

1

2

3

4

5

6

7

8

9

10

11

12

13

14

15

16

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35

36

37

38

39

40

41

42

43

44

45

46

47

48

49

50

51

52

53

54

55

56

57

58

59

60

61

62

63

64

65

**ZWITTERIONIC-HYDROPHILIC INTERACTION CAPILLARY LIQUID
CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY
FOR THE CHARACTERIZATION OF HUMAN ALPHA-ACID-
GLYCOPROTEIN N-GLYCAN ISOMERS**

**Montserrat Mancera-Arteu^a, Estela Giménez^a, José Barbosa^a, Rosa Peracaula^b,
Victòria Sanz-Nebot^{a*}**

^a Department of Chemical Engineering and Analytical Chemistry, University of
Barcelona, Diagonal 645, E-08028 Barcelona, Spain
^b Biochemistry and Molecular Biology Unit, Department of Biology, University of
Girona, Campus Montilivi s/n, 17071 Girona, Spain

*Corresponding author: vsanz@ub.edu, +34-934021283, Fax: +34-934021233

Running title: hAGP glycan isomers by μ ZIC-HILIC-MS/MS

Keywords: Isomer, glycan, AGP, tandem mass spectrometry, ZIC-HILIC

Abstract

In this work, a μ ZIC-HILIC-MS/MS methodology was established in negative ion mode for the characterization of glycan isomers. The possibility to separate the glycan isomers by the μ ZIC-HILIC strategy coupled to a high resolution tandem mass spectrometry detection permitted us to obtain valuable information about each glycan structure. The most important diagnostic ion fragments previously described to characterize structural features of glycans, were evaluated in this study using hAGP as model glycoprotein. The assignation of hAGP glycan isomers performed in our previous work using the GRIL strategy in combination with exoglycosidase digestion [1] was used in this paper to confirm or discard some ion fragments reported in the literature and delve into the structural characterization of glycan isomers. Sialic acid as well as fucose linkage-type glycan isomers were assigned using this approach and daughter ions with higher diagnostic value were determined. The location of α 2-3/ α 2-6 sialic acids on antennas and a deeper characterization of several highly sialylated tri- and tetraantennary glycans was also possible using the established MS/MS method. Moreover, relying on the characterization performed in [1], core and antenna fucosylation were differentiated in this work using specific ion fragments obtained in the tandem mass spectra. This methodology was also applied to hAGP purified from control and pathological serum samples, which corroborated its robustness and its potential for finding novel glycan-based biomarkers in patho-glycomic studies.

1. Introduction

Glycans play a fundamental role in the function and physicochemical properties of the proteins [2–4]. Moreover, modifications in their structure have been related to many diseases such as chronic inflammation, hereditary disorders and cancer [5–10]. Thus, the interest towards the structural analysis of these compounds has risen in recent years. Complex *N*-linked glycans contain a common trimannosyl-chitobiose core substituted in the two outer mannose residues with antennas, consisting mainly of *N*-acetylglucosamine-galactose (HexNAc-Hex) units [11, 12]. *N*-acetylneuraminic acids (NeuAc) are commonly found attached to the terminal galactose in an α 2-3 or an α 2-6 linkage. Additionally, other residues such as fucose can be linked to the core or antenna HexNAc as well as to the antennary galactose. The different possible linkages of each substituent has led to the existence of isobaric isomers in glycans [13–15]. Nowadays, the in depth characterization of these isomers is of great interest to many authors as overexpression of certain glycan linkage-types were reported in cancer [6, 10, 16–20].

Exoglycosidase digestion has classically been used for the structural characterization of glycans by monitoring the products of digestion by gel-filtration chromatography, high-performance liquid chromatography (HPLC), matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [21] and, more recently, liquid chromatography coupled to mass spectrometry detection (LC-MS) [22–24]. Nevertheless, commercially available enzymes can only cleave certain linkage-types and their specificity might not be absolutely guaranteed. MALDI-MS in combination with methyl esterification procedures was another technique extensively used that allows sialic acid linkage-specific derivatization to differentiate between α 2-3 and α 2-6 linked sialic acids. However, these methods require highly purified glycan samples, harsh conditions, long reaction times and, moreover, the complete derivatization and

linkage specificity are often not achieved [25, 26]. Therefore, the use of tandem mass spectrometry approaches to obtain structural information about the sequence and linkage of glycans was promoted [12, 13, 21, 27–35]. In last years, most authors have used MS/MS in negative ion mode as it yields less ambiguous spectra and contains ion fragments that are diagnostic of specific structural features, which cannot be easily obtained from the positive ion mode [21, 28–31]. Nevertheless, in many of these studies a liquid chromatography methodology able to separate the different isomers prior to the tandem mass spectrometry detection was not used [21, 28, 32]. Moreover, several authors did not previously characterize the linkage-types present in each glycan isomer with a complementary technique [29, 30, 33, 34] and, therefore, the specific ion fragments to diagnose certain structural features could not be clearly determined. MS³ approaches were also used to study fragmentation pathways of glycan structures. Despite the potential of these methods, creating a fingerprint catalogue is only possible for few structures and requires large amounts of purified glycan samples [13, 33, 35]. For all these reasons, MSⁿ fragmentation studies are usually performed with simple structures such as mono- or bisialylated biantennary glycans.

In a previous study, human alpha-1-acid glycoprotein (hAGP) N-glycans were characterized in serum samples from healthy volunteers and from patients with pancreatic cancer (PaC) and chronic pancreatitis (ChrP) by zwitterionic–hydrophilic interaction capillary liquid chromatography coupled to mass spectrometry detection (μ ZIC-HILIC-MS), observing an increase of several fucosylated glycans as well as changes in the proportion of certain glycan isomers in PaC samples [36]. These results agreed with those previously described in the literature for carcinomas, including changes in expression of α 2-3/6 linked sialic acids and over-expression of sialyl Lewis X (SLe^x) and sialyl Lewis A (SLe^a) epitopes [16–20, 37–39]. As main *N*-glycan

modifications reported in cancer are associated with variations in sialylation and fucosylation, deepening into the characterization of sialic acid and fucose linkage-type isomers is of crucial importance to find new glycan-based biomarkers. In a recent study, we developed a robust methodology to reliably identify the linkage-type of sialic acids and fucoses in hAGP *N*-glycan isomers using an stable isotope labelling strategy with [¹²C₆]/[¹³C₆] aniline in combination with exoglycosidase digestion [1]. Bi-, tri- and tetraantennary glycan isomers differing in the sialic acid linkage-type were assigned and, moreover, α1-3 antenna and α1-6 core fucosylations were both detected in hAGP glycans.

In this work, a μZIC-HILIC-MS/MS method in negative ion mode has been developed using hAGP as model glycoprotein in order to determine reliable diagnostic fragments useful for sialic acid and fucose linkage-type assignments. The exact mass of the daughter ions obtained using a LTQ-Orbitrap mass spectrometer, together with the characterization of hAGP glycan isomers performed in our previous study [1] have allowed us to confirm or discard some diagnostic ion fragments proposed in the literature. Furthermore, the fragments established with a higher diagnostic character have been used to delve into the structure and linkage-type characterization of some highly sialylated tri- and tetraantennary hAGP glycans that were not completely assigned with exoglycosidase digestion. Finally, this methodology was also applied to control and pathological serum samples from patients with pancreatic diseases to corroborate its robustness.

2. Materials and methods

2.1. Chemicals

All chemicals used in the preparation of buffers and solutions were of analytical reagent grade. Acetic acid (HAc, glacial), formic acid (FA 98-100%), dimethylsulphoxide (DMSO) and acetone were supplied by Merck (Darmstadt, Germany). Sodium phosphate dodecahydrate ($\text{Na}_3\text{PO}_4 \cdot 12\text{H}_2\text{O}$), sodium cyanoborohydride (NaBH_3CN), [$^{12}\text{C}_6$]-aniline ([$^{12}\text{C}_6$]AN), 2-mercaptoethanol (β -ME) and sodium dodecyl sulfate (SDS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid (HCl, 37%) was supplied by Panreac (Barcelona, Spain) and “NP-40 alternative” by Calbiochem (Darmstadt, Germany). Ammonium acetate (NH_4Ac), acetonitrile (ACN) and water LC-MS quality grade from Merck and Fluka (Madrid, Spain), respectively, were used for $\mu\text{ZIC-HILIC-TOF-MS}$ analysis. A standard sample of human $\alpha 1$ -acid-glycoprotein (hAGP, 99%) was purchased from Sigma-Aldrich. Peptide N-glycosidase F (PNGase F) and $\alpha 2$ -3,6,8 neuraminidase (total sialidase) were obtained from Roche Diagnostics (Basel, Switzerland) and, $\alpha 2$ -3 neuraminidase S ($\alpha 2$ -3 sialidase) from New England Biolabs (Ipswich, MA, USA).

