released from 25 μg of standard hAGP digested only with PNGase F and labelled with [$^{13}C_6$]-aniline (EICs in red), and 25 μg of standard hAGP digested with PNGase F and α2-3 sialidase and labelled with [$^{12}C_6$]-aniline (EICs in black). EICs of the biantennary (A), triantennary (B) and tetraantennary (C) non-fucosylated glycans.

Figure 3: EICs of some desialylated non-fucosylated (A) and fucosylated (B) glycans obtained from hAGP standard after α2-3,6,8 sialidase (total sialidase) digestion.

Figure 4: Equimolar mixture of aniline-labelled *N*-glycans released from 10 μg of hAGP standard sample digested with PNGase F and total sialidase and labelled with [$^{13}C_6$]-aniline (EICs in red), and 10 μg of standard hAGP digested with PNGase F, total sialidase and α1-3,4 fucosidase and labelled with [$^{12}C_6$]-aniline (EICs in black). EICs of the bi- (A), tri- and tetra-antennary (B) desialylated fucosylated glycans.

Figure 5: Equimolar mixture of aniline-labelled *N*-glycans released from 10 μg of standard hAGP digested with PNGase F and total sialidase and labelled with [$^{13}C_6$]-aniline, and 10 μg of standard hAGP digested with PNGase F, total sialidase and β1-4 galactosidase and labelled with [$^{12}C_6$]-aniline. EIC of the triantennary desialylated fucosylated glycan (shown in red) and EIC of the triantennary desialylated fucosylated glycans also digested with β1-4 galactosidase (shown in black).

