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Practical Proteomics

Supporting Information for

Practical Review

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Separation-based Glycoprofiling Approaches using Fluorescent Labels

Experimental Details

Supplemental Material

Supplementary Material to

Practical Review

Separation-based Glycoprofiling Approaches Using Fluorescent Labels

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Materials and Methods

Sample Preparation

Bovine fetuin glycan library was chosen as a model sample to analyse as it has well-characterised bi- and tri-antennary N- and O-glycosylation, there is much published work detailing its structures the most detailed of which is by Green *et al.* [1] using 500-megahertz 1H NMR spectroscopy. Bovine Fetuin glycoprotein was purchased from Sigma Aldrich and the glycan library was released by large-scale hydrazinolysis [2].

NP LC-Fluorescence

The method used is as described in "Methods in Molecular Biology" [3]. Normal phase chromatographic separations were carried out using Waters Alliance 2695 separation module with either a Waters 2475 or Jasco fluorescence detector (for the 2-AB excitation: λmax 330 nm, band width 16nm, emission λmax 420 nm, band width 16 nm). Normal phase column: TSK gel Amide-80 column 250 mm x 4.6 mm (Anachem). Data was analysed using Waters Empower Software. Ammonia solution 25% (Aristar grade) was purchased from Merck-BDH (Poole Dorset, UK). Acetonitrile E Chromasolv for HPLC far UV was purchased from Sigma Aldrich. AnalaR quality ammonium formate (Merck-BDH), Milli-Q water was used (18MΩcm^{-1}).

Samples were 2-AB labelled by reductive amination using the LudgerTag 2-AB labelling kit (Ludger Ltd) and post-labelling purification was performed using LudgerClean S cartridges (Ludger Ltd) as per the manufacturer's protocol.

2-AB labelled bovine fetuin glycans were analysed at a concentration of 0.156 μgmL^{-1} for repeatability and intermediate precision experiments. A calibration plot was established using a concentration range 0.004 to 0.156 μgmL^{-1}. A set of 7 0.156 μgmL^{-1} samples of 2-AB labelled bovine fetuin glycans were dried down and digested with exoglycosidase enzymes as described below. Subsequent digestion products were analysed using the same protocol. All samples were prepared in 80% AcN and injected in 95µl via a 100 µl loop.

The gradient applied is shown below, where Solvent A: 50mM ammonium formate, adjusted to pH 4.4 with ammonia and Solvent B: Acetonitrile.

Time (mins)	Flow Rate ($mLmin^{-1}$)	Solvent A (%)	Solvent B (%)
0	0.4	20	80
152	0.4	58	42
155	0.4	100	0
157	1	100	0
162	1	100	0
163	1	20	80
177	1	20	80
178	0.4	20	80
260	0	20	80

The 2-AB labelled dextran ladder sample was analysed, every 10 analyses as a system suitability tool and to produce a glucose unit (GU) scale using a fifth order polynomial fit. Sample peaks where then assigned a corresponding GU unit which were searched against the database “Glycobase” to assign the corresponding glycan structure.

CGE-LIF

All experiments were performed on a ProteomeLab PA800 system (Beckman Coulter, Fullerton, CA, USA) using LIF detection with an excitation wavelength of 488 nm and an emission band pass filter of 520 nm +/- 10 nm. All data was collected and analysed using 32 Karat software, version 7. The separation gel buffer, neutral coated capillary and APTS labelling solutions were purchased as a ProteomeLab Carbohydrate Labelling

and Analysis Kit (Beckman Coulter). For both sets of experiments the separation gel buffer from a ProteomeLab Carbohydrate Labelling and Analysis Kit (Beckman Coulter) was used as supplied. Reducing reagent sodium cyanoborohydride 1M/THF was purchased from Sigma Aldrich. The filter system (Corning 0.22μm pore size, sterile CA membrane non-pyrogenic, neck size 45 mm 500 mL / 70 mm membrane diameter), sodium acetate (anhydrous) and acetic acid were from Fisher Scientific. Sephadex G-10, and micro-spin columns were obtained from GE Healthcare. The sialylated triantennary glycan standard (A3G3S3) was supplied by Ludger Ltd. Milli-Q water was used ($18M\Omega cm^{-1}$).

