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Note

# The resolution of oligosaccharides by high pH anion exchange chromatography

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The natural heterogeneity of the oligosaccharide chains of glycoproteins requires that chromatography be an essential part of structural elucidation. Until recently, the rapid analysis of underivatised carbohydrates by HPLC was hampered, due to the extensive structural heterogeneity, by the absence of a chromatographic medium for satisfactory separation and by the lack of a sufficiently sensitive detection system. This problem has been overcome by the introduction of high pH anion exchange chromatography (HPAEC) with pulsed electrochemical detection (PED) which separates oligosaccharides as their oxyanions on micropellicular quaternary ammonium resins [1–10]. This technique resolves the linkage and branch isomers of neutral and anionic oligosaccharides to picomole sensitivity without pre- or post-column derivatisation. The DX-500 chromatography system is viewed as a major innovation in HPAEC-PED due to an improved sensitivity and resolution as a result of a new amperometric cell design and new digital detector signal processing. Our investigations have utilised the DX-500 in the analysis of various oligosaccharide mixtures in direct comparison with the DX-300 system that it has superceded.

## 1. Results and discussion

The neutral oligosaccharide profiles for the DX-300 and DX-500 are shown in Figs 1A and B, respectively. The components of the oligosaccharide mixture, which are

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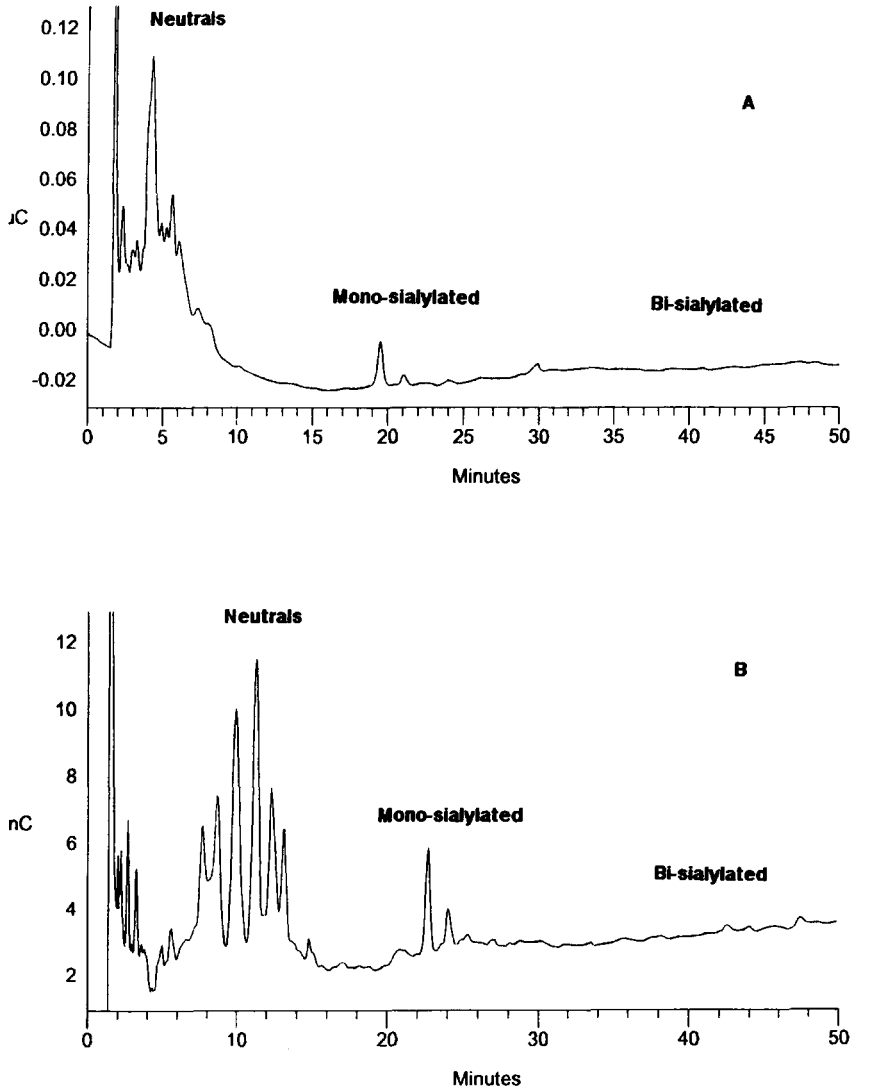