2.2. hAGP glycan samples

Control and pathological human serum samples were provided by Hospital Universitari Dr. Josep Trueta, Girona, Spain, following the standard procedures of its Ethics Committee. Three serum samples were analyzed: a healthy volunteer serum sample as control and two pathological serum samples, one from an individual with chronic pancreatitis (ChrP) and one from a patient suffering from pancreatic cancer (PaC) with an advanced stage (stage IV). 100 μL of each control and pathological serum samples were purified by immunoaffinity chromatography (IAC) using an anti-hAGP

column and following the method described in [36]. After IAC purification, eluted hAGP was quantified at 280 nm using a Nanodrop spectrophotometer (Thermo Scientific) following the recommendations of the manufacturer for hAGP standard. Then, hAGP samples were evaporated to dryness by Speed Vac and stored at -20 °C.

5-10 µg of hAGP (standard or purified from serum samples) were reduced with 0.5% β-ME in the presence of 0.5% of SDS in 50 mM Na₃PO₄ (pH 7.5) and boiled in a water bath for 30 min. When samples were at room temperature, a volume of 50 mM Na₃PO₄ (pH 7.5) with 1% (v/v) of NP-40 alternative was added to achieve a final concentration of 0.1% of SDS and β-ME in the samples. To release the *N*-glycans, 1µL of PNGase F (1 U) solution was added and the mixture was carefully vortexed and incubated at 37 °C for 18h. Digestion was stopped by heating in a thermo-block at 100 °C for 15 min and sample was stored at -20 °C until its use. Released *N*-glycans were purified by solid phase extraction (SPE) using Hypercarb cartridges (25 mg, 1 mL volume, Thermo Fisher Scientific). Firstly, SPE cartridges were conditioned and equilibrated with 1 mL of 60% ACN, 0.1% FA and with 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, 0.1% FA and the eluate was evaporated to dryness by Speed Vac. Dried *N*-glycans were stored at -20 °C until used.

Glycan labelling was carried out using a reaction mixture of 0.35 M aniline and 1 M NaBH₃CN in DMSO with 30% HAc. Dried glycans were mixed with 10 µL of the reaction mixture and incubated at 70 °C for 2h in a water bath. Samples were cooled to room temperature and labelled glycans were precipitated with acetone as described in [40]. Finally, samples were evaporated to dryness by Speed Vac to remove the excess of

166 acetone, and dried glycans were stored at -20 °C until their analysis. Centrifugations
167 were performed in a Mikro 220R centrifuge (Hettich Zentrifugen, Tuttlingen,
168 Germany).

169 **2.3. Sialidase digestion of hAGP *N*-glycans**

170 Sialic acids were released from hAGP *N*-glycans by enzymatic digestion with α 2-
171 3,6,8 sialidase (total sialidase) or α 2-3 sialidase. Sialidase digestion was performed
172 after PNGase F treatment in the same buffer solution of 50 mM Na₃PO₄ (pH 7.5) by
173 adding 1 μ L of total sialidase (50 mU) or 6.25 μ L of α 2-3 sialidase (50 U).
174 Subsequently, sample solution was incubated at 37 °C for 1h (α 2-3 sialidase) or 18h
175 (total sialidase). Digestions were stopped by heating in a thermo-block for 10 min at
176 100 °C, and samples were purified and labelled with aniline following the protocol
177 described in section 2.2. Samples were evaporated to dryness by Speed Vac and stored
178 at -20 °C until their analysis.

179 **2.4. μ ZIC-HILIC-MS/MS**

180 μ LC-MS/MS experiments were performed in a 1200 series capillary liquid
181 chromatography system coupled to a QTrap 6500 (AB Sciex) or a LTQ Orbitrap Velos
182 (Thermo Scientific). Separation was carried out using a ZIC-HILIC column (150 x 0.3
183 mm, 3.5 μ m, SeQuant, Umeå, Sweden). Experiments were performed at room
184 temperature with gradient elution at a flow rate of 4 μ L/min and injecting 0.25 μ L of
185 glycan sample labelled with aniline (glycan concentration: ~ 50-100 pmol/ μ L). Eluting
186 solvents were A: 1 mM NH₄Ac solution and B: acetonitrile. Gradient conditions were
187 used: solvent B from 90 to 80% (within 5 min) and from 80% to 65% (within 20 min)
188 as linear gradient, followed by cleaning and equilibration steps of B: 65→50% (within 5
189 min), 50→0% (within 5 min), 0% (over 15 min), 0→90% (within 5 min) and 90% (over

10 min). Tuning and calibration of the mass spectrometers followed the respective manufacturer's instructions. The MS/MS experiments were performed in the negative ion mode.

In ESI-QTrap MS/MS experiments, the optimum established parameters were: curtain gas flow (CUR) 25 , ion spray voltage (IS) 4500 V , source temperature 25°C , ion source gas 1 (GS1) 20 and ion source gas 2 (GS2) 0. MS spectra were collected between the mass range 600-1800 m/z and MS/MS spectra between 100 to 2000 m/z. An information dependent acquisition (IDA) method was used with seven different experiments: Enhanced MS (EMS), Enhanced resolution (ER) and five Enhanced product ion scans (EPI). Each EPI experiment selected automatically six precursor ions defined previously in an inclusion list with an intensity threshold of 500 and an exclusion time window of 20 s. The collision energy (CE) was set to 75% after optimization.

In ESI-LTQ-Orbitrap MS/MS experiments, the best operational conditions were: spray voltage 3 kV, capillary temperature 275 °C , sheath gas flow 20 , aux gas flow 10 , sweep gas flow 2 and s-lens RF level 67%. MS spectra, in this case, were obtained between 100-2000 m/z, while the MS/MS spectra were acquired between m/z 150 and 2000. Fragmentations obtained using collision induced dissociation (CID) and higher-energy collision dissociation (HCD) were compared. Finally, fragmentation experiments were performed using the HCD cell with an optimized collision energy of 70-75%. Six precursor ions, defined in the method, were automatically selected with an isolation window of 1.0 m/z.

Analyst 1.6.2 and Peak View 2.2 softwares (AB Sciex) were used for data acquisition and data analysis, respectively, in the ESI-QTrap instrument. Xcalibur 2.2

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

214 software (Thermo scientific) was used for ESI-LTQ-Orbitrap instrument control and
215 data analysis. The μ LC system was controlled by the ChemStation B.01.03 software
216 (Agilent Technologies). Interpretation and manual annotation of ion fragment exact
217 masses were performed using the SimGlycan 5 software (Premier Biosoft).

218

219

3. Results and Discussion

In the last years, some authors have proposed the use of diagnostic ion fragments for the characterization of glycan isomers. However, some of these studies were performed with low mass accuracy spectrometers [29] or they did not previously separate the different isomers by liquid chromatography [21, 28, 32]. Moreover, some authors could not be completely sure about the diagnostic value of the selected ion fragments because they did not have enough previous information about the linkage-type composition of each glycan isomer [29, 30, 33, 34], and most of them did not study MS/MS fragmentation of tri- and tetraantennary sialylated complex-type glycans. hAGP glycan isomers were characterized by stable isotope labelling with [$^{12}\text{C}_6$]/[$^{13}\text{C}_6$] aniline in combination with exoglycosidase digestion in our previous work [1], providing valuable information to confirm or discard diagnostic fragments for the identification of glycan isomers by tandem mass spectrometry. In this work, hAGP was used as model glycoprotein to establish a reliable $\mu\text{ZIC-HILIC-MS/MS}$ methodology but also delve into the structural characterization of its glycan isomers.

Two mass spectrometers were used in this study, i.e. a QTrap and a LTQ-Orbitrap. As can be observed in figure S1A and S1B for H5N4S2 glycan (isomer 2), similar ion fragments were obtained with both mass spectrometers in the analysis of hAGP glycans. Notwithstanding, the possibility to obtain the exact mass of daughter ions, the higher scan speed that permitted us to obtain more MS/MS scans for each chromatographic peak and the higher-energy collisions produced made LTQ-Orbitrap results more reliable. HCD cell was selected as more daughter ions, with high diagnostic value such as cross-ring fragment ions, were detected compared to CID fragmentation. Eight HCD energy values were evaluated ranging from 35 to 95%. Values below 55% did not produce fragmentation of the parent ion, while energy values from 85% caused the loss

of A- and D-ions. By way of an example, figure S1 compares the tandem mass spectra of H5N4S2 glycan at 55% of collision energy (Figure S1C) with the mass spectra of the same glycan at the optimal value established at 75% (Figure S1B). The ion fragments nomenclature used in this work was the proposed by Domon and Costello [41] and afterwards modified by Harvey and coworkers [31, 32]. A recompilation of most important characteristic ion fragments described in the literature for the characterization of complex-type *N*-glycans is summarized in Table S1 (supplementary material). Furthermore, ion fragments considered in the literature as a diagnostic of specific structural features (e.g. sialic acid or fucose linkage-type) are listed in Table S2. In both tables, we also enclosed those fragments generated after glycan desialylation. Moreover, it is important to remark that although some ion fragments were considered by several authors as specific of one structure [12, 13, 29, 30], in these tables we included different possible fragmentations that correspond to the same ion fragment exact mass (e.g. ion fragment at m/z 364.1243 could also correspond to B_3/Y_6 , not only to $^{3,5}A_2$ as suggested by [28]).

