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A general approach to desalting oligosaccharides released from glycoproteins

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Desalting of sugar samples is essential for the success of many techniques of carbohydrate analysis such as mass spectrometry, capillary electrophoresis, anion exchange chromatography, enzyme degradation and chemical derivatization. All desalting methods which are currently used have limitations: for example, mixed-bed ion-exchange columns risk the loss of charged sugars, precipitation of salt by a non-aqueous solvent can result in co-precipitation of oligosaccharides, and gel chromatography uses highly crosslinked packings in which separation of small oligosaccharides is difficult to achieve. We demonstrate that graphitized carbon as a solid phase extraction cartridge can be used for the purification of oligosaccharides (or their derivatives) from solutions containing one or more of the following contaminants: salts (including salts of hydroxide, acetate, phosphate), monosaccharides, detergents (sodium dodecyl sulfate and Triton X-100), protein (including enzymes) and reagents for the release of oligosaccharides from glycoconjugates (such as hydrazine and sodium borohydride). There is complete recovery of the oligosaccharides from the adsorbent which can also be used to fractionate acidic and neutral glycans. Specific applications such as clean-up of N-linked oligosaccharides after removal by PNGase F and hydrazine, desalting of O-linked glycans after removal by alkali, on-line desalting of HPAEC-separated oligosaccharides and β-eliminated alditols prior to electrospray mass spectrometry, and purification of oligosaccharides from urine are described.

***Keywords*: desalting oligosaccharides, graphitized carbon, solid phase extraction, PNGase F, electrospray mass spectroscopy**

***Abbreviations*: HPAEC-PAD, high performance anion exchange chromatography with pulsed amperometric detection; ESI-MS, electrospray ionization mass spectrometry; PNGase F, peptide:N-glycosidase F; HexNAc, N-acetyl hexosamine; Hex, hexose; GalNAc4S, N-acetylgalactosamine-4-sulfate; IduA, iduronic acid; TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; NP40, Nonidet P40**

Introduction

Most techniques used in the characterization of the carbohydrate moiety of glycoproteins, such as mass spectrometry, capillary electrophoresis, anion exchange chromatography, enzyme degradation and chemical derivatization require desalting of the released glycans prior to analysis.

In contrast to proteins which, because of their high molecular weight, are readily desalted by dialysis or gel chromatography, there is a surprising lack of effective methods for the desalting of the relatively smaller oligosaccharides. Methods which are most commonly used are dialysis, which requires arbitrary low molecular weight cut-off membranes and lengthy times; mixed-bed ion exchange medium, which risks the loss of charged sugars; salt precipitation, which can result in co-precipitation of oligosaccharides; and size exclusion gel chromatography, which needs long columns and small sample volumes for separation of small oligosaccharides (e.g., Bio-Gel P4 is susceptible to compression with high salt, and Sephadex G10 results in contamination of the samples with the dextran carbohydrate medium).

Solid-phase extraction cartridges are now commercially available for the extraction of hydrophobic substances from aqueous solution. Most commonly, these contain silica which has been modified by the covalent incorporation of alkyl groups (most commonly octadecyl chains) to confer reversed-phase separation characteristics. Such reversed-phase adsorbents show excellent retention of hydrophobic substances such as proteins, but have very little affinity for hydrophilic solutes such as oligosaccharides, and are

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therefore useful for the removal of salt from proteins but not from sugars.

Activated carbon is a long-established adsorbent for the purification of both gases and liquids and has extremely high chemical stability, as well as good mechanical properties. In the form of a 'charcoal-Celite' column, activated carbon has also been applied to the preparative chromatographic fractionation of mixtures of oligosaccharides which are obtained by partial hydrolysis of polysaccharides, such as starch [1]. With the recent availability of standardized graphitized carbon packings [2] a new separation mode for oligosaccharides has become available. HPLC separations of oligosaccharides and glycopeptides have been reported using the Hypercarb® column [3–6] in which the sugars are eluted using acetonitrile as the organic modifier, and trifluoracetic acid (TFA) as the acidic modifier, in water. A hitherto unrecognized aspect of this procedure is the value of carbon columns for the rapid desalting of a range of sugar solutions.

In this paper we demonstrate that graphitized carbon can be used for the isolation and clean-up of mixtures of oligosaccharides (or their derivatives such as hydrazones and alditols) released from glycoproteins by commonly used methods. The oligosaccharides can be separated quickly and cleanly from solutions containing one or more of the following common contaminants: salts, detergents, protein and reagents for the release of oligosaccharides (such as hydrazine and sodium borohydride). Moreover, the desalting procedure also can be used to fractionate neutral oligosaccharides from acidic oligosaccharides which are sialylated, sulfated or phosphorylated. Importantly, the adsorbent can be used in an on-line cartridge to desalt glycan samples before their introduction into an electrospray mass spectrometer.

Materials and methods

Glycoproteins and glycans

Bovine fetuin, human glycophorin A, ovalbumin, bovine serum albumin, glucose, glucosamine, N-acetylglucosamine N-acetylglucosamine-6-PO_4, glucose-6-PO_4, glucosamine-2-SO_4, glucosamine-3-SO_4 and galacturonic acid were obtained from Sigma (St. Louis, MO, USA). Recombinant human enzyme N-acetylglucosamine-4-sulphatase, produced in Chinese Hamster Ovary cells, and a proteoglycan-derived sulfated tetrasaccharide (GalNAc4S-IduA-GalNAc4S-IduA), were provided by Peter Clements and Peter Meikhle, respectively, of the Adelaide Children's Hospital, South Australia.

Graphitized Carbon

Non-porous graphitized carbon black (Carbograph) SPE columns and porous graphitized carbon (Hypercarb®, Shandon Scientific, Cheshire, UK) HPLC guard cartridges were purchased from Alltech Associates. The Carbograph packing is supplied in 150 mg (approximately 0.5 ml column volume) and 300 mg (1 ml column volume) solid phase extraction (SPE) polypropylene columns. A 150 mg carbon column has the capacity to bind 40 mg of the trisaccharide, raffinose (Packer NH, Redmond JW, submitted for publication), so that this size column has been found to be sufficient for all glycoconjugate applications tested so far. Hypercarb® packing is contained in pre-column HPLC cartridges (70 μm, 10×4.6 mm).

