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Use of a porous graphitised carbon column for the high-performance liquid chromatography of oligosaccharides, alditols and glycopeptides with subsequent mass spectrometry analysis

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ABSTRACT

HPLC using a porous graphitised carbon (PGC) column eluted in acetonitrile–aqueous trifluoroacetic acid has been shown to give complementary chromatography to reversed-phase (ODS) HPLC for separation of peptides and glycopeptides. The PGC column can also be used for separation of oligosaccharides and oligosaccharide alditols released from protein by enzymes (N-linked chains) or base–borohydride degradation (O-linked chains). The advantages are that peptides, glycopeptides, reducing oligosaccharides, sialylated oligosaccharides and oligosaccharide alditols can be chromatographed under the same conditions. The samples can be readily recovered by evaporation for sensitive liquid secondary ion mass spectrometric (LSI-MS) analysis and there is no contamination or deterioration of chromatography from column leakage. LSI-MS analysis revealed that complete peak separation of all of the possible oligosaccharide components of the standard glycoproteins fetuin and bovine submaxillary mucin was not achieved. However, PGC remains as a useful adjunct to other HPLC profiling and separation techniques in particular where subsequent MS analysis is desired.

INTRODUCTION

Several different methods have been proposed for the purification of reducing oligosaccharides, alditols and glycopeptides which include reversed-phase [RP-HPLC; usually on octadecylsilyl (ODS) columns], normal-phase (NP-HPLC) chromatography on amino-bonded columns [NH_2, aminopropyl silica (APS), etc] and anion-exchange HPLC (AX-HPLC). Four types of solvent elution have been applied, either (i) aqueous–organic, (ii) buffer–organic, (iii) aqueous buffer gradients, or (iv) mild acid/aqueous acetonitrile, depending on the molecules to be separated. As a generalisation, neutral oligosaccharides and alditols have been eluted by RP- or NP-HPLC with solvent system i [1–3], sialylated oligosaccharides by NP-HPLC with

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solvent system ii [4–6] or by AX-HPLC with solvent system iii [7,8], sulphated oligosaccharides by NP-HPLC [9,10] or AX-HPLC [11] with solvent system iv or by the use of RP-HPLC with ion-pairing reagents [12] and glycopeptides by NP- or RP-HPLC with solvent system iv [4,13]. In general initial detection could be achieved by UV absorbance at a sensitivity down to 1 μg but after solvent systems ii or iii a desalting step has to be introduced for further analysis, for example by mass spectrometry (MS) The disadvantage of these methods is the need for more than one column for different applications and their limited ability to isolate isomeric oligosaccharides. Additional systems have therefore been explored.

High pH anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) has been shown to separate a wide range of oligosaccharide and alditol isomers both varying in monosaccharide sequence and/or linkage) [14–18]. The major drawback of this technique is that it requires high concentrations of sodium hydroxide and sodium acetate which are then difficult to remove sufficiently for further analysis. There is also a risk of epimerisation and degradation of reducing sugars. Ion suppression membranes have been used successfully [19] to desalt monosaccharides separated by HPAEC–PAD with on-line detection by thermospray MS. The separation of oligosaccharides by HPAEC–PAD requires the use of stronger sodium hydroxide and sodium acetate which are difficult to remove completely with these suppression membranes. Separation of malto-oligosaccharides and high-mannose oligosaccharides by HPAEC–PAD with membrane desalting and on-line ionspray MS has been achieved with a custom-packed 2 mm I.D. Carbopac PA1 column but it was reported [20] that acetic acid formed by the desalting process still interfered with the ionisation for MS. Alternative to the use of a membrane, the sugar-containing fractions can be collected and desalted using a cation-exchange column or Bio-Gel P2 minicolumns [21]. All of these desalting mechanisms can result in considerable losses of required material.