Fig. 1. The separation of 10 $\mu$g of an IgG oligosaccharide library using a 100 mM NaOH/20–140 mM NaOAc gradient Dionex DX-300 (A) and Dionex DX-500 (B) systems. The separation conditions are summarised in the text.

predominantly desialylated bi-antennary chains with 0, 1 or 2 galactose residues on the outer branches, are separated to a greater extent by the DX-500 system. Additionally, the structures are retained much longer on the column resulting in distinct separation from the solvent front. The sialylated oligosaccharide profiles for the DX-300 and DX-500 are shown in Figs 2A and B, respectively. The gradient separates the mixture on the basis of the number of charges per oligosaccharide chain [4–9] which correlates to the number of

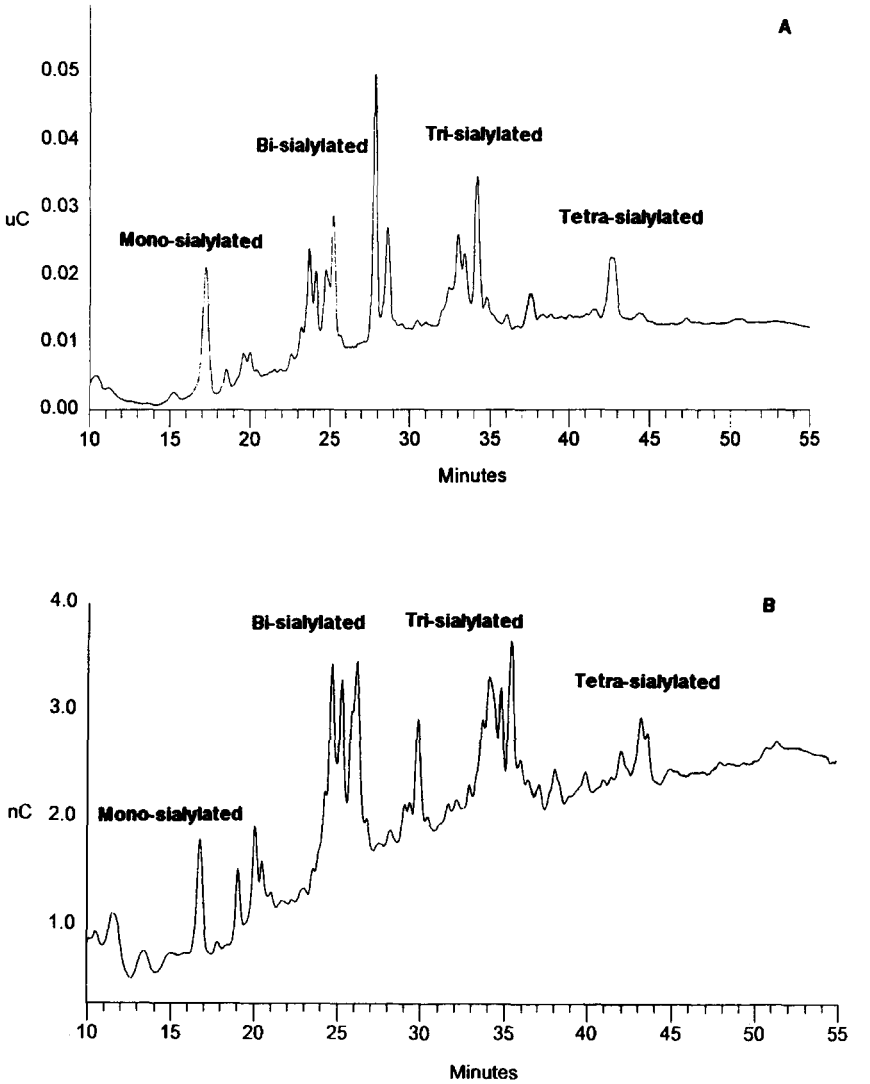


Fig. 2. The separation of 10 $\mu$g of an AGP oligosaccharide library using a 100 mM NaOH/50–200 mM NaOAc gradient Dionex DX-300 (A) and Dionex DX-500 (B) systems. The separation conditions are summarised in the text.