3.1 Ion fragments for the characterization of complex-type *N*-glycans

Prior to the characterization of the glycans in terms of sialic acid and fucose linkage-type isomers, desialylation with $\alpha 2$ -3,6,8 sialidase (total sialidase) permitted to reveal easily information about antenna sequence and composition of complex-type glycans. In this regard, as can be observed in Figure 1A for the desialylated biantennary glycan of hAGP (H5N4), B- and C-ions at m/z 179.1 (C_1) and 364.1 (B_2) as well as several cross-ring fragments such as 424.1 ($^{1,3}A_3$) were abundant and provided sequencing characterization of antennas. For higher complex structures such as tri- and tetraantennary desialylated glycans (H6N5 and H7N6, respectively), some of the specific ions listed in Table 1 revealed the existence of a branched antenna. The

observed fragments that disclosed this feature were 508.2 for H6N5 glycan and 609.2, 771.3 and 789.3 for H7N6 glycan as well as the ions at m/z 951.3 and 933.3 for both glycan structures (see Figure 1B and 1C). Moreover, as was previously reported in many studies [12, 21, 29, 30], D-ions provided information about the 6-antenna and were diagnostic of its composition. In this study, D and $[D-H_2O]^+$ ions at m/z 688.2 and 670.2 were used to identify the position of the unbranched antenna and were detected in the tandem mass spectra of H5N4 and H6N5 glycans while they were not present in H7N6 (see Table 1 and Figure 1). The existence of these D-ions in the case of the H6N5 revealed that, in hAGP, the branched antenna is located on the 3-antenna. These results were also corroborated later for the sialylated glycans by observing D-ions at m/z 979.3 and 961.3 (see Table 2). Even though it was expected to observe D-ions at m/z 1053.3 or 1035.3 for the H7N6 glycan to confirm the branching also on the 6-antenna, only the absence of D-ions at m/z 688.2 and 670.2 were observed in this case.

On the other hand, some authors have reported E-ions as a diagnostic of the 3-antenna composition at m/z 466.1 or 831.3, in glycans with the branching located on the 3-antenna [12, 21]. Nevertheless, in this study, the E-ion 466.1 was also observed in tri- and tetraantennary glycans showing its lack of specificity to unbranched 3-antenna. Furthermore, E-ions correspond to other structures, such as $^{0,4}X_3/B_3$ or $^{2,4}X_3/B_3$, rather than to the $^{0,4}A_3$ structure previously described [12]. For this reason, E-ions were not considered in this work as specifics of 3-antenna composition. Thus, the branched 3-antenna structure of the H6N5 glycan was concluded using only the D-ions observed.

3.2 Characterization of sialic acid linkage-type N-glycan isomers

In a previous work, a methodology to identify sialic acid linkage-type glycan isomers in hAGP glycans was established combining total and $\alpha 2$ -3 sialidase digestions

with glycan reductive isotope labelling (GRIL) using [$^{12}\text{C}_6$]/[$^{13}\text{C}_6$] aniline [1]. Although some authors have pointed out problems with the specificity of enzymes to cleavage one single linkage-type, the combination of exoglycosidase digestions with the GRIL methodology enabled a reliable assignment of sialic acid linkages. Hence, hAGP glycans were used in this study to evaluate certain ion fragments for the characterization of sialic acid linkage-type isomers by tandem mass spectrometry.

For this purpose, hAGP glycan isomers were analyzed by ZIC-HILIC-MS/MS and, moreover, some glycans were also digested with α 2-3 sialidase in order to completely characterize the greatest number of isomers. The ion fragments 306.1, 408.1 and 470.1 proposed by Wheeler *et al.* [28] to assign sialic acid linkage-type were also detected in hAGP glycans. Nevertheless, ions 350.1 and 364.1 described afterwards for the same purpose [30], were not considered by us as several structures were found to produce the same exact mass, showing their lack of specificity (e.g. $^{2,4}\text{A}_2$ or $^{1,3}\text{A}_2$ fragments that could be specific of α 2-3 NeuAc show the same exact mass of 350.1087 as $^{0,4}\text{A}_2$ that could be specific of α 2-6 NeuAc, see supplemental Table S2). Moreover, the ion fragment 364.1 was also observed in the tandem mass spectra of the desialylated *N*-glycans demonstrating its low diagnostic value for α 2-6 NeuAc (see Figure 1).

Although the native H5N4S2 glycan of hAGP showed two isomers, after α 2-3 sialidase digestion the first isomer disappeared and turned into the H5N4S1, increasing its native peak area, as was described in more detail in our previous work [1]. Hence, isomer 2 of H5N4S2 and H5N4S1 glycan showed all sialic acids linked α 2-6. Figure 2A and 2B show the tandem mass spectra of isomers 1 and 2 of the native H5N4S2 glycan, and the H5N4S1 glycan obtained after α 2-3 sialidase digestion, respectively. As can be observed, similar MS/MS spectra were obtained for isomer 2 of H5N4S2 and H5N4S1 glycans (Figure 2A(ii) and 2B(i), respectively). In both cases, an abundant ion at m/z

306.1 and the ion fragment 470.1 were observed, which have been previously reported as characteristic of α 2-6 linked sialic acids [28]. In addition, the absence of the ion fragment 408.1 in these glycans confirmed these three ion fragments (306.1, 408.1 and 470.1) as diagnostic for sialic acid linkage-type assignation. Moreover, the presence of D and D-H₂O ions at m/z 979.3 and 961.3 in H5N4S1 glycan revealed the localization of the α 2-6 NeuAc on the 6-antenna (see Table 2 and Figure 2B). Therefore, the first isomer of the H5N4S2 glycan, which turned into H5N4S1 glycan after α 2-3 sialidase digestion, showed one α 2-3 NeuAc located on the 3-antenna and one α 2-6 NeuAc located on the 6-antenna. This assignation was also corroborated by observing the ion fragments 306.1 and 408.1 in the MS/MS spectra of the native isomer 1, despite the low abundance of these ion fragments as a consequence of its coelution with the more abundant H6N5S3 glycan (see Table 2 and Figure 2A(i)).

Regarding the native trisialylated triantennary glycan (H6N5S3), which showed three isomers, ion fragments diagnostic of α 2-6 and α 2-3 NeuAc were observed in isomer 1 and 2 (m/z 306.1 and 408.1, see Table 2 and Figure 3A (i) and (ii)). In contrast, isomer 3 only presented α 2-6 NeuAc fragments (m/z 306.1 and 470.1, see Table 2 and Figure 3A (iii)), showing higher relative intensity of 306.1 ion in this isomer compared to the others. These diagnostic ion fragments confirmed our previous assignation [1]. Isomers 1 and 2 presented both α 2-3 and α 2-6 sialic acid linkage-types and only the third isomer showed all sialic acids α 2-6 linked. Additionally, some authors have pointed out that α 2-3 NeuAc are more labile than α 2-6 ones and they have described the relative intensity between ions 290.1 (NeuAc) and 655.2 (NeuAc-Hex-HexNAc) as a parameter to evaluate the linkage-type composition in each glycan isomer [13, 29]. In our case, similar relative intensities were obtained for all isomers of H6N5S3 glycan, which demonstrated this relation is independent of the proportion of

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

344 α 2-3 and α 2-6 linkages. On the other hand, $[D-H_2O]^-$ ions at m/z 961.3 were also
345 observed in the three isomers (see Table 2), corroborating that H6N5S3 glycan was
346 branched on the 3-antenna.