We have previously explored porous graphitised carbon (PGC) HPLC as a method of separation of glycopeptides and oligosaccharide alditols [22]. HPLC with PGC columns exhibits similar properties to RP-HPLC but in addition is able to separate closely related oligosaccharide isomers. We now extend the applications of HPLC with PGC columns to encompass separation of N-linked oligosaccharides and mucin-derived oligosaccharide alditols. The solvent systems used with HPLC on PGC columns do not utilise salts, thus the separated fractions require no clean up prior to further analysis. For example LSI-MS of peptides and native oligosaccharides are readily obtainable. Comparisons are also made with HPAEC–PAD both as a means of separation and as a method of preparing oligosaccharides for MS analysis. We have previously shown that PGC gives more reliable chromatography for neutral oligosaccharide alditols [22] compared to HPAEC as these are eluted away from the solvent front. For sialylated alditols adequate retention is observed on HPAEC [16,17] but an improved HPLC system is required which will separate both sialylated and non-sialylated molecules in one run and which, for preparative purposes, does not require high salt concentrations.

EXPERIMENTAL

Materials

Peptide-N-glycosidase F (PNGase F) was purchased from Boehringer Mannheim (Lewes, UK). Fetuin, asialofetuin, RNAase B, bovine submaxillary mucin (BSM) and L-1-tosylamide-2-phenyl ethyl chloromethyl ketone (TPCK)-treated trypsin were all from Sigma (Poole, UK). The PBA Bond Elut column was from Jones Chromatography (Hengoed, Glamorgan, UK). HPLC-grade trifluoroacetic acid (TFA) was from Pierce (Chester, UK). The PGC column was a Hypercarb S (100 mm × 6.4 mm) fitted with a Hypercarb guard cartridge, both from Shandon Scientific (Runcorn, UK). HPAEC was performed using a Carbopac PA-1 column (250 mm × 4 mm) and a PA-1 guard column (50 mm × 4 mm) both from Dionex (Camberley, UK) with on-line desalting with an anion micromembrane suppressor (AMMS) and autoregen accessary (Dionex).

Methods

Preparation of peptides, glycopeptides, oligosaccharides and oligosaccharide alditols. Peptides and glycopeptides from the bovine serum glycoprotein, fetuin, were generated by trypsin digestion and fractionated by RP-HPLC on an ODS column and HPLC on a PGC column as previously described [13,22].

N-Linked oligosaccharides of asialofetuin, fetuin and RNAase B were prepared by digestion with PNGase F. The glycoproteins were dissolved in 200 μl 40 mM KH_2PO_4–10 mM EDTA pH 6.2 with 5 μl toluene and 1 unit PNGase F per 10 nmol protein. The mixtures were then incubated for 72 h at 37°C. Protein was precipitated with a 2-fold excess of ice-cold ethanol followed by two washes with ice-cold ethanol. The oligosaccharides were retained in the supernatant which was dried for further analysis.

Oligosaccharide alditols were released from BSM by treatment with alkaline borohydride (1 M $NaBH_4$ in 0.05 M NaOH) for 16 h and at 50°C and purified on a PBA column as described previously [23]. In brief the column was equilibrated consecutively with: 2 × 1 ml methanol; 1 × 1 ml 0.1 M HCl; 2 × 1 ml water; 4 × 1 ml 0.2 M NH_4OH. The sample was then added in 100 μl 0.2 M NH_4OH, washes of 2 × 100 μl 0.2 M NH_4OH and 2 × 100 μl water were carried out and the alditols were then eluted in 6 × 100 μl 0.1 M acetic acid and the column washed with 1 × 1 ml 0.1 M HCl and 2 × 1 ml water.

HPLC on PGC column. HPLC on a PGC column was carried out using a Gilson system which consisted of two Model 302 pumps, a Model 802C manometric module, a Model 811 dynamic mixer, Model 201 fraction collector and a Model 116 detector (all from Gilson, Anachem, Luton, UK) controlled by Gilson 715 software (Anachem) on an IBM personal computer (PS2) model 55 SX. The solvent system for peptides/glycopeptides was a gradient from 0.1% TFA in water–acetonitrile (98:2), pH 2.2 to 0.1% TFA in water–acetonitrile (18:82), pH 2.2 in 80 min. The column was initially equilibrated with 0.1%TFA in water–acetonitrile (98:2), pH 2.2 for 30 min between each run. (The flow-rate was 1 ml/min and detection was by UV absorbance at 206 or 210 nm).