sialic acid (SA) residues, ie 20–30 mins (2 SA normally biantennnary), 30–40 min (3 SA normally triantennary) and 40–50 min (4 SA normally tetraantennary). Thereafter, each sialylation band is further resolved on the basis of subtle isomeric differences. For example, chains terminating in $\alpha$2–3 linked sialic acid are known to have increased retention time relative to chains terminating $\alpha$2–6 [11]. Moreover peripheral fucose has been shown to further influence retention time [12] by virtue of its charge masking

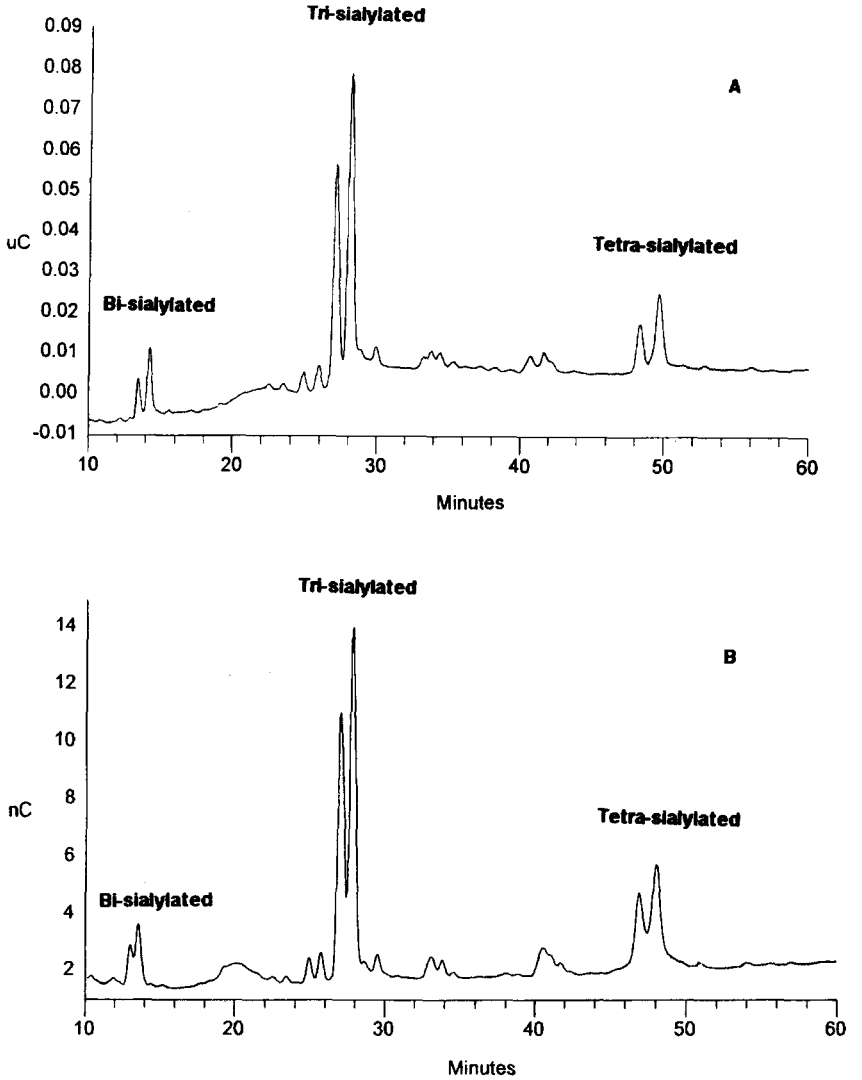


Fig. 3. The separation of 1 nmol of a fetuin reduced N-linked oligosaccharide library using a 100 mM NaOH/50–170 mM NaOAc gradient Dionex DX-300 (A) and Dionex DX- 500 (B) systems. The separation conditions are summarised in the text.