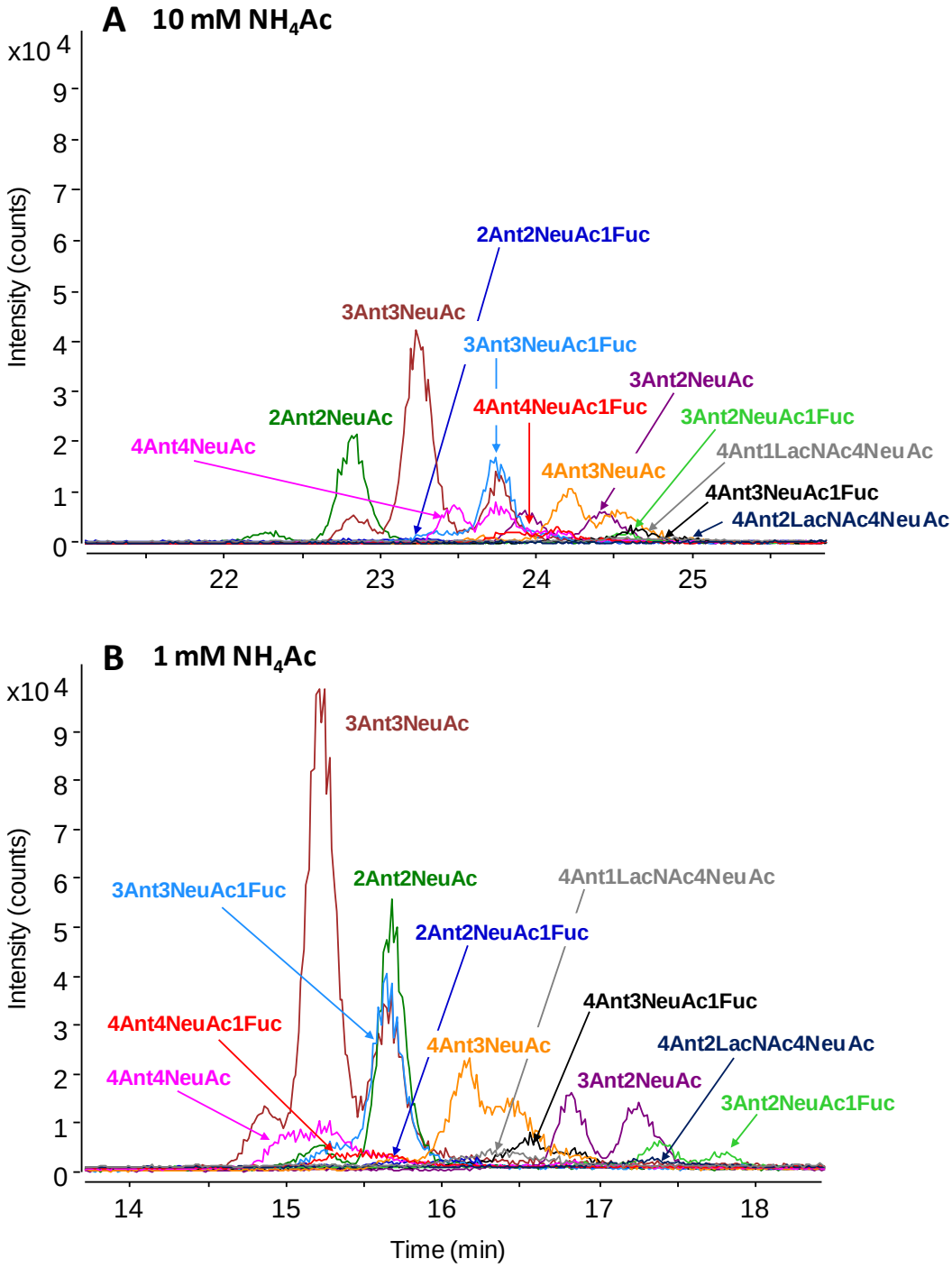

A 10 mM NH4Ac
x10 4
Intensity (counts)
2Ant2NeuAc1Fuc
3Ant3NeuAc
3Ant3NeuAc1Fuc
3Ant2NeuAc
4Ant4NeuAc1Fuc
2Ant2NeuAc
3Ant2NeuAc1Fuc
4Ant1LacNAc4NeuAc
4Ant4NeuAc
4Ant3NeuAc
4Ant3NeuAc1Fuc
4Ant2LacNAc4NeuAc
22
23
24
25
B 1 mM NH4Ac
x10 4
Intensity (counts)
3Ant3NeuAc
3Ant3NeuAc1Fuc
2Ant2NeuAc
4Ant1LacNAc4NeuAc
2Ant2NeuAc1Fuc
4Ant3NeuAc1Fuc
4Ant4NeuAc1Fuc
4Ant3NeuAc
4Ant2LacNAc4NeuAc
4Ant4NeuAc
3Ant2NeuAc
3Ant2NeuAc1Fuc
14
15
16
17
18
Time (min)