The neutral coated capillary employed has an i.d. of 50 μm, a total length of 52 cm and an effective length of 40 cm. The laser was allowed to warm up for 15 minutes prior to use and calibrated each time a new capillary was installed (as described in the instrument manufacturer's maintenance manual). New capillaries were pre-rinsed with carbohydrate separation gel buffer for 10 minutes at 30 psi.

Bovine fetuin glycan samples labelled with APTS, were prepared following the instructions given in the Proteome Lab "Carbohydrate Labelling and Analysis" kit. Great care needs to be taken when handling the sodium cyanoborohydride and the addition of the labelling reagents must be carried out in a fume hood. Having whirl mixed and spun (this is necessary due to the small volumes of reagent used), samples were then left in the dark, at room temperature overnight to react with labelling reagents. A faster labelling protocol (6 hours) was investigated, however this resulted in loss of material, as peak areas where substantially reduced.

For repeatability and intermediate precision experiments a starting dry sample weight of 5 μg of bovine fetuin glycans was used. A calibration plot was established using a range of 0.1 to 20 μg of bovine fetuin glycans. A standard of triantennary (A3G3S3) glycan was also prepared at 2 μg to identify the major peaks present. 5 μL of 1 $nmol\mu L^{-1}$ maltose was spiked into each sample as an internal standard, against which peak area data could be normalised. Every seventh analysis, an APTS labelled dextran ladder sample was analysed. Exoglycosidase array digestion products were also analysed for APTS labelled bovine fetuin with a starting amount of 5 μg. For samples undergoing digestion it was necessary to remove any residual APTS labelling chemicals as they

appeared to block the action of the SPG enzyme. The cleaning method described by Callewaert *et al.* [4] was applied to all the samples post labelling as described below.

1) Weigh 1.6 g of dry Sephadex G-10 into a flask (this will make 8 mini-spin cartridges).
2) Add 10 mL of incubation buffer; 50 mM sodium acetate, adjusted to pH 5.5 with acetic acid (as used for the exoglycosidase digests) filtered through a 0.22 μm filter system.
3) Leave for 3 hours to allow material to swell.
4) Fill the 300 μL mini-spin column with approximately 75% slurry, top up to 100% with buffer.
5) Micro-centrifuge at 6500 rpm (1400 xg) for 1 minute, add further buffer and repeat centrifugation.
6) To dry the packed bed the cartridge is then centrifuged for a further 2 minutes.
7) Dilute the 4 μl APTS labelled glycan sample (prepared as described above) with an additional 96 μL of incubation buffer.
8) Add diluted sample to the mini-spin column, centrifuge at 6500 rpm for 1 minute.
9) Add a further 100 μL of incubation buffer to the cartridge and repeat centrifugation.
10) Dry down eluant using a rota evaporator, samples are now ready for exoglycosidase digestion step.

The prewritten Proteome Lab "Carbohydrate Labelling and Analysis" method was used to analyse samples, applying a constant voltage of -30 kV, with the cathode at the inlet and anode at the outlet, which generated a stable current of -17 μA. Capillary temperature was controlled at 30°C for the initial experiments, being reduced to 20°C for digestion experiments to enhance resolution (on the advice of Beckman Coulter) and samples trays were cooled to 10°C. Samples were introduced via the application of pressure to the inlet of the capillary of 0.5 psi for 3 seconds. The capillary was flushed with capillary gel separation buffer for 2 minutes, prior to each analysis. Again the dextran ladder sample was used to produce a glucose unit (GU) scale, for which the shift of digestion products can be measured against.

Exoglycosidase Digests

Samples of bovine fetuin glycans were analysed using the same exoglycosidase arrays for both NP LC fluorescence and CGE-LIF experiments. As we had prior knowledge of the type of glycans present in bovine fetuin a reduced set of enzymes was used. For unknown samples an array enzymes is required as described in "Methods in Molecular Biology" [3]. All enzymes were purchased from Prozyme and were used in the relative volumes detailed in Table 1, with the incubation buffer of 50 mM sodium acetate, adjusted to pH 5.5 with acetic acid prepared in-house (reagent suppliers detailed in CGE-LIF section). Samples were incubated overnight (16-18 hours at 37°C) as described by Royle *et al.* [3].

