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Comparison of separation modes of high-performance liquid chromatography for the analysis of glycoprotein- and proteoglycan-derived oligosaccharides

Michael J. Davies, Elizabeth F. Hounsell*

Glycoprotein Structure/Function Group, Department of Biochemistry and Molecular Biology, University College London, Gower Street, London WC1E 6BT, UK

Abstract

Porous graphitised carbon (PGC) has been explored for high-performance liquid chromatography (HPLC) of mono- and di-saccharides released from proteoglycans and of fluorescently labelled oligosaccharide derivatives for high-sensitivity detection. Sulphated oligosaccharides show good retention and separation behaviour on PGC–HPLC, and compared to anion-exchange or reversed-phase ion-pair chromatography the chromatography is carried out in the absence of salt. Due to their poor retention on PGC–HPLC the analysis of single uronic acids has been optimised with high pH anion-exchange chromatography. Fluorescent labelled derivatives formed by reductive amination of neutral oligosaccharides with 2-aminobenzamide have been chromatographed on PGC–HPLC and by BioGel P4 gel filtration.

1. Introduction

Oligosaccharides derived from glycoproteins and proteoglycans are a diverse set of molecules with significant size, charge, composition and linkage heterogeneity. Strategies for their analysis are based on two approaches; (a) optimisation of chromatography for particular molecular families and (b) column systems that will support more than one type of application. Thus, as reviewed previously [1,2], reversed-phase chromatography has been applied to neutral oligosaccharides, using aqueous organic solvents or with ion-pair reagents for sulphated oligosaccharides, and normal-phase amine-bonded chromatography has been applied to neutral oligosaccharides in aqueous organic solvents and with buffers for sialylated and sulphated oligosaccharides. These are particularly useful for reduced mono- and oligosaccharides (alditols) as this alleviates the problem of anomeric separation. High-resolution gel filtration (Biogel P4) chromatography has been optimised for neutral oligosaccharide separation by size with water as eluent and for sialylated oligosaccharides with buffers. Again alditols have been used, this time in order to introduce a tritium label at C-1 for sensitive radioactive detection. High-pH anion-exchange chromatography (HPAEC) has its primary applications for N-linked chains, in particular sialylated oligosaccharides, but can also be used for neutral reducing oligosaccharides and neutral or sialylated/sulphated alditols [3–6], chondroitin disaccharides [7] and oligosac-

* Corresponding author.

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charides of hyaluronan [8]. Multiply sulphated oligosaccharides have been mainly chromatographed on strong anion-exchange columns [9,10] or reversed-phase columns [11,12] with elution in non-volatile buffer systems. The further analysis of oligosaccharides is often dependent on salt-free samples, and thus the use of volatile buffers for purification is an important consideration. To this aim we have explored the use of porous graphitised carbon (PGC) columns for oligosaccharide analysis and purification. The underlying mechanism of separation on PGC–HPLC is by reversed-phase [13,14], but there are additional capabilities for isomer separation and chromatography of neutral, sialylated and sulphated oligosaccharides, all in the absence of salt [14]. These have so far been detected by UV absorbance. However, post-column addition of salt is an option to obtain the sensitivity of pulsed amperometric detection and has also been recommended for ion-pair HPLC on PGC columns [15]. In the past considerable success has been achieved with fluorescence detection and established HPLC methods. In addition, many fluorescent conjugates also exhibit UV chromophores, for example HPLC of aminopyridine derivatives with UV detection at 290 nm [16], 9-fluorenylmethoxycarbonyl (FMOC) oligosaccharides on reversed-phase HPLC with UV detection at 263 nm [17] and anthranilic acid derivatives on reversed-phase HPLC with detection at 220 nm [18]. 2-Aminobenzamide (2-AB) has been developed as a fluorescent conjugate [19,20] for use on an automated Biogel P4 system [21], with fluorescence detection down to a sensitivity of 20 pmol. Thus fluorescence derivatives offer a highly sensitive alternative which relies on reductive amination methods (similar to alditol formation but with addition of a hydrophobic chromophore). The present paper explores new options in the context of previous chromatographic strategies. It compares the use of high-pH anion-exchange chromatography and PGC–HPLC for uronic acid determination and the separation of sulphated oligosaccharides and of PGC–HPLC and Biogel P4 for the detection of fluorescent labelled derivatives.