347 With respect to the native H6N5S2 glycan, two isomers were detected but, after α 2-
348 3 sialidase treatment, the first isomer disappeared, the peak area of the second isomer
349 increased considerably and a third isomer appeared. As was justified in [1], after α 2-3
350 sialidase digestion, isomers 1 and 2 of H6N5S3 glycan turned into isomers 3 and 2 of
351 H6N5S2 glycan, respectively, and only the third isomer remained. Hence, isomers 1 and
352 2 of H6N5S3 showed two α 2-6 NeuAc and one α 2-3 NeuAc, while isomer 3 presented
353 the three sialic acids α 2-6 as was discussed before [1]. In this work, the tandem mass
354 spectra of isomers 2 and 3 of H6N5S2 glycan, obtained after α 2-3 sialidase digestion,
355 were studied in order to identify the structural differences between them, which could
356 not be determined previously with exoglycosidase digestion. Figure 3B (i) and (ii) show
357 the tandem mass spectra obtained for both isomers. Only diagnostic fragments of α 2-6
358 NeuAc (306.1 and 470.1) with similar relative intensities were detected in both isomers,
359 which corroborated the same NeuAc linkage-type composition. These results were
360 consistent with our previous study with exoglycosidase digestion and GRIL analysis
361 [1]. Furthermore, the MS/MS method provided additional information about the
362 position of these α 2-6 sialic acids as D-ions detected at m/z 979.3 and 961.3 (see Table
363 2) confirmed that in both isomers one NeuAc was located on the unbranched 6-antenna,
364 and consequently, the other one was on the branched 3-antenna . Thus, the difference
365 among isomer 2 and 3 had to be associated with the different localization of the α 2-6
366 NeuAc on the branched 3-antenna (2- or 4- branching). The presence of the ion at m/z
367 775.3 ($^{0,2}A_4$) in isomer 2 of the H6N5S2 glycan and its absence in isomer 3 (see Table
368 2) suggested that the α 2-6 NeuAc present in the branched 3-antenna was located on the

1 369 4- and 2-branching respectively. Moreover, the presence of this specific ion in all
2 370 isomers of H6N5S3 (see Table 2), corroborated the specific character of this ion
3
4 371 fragment to characterize the NeuAc position on the 4-branching. Furthermore, it also
5
6 372 confirmed that the α 2-3 NeuAc in isomers 1 and 2 of this glycan was located on the 4-
7
8 373 and 2-branching respectively (as they are related to isomers 3 and 2, respectively, of
9
10 H6N5S2 glycan after α 2-3 sialidase digestion, see Figure 3). The assignation of the
11
12 374 branching position of the α 2-3 NeuAc would not have been possible without the
13
14 375 combined strategy of specific sialidase digestion and MS/MS analysis. On the other
15
16 376 hand, it is important to note that, while the ion 306.1 was present in all isomers
17
18 377 containing at least one α 2-6 NeuAc, and its relative abundance could be used to estimate
19
20 378 the proportion of α 2-6 NeuAc in each isomer, the ion 470.1 was only observed when all
21
22 379 sialic acids were α 2-6 . This fact was concluded because the ion at m/z 470.1 was not
23
24 380 observed in isomers 1 and 2 of the native H6N5S3 but it was observed in the
25
26 381 corresponding glycan isomers obtained after α 2-3 sialidase digestion (isomers 3 and 2
27
28 382 of H6N5S2 glycan, respectively). Hence, ion 470.1 could be used for screening
29
30 383 purposes to identify those glycan isomers with all sialic acids α 2-6.
31
32
33
34
35
36
37
38
39

40 385 Although, characterization of highly branched sialylated glycans is still a challenge
41
42 386 and therefore less addressed by glycomic researchers, in the particular case of hAGP,
43
44 387 tetraantennary glycans are less abundant and as they are also more difficult to ionize,
45
46 388 their characterization is even more complicated. Nevertheless, in this work, an
47
48 389 estimation of the α 2-6 NeuAc composition in H7N6S3 glycan isomers was carried out.
49
50 390 For this purpose, only the ion fragment at m/z 306.1 was used as other diagnostic ion
51
52 391 fragments (such as 408.1 or 470.1) were not observed due to the lower abundance of
53
54 392 this glycan. In agreement with our previous study [1], isomer 4 only presented α 2-6
55
56 393 NeuAc with the highest intensity of 306.1 ion with respect to the other isomers (data not
57
58
59
60
61
62
63
64
65

shown). Furthermore, the relative intensity of this diagnostic ion also suggested that the proportion of α 2-6 NeuAc was higher in isomer 3 compared to isomer 2. Thus, our previous assignation [1] was complemented identifying isomer 2 with two α 2-3 NeuAc and one α 2-6 NeuAc, and isomer 3 with one α 2-3 NeuAc and two α 2-6 NeuAc. Nevertheless, contrary to the tentative characterization carried out in our previous work [1], the ion at m/z 306.1 was not observed in isomer 1, which could reveal that all sialic acids present in this isomer are α 2-3 linked. These results deepened the partial assignation of this glycan performed previously with exoglycosidase digestion [1], identifying tentatively the linkage-type of the three sialic acids in all isomers of H7N6S3. In addition, the characterization of all hAGP glycan isomers performed in our previous and present studies could also reveal that the proportion of α 2-6 NeuAc is strongly related with the elution order of the ZIC-HILIC column, demonstrating that glycan isomers with a higher proportion of α 2-6 linked NeuAc are eluted later.

3.3 Characterization of fucose linkage-type *N*-glycan isomers

Fucose linkage-type characterization of the major glycans of hAGP was also performed in this work using several diagnostic ion fragments, some of them previously reported in the literature [12, 21, 29] (Table S2 of the supplementary material). In general, diagnostic fucose ion fragments are usually present at low abundance or are not directly observed in the tandem mass spectra as the fucose, mainly the antennary fucose, is preferably removed from the glycan structure during fragmentation [21]. Thus, few studies have been able to identify fucose linkage-type of complex *N*-glycans [12, 21, 42], which demonstrated finding novel specific ion fragments to differentiate between Fuc Core and Fuc Antenna is still necessary. As in the characterization of sialic acids, the assignation performed in [1] was used to verify the reliability of certain ion fragments described in the literature for fucose isomer identification. In this case, total

sialidase digestion was also carried out in order to remove heterogeneity and obtain the isomers resulting only from different fucose linkages. Isomers 1 and 2 of H5N4F1 and H6N5F1 glycans, which were previously assigned as core fucosylated [1], and isomer 3 of H6N5F1 glycan, which was previously assigned as α 1-3 antennary fucosylated [1], were studied in this work as they were intense enough to delve into the differentiation between Fuc Core and Fuc Antenna using tandem mass spectra ion fragments. Figure 4 shows the tandem mass spectra of these glycans in hAGP. Fragments diagnostic of core fucosylation such as Y₁ at m/z 461.2 and 443.2 were found in isomers 1 and 2 of the biantennary glycan (H5N4F1) but at very low intensity and, thus, with higher error mass. Moreover, the ion at m/z 443.2 was detected in isomer 1 and 2 of the triantennary glycan (H6N5F1) but only in half of the runs. These results suggested the difficulty of detecting these diagnostic ions when the complexity of the glycan increases, and thus, their absence in the MS/MS spectra could not always be indicative of lack of core fucosylation in tri- and tetraantennary glycans. However, the absence of the ion fragment Y₁ at m/z 297.1 in the tandem mass spectra of isomers 1 and 2 of H5N4F1 and H6N5F1 glycans (see Figure 4A and 4C), which was abundant in the tandem mass spectra of the non-fucosylated glycans (Figure 1A and 1B), revealed that the fucose unit could be linked to the core. Therefore, the previous assignation of these isomers as core fucosylated [1] was confirmed and the diagnostic potential of the ion fragment 297.1 to differentiate between Fuc Core and Fuc Antenna was demonstrated. Moreover, other ion fragments such as ^{2,4}A₅/Y₅ at m/z 1095.3 suggested that the fucose unit was not located on any antenna (see Table 1 and Figure 4A). Additionally, in the tandem mass spectra of H5N4F1 and H6N5F1 (isomers 1 and 2), specific ion fragments of antennary fucosylation were not observed.

With respect to isomer 3 of H6N5F1 glycan, as can be observed in Figure 4B, the

ion fragment at m/z 297.1 was observed with practically the same relative intensity than in the tandem mass spectrum of H6N5 glycan (Figure 1B), which demonstrated, in this case, that the fucose was not linked to the core HexNAc. Furthermore, the ion 364.1 (which corresponds to the antennary Hex-HexNAc unit) presented lower relative intensity in comparison with the tandem mass spectrum of the H6N5 glycan, showing that the fucose unit could be located at one antenna. This fact was definitely confirmed with the presence of the fragment 570.2 ($^{0,4}A_4/Y_5$, see Figure 4B and Table 1)), despite its low intensity. Moreover, the absence of 325.1 (C_1 , see Supplementary Table 2) revealed that the fucose was linked to the antennary HexNAc, as was described in our previous assignation [1], rather than to the Hex (galactose). Additionally, D-ion at m/z 670.2 was observed (see Figure 4B and Table 1) showing that the fucose could be located at 2- or 4-branching of the 3-antenna. The considerable decrease in the relative intensity of the ion fragments 933.3 ($^{0,4}A_4$) and 1095.3 ($^{2,4}A_5/Y_3$) with respect to the tandem mass spectrum of the H6N5 glycan corroborated that the fucose was linked to a HexNAc of the branched 3-antenna (compare Figure 4B with Figure 1B). The presence of these ions in the tandem mass spectra could be attributed to the preferable elimination of the fucose linked to the 3-position of the antennary HexNAc [21], as was previously mentioned.