Figure 1

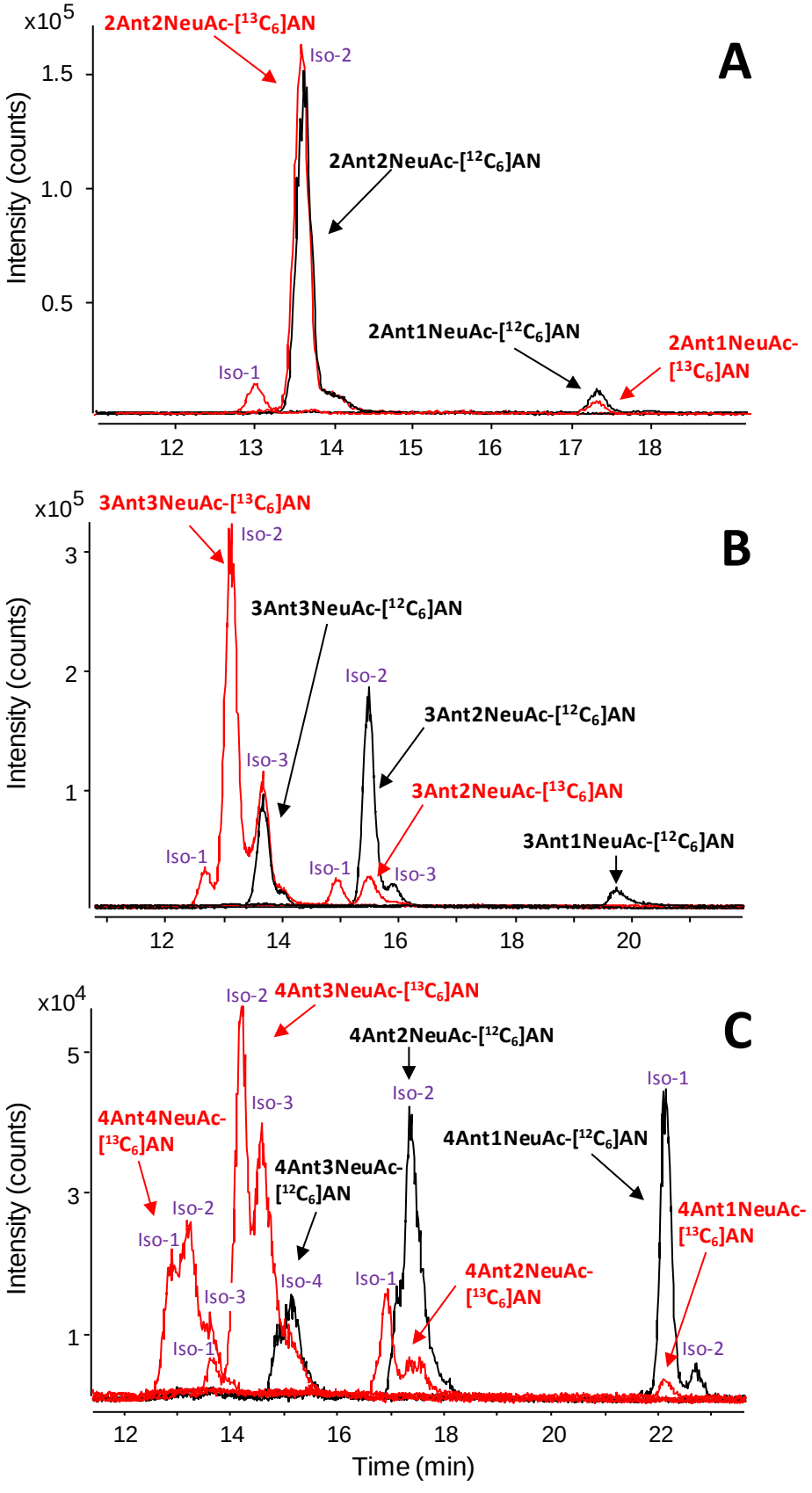

A
x10^5
Intensity (counts)
2Ant2NeuAc-[13C6]AN
Iso-2
2Ant2NeuAc-[12C6]AN
2Ant1NeuAc-[12C6]AN
2Ant1NeuAc-[13C6]AN
Iso-1
B
x10^5
3Ant3NeuAc-[13C6]AN
3Ant3NeuAc-[12C6]AN
3Ant2NeuAc-[12C6]AN
3Ant2NeuAc-[13C6]AN
3Ant1NeuAc-[12C6]AN
C
x10^4
4Ant3NeuAc-[13C6]AN
4Ant2NeuAc-[12C6]AN
4Ant4NeuAc-[13C6]AN
4Ant3NeuAc-[12C6]AN
4Ant1NeuAc-[12C6]AN
4Ant1NeuAc-[13C6]AN
4Ant2NeuAc-[13C6]AN
Time (min)
PNGase F
PNGase F + α2-3 Sialidase