Following incubation the digestion products were cleaned up using a Micropure-EZ enzyme removal cartridge pre-rinsed with 200μL water in a microcentrifuge at half speed (~7000 x g) for 10 minutes, discarding the washings. The digested glycan sample was then applied and centrifuged at full speed (~14000 x g) for two minutes, the digestion tube was rinsed with 20 μL of water which was then applied to the filter and centrifuged for 2 minutes. Finally 100 μL of water was applied to the filter and centrifuged for a further 5 minutes. The sample was then dried down and re-dissolved in the appropriate volume of water depending on the analysis to be carried out.

Table 1. Reagents used for exoglycosidase digestion steps of glycan samples.

	Volume added		
Sample number	**Enzyme**	**Buffer**	**Ultra pure water**
1	UNDIGESTED		
2	2μL NAN1	1μL	7μL
3	1μL ABS	1μL	8μL
4	1μL ABS + 2μL SPG	1μL	6μL
5	1μL ABS + 2μL BTG	1μL	6μL
6	1μL ABS + 2μL SPG + 2μL GUH	1μL	4μL
7	1μL ABS + 2μL BTG + 2μL GUH	1μL	4μL

Results and Discussion

NP LC-Fluorescence

Using the standard NP LC approach ten peaks can clearly be identified in the N-linked glycan region of the chromatogram (Figure 1) for the undigested sample of bovine fetuin glycans. Figure 1 also shows the chromatograms obtained for the different exoglycosidase digestion products. Structures were assigned by matching the GU values obtained to values in the Glycobase database. The digestion with NAN 1, shows the presence of both α2-6 and α2-3 non-reducing terminal sialic acids. On digesting with ABS all the non-reducing terminal sialic acids are removed and the two remaining peaks have GU values corresponding to trigalactosylated triantennary (A3G3) glycan (major) and bigalactosylated biantennary (A2G2) glycan. On examining the differences between digests containing galactosidases of different specificities (SPG and BTG) it becomes apparent that some of the triantennary glycans have one of the galactose units β1-3 linked. This also explains the shouldering observed in the sialidase (ABS) digest. This is in agreement with previously published work characterising the different glycan structures present in bovine fetuin [1].

CGE-LIF

The CGE-LIF analysis of APTS labelled bovine fetuin and its corresponding trisialylated triantennary glycan standard (A3G3S3) is shown in Figure 2. Ten peaks can be observed for the bovine fetuin, this agrees well with the NP LC experiments. A number of minor peaks are also present, though it is difficult to distinguish these from the baseline noise. The analysis time is considerably shorter than with the liquid chromatography approaches, with samples eluting in less than 10 minutes. These short run times are in part due to the presence of the negative charges from the three sulphate groups on the APTS label which increase the electrophoretic mobility of the analytes.

The resultant electropherograms for undigested and the exoglycosidase digestion products of APTS labelled bovine fetuin glycans are shown in Figure 3, with analysis times of less than 12 minutes. The identity of peaks has been assigned based on the relative shifts observed with each of the enzymes and comparison with results observed

with the NP separation (in terms of number of peaks and relative peak heights). To increase the ease of analysis of data from CGE-LIF a database analogous to that produced for NPLC would need to be established. The same structures were observed as with the HPLC route, but with the different isoforms (1-4 and 1-3 linked galactose) of the trigalactosylated triantennary glycan being resolved. The O-linked glycan structures have not been assigned using this approach as the appropriate standards, database were not available, as it was not possible to compare these peaks to those observed by NP-LC.

Migration time increases on the removal of sialic acid, as this reduces the electrophoretic mobility of species towards the cathode. Subsequent digestions then give a gradual decrease in size, reducing migration time. It is therefore necessary to monitor migration time shifts carefully.

References

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[4] Callewaert, N., Geysens, G., Molemans, F., Contreras, R., Ultrasensitive profiling and sequencing of N-linked oligosaccharides using standard DNA-sequencing equipment. *Glycobiology*, 2001, *11*, 275-281.

Figure Legends

Figure 1. Sequencing N-linked glycans from bovine fetuin labelled with 2-AB by HPLC-fluorescence. ■ = GlcNAc, ○ = Maltose and ◊= Galactose (for enzyme abbreviations see Figure 4 of main article).

Figure 2. CGE-LIF analysis of APTS labelled bovine fetuin and A3G3S3 standard.