3.4 Application of the MS/MS methodology to serum samples

Once the μ ZIC-HILIC-MS/MS methodology was optimized and used to characterize sialic acid and fucose linkage-type isomers of several glycans released from hAGP standard, the same method was applied to hAGP serum samples to corroborate its robustness. Figure 5 shows the extracted ion chromatogram (EIC) of the H6N5S3 glycan obtained in three serum samples: one control, one pathological sample with chronic pancreatitis (ChrP) and one with an advanced stage of pancreatic cancer

(PaC, stage IV) and the corresponding MS/MS spectra obtained for the third isomer. As can be observed, the diagnostic ion fragments obtained in the tandem mass spectra of isomer 3 of H6N5S3 in real samples (Figure 5 (ii)) were the same as the ones obtained in hAGP standard (see Table 2 and Figure 3C (iii)). The same results were obtained for all studied glycans demonstrating the robustness of the established methodology. Even though in this work the analysis of these pathological samples was performed only with the aim of evaluating the MS/MS method in serum samples, the tendency to increase the relative abundance of the isomer 3 of the triantennary glycan in PaC with respect to the control and to the ChrP samples was again revealed (see Figure 5). This fact was reported in a previous work [36] in which a larger number of PaC and ChrP samples were analyzed and could be again related with our assignation, as isomer 3 was the only one that presented all sialic acids α 2-6 linked and the overexpression of this linkage-type was reported in cancer by several authors [8, 20, 43]. The glycans obtained from these hAGP serum samples were also treated with total sialidase in order to study the future applicability of this methodology also in the characterization of fucose isomers. Similar tandem mass spectra were obtained for all fucosylated glycan isomers compared to the ones obtained previously in hAGP standard (data not shown). These results revealed again the robustness of this approach and demonstrated that the analysis of additional PaC samples is still pending to evaluate the potential of some glycan isomers as possible biomarkers of this pathology.

Conclusions

In this paper, a μ ZIC-HILIC-MS/MS methodology was established in negative ion mode for the characterization of glycan isomers. With this strategy, the separation of the glycan isomers by μ ZIC-HILIC combined with high resolution tandem mass spectrometry detection enabled to unequivocally assign the ion fragments to a particular glycan structure. Firstly, the most important diagnostic ion fragments, previously reported in [12, 13, 21, 27–34] and used for the characterization of glycans, were evaluated with hAGP. Sequencing of *N*-glycan antennas was carried out using B- and C-ions and, structural features such as branching location were determined using D-ions. This method was also applied in this work for the assignation of the sialic acid linkage-type. In this regard, characterization of bi- and triantennary hAGP glycans was carried out in this study using several specific ion fragments. The exact mass of daughter ions provided by the LTQ-Orbitrap mass spectrometer and our previous results using exoglycosidase digestion in combination with the GRIL methodology [1] permitted us to confirm or discard some diagnostic ion fragments proposed in the literature. Moreover, unlike our previous work [1], the established MS/MS methodology enabled the location of α 2-3 and α 2-6 sialic acids on antennas and also the complete characterization of some tetraantennary glycans such as H6N7S3, which were only partially characterized with exoglycosidase digestion [1]. Furthermore, based on the characterization performed previously in [1], differentiation between Fuc core and Fuc antenna isomers was also performed in this work using specific ion fragments obtained in the tandem mass spectra. Y_1 ions at m/z 461.2 and 443.2 were not considered reliable diagnostic fragments of core fucosylation in the case of tri- and tetraantennary glycans as they were hardly detected in those isomers previously identified as core fucosylated [1]. On the other hand, the ion fragment 297.1, which describes the absence of core

1 515 fucosylation, was established as an important diagnostic ion for fucose linkage-type
2 516 assignation. Finally, robustness and future applicability of the MS/MS method was
3
4 517 demonstrated for the study of control and pathological serum samples in order to find
5
6
7 518 novel glycan isomers that could be used as biomarkers in cancer.
8
9

10 519
11
12

13
14 520
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

521 **Acknowledgments**

522 This study was supported by Spanish Ministry of Economy and Competitiveness
523 (CTQ2014-56777-R awarded to V. S-N and BIO2015-66356-R awarded to R.P.).
524 Montserrat Mancera-Arteu acknowledges the University of Barcelona for an ADR
525 fellowship. The authors thank the Separation Techniques Unit of the Scientific and
526 Technological Centres of the University of Barcelona (CCiTUB) for technical
527 assistance and advice.

528

References

- [1] M. Mancera-Arteu, E. Giménez, J. Barbosa, V. Sanz-Nebot, 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, *Anal. Chim. Acta* 940 (2016) 92–103.
- [2] R. Dwek, Glycobiology: towards understanding the function of sugars, *Chem. Rev.* 96 (1996) 683–720.
- [3] W. Morelle, J.C. Michalski, Analysis of protein glycosylation by mass spectrometry, *Nat. Protoc.* 2 (2007) 1585–1602.
- [4] K. Mariño, J. Bones, J.J. Kattla, P.M. Rudd, A systematic approach to protein glycosylation analysis: a path through the maze, *Nat. Chem. Biol.* 6 (2010) 713–723.
- [5] T. Cartwright, R. Schwalbe, Atypical sialylated N-glycan structures are attached to neuronal voltage-gated potassium channels, *Biosci. Rep.* 29 (2009) 301–313.
- [6] M.J. Kailemia, D. Park, C.B. Lebrilla, Glycans and glycoproteins as specific biomarkers for cancer, *Anal. Bioanal. Chem.* (2016).
- [7] A. Lee, J.M. Chick, D. Kolarich, P. Haynes, G.R. Robertson, M. Tsoli, L. Jankova, S.J. Clarke, N.H. Packer, M.S. Baker, Liver membrane proteome glycosylation changes in mice bearing an extra-hepatic tumor, *Mol. Cell. Proteomics* 10 (2011).
- [8] Y. Mechref, Y. Hu, A. Garcia, A. Hussein, Identifying cancer biomarkers by mass spectrometry-based glycomics, *Electrophoresis* 33 (2012) 1755–1767.
- [9] M. Nakano, R. Saldanha, A. Gobel, M. Kavallaris, N.H. Packer, Identification of glycan structure alterations on cell membrane proteins in desoxyepothilone B resistant leukemia cells, *Mol. Cell. Proteomics* 10 (2011).
- [10] M.K. Sethi, W.S. Hancock, S. Fanayan, Identifying N-glycan biomarkers in colorectal cancer by mass spectrometry, *Am. Chem. Soc.* 49 (2016) 2099–2106.
- [11] P.H. Jensen, N.G. Karlsson, D. Kolarich, N.H. Packer, Structural analysis of N- and O-glycans released from glycoproteins, *Nat. Protoc.* 7 (2012) 1299–1310.
- [12] D.J. Harvey, L. Royle, C.M. Radcliffe, P.M. Rudd, R.A. Dwek, Structural and quantitative analysis of N-linked glycans by matrix-assisted laser desorption ionization and negative ion nanospray mass spectrometry, *Anal. Biochem.* 376 (2008) 44–60.
- [13] K. Deguchi, Y. Takegawa, H. Ito, N. Miura, S. Yoshioka, S. Nagai, H. Nakagawa, S.I. Nishimura, Structural assignment of isomeric 2-aminopyridine-derivatized monosialylated biantennary N-linked oligosaccharides using negative-ion multistage tandem mass spectral matching, *Rapid Commun. Mass Spectrom.* 20 (2006) 412–418.
- [14] F. Tousi, J. Bones, W.S. Hancock, M. Hincapie, Differential chemical derivatization integrated with chromatographic separation for analysis of isomeric sialylated N-glycans: A nano-hydrophilic interaction liquid chromatography-MS platform, *Anal. Chem.* 85 (2013) 8421–8428.
- [15] M. Pabst, J. Grass, S. Toegel, E. Liebminger, R. Strasser, F. Altmann, Isomeric analysis of oligomannosidic N-glycans and their dolichol-linked precursors, *Glycobiology* 22 (2011) 389–399.