Figure 2

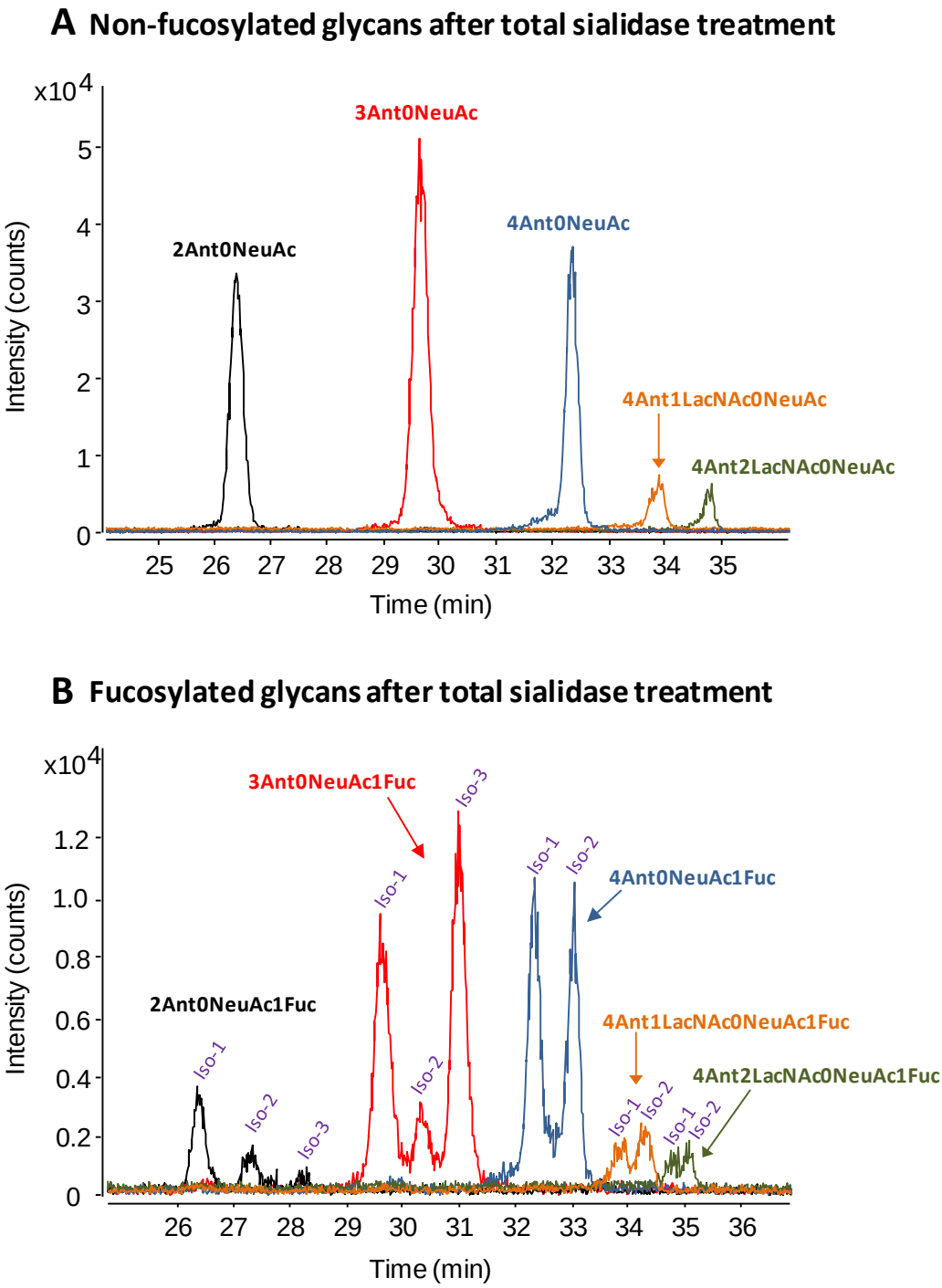

A Non-fucosylated glycans after total sialidase treatment
x10^4
Intensity (counts)
2Ant0NeuAc
3Ant0NeuAc
4Ant0NeuAc
4Ant1LacNAc0NeuAc
4Ant2LacNAc0NeuAc
Time (min)
B Fucosylated glycans after total sialidase treatment
x10^4
Intensity (counts)
3Ant0NeuAc1Fuc
4Ant0NeuAc1Fuc
2Ant0NeuAc1Fuc
4Ant1LacNAc0NeuAc1Fuc
4Ant2LacNAc0NeuAc1Fuc
Iso-1
Iso-2
Iso-3
Time (min)