Figure 3. Sequencing N-linked glycans from bovine fetuin labelled with APTS by CGE-LIF. ■ = GlcNAc, ○ = Maltose and ◊= Galactose (for enzyme abbreviations see Figure 4 of main article).

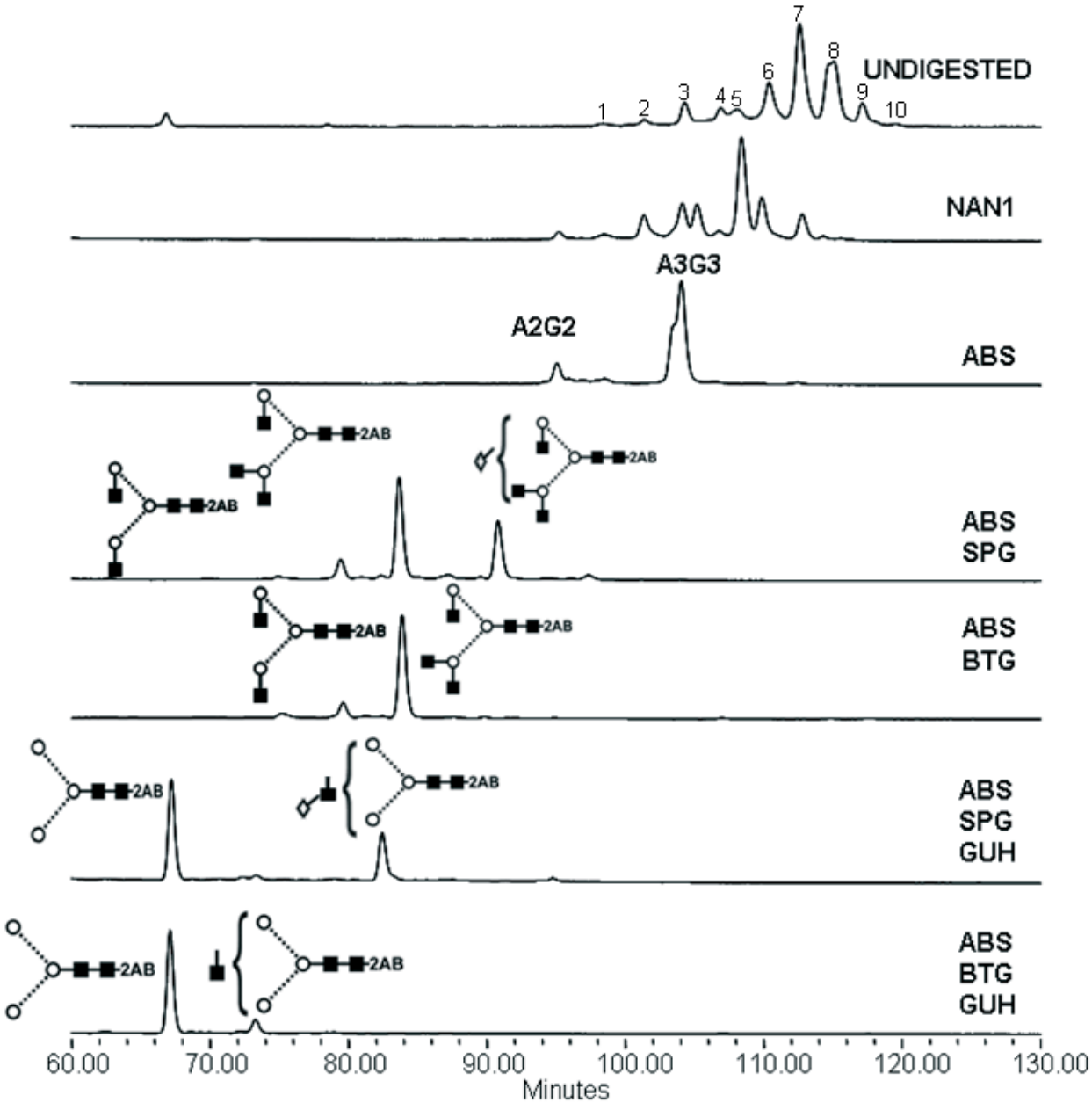
UNDIGESTED
1
2
3
4
5
6
7
8
9
10
NAN1
A3G3
A2G2
ABS
2AB
ABS
SPG
ABS
BTG
ABS
SPG
GUH
ABS
BTG
GUH
60.00
70.00
80.00
90.00
100.00
110.00
120.00
130.00
Minutes