Prior to use, the packing should be washed with 80%(v/v) acetonitrile in 0.1%(v/v) TFA (three column volumes) followed by three column volumes of water. Aqueous solutions to be desalted are applied to the SPE carbon columns and allowed to run into the adsorbent. The volume of the sample is not critical and the flow rate should be about 0.5–1 ml min^{-1}. Salts are washed off with approximately three column volumes of water, while the glycans are adsorbed to the carbon and are eluted batchwise with three column volumes of the appropriate concentration of acetonitrile in water or dilute acid.

Regeneration of graphitized carbon

The manufacturers (Shandon) of porous graphitized carbon recommend several procedures for the regeneration of the carbon packing which in principle is an inert support capable of withstanding extremes of pH and organic solvent. They suggest that the chromatographic packing can be regenerated using alkali (e.g., 50%(v/v) tetrahydrofuran: 50%(v/v) water containing 0.1%(w/v) NaOH), acid (e.g., 50%(v/v) tetrahydrofuran: 50%(v/v) water containing 0.1%(v/v) TFA) or appropriate solvents (e.g., acetone, dichloromethane, methanol) followed by copious washing with water. We however recommend that, because of their low cost, the SPE columns are not re-used. This removes any risk of cross contamination and the possibility of a change in chromatographic behavior due to adsorption of contaminating material. For example, stearic acid was observed to change the elution characteristics of the early carbon-Celite columns [1].

The porous graphitized carbon on-line guard cartridge is, however, more expensive and should be checked routinely by desalting of a relevant glycan standard into the electrospray mass spectrometer. If the separation characteristics of the cartridge change, the choice of clean-up procedure (as recommended by the manufacturer) will depend on the properties of the individual samples which were being desalted.

PNGase F release of oligosaccharides

Oligosaccharides were released by peptide N-glycosidase F (PNGase F, Boehringer-Mannheim, Mannheim, Germany) from 1 nmole of glycoprotein in 100 μl 100 mM sodium phosphate buffer pH 7.6, containing 5 mM dithiothreitol or mercaptoethanol, and 0.1%(w/v) SDS or 0.1%(v/v) Triton

X-100. The enzyme reaction (16 h, 37 °C, 5 U) was carried out after denaturation of the protein by boiling for 10 min, and where SDS was used as the detergent, 0.75%(v/v) NP40 was added before the addition of enzyme. The sample was then applied to a carbon column (150 mg). The column was eluted with 2 ml of water to remove salts and detergent, then with 2 ml of 25%(v/v) acetonitrile to elute neutral oligosaccharides and then with 2 ml 25%(v/v) acetonitrile containing 0.05%(v/v) trifluoroacetic acid to elute sialylated oligosaccharides. Neutral and sialylated glycans can be recovered together in the same fraction by using only the acidified acetonitrile eluant.

Hydrazinolysis of glycoproteins

Proteins (10 nmole) were subjected to hydrazinolysis by heating in anhydrous hydrazine (100 µl) at 95 °C for 4 h [7]. Excess hydrazine was removed by lyophilization and the mixture of products was dissolved in 200 µl water, and applied to a carbon column (150 mg). The column was eluted with 5 ml of water to remove the excess residual hydrazine, then with 2 ml of water containing 25%(v/v) acetonitrile to elute neutral glycan hydrazones, then with the same eluant containing 0.05%(v/v) trifluoroacetic acid to elute sialylated glycan hydrazones (or only with the latter eluant if separation of neutral and acidic glycans is not required). After lyophilization of the samples, the acetylated glycan hydrazones were prepared by the addition of 2 ml 1.6%(w/v) sodium acetate and 200 µl acetic anhydride for 30 min at room temperature. The released hydrazones were then converted to reducing glycans by reaction for 30 min at room temperature with 100 µl 2%(w/v) aqueous copper(II) acetate [7] and were passed through a 1 ml H^+ anion exchange resin (AG 50W-X8 Bio-Rad, Hercules, CA, USA) before being separated by HPAEC.

β-elimination of oligosaccharides

O-linked sugars were β-eliminated from 0.5 nmole glycophorin A with 100 µl 0.1 M NaOH in the presence of 1 M sodium borohydride at 45 °C overnight [8] and the reaction was terminated by acidification with acetic acid. The sample can be desalted on a graphitized carbon SPE column or the released alditols can be injected directly on-line onto a graphitized carbon guard cartridge attached to the electrospray source of the mass spectrometer.

HPAEC–PAD

To monitor recoveries and to collect oligosaccharides, the carbon column eluants were lyophilized (SpeedVac, Savant, Framingdale, NY, USA) and the sugars separated by high pressure anion exchange chromatography (HPAEC) on a Dionex DX 500 Carbohydrate System or on a Waters 625 metal – free LC System. A CarboPac PA1 column (4 × 250 mm) eluted with a gradient of 0–200 mM sodium acetate in 250 mM NaOH over 30 min at 1 ml min^{-1} was used.

Recoveries of the glycans were quantitated by determining the monosaccharide content, before and after elution from the graphitized carbon SPE column. Monosaccharide analysis was performed after hydrolysis in 100 µl 2 M TFA or 4 N HCl at 100 °C for 4 h. The monosaccharides were separated isocratically on a CarboPac PA10 column (Dionex, 4 × 250 mm) using 12 mM NaOH and quantitated using an internal standard of 2-deoxyglucose. Separations were monitored by pulsed amperometric detection (ED40, Dionex; PED 464, Waters).

Electrospray ionisation mass spectrometry (ESI-MS)

The on-line carbon cartridge for desalting and clean up of sugar samples was inserted into the sample loop of a Rheodyne 7125 injection valve. The sample was injected and the salts were washed off the cartridge with water for 10 min at a flow rate of 50 µl /min^{-1} and diverted to waste. This wash volume was increased to 1 ml for samples collected in the very high salt buffers used in HPAEC. The sugars were then eluted at 50 µ min^{-1} with 25–50%(v/v) acetonitrile in 0.1%(v/v) TFA directly into the ESI source.

Electrospray ionization (ESI) spectra in the negative ion mode were recorded using a Quattro II triple quadrupole MS (Micromass, Manchester, UK). Alternating scan functions were performed: function 1 at low cone voltage (30–35V) to allow detection of intact sugar ions; Function 2 at high cone voltage (100V) and scanned at the low mass region (m/z 100 to 400) to detect sugar fragments (e.g., 290 m/z for sialic acid in negative ion mode).