2. Experimental

2.1. Materials

The glycoproteins fetuin and asialo-fetuin and the oligosaccharides chitotriose (CT) GalA, GlcA, α(2-6)sialyllactose, GlcNAc(3S), GlcNAc(6S), N-acetylneuraminic acid (NeuAc), N-glycolylneuraminic acid (NeuGc), ΔUAβ1-3GalNAc, ΔUAβ(1-3)GalNAc(4S), ΔUAβ1-3GalNAc(6S) and ΔUAβ1-3GalNAc(4S, 6S) were from Sigma (Poole, UK), where S is sulphate and ΔUA is 4,5-unsaturated uronic acid (4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid). Galβ1-3GlcNAc (G3GN) was obtained by chemical synthesis and kindly supplied by Dr. A. Veryières (Paris, France). Peptide-N-Glycanase F (PNGaseF) and neuraminidase (*A. uraefaciens*) were from Boehringer Mannheim (Lewes, UK). Triethylamine was obtained from Fluka (Poole, UK) and the oligosaccharide labelling kit (signal labelling kit) was from Oxford Glycosystems (Abingdon, UK). HPLC on a PGC column (Hypercarb S, 100 mm × 4.6 mm I.D., Shandon, Runcorn, UK) was carried out using a Gilson system which consisted of two Model 302 pumps, a Model 802C manometric module, a Model 811 dynamic mixer and a Model 116 UV detector set at 206 nm or 220 nm. High-pH anion-exchange chromatography (HPAEC) was performed on a titanium Gilson system comprising of Model 302 pumps, a 802Ti manometric module, a 811B mixer, and a Dionex PED-2 detector with a CarboPac PA-1 column as previously described [2]. The Biogel P4 chromatography was carried out using a RAAM (reagent arrray analysis method) 2000 sequencer (Oxford Glycosystems). All solvents were HPLC grade (Merck, Poole, UK and Pierce, Warrington, UK)

2.2. Methods

Glycoproteins were suspended in 100 μl sodium acetate, pH 5.5, and digested with 50 mU of neuraminidase at 37°C for 18 h. The proteins were precipitated with ice cold ethanol and the

dried pellets resuspended in 200 μl 40 m*M* K_2HPO_4 containing 10 m*M* EDTA and further digested with 1 U of PNGaseF at 37°C for 72 h. The proteins were again precipitated with ice cold ethanol and the oligosaccharide-containing supernatants dried. The supernatants were desalted on a minicolumn of Biogel P2 eluted with water. Oligosaccharides eluted in the void volume were chromatographed on a Dowex 50W X8 (cation-exchange) minicolumn eluted with water and dried ready for labelling.

Labelling with 2-aminobenzamide (2-AB) was carried out as in the manufacturers instructions; briefly, oligosaccharides were suspended in 5 μl of 0.35 *M* 2-AB containing 1.0 *M* sodium cyanoborohydride in 30% (v/v) acetic acid in dimethylsulphoxide. The tubes were sealed and incubated at 60°C for 2 h. The reaction mixture was then purified by paper chromatography in a solvent system of butanol–ethanol–water (4:1:1, v/v/v). The origin of the paper was excised and the labelled oligosaccharides eluted in water. These oligosaccharides were further purified on a mixed-bed anion-exchange resin (supplied with the labelling kit), the anion-exchange resin being washed first with 1.5% (v/v) triethylamine, before elution of the oligosaccharides in water. The oligosaccharides were dried prior to further analysis.

Fluorescent labelled and anionic oligosaccharides were chromatographed on PGC–HPLC with a flow-rate of 1 ml/min in solvent system PGCa as follows: eluent A, H_2O–0.05% trifluoroacetic acid (TFA); eluent B, acetonitrile–0.05% TFA with the gradient 0–95 min 0–100% B, 95–100 min 100% B, 100–102 min 100–0% B. Anionic monosaccharides were also eluted with solvent system PGCb using eluent A and B as above with a gradient of 0–5 min 100% A, 5–35 min 0–40% B, 35–40 min 40% B, 40–41 min 40–0% B, at a flow-rate of 0.75 ml/min [2]. HPAEC of acidic monosaccharides used a gradient of 100 m*M* NaOH (A) and 100 m*M* NaOH–1.0 *M* NaOAc (B):95% A for 5 min followed by a linear gradient to 60% B over 35 min before column regeneration in 100% A for 10 min. Biogel P4 chromatography was performed on the RAAM 2000 system at a temperature of 55°C with eluent water at a flow-rate of 150 μl/min for 11 ml ramped to 160 μl/min over 27 ml and held for 7 ml. Detection was by fluorescence at $\lambda_{max} = 330$ nm and $\lambda_{max} = 420$ nm for excitation and emission, respectively, for labelled oligosaccharides and refractive index for the dextran ladder internal standard as per the manufacturers instructions. Data was analysed by the Glyco-link software supplied with the RAAM 2000 by Oxford Glycosystems.