Figure 3

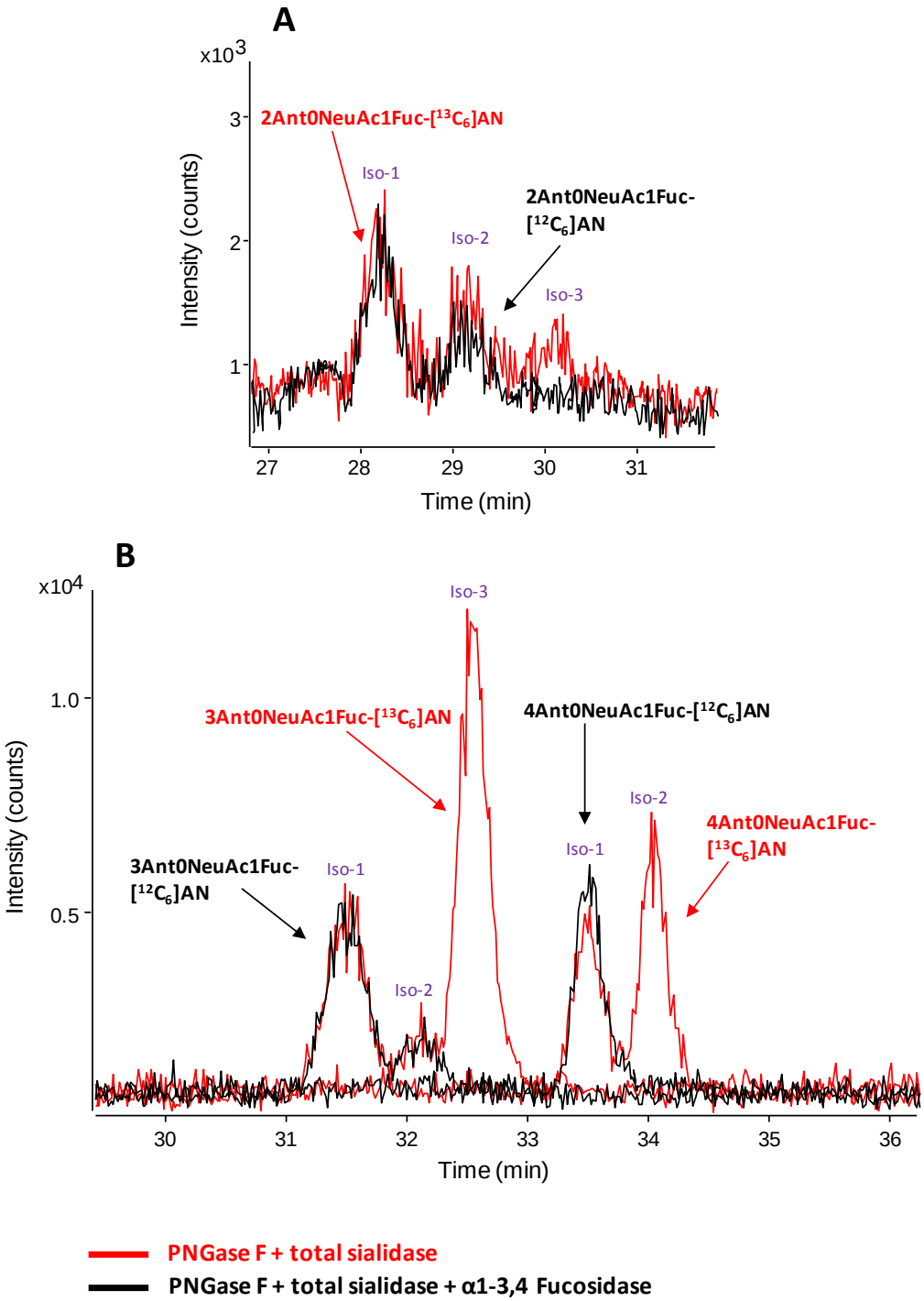

A
x10^3
3
2
1
Intensity (counts)
2Ant0NeuAc1Fuc-[13C6]AN
Iso-1
Iso-2
Iso-3
2Ant0NeuAc1Fuc-
[12C6]AN
27
28
29
30
31
Time (min)
B
x10^4
1.0
0.5
Intensity (counts)
Iso-3
3Ant0NeuAc1Fuc-[13C6]AN
4Ant0NeuAc1Fuc-[12C6]AN
Iso-2
Iso-1
4Ant0NeuAc1Fuc-
[13C6]AN
3Ant0NeuAc1Fuc-
[12C6]AN
Iso-1
Iso-2
30
31
32
33
34
35
36
Time (min)
PNGase F + total sialidase
PNGase F + total sialidase + α1-3,4 Fucosidase