Amino acid analysis

The peptide content of fractions eluted from the carbon columns was monitored by amino acid analysis using pre-column FMOC derivatisation [9]. Protein was quantitated by the DC Protein Assay kit (Bio Rad).

Spot tests

SDS. The elution of sodium dodecyl sulfate (SDS) was checked by applying a 0.5%(v/v) SDS aqueous solution (1 ml) to a carbon column (150 mg) and mixing a few drops of the eluants with 100 µl 2 M guanidine HCl [10]. If SDS is present a precipitate is observed.

NaCl. As a check on the retention of salt by graphitized carbon, the presence of sodium chloride in the eluants after applying 1 ml of a 1%(w/v) solution of NaCl was detected by mixing 100 µl with a few drops of acidified dilute aqueous silver nitrate. A white precipitate is seen in the presence of chloride.

Hydrazine. The elution of free hydrazine was detected by applying 5 µl anhydrous hydrazine to the carbon column and adding 5–6 drops of saturated aqueous salicylaldehyde to the eluants [11].The formation of a precipitate indicates the presence of hydrazine.

Results

Adsorption of sugars on graphitized carbon

To test the adsorption characteristics of graphitized carbon, several sugar moieties which may result from the acid or enzymic hydrolysis of glycoconjugates were applied to a solid phase extraction column. Neutral monosaccharides (e.g., glucose, glucosamine, N-acetylglucosamine) were not retained on the carbon column and were eluted with salts, such as sodium chloride, in the water wash (Figure 1a).

Monosaccharide phosphates, sulfates and acids were retained by the graphitized carbon and complete recoveries, as monitored by HPAEC-PAD, of N-acetylglucosamine-6-PO_4, glucose-6-PO_4, glucosamine-2-SO_4, glucosamine-3-SO_4 and galacturonic acid were obtained by elution with 25%(v/v) acetonitrile in 0.05%(v/v) TFA (Figure 1a, data not shown). The proteoglycan-derived sulfated tetrasaccharide (GalNAc4S-IduA-GalNAc4S-IduA) was retained by the graphitized carbon and was eluted by 25%(v/v) acetonitrile in 0.05%(v/v) TFA, as judged by the complete recovery of galactosamine in a hydrochloric acid hydrolysate of the eluant.

Desalting and fractionation of glycans released by PNGase F

The most commonly used method for releasing oligosaccharides from glycoproteins is the use of endoglycosidases

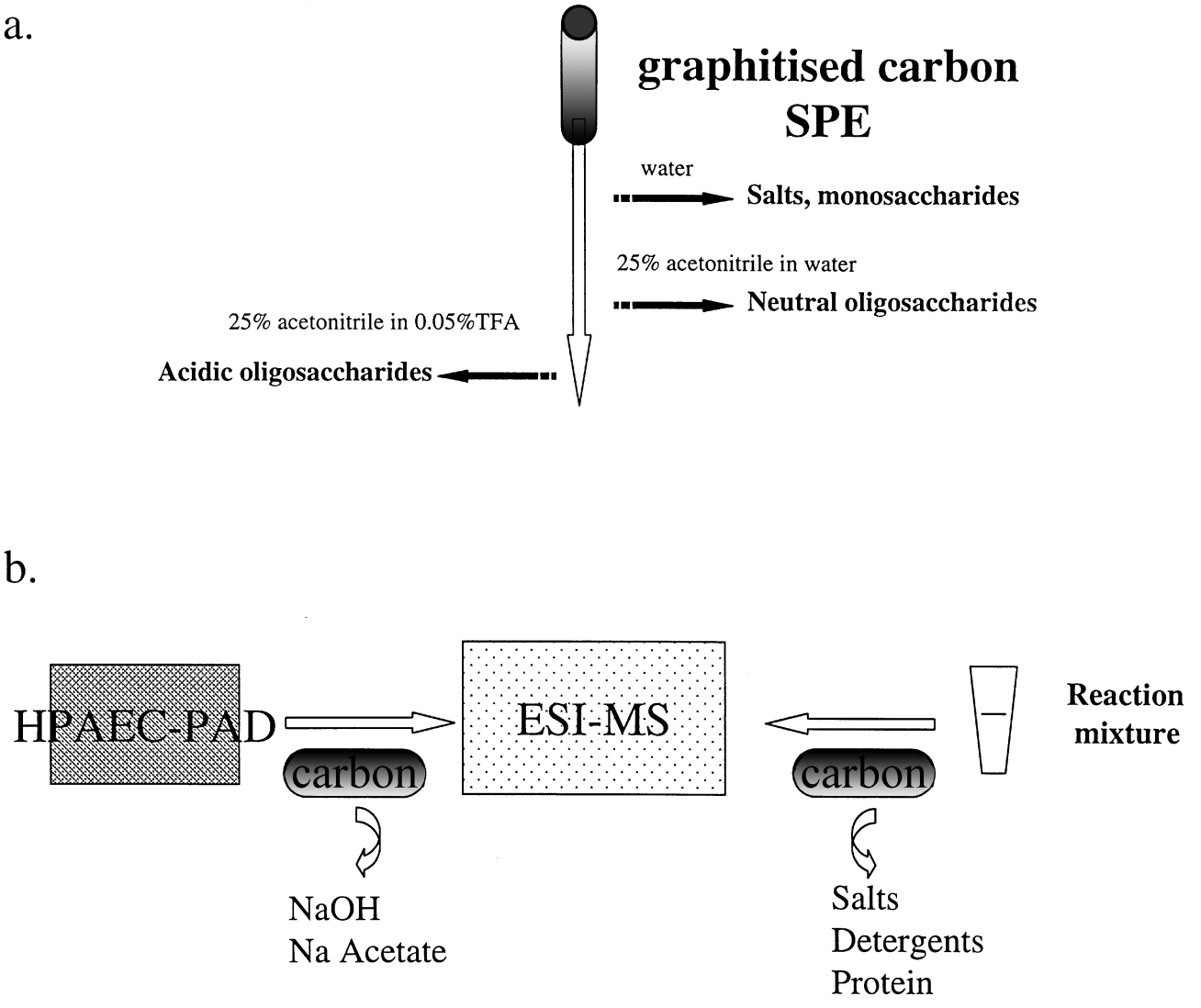


Figure 1. Strategies for using graphitized carbon in the sample preparation of oligosaccharide samples: a) desalting and separation into neutral and acidic oligosaccharide classes by Carbograph solid-phase extraction columns (if fractionation of neutral and acidic glycans is not required total oligosaccharides can be eluted using only the acidic acetonitrile eluant); b) on-line desalting of oligosaccharides with Hypercarb® PGC guard cartridges for analysis by ESI-MS of fractions separated by HPAEC or in solution.