- [16] C. a Reis, H. Osorio, L. Silva, C. Gomes, L. David, Alterations in glycosylation as biomarkers for cancer detection., *J. Clin. Pathol.* 63 (2010) 322–329.
- [17] M. Balmaña, E. Giménez, A. Puerta, E. Llop, J. Figueras, E. Fort, V. Sanz-Nebot, C. de Bolós, A. Rizzi, S. Barrabés, M. de Frutos, R. Peracaula, Increased α 1-3 fucosylation of α -1-acid glycoprotein (AGP) in pancreatic cancer, *J. Proteomics* 132 (2016) 144–154.
- [18] S. Singh, K. Pal, J. Yadav, H. Tang, K. Partyka, D. Kletter, P. Hsueh, E. Ensink, B. KC, G. Hostetter, H.E. Xu, M. Bern, D.F. Smith, A.S. Mehta, R. Brand, K. Melcher, B.B. Haab, Upregulation of glycans containing 3' fucose in a subset of pancreatic cancers uncovered using fusion-tagged lectins, *J. Proteome Res.* 14 (2015) 2594–2605.
- [19] L.R. Ruhaak, S. Miyamoto, C.B. Lebrilla, Developments in the identification of glycan biomarkers for the detection of cancer., *Mol. Cell. Proteomics* 12 (2013) 846–55.
- [20] M. Hedlund, E. Ng, A. Varki, N.M. Varki, α 2-6-linked sialic acids on N-glycans modulate carcinoma differentiation in vivo, *Cancer Res.* 68 (2008) 388–394.
- [21] D.J. Harvey, Fragmentation of negative ions from carbohydrates: Part 3. Fragmentation of hybrid and complex N-linked glycans, *J. Am. Soc. Mass Spectrom.* 16 (2005) 647–659.
- [22] J.L. Abrahams, N.H. Packer, M.P. Campbell, Relative quantitation of multi-antennary N-glycan classes: combining PGC-LC-ESI-MS with exoglycosidase digestion, *Analyst* (2015) 5444–5449.
- [23] L. Mauko, N.A. Lacher, M. Pelzing, A. Nordborg, P.R. Haddad, E.F. Hilder, Comparison of ZIC-HILIC and graphitized carbon-based analytical approaches combined with exoglycosidase digestions for analysis of glycans from monoclonal antibodies, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 911 (2012) 93–104.
- [24] S. Tao, Y. Huang, B.E. Boyes, R. Orlando, Liquid Chromatography-Selected Reaction Monitoring (LC-SRM) Approach for the Separation and Quantitation of Sialylated N-Glycans Linkage Isomers, *Anal. Chem.* 86 (2014) 10584–10590.
- [25] K.R. Reiding, D. Blank, D.M. Kuijper, A.M. Deelder, M. Wührer, High-throughput profiling of protein N-glycosylation by MALDI-TOF-MS employing linkage-specific sialic acid esterification, *Anal. Chem.* 86 (2014) 5784–5793.
- [26] S.F. Wheeler, P. Domann, D.J. Harvey, Derivatization of sialic acids for stabilization in matrix-assisted laser desorption/ionization mass spectrometry and concomitant differentiation of α (2-3)- and α (2-6)-isomers, *Rapid Commun. Mass Spectrom.* 23 (2009) 303–312.
- [27] D.J. Harvey, Structural determination of N-linked glycans by matrix-assisted laser desorption/ionization and electrospray ionization mass spectrometry, *Proteomics* 5 (2005) 1774–1786.
- [28] S.F. Wheeler, D.J. Harvey, Negative ion mass spectrometry of sialylated carbohydrates: Discrimination of N-acetylneuraminic acid linkages by MALDI-TOF and ESI-TOF mass spectrometry, *Anal. Chem.* 72 (2000) 5027–5039.
- [29] A. V. Everest-Dass, J.L. Abrahams, D. Kolarich, N.H. Packer, M.P. Campbell, Structural feature ions for distinguishing N- and O-linked glycan isomers by LC-ESI-IT MS/MS, *J. Am. Soc. Mass Spectrom.* 24 (2013) 895–906.

- [30] C. Michael, A.M. Rizzi, Tandem mass spectrometry of isomeric aniline-labeled N-glycans separated on porous graphitic carbon: Revealing the attachment position of terminal sialic acids and structures of neutral glycans, *Rapid Commun. Mass Spectrom.* 29 (2015) 1268–1278.
- [31] D.J. Harvey, Fragmentation of negative ions from carbohydrates: Part 2. Fragmentation of high-mannose N-linked glycans, *J. Am. Soc. Mass Spectrom.* 16 (2005) 631–646.
- [32] D.J. Harvey, R.L. Martin, K.A. Jackson, C.W. Sutton, Fragmentation of N-linked glycans with a matrix-assisted laser desorption/ionization ion trap time-of-flight mass spectrometer, *Rapid Commun. Mass Spectrom.* 18 (2004) 2997–3007.
- [33] H. Ito, K. Yamada, K. Deguchi, H. Nakagawa, S.-I. Nishimura, Structural assignment of disialylated biantennary N-glycan isomers derivatized with 2-aminopyridine using negative-ion multistage tandem mass spectral matching, *Rapid Commun. mass Spectrom.* 21 (2007) 212–218.
- [34] S. Zhou, X. Dong, L. Veillon, Y. Huang, Y. Mechref, LC-MS/MS analysis of permethylated N-glycans facilitating isomeric characterization, *Anal. Bioanal. Chem.* (2016).
- [35] A. V. Everest-Dass, D. Kolarich, M.P. Campbell, N.H. Packer, Tandem mass spectra of glycan substructures enable the multistage mass spectrometric identification of determinants on oligosaccharides, *Rapid Commun. Mass Spectrom.* 27 (2013) 931–939.
- [36] E. Giménez, M. Balmaña, J. Figueras, E. Fort, C. de Bolós, V. Sanz-Nebot, R. Peracaula, A. Rizzi, Quantitative analysis of N-glycans from human α -acid-glycoprotein using stable isotope labeling and zwitterionic hydrophilic interaction capillary liquid chromatography electrospray mass spectrometry as tool for pancreatic disease diagnosis, *Anal. Chim. Acta* 866 (2015) 59–68.
- [37] V. Padler-Karavani, Aiming at the sweet side of cancer: Aberrant glycosylation as possible target for personalized-medicine, *Cancer Lett.* 352 (2014) 102–112.
- [38] A. Sarrats, R. Saldoval, E. Pla, E. Fort, D.J. Harvey, W.B. Struwe, L.R. De, P.M. Rudd, R. Peracaula, Glycosylation of liver acute-phase proteins in pancreatic cancer and chronic pancreatitis, *Proteomics.Clin.Appl.* 4 (2010) 432–448.
- [39] K.D. Smith, J. Behan, G. Matthews Smith, A.M. Magliocco, Alpha-1-acid glycoprotein (AGP) as a potential biomarker for breast cancer, *Glycosylation* (2012) 201–222.
- [40] E. Giménez, V. Sanz-Nebot, A. Rizzi, Relative quantitation of glycosylation variants by stable isotope labeling of enzymatically released N-glycans using $[^{12}\text{C}]/[^{13}\text{C}]$ aniline and ZIC-HILIC-ESI-TOF-MS, *Anal. Bioanal. Chem.* 405 (2013) 7307–7319.
- [41] B. Domon, C.E. Costello, A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates, *Glycoconjugate J* 5 (1988) 397–409.
- [42] C. Nwosu, H.K. Yau, S. Becht, Assignment of core versus antenna fucosylation types in protein N-glycosylation via procainamide labeling and tandem mass spectrometry, *Anal. Chem.* 87 (2015) 5905–5913.

657 [43] B.N. Vajaria, K.R. Patel, R. Begum, P.S. Patel, Sialylation: an Avenue to Target
658 Cancer Cells, *Pathol. Oncol. Res.* 22 (2016) 443–447.

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

Figure legends

Figure 1: Tandem mass spectra of the biantennary (A), triantennary (B) and tetraantennary (C) desialylated *N*-glycans obtained from hAGP standard after total sialidase digestion and labelled with [$^{12}\text{C}_6$]-aniline. (*) This ion fragment could correspond to other possible structures listed in Table S1 or S2 of the supplementary material.

Figure 2: (A) Extracted ion chromatogram (EIC) of the native H5N4S2 glycan obtained from hAGP standard and the MS/MS spectra obtained for each isomer (isomer 1 (i) and isomer 2 (ii)). (B) EIC of the H5N4S1 glycan, obtained from hAGP standard after α 2-3 sialidase digestion, and its corresponding MS/MS spectrum (i). (*) This ion fragment could correspond to other possible structures listed in Table S1 or S2 of the supplementary material.

Figure 3: (A) Extracted ion chromatogram (EIC) of the native H6N5S3 glycan obtained from hAGP standard and the corresponding MS/MS spectra of each isomer (isomer 1 (i), isomer 2 (ii) and isomer 3 (iii)). (B) EIC of the H6N5S2 glycan, obtained from hAGP standard after α 2-3 sialidase digestion, and the corresponding MS/MS spectra gained for each isomer (isomer 3 (i) and isomer 2 (ii)). (*) This ion fragment could correspond to other possible structures listed in Table S1 or S2 of the supplementary material.

Figure 4: Tandem mass spectra of isomers 1 and 2 of the H5N4F1 glycan (A), isomer 3 of the H6N5F1 glycan (B) and isomers 1 and 2 of the H6N5F1 glycan (C) obtained from hAGP standard after total sialidase digestion. (*) This ion fragment could correspond to other possible structures listed in Table S1 or S2 of the supplementary material.

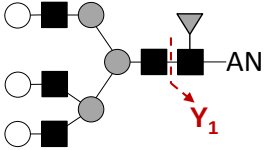
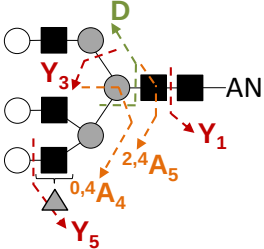
Figure 5: EICs of the H6N5S3 glycan and the corresponding MS/MS spectra of the isomer 3 obtained from three hAGP serum samples: one control (A), one pathological

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60
61
62
63
64
65

698 sample with chronic pancreatitis (B) and one pathological sample with an advanced
699 stage of pancreatic cancer (C). (*) This ion fragment could correspond to other possible
700 structures listed in Table S1 or S2 of the supplementary material.