Figure 1

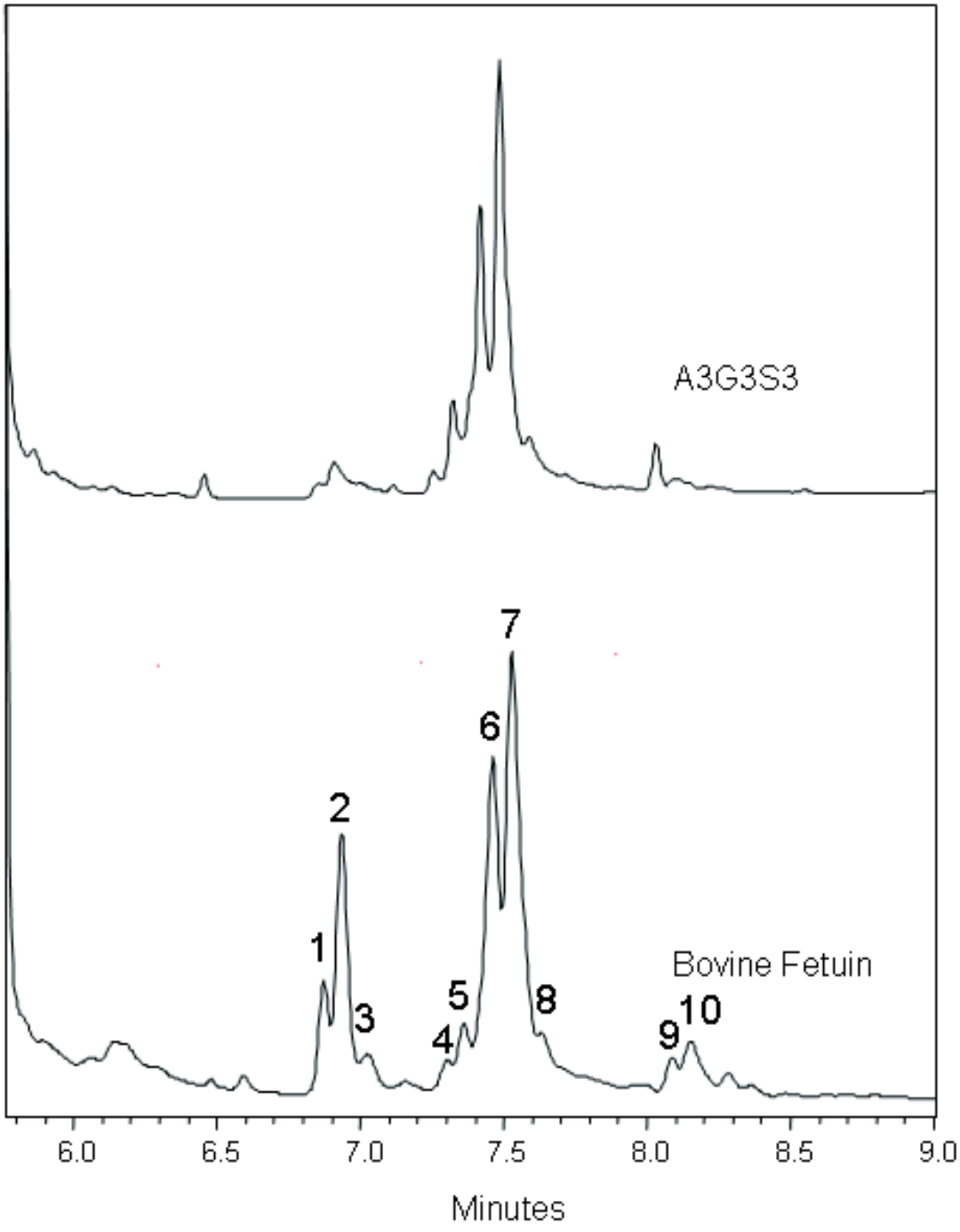
A3G3S3
Bovine Fetuin
1
2
3
4
5
6
7
8
9
10
6.0
6.5
7.0
7.5
8.0
8.5
9.0
Minutes