such as PNGase F, Endo F and Endo H. The N-linked glycans are released in high yield by the denaturation of the glycoprotein with detergents at high temperature, which increases the accessibility of the enzyme to the glycosylated sites. When Triton X-100 was used as the detergent, it was removed from the carbon column by elution with water. Aqueous SDS however was retained by the carbon adsorbent and required elution with 25–50%(v/v) acetonitrile to remove it, as judged by precipitation with guanidine HCl. However, in the presence of proteins which bind the SDS it was found that the addition of NP40, or a similar non-ionic detergent, in excess of the SDS, enabled water to wash the SDS micelles which are formed, from the carbon column. Thus, the endoglycosidase incubation mixture can be applied to a carbon column and water can be used to remove both the detergents and buffer salts. The column can then be washed with 25%(v/v) acetonitrile to elute neutral glycans, and then with the same eluant containing 0.05%(v/v) trifluoroacetic acid to elute sialylated glycans, or only with the latter eluant to obtain both neutral and charged species.

Two glycoproteins, bovine fetuin (consisting of three O-linked sites and three sites with sialylated N-linked oligosaccharides [12] and recombinant enzyme human N-acetylglucosamine-4-sulphatase (comprising six potential sites of N-linked oligosaccharides [13]), were treated with PNGase F to release the asparagine-linked (N-linked) glycans. The neutral oligosaccharides of the N-acetylglucosamine-4-sulphatase were recovered in the 25%(v/v) acetonitrile eluant as shown in the HPAEC separation of the fraction (Figure 2a), with no further evidence of sugars in the acidic acetonitrile fraction. The acidic acetonitrile eluant was required to elute the PNGase F released oligosaccharides of fetuin and the HPAEC separated glycan profile obtained is shown (Figure 2b) and is a typical profile of the sialylated fetuin N-linked oligosaccharides [14].

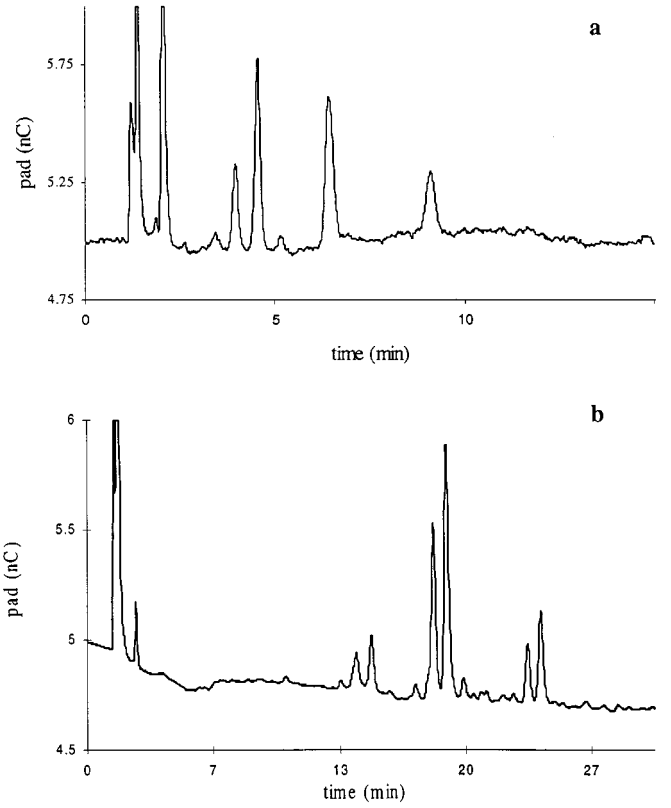


Figure 2. HPAEC-PAD chromatography of the PNGase F released N-linked a) neutral oligosaccharides of N-acetylglucosamine-4-sulphatase eluted with 25%(v/v) acetonitrile from a Carbograph SPE column and b) acidic oligosaccharides of bovine fetuin eluted with 25%(v/v) acetonitrile containing 0.05%(v/v) TFA from a Carbograph SPE column after removal of SDS, buffer salts and enzyme by water.

Recovery of glycans from graphitized carbon

The recovery of the fetuin oligosaccharides was quantitated in two ways. There was complete recovery (97% and 100% respectively) of the oligosaccharides released by PNGase F from fetuin in the presence of Triton X-100 or SDS, as judged by the monosaccharide content of hydrolyzed samples before and after the carbon column clean-up. This was further substantiated by spiking a sample of fetuin oligosaccharides derived from 30 μg of protein with 0.2 μg of an internal standard of maltotriose, before applying the glycans to the carbon SPE column. When the areas of the resolved oligosaccharides which were eluted by 25% (v/v) acetonitrile in 0.5%(v/v) TFA were integrated relative to the internal standard, before and after the carbon column, the recoveries of the di-, tri-, and tetra-sialylated glycans were in the range of 90 to 97%.

Furthermore, to check the linearity of recovery, ovalbumin (20, 30, 40 and 60 μg) was treated with PNGase F. The digests were desalted on carbon SPE columns, and the neutral glycans were eluted with 25%(v/v) acetonitrile and were separated by HPAEC. Integration of the peak eluting at 7.8 min in each sample showed a linear recovery of the neutral oligosaccharide from the graphitized carbon.

Adsorption of protein (deproteinisation of solutions)

The fate of the deglycosylated protein was investigated in these experiments by monitoring the behavior of bovine serum albumin (a non-glycosylated protein) on the carbon cartridge. A solution of bovine serum albumin (BSA, 0.5 mg ml^{-1}) in 100 mM sodium phosphate buffer pH 7.6 (1 ml) was applied to a carbon cartridge. Monitoring of the fractions showed that no protein was eluted using three bed volumes of 0, 25, 50, 100%(v/v) acetonitrile or 0, 25, 50, 80%(v/v) acetonitrile containing 0.1%(v/v) TFA. Under these conditions all glycans tested can be eluted. The protein could not be eluted with a range of other solvents such as propanol, water-saturated butanol or alkali. The carbon column can thus be used for deproteinising solutions of oligosaccharides.