3. Results

We have explored the use of PGC–HPLC for the separation of sulphated and acidic mono- and disaccharides in two solvent systems, named PGCa and PGCb. While unsulphated GlcNAc eluted close to the solvent front in the starting eluant of solvent system PGCa (100% aqueous TFA), mono-sulphated GlcNAc residues (GlcNAc-3-SO_4 and GlcNAc-6-SO_4) were significantly retained and eluted with increasing acetonitrile concentration within the same region of the gradient as non-sulphated neutral disaccharides, e.g. Galβ1-3GlcNAc (G3GN; Table 1). Two of the anomers of GlcNAc-3-SO_4 and GlcNAc-6-SO_4 were clearly resolved, although the other two co-elute (Fig. 1). Disaccharides from enzymatic digests of chondroitin sulphate

Table 1
Separation of neutral and sulphated mono-, di- and trisaccharides on a PGC column eluted in a 95 min linear gradient from 0–100% acetonitrile–0.05% TFA in aqueous 0.05% TFA (solvent system PGCa, flow-rate 1 ml/min)

Oligosaccharide	Retention time (min)
CT	12.3, 13.7
G3GN	8.93, 10.02
GlcNAc-3-SO_4	8.24, 9.85
GlcNAc-6-SO_4	6.5, 8.34
ΔUA-GalNAc	11, 13.05
ΔUA-GalNAc-4-SO_4	12.2, 13.2
ΔUA-GalNAc-6-SO_4	15.26, 18.37
ΔUA-GalNAc-4,6-SO_4	18.95, 20.52

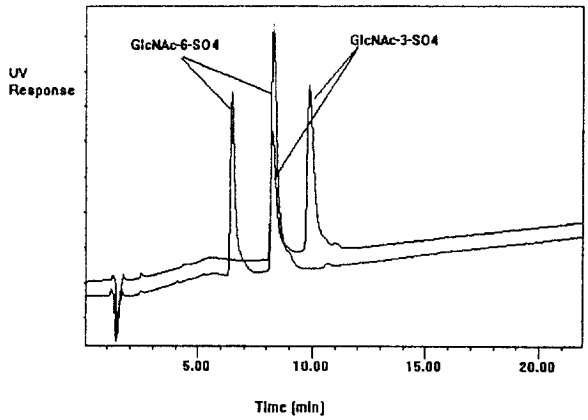

Fig. 1. PGC chromatography of 2-deoxy-2-N-acetyl-3-O-sulpho-D-glucopyranose (GlcNAc-3-SO_4) and 2-deoxy-2-N-acetyl-6-O-sulpho-D-glucopyranose (GlcNAc-6-SO_4).

(ΔUAβ1-3GalNAc) were retained longer than GlcNAc, GlcNAc-*n*SO_4 or the disaccharide G3GN (Table 1). The -4-SO_4 and -6-SO_4 unsaturated chondroitin disaccharides separated from each other (Fig. 2) with elution order 4-SO_4 < 6-SO_4 < 4,6-SO_4 (when sulphation is on the GalNAc residue). This is similar to the elution pattern of other neutral disaccharides with respect to their glycosidic linkage β1-4 < β1-6 [12]. However, the elution of GlcNAc-6-SO_4 before GlcNAc-3-SO_4 more closely resembles the elution of α2-6 and α2-3 linked

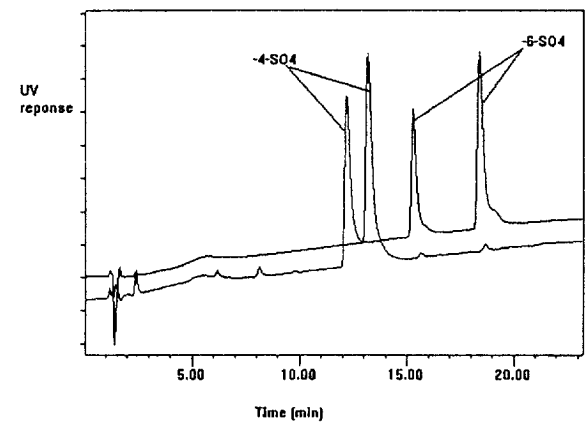

Fig. 2. PGC chromatography of 2-deoxy-2-N-acetyl-3-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-O-sulpho-D-galactopyranose (-4-SO_4) and 2-deoxy-2-N-acetyl-3-O-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-O-sulpho-D-galactopyranose (-6-SO_4).

sialylated oligosaccharides [2]. Due to the possibility of the loss of the more labile sulphate groups on reduction (M.J.D. and E.F.H., unpublished data) we have not reduced the sulphated disaccharides to alditols. This also offers the advantage of having two peaks for unambiguous identification of unreduced sulphated oligosaccharides in non-aggressive eluents.