The solvent systems for oligosaccharides and oligosaccharide alditols used a gradient of 0.05% TFA in water to 0.05% TFA in water–acetonitrile (60:40) in 35 min with re-equilibration in 0.05% TFA in water between runs.

HPAEC–PAD

HPAEC–PAD was carried out on a titanium-lined Gilson system consisting of two Model 302 pumps, a Model 802TI manometric module, a Model 811B dynamic mixer and a Model 201-202 fraction collector (all from Gilson, France) controlled by an IBM personal computer (PS2) Model 70 running Gilson 712 software (Anachem). Detection utilised a pulsed electrochemical detector fitted with a gold working electrode (Dionex) using the following pulse potentials and durations: $E_1 = 0.1$ V ($t_1 = 0.5$ s), $E_2 = 0.6$ V ($t_2 = 0.11$ s), $E_3 = -0.8$ V ($t_3 = 0.11$ s). The oligosaccharide sample was applied to the column equilibrated with the starting eluent at room temperature. Three gradients were run:

(1) Solvent A (water)–solvent B (50 mM NaOH–1.5 mM NaOAc) (98:2) for 20 min followed by a linear gradient of A–B (98:2) to A–B (85:15) over 15 min at a flow-rate of 0.75 ml/min. The limit solvent was then continued for a further 4 min before the column was regenerated in 100 mM NaOH and re-equilibrated in A–B (98:2).

(2) Solvent A (200 mM NaOH)–solvent B (200 mM NaOH–1.0 M NaOAc) (100:0) for 5 min followed by a linear gradient of 100% A to A–B (50:50) over 30 min at a flow-rate of 1 ml/min. The limit solvent was continued for a further 5 min before re-equilibration in 100% A.

(3) Solvent A (100 mM NaOH)–solvent B (100 mM NaOH–500 mM NaOAc) (95:5) for 15 min followed by a linear gradient of A–B (95:5) to A–B (40:60) over 35 min with a flow-rate of 1 ml/min. The limit solvent was continued for a further 5 min before the column was regenerated by moving to 100% A in 3 min and holding for a further 10 min. Thereafter the initial conditions A–B (95:5) were resumed.

On-line desalting was performed with a AMMS ion suppression membrane with a regenerant of 50 mM reagent-grade H_2SO_4 pumped at a flow-rate of 10 ml/min.

Liquid secondary ion mass spectrometry. LSI-MS was carried out on a VG Analytical (Manchester, UK) ZAB2-E mass spectrometer employing a caesium gun operated at 25 keV with an emission current of 0.5 μA. Mass spectra were obtained at 8 keV accelerating voltage and 1000 resolving power. The data were processed by the 11-250J data system. Mass scans were made at 30 s/decade. Fractions from HPLC were evaporated to dryness, re-evaporated with water (2 × 100 μl) and taken up in 0.5% aqueous TFA pH 2.2 at a concentration of 200 *μM*. A 1-μl (*ca.* 200 pmol) sample aliquot was loaded onto the MS probe tip containing the matrix, thioglycerol–TFA (10:1) or thioglycerol.

RESULTS AND DISCUSSION

We have previously shown the use of HPLC on a PGC column for the separation of standard oligosaccharide alditols and for the separation of peptides and glycopeptides [22]. During the latter analysis we noted that the order of elution and MS detection of the non-glycosylated peptides separated by chromatography on a PGC column gave complementary information to that obtained from RP-HPLC using an ODS column. A further example of the use of PGC columns to separate glycopeptides and peptides which cochromatographed in RP-HPLC is described in Fig. 1 for one area of the chromatogram of a protease digest of the standard glycoprotein, fetuin. In brief two glycopeptides (Fig. 1, panel A, peak 1) and also their peptide moieties obtained after PNGase F treatment (Fig. 1, panel B, peak 2), coeluted in each case on an ODS column whereas the original glycopeptides were well separated on a PGC column (Fig. 1, panel C, peaks 3 and 5) and from two peptides (Fig 1, panel C, peaks 4 and 6) enabling the component peptide sequence and oligosaccharide chains to be readily identified. Chromatography on a PGC column was also investigated for preparative separation of the released N-linked chains of the standard glycoprotein fetuin. Fig. 2 shows the superimposed PGC chromatograms of oligosaccharides released by PNGase F from fetuin and asialofetuin. The peaks a–m were fractionated, evaporated to dryness, re-evaporated once with water and added to the matrix for LSI-MS analysis. Table I shows the molecular ions and the assigned structures of fractions a–d and l–m. No ions consistent with oligosaccharide masses were given in the spectra of fractions e–g. Fig. 3 shows representative spectra of fractions b–d and j–l. High-mannose type