Post-hydrazinolysis clean up

Anhydrous hydrazine is used for the removal of N- and O-linked oligosaccharides from glycoproteins. The hydrazine needs to be removed before the reducing terminus is regenerated. To date this has involved exhaustive paper chromatography [7] or more recently, cellulose chromatography has been incorporated into hydrazinolysis kits marketed by Oxford GlycoSciences (UK).

Free hydrazine was shown, by precipitation with salicylaldehyde, to elute from the carbon SPE column with water. To test the elution behavior of released sugar hydrazones, bovine fetuin was treated with anhydrous hydrazine. It was found advisable to evaporate the excess hydrazine by lyophilization or under a stream of nitrogen before desalting the residual hydrazine with the carbon column. After rehydration the dried hydrazinolysate was applied to a carbon column, the residual hydrazine washed off with water and the hydrazones in the eluants were acetylated and their reducing glycans regenerated and monitored by HPAEC-PAD. The chromatogram of the fraction of the hydrazine released oligosaccharides eluted by 25%(v/v) acetonitrile containing 0.05%(v/v) TFA from the carbon column shows the typical sialylated O-linked and di-, tri- and tetra-sialylated N-linked oligosaccharides (Figure 3) of bovine fetuin and compares very favorably with the published data obtained using paper chromatography to remove the hydrazine [7].

Amino acid analysis of the step-wise eluted fractions of the hydrazinolysate of bovine serum albumin from the carbon column showed that the sum of all the amino acids (26879 pmol) recovered (Table 1) was only about 1% of the protein (as amino acids) applied to the column. These amino acids or peptides were eluted predominantly by 50%(v/v) acetonitrile in water and 50%(v/v) acetonitrile in 0.05%(v/v) TFA.

Desalting of oligosaccharides for ESI-MS analysis

On-line ESI-MS of PNGase F released oligosaccharides

An obvious need in the modern analysis of oligosaccharides is the capacity to desalt glycan solutions for analysis by mass spectrometry (Figure 1b). The total neutral oligosaccharides released by PNGase F from ovalbumin were desalted on a carbon SPE column and eluted with 25%(v/v) acetonitrile. The eluant was dried, reconstituted in water and applied directly to a carbon on-line cartridge inserted in the injection loop of the electrospray mass spectrometer. The cartridge was washed to waste with at least 1 ml of water before the oligosaccharides, which are concentrated by the carbon pre-column, were eluted directly into the source with 50%(v/v) acetonitrile in 0.1%(v/v) TFA. The multiple masses observed (Figure 4) correlated with most of the structures which have been reported to occur on the single site of N-glycosylation on ovalbumin [15,16].

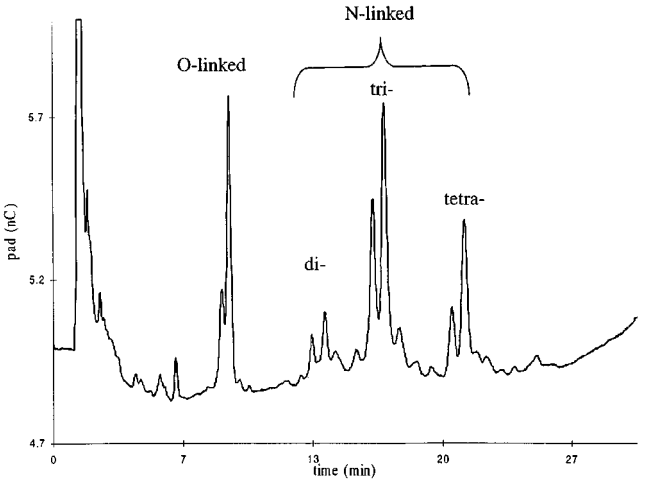


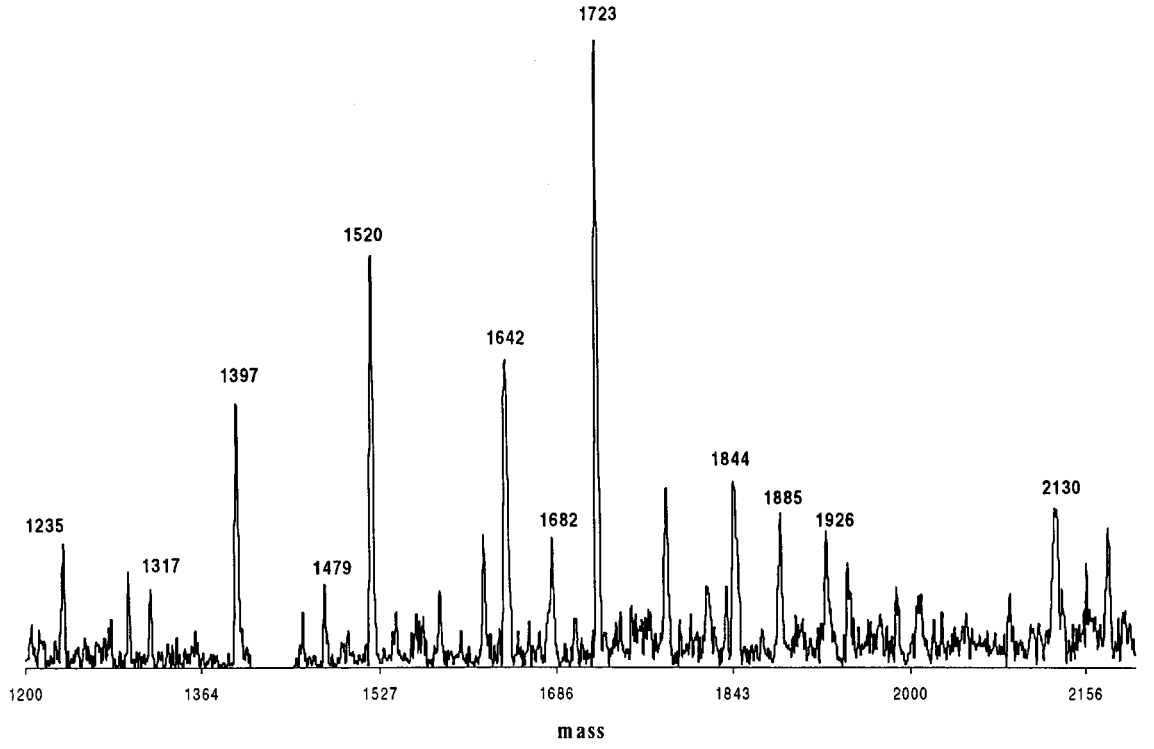
Figure 3. HPAEC-PAD chromatography to monitor the clean-up by a Carbograph SPE column of oligosaccharides released by the hydrazinolysis of 10 nmoles of bovine fetuin. Free hydrazine was eluted in water and the typical O-linked and N-linked oligosaccharide hydrazones (di-, tri- and tetrasialylated classes) were recovered in the 25%(v/v) acetonitrile containing 0.05%(v/v) TFA fraction and were subjected to acetylation and regeneration of the reducing terminus prior to chromatography.