effect. The separation of the mixture by the DX-300 is consistent with published results [13,14]. The profile illustrated in Fig. 2B was reproducible using the DX-300 column (which had produced the profile in Fig. 2A) on the DX-500 system. The profiles of the reduced oligosaccharides of fetuin are shown in Figs 3A and B, respectively for the DX-300 and DX-500 and are consistent with that expected [15]. The mixture is separated on a gradient similar to the sialylated structures however, there is a general decrease in retention times resulting from the oligosaccharide chains being present in a

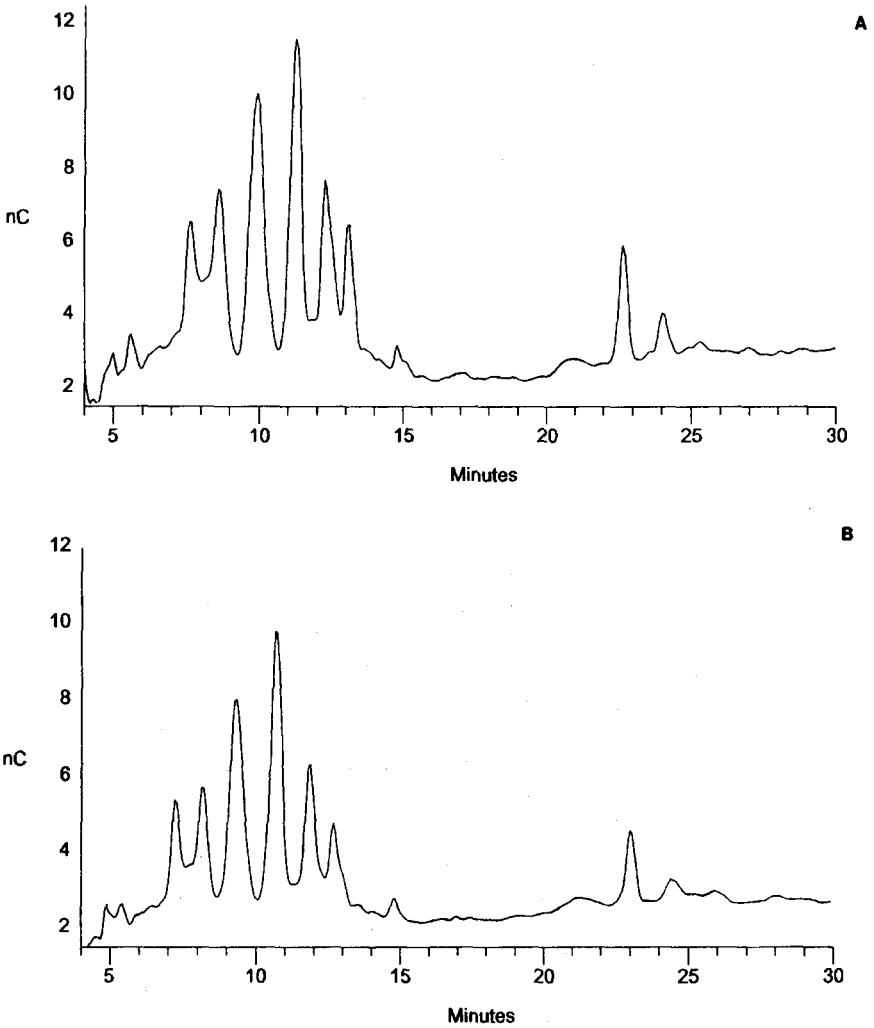


Fig. 4. A comparison of the separation of 10 $\mu$g of an IgG oligosaccharide library by two different CarboPAc PA-100 columns. Profile A utilised the column used to generate all the other DX-500 traces; profile B utilised the column that had been previously used on the DX-300 system.

reduced form as alditols. The similarity of the profiles appears to indicate that both systems exhibit identical behaviour in the resolution of reduced structures.