Figure 4

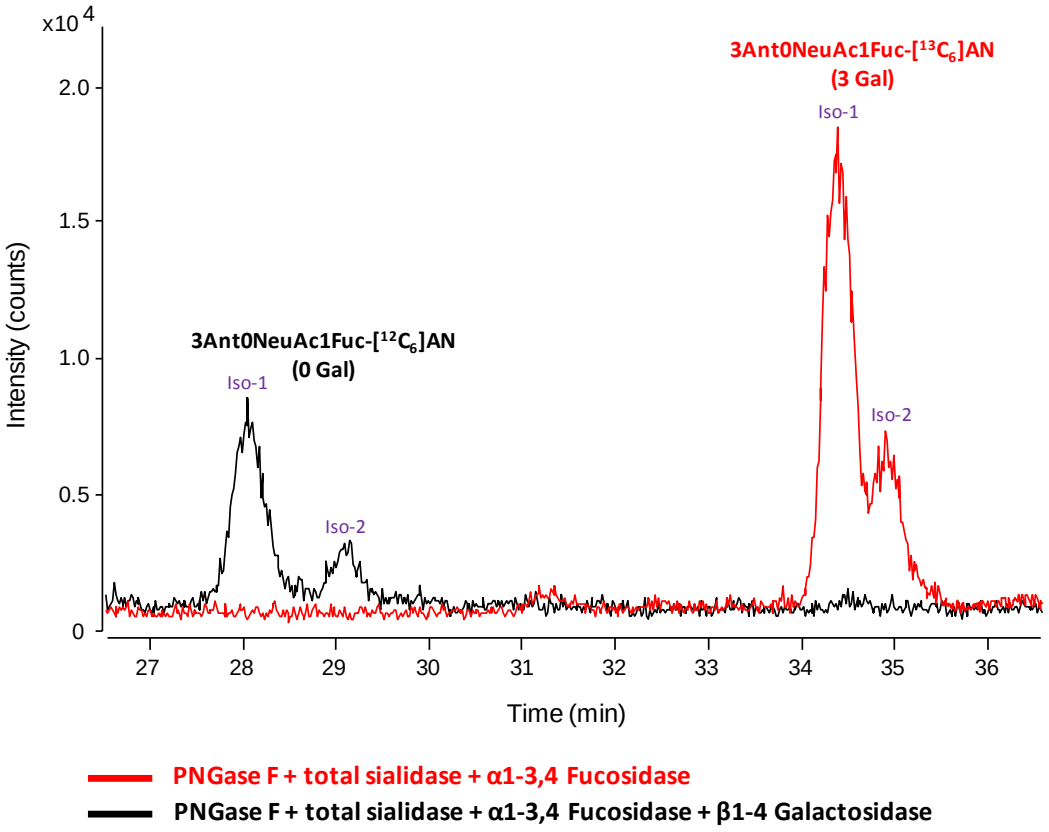

x10 4
2.0
1.5
1.0
0.5
0
Intensity (counts)
3Ant0NeuAc1Fuc-[12C6]AN
(0 Gal)
Iso-1
Iso-2
3Ant0NeuAc1Fuc-[13C6]AN
(3 Gal)
Iso-1
Iso-2
27
28
29
30
31
32
33
34
35
36
Time (min)
PNGase F + total sialidase + α1-3,4 Fucosidase
PNGase F + total sialidase + α1-3,4 Fucosidase + β1-4 Galactosidase

Figure 5