Table 1
Amino acid analysis of the fractions eluted from a graphitized carbon column of a hydrazinolysate of bovine serum albumin (10 nmole). The column was eluted stepwise with 3 ml of each eluant, the fractions dried and hydrolyzed for amino acid analysis. The total amino acids recovered represent only 1% of the amount of protein subjected to hydrazinolysis.

Eluant	*pmole amino acids*	*% of total amino acids recovered*
Water	1075	4
10%(v/v) acetonitrile	1637	6
25%(v/v) acetonitrile	3510	13
50%(v/v) acetonitrile + 0.05%(v/v) TFA	5231	20
10%(v/v) acetonitrile + 0.05%(v/v) TFA	1089	4
25%(v/v) acetonitrile + 0.05%(v/v) TFA	525	2
50%(v/v) acetonitrile + 0.05%(v/v) TFA	1201	4
80%(v/v) acetonitrile + 0.05%(v/v) TFA	10683	40
	1928	7

On-line ESI-MS of HPAEC-PAD separated fractions

Commonly the oligosaccharides released enzymically or chemically from glycoproteins are separated by HPAEC-PAD, which is capable of resolving complex glycan



1235 Da	1317 Da	1397 Da	1520 Da	1642 Da	1682 Da	1723 Da	1844 Da	1885 Da	1926 Da	2130 Da
$HexNAc_2$ Hex_5	$HexNAc_4$ Hex_3	$HexNAc_2$ Hex_6	$HexNAc_5$ Hex_3	$HexNAc_4$ Hex_5	$HexNAc_5$ Hex_4	$HexNAc_6$ Hex_3	$HexNAc_5$ Hex_5	$HexNAc_6$ Hex_4	$HexNAc_7$ Hex_3	$HexNAc_8$ Hex_3

Figure 4. Transformed electrospray mass spectrum of the PNGase F released oligosaccharides from 1 nmole ovalbumin after clean-up by graphitized carbon. The sample was desalted with a Carbograph SPE column and the 25%(v/v) acetonitrile fraction was dried, reconstituted in water and concentrated with a Hypercarb® PGC guard cartridge on-line to the ESI-MS.

mixtures. The separation of the oligosaccharides in this type of analysis requires the use of high salt solutions at high pH which are incompatible with mass spectrometric methods. The carbon cartridge can be placed on-line to the ESI-MS by simply inserting it into the Rheodyne injection sample loop. We collected the samples manually from the HPAEC outlet before applying it to the on-line cartridge (Figure 1b) but if necessary it would be a simple matter to automate the process.

To demonstrate the applicability of this technique, the trisialylated oligosaccharides released by PNGase F from bovine fetuin were separated and collected (Figure 5a) from the CarboPac PA1 HPAEC column in about 0.5 ml of approximately 0.2 M sodium acetate in 0.25 M sodium hydroxide and were applied directly to a carbon cartridge on-line to the ESI-MS. The cartridge was washed with at least 1 ml of water before the oligosaccharides were eluted directly into the ESI-MS with 50%(v/v) acetonitrile in 0.1%(v/v) TFA. The correct mass of the fetuin trisialic oligosaccharides was obtained (Figure 5b; 2880 Da, $HexNAc_5Hex_6NeuAc_3$). It should be noted that whereas three or four peaks were collected from the HPAEC only one major mass was observed, since the trisialylated glycan structures of fetuin are different in linkage but the isomers are isobaric [14]. The mass spectrum showed evidence of a small amount of peeling in the alkaline HPAEC chromatography as evidenced by the loss of mass equal to one N-acetylglucosamine moiety (peak of mass 2677Da, $HexNAc_4Hex_6NeuAc_3$). The observed mass of 2837Da could not be assigned to a glycan structure which has been previously reported in bovine fetuin.

ESI-MS after β-elimination of O-linked oligosaccharides

Chemical release of O-linked oligosaccharides from glycoproteins is typically by beta elimination. Glycophorin A, which has 16 O-linked sites [17] was subjected to beta

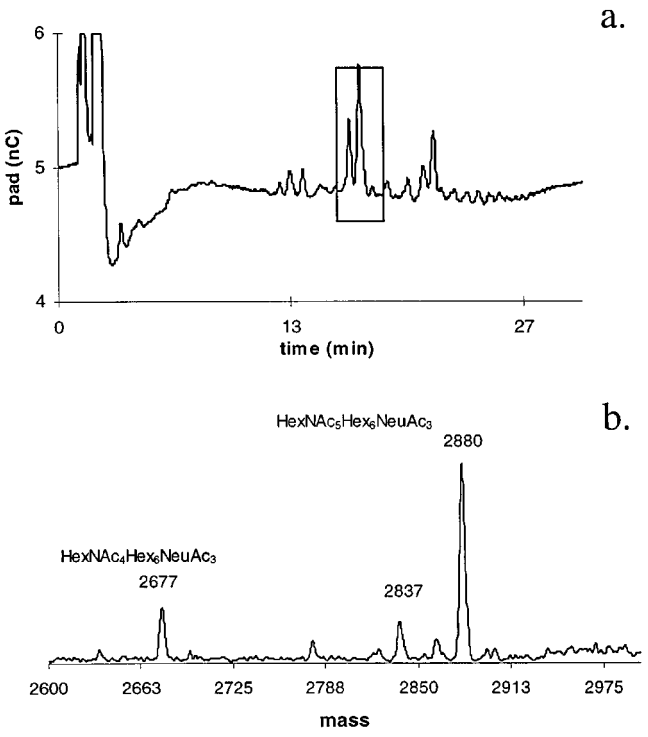


Figure 5. The trisialylated oligosaccharides released by PNGase F from 1 nmole bovine fetuin were a)separated by HPAEC, collected (box) and applied to an on-line PGC cartridge which was washed with water before switching to 50%(v/v) acetonitrile in 0.1%(v/v) TFA to b) obtain the transformed electrospray mass spectrum of the eluted oligosaccharides by ESI-MS.