Our results indicate that the DX-500 is better at resolving mixtures of neutral oligosaccharides than the DX-300 however there was no significant increase in the resolution of sialylated oligosaccharides or alditols. No improvement in the separation of the latter two mixtures could be obtained either by altering the gradient conditions or the flow rate. The traces obtained using the DX-500 system were reproducible and independent of the column used (Fig. 4). Although from unit to unit comparison of the profiles,

it appears that the PED detector is the more sensitive (e.g. major monosialylated oligosaccharide peak is 20nC in Fig. 1A compared to 3.5nC in Fig. 1B), absolute comparison of scales between the systems is difficult. This is due to variations in software control between the DX-500 PeakNet and the DX-300 AI 450 software systems which result in the production of different output scales for each system. There was no major improvement in the signal to noise ratio between the two systems.

## 2. Conclusion

We demonstrate that the most recent HPAEC-PED system, the DX-500, is better at resolving mixtures of neutral oligosaccharides than its predecessor, the DX-300, but not alditols or sialylated oligosaccharides.

## 3. Experimental

Both chromatography systems were supplied by Dionex (Camberley, UK). The DX-300 system consisted of an advanced gradient pump, pulsed electrochemical detector and an anion micromembrane suppressor controlled via an advanced computer interface by a Vtech 486SX 25 (Viglen, UK) using AI-450 software. The DX-500 system consisted of a GP40 gradient pump, ED40 electrochemical detector and an anion self regenerating suppressor controlled by a Vtech 486SX 25 (Viglen, UK) using PeakNet software. For all analyses, the oligosaccharide sample was applied to a CarboPac PA-100 column ($25 \times 0.4$ cm I.D.) equilibrated with an eluent mixture, corresponding to the initial conditions of the separation gradient, at a flow rate of 1 m $min^{-1}$ at room temperature. The chromatography eluents were prepared using sodium hydroxide and sodium acetate purchased from BDH (Poole, UK) and HPLC grade water purchased from Rathburn (Murrayburn, UK). Detection was by a pulsed electrochemical detector using the following pulse potentials and durations : Time = 0 s, $E = +0.05$ V; 0.29 s, +0.05 V; 0.49 s, +0.05 V; 0.50 s, 0.05 V; 0.51 s, +0.6 V; 0.6 s, +0.6 V; 0.61 s, −0.6 V; 0.65 s, −0.6 V; 0.66 s, +0.05 V.

N-linked oligosaccharides (10 $\mu$g of an immunoglobulin (Ig) G oligosaccharide library from Oxford Glycosystems; Abingdon, UK) were chromatographed on the column equilibrated in 10% solvent A (1 M sodium hydroxide)/2% solvent B (1 M sodium acetate)/88% solvent C (HPLC water). This solvent elution was continued for 3 min after which a linear gradient of 10%A/2%B/88%C to 10%A/14%B/76%C was achieved over 57 min. The limit solvent was continued for a further 5 min before the column was regenerated by moving to 50%A/50%C in 1 min and holding for a further 10 min. Thereafter, the initial conditions were resumed.

Sialylated N-linked oligosaccharides (10 $\mu$g of an $\alpha_1$ acid glycoprotein (AGP) oligosaccharide library from Oxford Glycosystems; Abingdon, UK) were chromatographed on the column equilibrated with a mixture of 10% solvent A (1 M sodium hydroxide)/5% solvent B (1 M sodium acetate)/85% solvent C (HPLC Grade Water). This solvent elution was continued for 10 min after which a linear gradient of

10%A/5%B/85%C to 10%A/20%B/70%C was achieved over 40 min. The limit solvent was continued for a further 5 min before the column was regenerated by moving to 50%A/50%C in 1 min and holding for a further 10 min. Thereafter, the initial conditions were resumed.

The resolution of reduced, sialylated N-linked oligosaccharides by each system was compared by simultaneously injecting 1 nmol of fetuin N-linked oligosaccharide alditols (Dionex, UK) onto the column equilibrated with a mixture of 10% solvent A (1 M sodium hydroxide)/5% solvent B (1 M sodium acetate)/85% solvent C (HPLC Grade Water). This solvent elution was continued for 10 min after which a linear gradient of 10%A/5%B/85%C to 10%A/17%B/73%C was achieved over 60 min. The limit solvent was continued for a further 5 min before the column was regenerated by moving to 50%A/50%C in 1 min and holding for a further 10 min. Thereafter, the initial conditions were resumed.

Each run was repeated three times on each system in order to determine reproducibility.

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