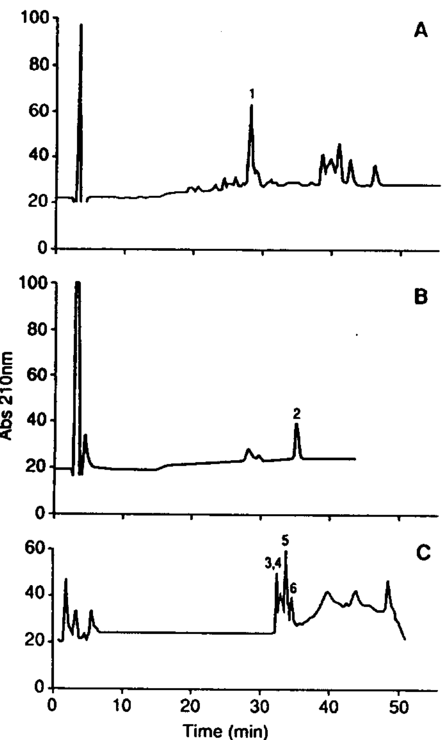

Fig. 1. (A) RP-HPLC of a thermolysin digest of the tryptic glycopeptide LCPDCPLLAPLN*DSRVVHAVEVALATFNAESN*GSYLQLVEISR representing amino acids 127 to 169 of bovine serum fetuin where * denotes a glycosylation site. Peak 1 was hexose positive. (B) Analysis of peak 1 from panel A after PNGase F digestion showing the deglycosylated peptides at peak 2 and released oligosaccharides in the solvent front. LSI-MS analysis showed the presence of the sequence LAPLD̊DSR (amino acids 134–141) where D̊ is the aspartic acid formed after deglycosylation (*m/z* 886.7) and peptide sequencing indicated the presence of ALATFNAESDGSY (amino acids 149–161). (C) Analysis of peak 1 in panel A above using a PGC column eluted in the same gradient as the RP-HPLC. Peaks 3 and 5 were hexose positive and shown by peptide sequencing to be amino acids 149–161 and 134–141, respectively. The oligosaccharides released from 3 and 5 gave a similar profile on HPAEC–PAD to the oligosaccharides released from intact fetuin showing that both sites were glycosylated by a mixture of disialo-, trisialo- and tetrasialo-oligosaccharides.

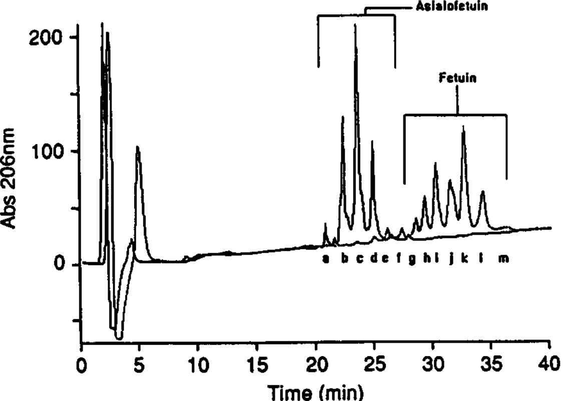

Fig. 2. Superimposed chromatograms of oligosaccharides released from asialofetuin and fetuin by PNGase F and separated by HPLC on a PGC column.

chains of RNAase B were also investigated and these were found to elute between 19 and 29 min (results not shown).