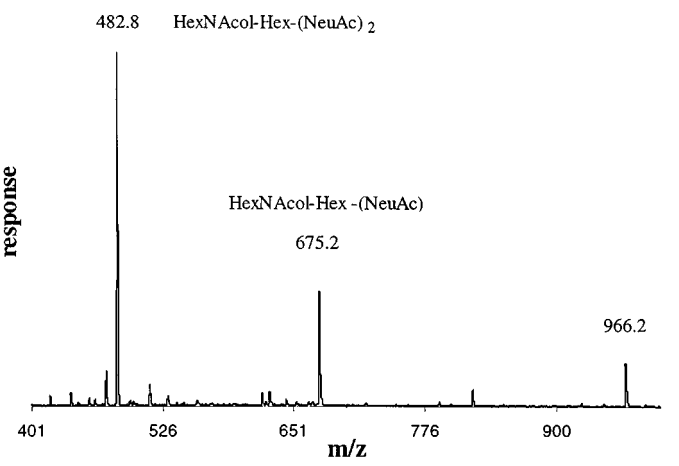


Figure 6. Electrospray mass spectrum of oligosaccharide alditols released by β-elimination of 0.5 nmole glycophorin A. The reaction mixture was neutralized, applied to an on-line PGC cartridge and desalted with water before elution of the oligosaccharides into the ESI-MS with 50%(v/v) acetonitrile in 0.1%(v/v) TFA.

elimination and the solution was applied directly onto a carbon cartridge on-line to the ESI-MS. The cartridge was first washed with water to remove the salts and the alditols were eluted directly into the mass spectrometer with 50%(v/v) acetonitrile containing 0.1%(v/v) TFA (Figure 6). The major peak corresponded to the doubly charged ion (m/z) of the disialylated disaccharide alditol (HexNAcolHex-$NeuAc_2$, 482.8 m/z) with the singly charged ion seen (966.2 m/z) as well as the monosialylated disaccharide alditol (HexNAcolHexNeuAc, 675.2 m/z).

The recovery of alditols from the graphitized carbon column was measured by β-elimination of 100 μg porcine gastric mucin. A comparison of the monosaccharide content before and after elution of the alditols from a carbon SPE column with 25%(v/v) acetonitrile in 0.05%(v/v) TFA resulted in almost complete recoveries of fucose (100%), glucosamine (100%) and galactose (88%). A decreased recovery of galactosamine (52%) reflected the conversion of the reducing terminus of the O-linked glycan to an alditol.

Desalting of urine

HPAEC analysis of the urine of patients who accumulate oligosaccharides in their urine in lysosomal storage diseases, such as mannosidosis, has been difficult because of the high salt and glucose content of urine [18]. Urine (5 ml) collected from a mannosidosis patient was applied to a carbon column, the salts and glucose were washed off with water and the neutral oligosaccharides were eluted with 25%(v/v) acetonitrile. The eluted sugars were dried, redissolved in water and applied directly to a HPAEC column (Figure 7). The mannose glycans which accumulate in this condition elute in the first 12 min. This example of the usefulness of the graphitized carbon column is given to demonstrate that this simple desalting technique would enable the previously difficult automation of the quantitative diagnosis of these types of genetic disease to be performed by chromatography and/or mass spectrometry.

Discussion

The non porous graphitized carbon black (GCB, Carbograph) which is used in the SPE columns in this paper is formed by heating carbon to temperatures above 2000 °C and has been extensively used to remove polar pesticides and other organic pollutants from water [19]. Shandon (UK) changed the structure of the carbon to form porous graphitized carbon (PGC, Hypercarb®) which is robust enough to be used for HPLC applications, and in our case a guard cartridge containing Hypercarb® was used on-line to the electrospray mass spectrometer. The two dimensional graphite structure of both adsorbents is the same [20], but they differ in that the PGC has a three dimensional sponge-like structure capable of withstanding considerable shear forces. The PGC HPLC column has been used to separate a range of glycans [5, 6, 21] but the mechanism of carbon interaction with carbohydrates is poorly understood and

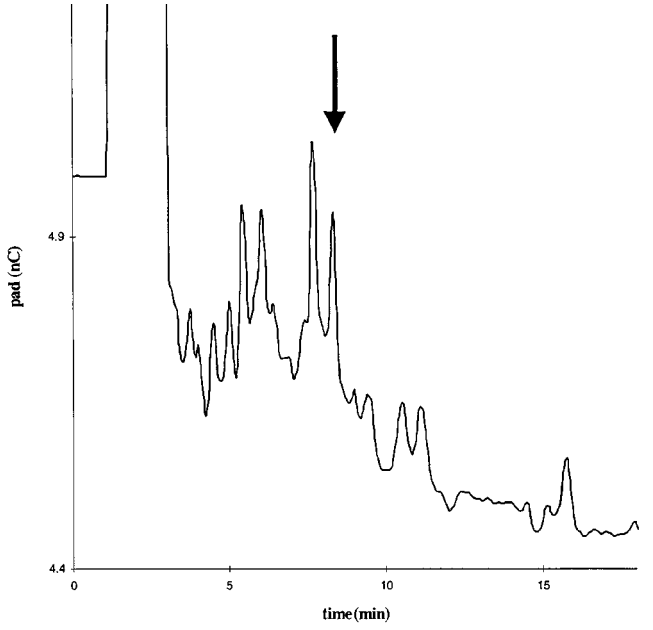


Figure 7. HPAEC-PAD chromatogram of the 25%(v/v) acetonitrile fraction from a Carbograph SPE column after desalting 5 ml of urine. → Typical retention of neutral mannose oligosaccharides.

involves both absorption and hydrophobic interaction. The elution is based on the size, charge and linkage or planarity of the sugars and it is able to separate isomeric and closely related structures [22]. In practice, the separation of the four possible anomers (α,β, pyranose and furanose) of the constituents of oligosaccharides can make interpretation of the complex chromatogram difficult.