Table 1. Characteristic ion fragments observed in the tandem mass spectra and used to characterize aniline-labelled desialylated hAGP N-glycan isomers. The nomenclature, assignation and structure of each ion fragment are also included.

Glycan	Isomer	Ion fragments observed (m/z, m/z – H ₂ O)	Nomenclature	Diagnostic	Structure
H5N4	Isomer 1	297.1450 688.2311, 670.2173	Y ₁ D	Absence of core fucosylation Unbranched 6-antenna	
H6N5	Isomer 1	297.1443 508.1652 688.2310, 670.2164 951.3377, 933.3153	Y ₁ B ₃ /Z ₄ D ^{0,4} A ₄ [*]	Absence of core fucosylation Branched antenna Unbranched 6-antenna Branched antenna	
H7N6	Isomer 1	297.1446 609.2148 789.2747, 771.2657 951.3221, 933.3160	Y ₁ ^{0,2} X ₄ /B ₃ ^{0,4} A ₄ /Y ₅ [*] ^{0,4} A ₄ [*]	Absence of core fucosylation Branched antenna Branched antenna Branched antenna	
H5N4F1	Isomer 1 and 2	461.2251, 443.2035 1095.3708	Y ₁ ^{2,4} A ₅ /Y ₅ [*]	Core fucosylation Absence of antenna fucosylation	

Isomer 1 and 2		443.2040 [#]	Y ₁	Core fucosylation	
H6N5F1		297.1441	Y ₁	Absence of core fucosylation	
		570.2033	^{0,4} A ₄ /Y ₅ [*]	Fucosylated antenna	
Isomer 3		670.2611	D	Unbranched 6-antenna	
		933.3098	^{0,4} A ₄ [*]	Branched antenna	
		1095.3689	^{2,4} A ₅ /Y ₃ [*]	Branched antenna	

* This ion fragment could correspond to other possible structures listed in Table 1 or 2 of the supplementary material.

[#] Detected only in half of the runs.

Figure1
[Click here to download high resolution image](#)

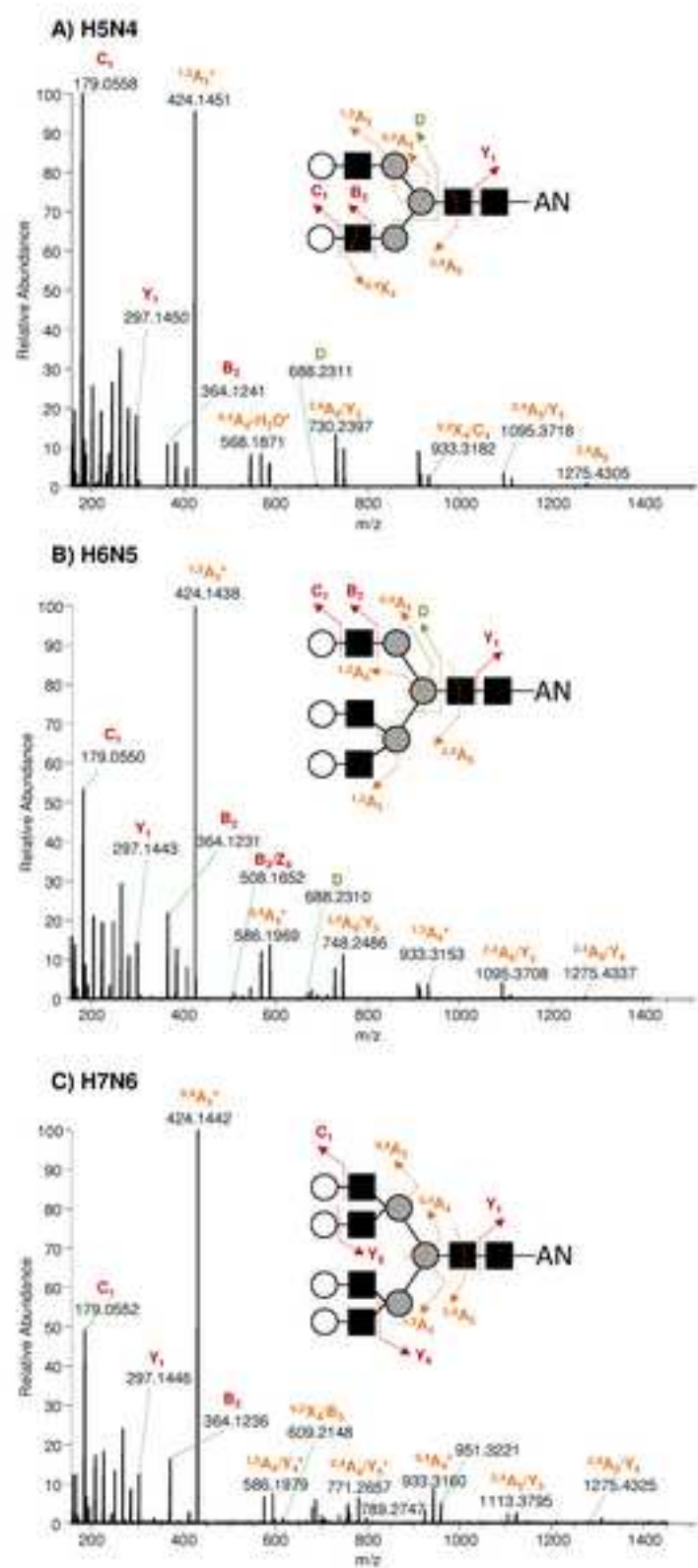


Figure 1

Figure3
[Click here to download high resolution image](#)

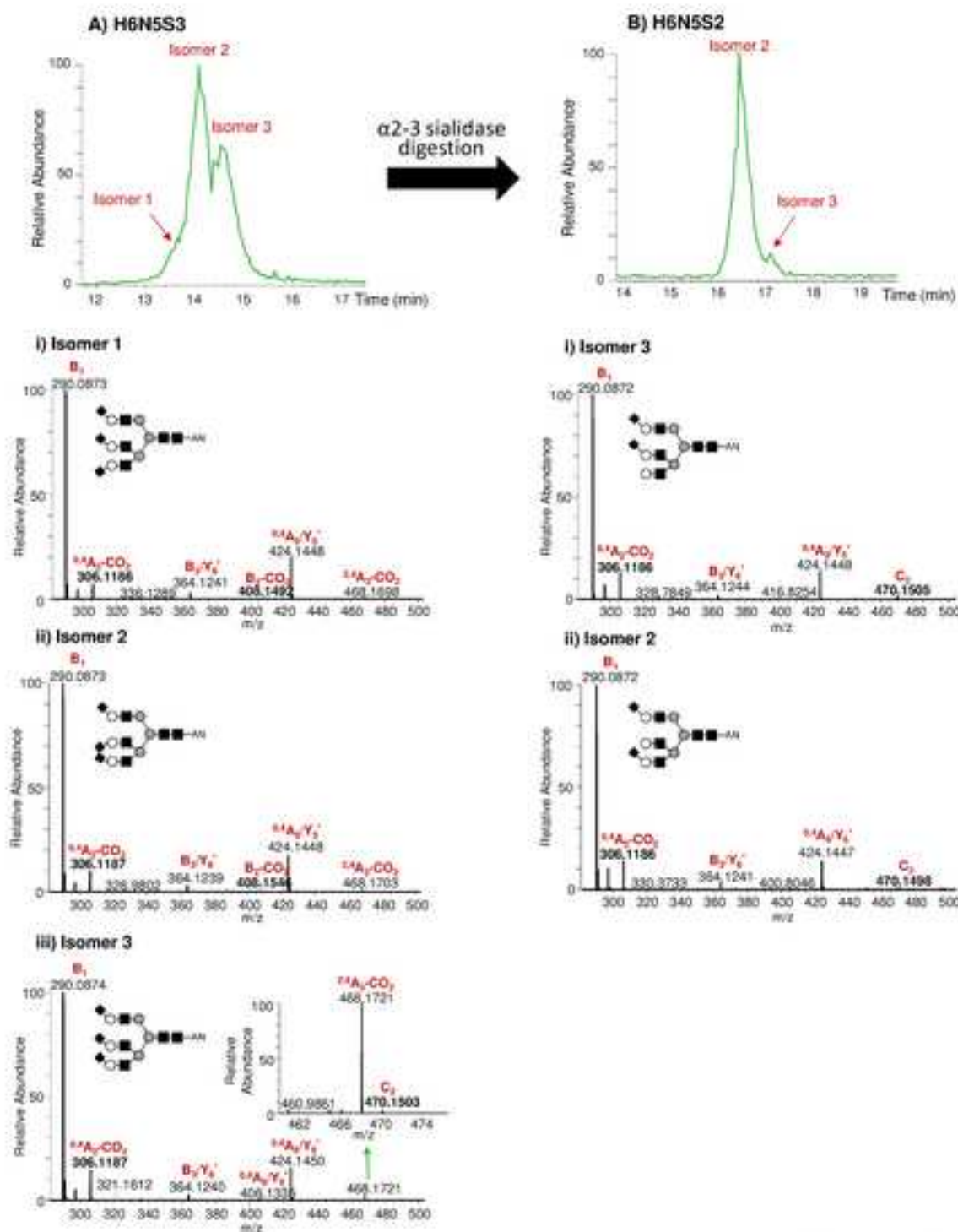
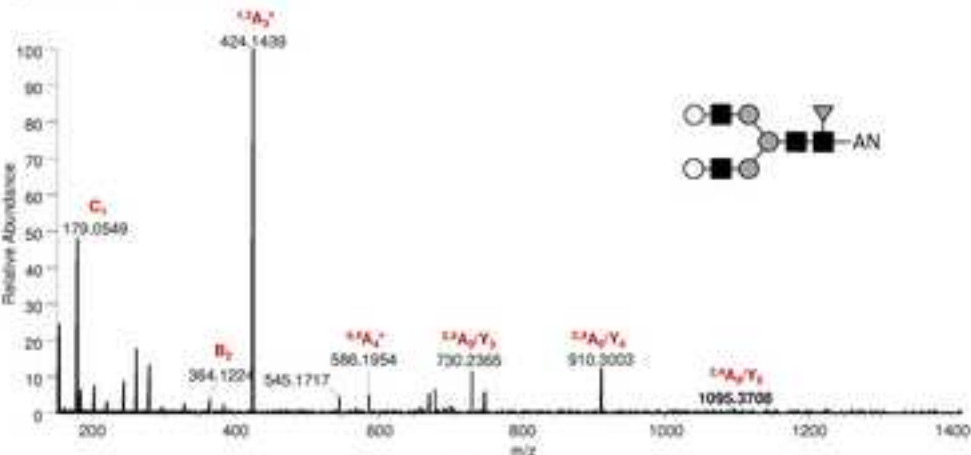