These results show that both non-sialylated and sialylated oligosaccharides can be chromatographed using the same gradient elution on PGC, unlike HPAEC where non-sialylated oligosaccharides are poorly retained compared to sialylated ones. Chromatography on a PGC column could separate biantennary from triantennary non-sialylated oligosaccharides (Table I), although the absence of a Gal from one of the branches could not be distinguished and mono-sialo-oligosaccharides eluted with the same retention time as non-sialylated oligosaccharides. It should be noted that asialofetuin obtained

Fig. 3. Representative negative ion LSI-MS spectra of underivatised oligosaccharides obtained by PNGase F digestion of fetuin and asialofetuin analysed after separation by HPLC on a PGC column (Fig. 2) and assigned as shown in Table I.

TABLE I

THE PEAK ASSIGNMENTS AND MOLECULAR IONS FROM LSI-MS ANALYSIS OF THE OLIGOSACCHARIDES RELEASED FROM FETUIN AND ASIALOFETUIN FROM HPLC ON A PGC COLUMN AS SHOWN IN FIG. 2

Structure	M/Z $[M-H]^-$	Peak where present
Gal$_1$ [GlcNAc-Man Man-GlcNAc$_2$ GlcNAc-Man]	1479	a, b
Gal-GlcNAc-Man Man-GlcNAc$_2$ Gal-GlcNAc-Man	1640	a, b
Gal-GlcNAc-Man GlcNAc-Man-GlcNAc$_2$ Gal-GlcNAc-Man Gal$_2$ [GlcNAc↘ Man GlcNAc↗ Man-GlcNAc$_2$ GlcNAc-Man]	1843	b, c
Gal-GlcNAc↘ Man Gal-GlcNAc↗ Man-GlcNAc$_2$ Gal-GlcNAc-Man	2005	b, c
NeuAc$_1$ [Gal-GlcNAc↘ Man Gal-GlcNAc↗ Man-GlcNAc$_2$ Gal-GlcNAc-Man]	2296	b, c, d
NeuAc$_1$ [Gal-GlcNAc-Man Man-GlcNAc$_2$ Gal-GlcNAc-Man]	1931	c, k, l
NeuAc$_1$ [Gal- GlcNAc↘ Man Gal-GlcNAc↗] Man-GlcNAc$_2$ GlcNAc-Man NeuAc$_1$ [Gal-GlcNAc-Man GlcNAc-Man-GlcNAc$_2$ Gal-GlcNAc-Man]	2134	c, d

TABLE I *(continued)*

Structure	M/Z [M − H]⁻	Peak where present
NeuAc-Gal-GlcNAc-Man | Man-GlcNAc$_2$ | NeuAc-Gal-GlcNAc-Man	2222	h, j, k, l
NeuAc$_2$ [Gal-GlcNAc↘ Man ↗Gal-GlcNAc	Man-GlcNAc$_2$	Gal-GlcNAc-Man]	2588	i, j
NeuAc-Gal-GlcNAc↘ Man NeuAc-Gal-GlcNAc↗ | Man-GlcNAc$_2$ | NeuAc-Gal-GlcNAc-Man	2878	i, j, m

from Sigma has some so-called Gal$_0$ structures and some remaining sialic acid showing the inefficiency of desialylation with the lability of underlying Gal. The results suggest that the PGC column is capable of distinguishing oligosaccharide isomers varying in sialic acid linkage as monosialo-oligosaccharides eluted in peaks b, c and d. Peak d also contained a disialylated biantennary chain showing that the PGC type separation is not by size or charge alone but also by linkage.

For oligosaccharides released from sialylated fetuin those in peaks i–m had LSI-MS spectra with assignable ions. Peaks i and j contained di- and triantennary-oligosaccharides with two or three sialic acids, whereas k and l contained only biantennary chains but having one or two sialic acids. Only peaks h and m contained single-chain isomers being, respectively, a disialylated biantennary and a trisialylated triantennary chain. From previous studies of fetuin sialyloligosaccharides (reviewed in refs. 13–15) it is known that oligosaccharides with the same composition but varying in linkage positions are present (*i.e.* sialic acid linked either 2–3 or 2–6 to galactose and Galβ1–4GlcNAc sequences linked either β1–2, 4 or 6 to Man) and that these isomers have distinct elution patterns. Minor tetra-sialylated oligosaccharides which have a Galβ1–3GlcNAc sequences sialylated on both Gal and GlcNAc also present in fetuin [24] were not detected in the present study. The additional variation of mono- or non-sialylated Galβ1–3GlcNAc sequences may be present in either the asialo- or sialo-fetuin derived oligosaccharides.