We have applied the properties of the graphitized carbon packing to the separation of contaminants from a range of carbohydrate containing solutions. Desalting of the oligosaccharide components of glycoconjugates and complex polysaccharides has been a major impediment to the success of different chemical, separation and spectroscopic techniques which are used for the preparation, isolation and analysis of the glycan component of glycoconjugates.

As an illustration, glycans are removed from the glycoprotein by endoglycosidases and further analyzed by the use of the exoglycosidases used for sequencing oligosaccharides. In this event, it is usually necessary to remove the buffer salts and the protein prior to further analysis. In particular, the detergents usually used for denaturing the protein to obtain maximum yield in this process make the post-reaction clean-up difficult. The detergent, the monosaccharides released and the buffer salts can be removed on the carbon solid-phase extraction cartridge with water, the released neutral or acidic oligosaccharides easily recovered for further sequencing by elution with water-acetonitrile mixtures (with and without the addition of TFA, respectively), while the protein is retained by the adsorbent.

Alternatively, N-linked glycans are released from a glycoprotein by treatment with hydrazine [7]. For the analysis to proceed, it is necessary to separate the glycans from the excess of hydrazine and from the protein and derived peptide hydrazides. This separation is typically achieved by a combination of evaporation (to remove most of the hydrazine) and size-exclusion or reversed phase chromatography to separate the glycans from the proteinaceous materials. Neither of these procedures is particularly effective because of the heterogeneity in size of the sugars and the contaminating peptide products. Using graphitized carbon after evaporation of the bulk of the hydrazine, the remaining hydrazine can be removed and the retained oligosaccharides can then be eluted or fractionated into classes of neutral and acidic glycans. Under these elution conditions, most of the peptide hydrazides and proteinaceous material are retained by the column.

The next step in the analysis of released glycoforms from glycoconjugates is commonly a separation of the glycans using high-performance anion-exchange chromatography (HPAEC-PAD) at high pH, which involves using an eluant which contains sodium hydroxide and (usually) a gradient of sodium acetate. The CarboPac (Dionex) column used in this system is arguably still the best chromatographic medium for the separation of complex mixtures of oligosaccharides. When the fractions are isolated, they are therefore very alkaline and contain high levels of salt which greatly complicate further analysis of the glycans. To date, desalting of the separated fractions has been difficult, with dialysis, ion-exchange and gel filtration being used with varying associated problems [22]. It was necessary to sequentially desalt HPAEC-separated fractions by using an anionic micro-membrane suppressor (AMMS-II, Dionex), followed by a cation-exchange resin column, to enable MALDI-MS analysis of the glycans [23]. Dionex have recently released a cation-exchange membrane desalter (CMD™) to remove sodium from the eluant of the HPAE column but the sample is exchanged into acetic acid which may result in some desialylation of the oligosaccharides, particularly when being concentrated. The carbon adsorbent provides a cheaper, simpler, more convenient and reproducible alternative, applicable to a wide range of clean-up procedures. It is not necessary to neutralize the eluant with acid; the collected fraction containing one or more oligosaccharides can be applied directly to the on-line carbon extraction cartridge, the inorganic salts eluted with water, and the glycans eluted with water containing acetonitrile and a low concentration of the volatile acid, TFA, directly into the electrosray mass spectrometer. Alternatively, the desalted HPAEC-separated glycans can be further analyzed by a second dimension of chromatography, or electrophoresis, following derivatization.

Mass spectrometry provides a sensitive technique to assist in the determination of the structure of glycans but it is essential that the samples are desalted to achieve maximum

ionization. Pretreatment of oligosaccharides by microdialysis using a cation exchange membrane (Nafion™) [24] or low molecular weight cut-off cellulose ester membrane [25] and on-the-probe desalting of oligosaccharides with a range of chromatographic media [26] has been shown to improve the detection of oligosaccharides by matrix-assisted laser desorption ionization mass spectrometry. The capacity of these techniques to desalt solutions however is low (10 μM salt), uses small (μl) quantities of sample and requires skilled manipulation.

The benefit of electrospray mass spectrometry is the capacity to perform on-line liquid sample pre-treatment. The use of carbon for desalting on-line has the advantage of using solvents which are ideal for this mode of ionization (acidic acetonitrile) and utilizes a medium which has a high capacity to retain the sugars while removing salts, detergents and proteins. The medium, used as a stepwise clean-up procedure prior to ESI-MS, has a wide range of potential applications. We have shown here, as examples, the application of the carbon column to desalting the products of the enzymic removal of N-linked oligosaccharides and of the β-elimination of O-linked oligosaccharides, on-line into the ESI-MS, without any further clean-up. The packing has been used for sample clean-up on-line in a mass spectrometric assay for chitobiose after the enzymatic hydrolysis of chitin [27]. We have shown that carbon can desalt solutions of oligosaccharides released by PNGase F from glycoproteins which have been separated by two dimensional electrophoresis and electroblotted to PVDF membrane. In this case octyl glucoside was used as detergent and the released oligosaccharides were passed through a C8 reversed phase cartridge and a serial carbon cartridge on-line to the electrospray mass spectrometer and the masses of the oligosaccharides successfully obtained [28]. The graphitized carbon clean-up of oligosaccharides released by PNGase has also been applied successfully by us to the analysis of the effect of sialylation on the LDL receptor, by allowing the separation of the neutral and sialylated oligosaccharides present on low density lipoprotein [29].

The high salt and glucose content of urine often makes analysis of excreted metabolites difficult. One such case is in the analysis of the group of lysosomal storage diseases characterized by the excretion in the urine of excess oligosaccharides resulting from a deficiency in various glycosidase enzymes. At present the urine of these patients is analyzed by TLC techniques which, compared with HPLC, are difficult to quantitate and automate. The usefulness of HPAEC for screening of these diseases was limited by the difficulty of cleaning up the urine samples [19]. Use of the carbon column, which can in one step desalt and remove both glucose and protein, would facilitate the improved analysis of these diseases.

In summary, salt contamination of solutions of oligosaccharides has been a constant hindrance to the success of many of the analytical methods used in glycoconjugate research. The use of graphitized carbon for desalting promises to be a universal method which will solve many of the problems of glycoanalysis.

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