Figure 2

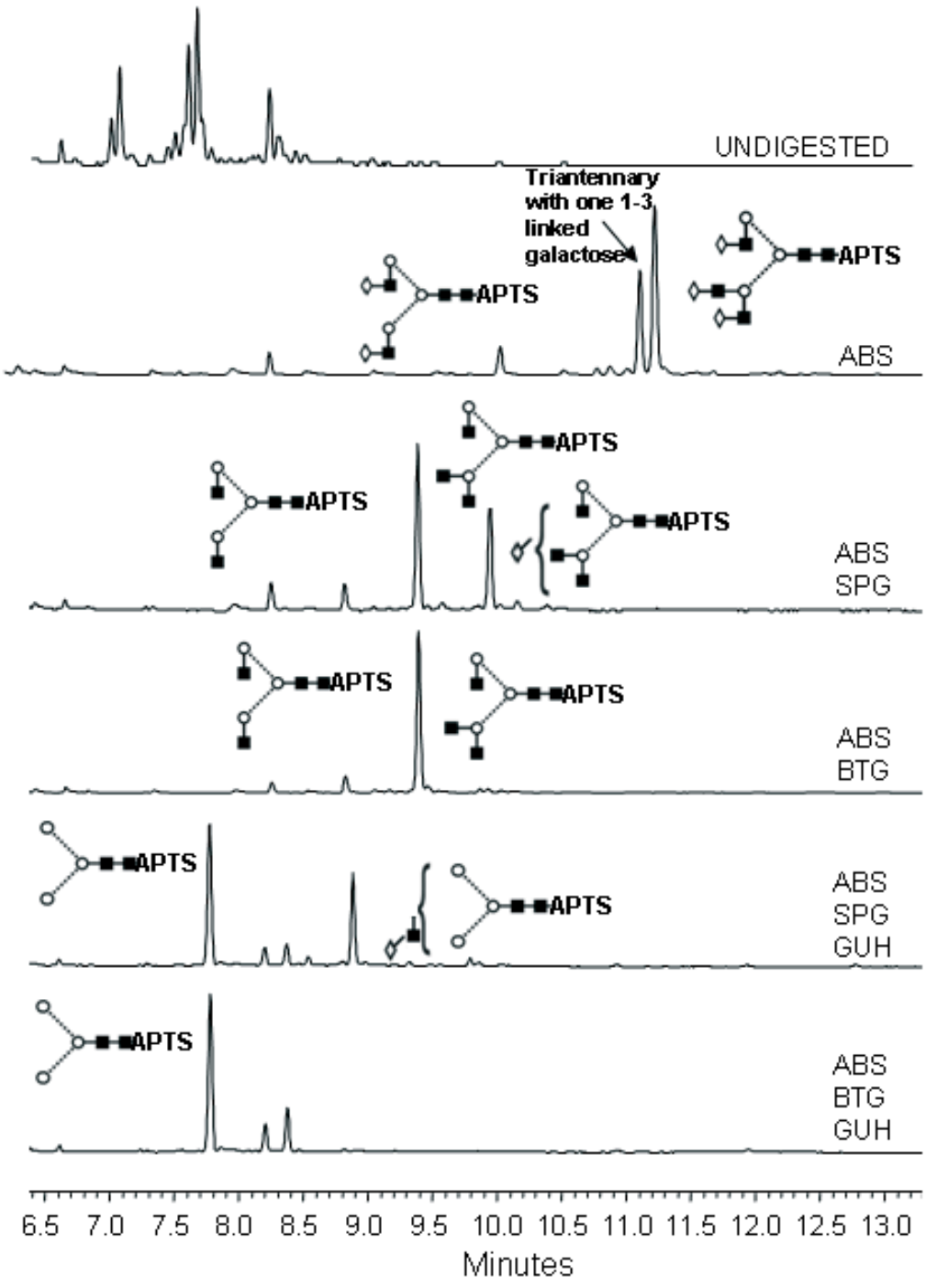
UNDIGESTED
Triantennary with one 1-3 linked galactose
APTS
APTS
ABS
APTS
APTS
APTS
ABS
SPG
APTS
APTS
ABS
BTG
APTS
APTS
ABS
SPG
GUH
APTS
ABS
BTG
GUH
6.5 7.0 7.5 8.0 8.5 9.0 9.5 10.0 10.5 11.0 11.5 12.0 12.5 13.0
Minutes

Figure 3