Uronic acid and sialic acid monosaccharides were retained on PGC–HPLC, although the resolution of GlcA and GalA, and NeuAc and NeuGc was less than by HPAEC (Table 2). The separation of NeuAc and NeuGc on PGC–HPLC differs from HPAEC, with NeuGc eluting just prior to NeuAc on PGC–HPLC, but NeuGc being significantly more retained on HPAEC. Similarly, by high-pH anion-exchange chromatography, uronic acids were retained more strongly than NeuAc and eluted close to NeuGc, whilst on PGC–HPLC they are only slightly more retained than GlcNAc.

Due to the difficulties inherent in post-column addition of alkali for increasing the sensitivity of PGC–HPLC analyses by pulsed amperometric detection (M.J.D. and E.F.H., unpublished data), we also investigated chromatography of fluorescent labelled derivatives and compared the data with those of the new technique of gel filtration chromatography of 2-aminobenzamide (2-AB) derivatives. On PGC–HPLC, chitotriose

Table 2
Comparison of the separation of sialic and uronic acids by PGC–HPLC and HPAEC

Oligosaccharide	Retention time		
	PGCa (min)	PGCb (min)	HPAEC[a]
NeuAc	6.1	13.2	0.69
NeuGc	5.5	12.5	1.68
GalA	3.9	6.27	1.39
GlcA	3.4	5.49	1.62

Solvent systems PGCa, a 95 min linear gradient from 0–100% acetonitrile–0.05% TFA in aqueous 0.05% TFA; PGCb, 5–35 min linear gradient from 5–40% acetonitrile–0.05% TFA; HPAEC 5–35 min gradient from 0–60% 0.1 *M* NaOH–1 *M* NaOAc in 0.1 *M* NaOH.

[a] Relative to the elution of α(2 − 6)sialyllactose.

(CT) labelled with 2-AB (CT-2AB) has a significantly longer retention than unlabelled CT (Fig. 3a), as expected from the increased hydrophobicity of the 2-AB group. The peaks at 11.5 and 13 min equate to unlabelled CT anomers, whilst the single peak at 33 min equates to CT-2AB. This is supported by the stronger UV absorbance of the 37-min peak at 220 nm than at 206 nm, i.e., the stronger UV absorbance of 2-AB-like chromophores at 220 nm than the N-acetyl group of amino sugars. Detection by UV absorbance, although less sensitive than fluorescence detection, can be useful for gauging the efficiency of derivatisation. In contrast, on Biogel P_4 the 2-AB-labelled CT (Fig. 3b) has a very similar elution profile to unlabelled CT, the label slightly reducing the apparent hydrodynamic volume compared to reduced and radioactivity labelled glycan [17,18] resulting in a slightly greater elution volume (Table 3). The elution of desialylated, 2-AB-labelled fetuin oligosaccharides on PGC–HPLC shows increased retention in relation to unlabelled neutral fetuin oligosaccharides (Table 3, Fig. 4a). The elution of 2-AB-fetuin oligosaccharides prior to CT-2AB is surprising and may indicate a greater influence of the aromatic 2-AB group in the separation. 2-AB-labelled neutral fetuin oligosaccharides on Biogel P4 (Fig. 4b) behave (similarly to labelled CT) with reduced retentions. If the experimental glucose units (GU_{AB}) are converted to glucose units of unreduced oligosaccharides (UGU) by the formula: $UGU = GU_{AB} \times 0.98 + 1.95$ [19], we can detect the presence of N-linked oligosaccharides of the classes biantennary (11.18 GU), triantennary (14.10 GU) and tetraantennary (16.35 GU).