In order to show the applicability of HPLC on a PGC column for the separation of additional oligosaccharides having blood group-type terminal substituents and N-glycolyl as well as N-acetylneuraminic acid, we have fractionated the oligosaccharide alditols obtained from base-borohydride degradation of BSM. The chromatography was interpreted with knowledge of the oligosaccharides of BSM from detailed structural studies [25,26] Table II shows the molecular ions and structural assignment from analysis of the PGC fractions compared to their HPAEC using three different gradients. Interestingly on PGC, N-glycolylneuraminic acid itself has a slightly reduced retention time compared to N-acetylneuraminic acid whereas the former has twice the retention time on HPAEC. This is reflected in the retention times of the sialylated oligosaccharide alditols present in BSM *e.g.* NeuAc-α2–6[GlcNAcβ1–3]GalNAcol and NeuGcα2–6-[GlcNAcβ1–3]GalNAcol had the same retention

TABLE II

RETENTION TIMES OF SIALIC ACIDS AND SIALYLATED OLIGOSACCHARIDE ALDITOLS

Peaks a–f are from HPLC on a PGC column of released chains of bovine submaxillary mucin (BSM).

Structure	M/Z [M − H]⁻	Retention time (min)			
		PGC	HPAEC		
			Gradient 1	Gradient 2	Gradient 3
BSM-derived alditols					
NeuGc	324	12.5	58	28	35
NeuAc	308	13.2	50	14	12
a GlcNAc–GalNAcol (Neu on GalNAcol)	675	14.3	46	8	20
b NeuAc–GalNAcol	513	16.4	48	13	23
b NeuGc–GalNAcol	529	16.4	53	14	23
c GlcNAc–GalNAcol (NeuAc on GalNAcol)	716	17.4	39	12	29
c GlcNAc–GalNAcol (NeuGc on GalNAcol)	732	17.4	50	15	32
d Gal–GlcNAc–GalNAcol (NeuAc on GalNAcol)	878	20.9	37	15	24
e Fuc–Gal–GlcNAc–GalNAcol (NeuAc on GalNAcol)	1024	23.5	NT[a]	12	23
e Fuc–Gal–GlcNAc–GalNAcol (NeuGc on GalNAcol)	1040	23.5	NT	14	33
f Fuc–Gal–GlcNAc–GalNAcol	733	26.4	NR[b]	NR	NR
f Fuc–Gal–GlcNAc–GalNAcol (Fuc on Gal)	879	26.4	NR	NR	NR
f Gal–GlcNAc / Fuc–Gal → GalNAcol	895	26.4	NR	NR	NR
f Fuc–Gal–GlcNAc / GlcNAc → GalNAcol	936	26.4	NR	NR	NR
Non-mucin oligosaccharides					
NeuAcα2–6Galβ1–4Glcol	634	18.5	34.3	16.0	21
NeuAcα2–3Galβ1–4Glcol	634	24.6	37.7	19	27
Galβ1–3GlcNAcβ1–3Galβ1–4Glcol (NeuAc α2–3 on Gal; NeuAc α2–6 on GlcNAc)	1290	27.5	39.1	27	30

[a] NT = Not tested.
[b] NR = Not retained.