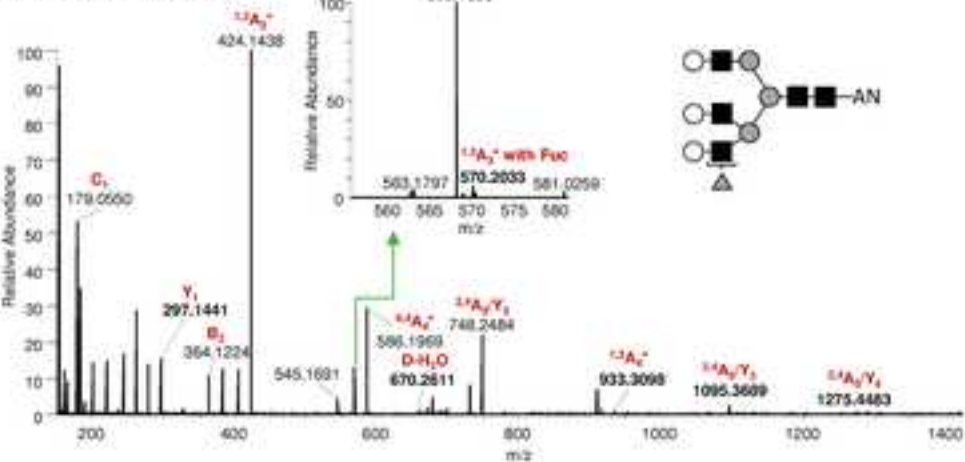
Figure 3

Figure4
[Click here to download high resolution image](#)

A) H5N4F1 – Isomer 1 and 2



B) H6N5F1 – Isomer 3



C) H6N5F1 – Isomer 1 and 2

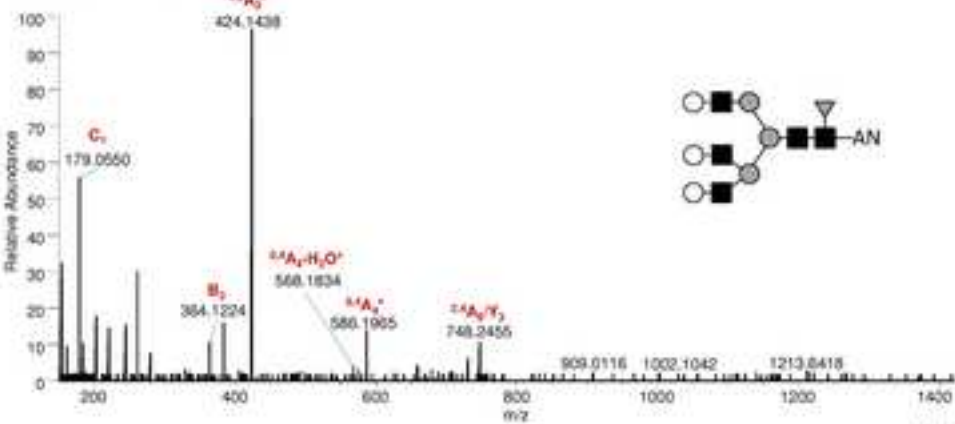


Figure 4

Figure5
[Click here to download high resolution image](#)

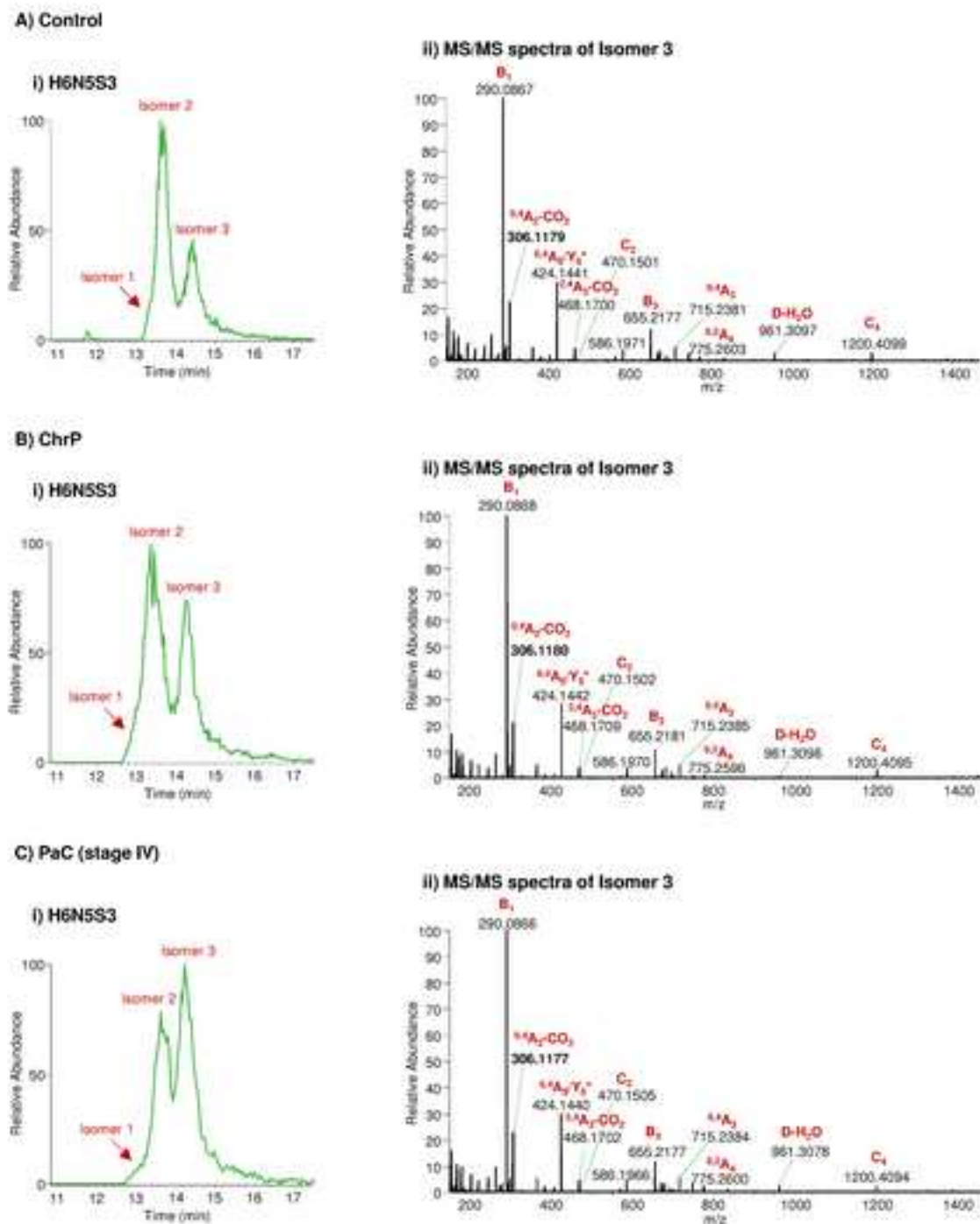


Figure 5