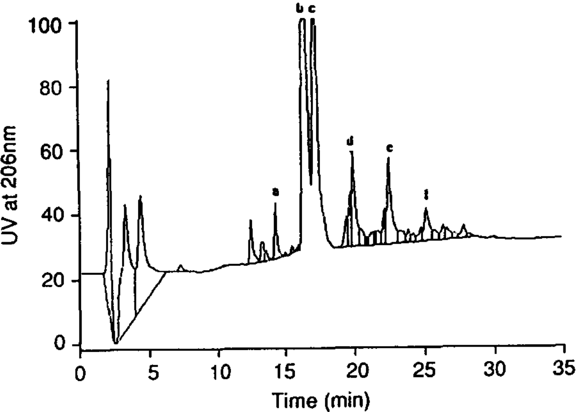

Fig. 4. Chromatography on a PGC column of oligosaccharide alditols obtained from BSM by base–borohydride degradation. Peaks a–f were those identified by LSI-MS as containing oligosaccharide alditols (Fig. 5, Table II).

on PGC but the latter had a greater retention on HPAEC. Identification of the coeluting NeuAc/NeuGc pairs could easily be detected by LSI-MS (Figs. 4 and 5) or by hydrolysis and sialic acid analysis [27]. The advantage of the PGC column was the ability to analyse sialylated and non-sialylated alditols from one HPLC run which cannot be achieved reliably by HPAEC. In particular separation of fucosylated alditols was facilitated which chromatograph near to the solvent front in both HPAEC [15] and RP-HPLC [6]. In addition LSI mass spectra of molecules prepared by chromatography on a PGC column were easily obtained at high sensitivity. PGC has the additional advantages over classical RP-, NP- and HPAEC columns of stability over a large pH range, long column half life, and low day-to-day and column-to-column variability.

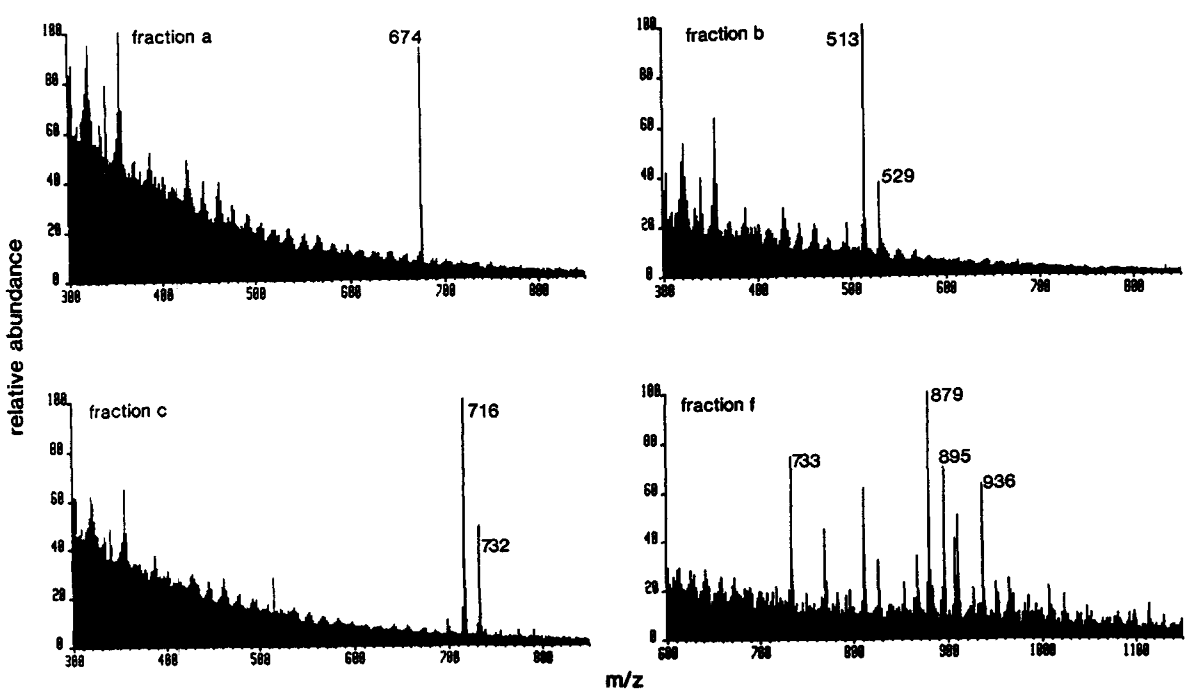

Fig. 5. Representative negative ion LSI-MS of oligosaccharide alditols of BSM as assigned in Table II and Fig. 4.

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