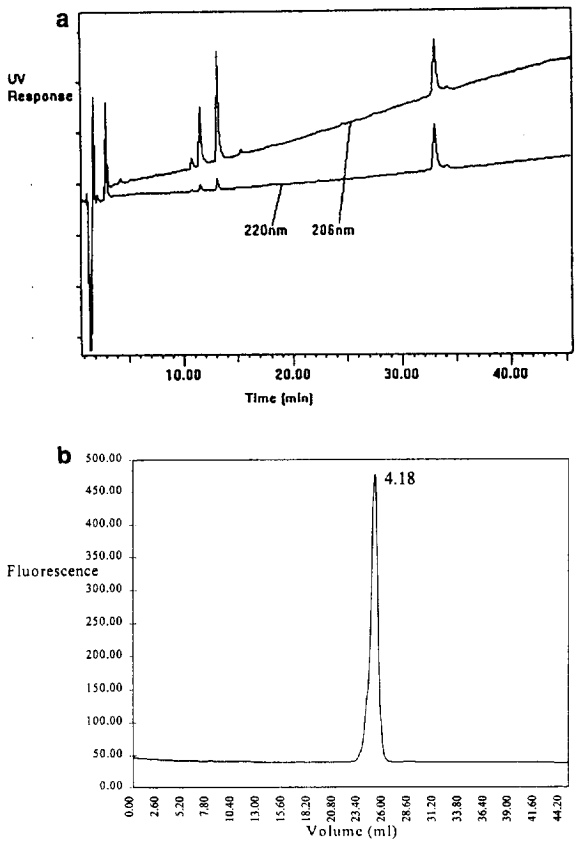

Fig. 3. PGC chromatography (a) and Biogel P4 chromatography (b) with UV and fluorescence detection, respectively, of chitotriose derivatised by reductive amination with 2-aminobenzamide.

Table 3
Comparison of the chromatography of native and 2-AB-derivatised oligosaccharides on BioGel P4 and PGC, the former run in water and the latter in solvent system PGCa

Oligosaccharide	Retention time	
	Biogel P4 (GU)	PGCa (min)
CT	6.0	11.5, 13.1
CT-ol	6.5	11
CT-2-AB	4.18	33
Asialo-Fetuin	13–18	
Asialo-Fetuin-2-AB	11.18–16.35	

4. Discussion

Sulphated oligosaccharides and components of glycosaminoglycans (GAGS) have previously been fractionated by strong anion-exchange HPLC or reversed-phase ion-pair HPLC in salt-based buffers [9–12]. We now show that these can be chromatographed in the absence of salt by PGC–HPLC in aqueous–TFA/acetonitrile–TFA gradient. The relatively early elution of the sulphated disaccharides (approximately 20% acetonitrile) means that larger or more heavily

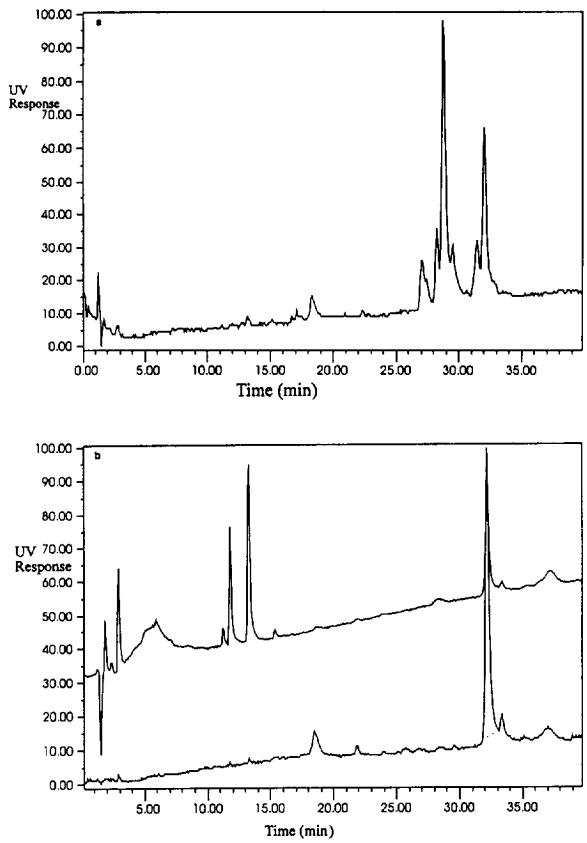

Fig. 4. PGC chromatography (a) and Biogel P4 chromatography (b) with UV and fluorescence detection, respectively, of oligosaccharides released from sialidase-treated bovine serum fetuin by PNGaseF digestion and derivatized by reductive amination with 2-aminobenzamide.

sulphated oligosaccharides should be resolvable by this method (either by increased acetonitrile or TFA concentrations). In contrast, HPAEC would appear to be the method of choice for the analysis of non-sulphated acidic monosaccharides, exhibiting greater retention and resolution than PGC–HPLC. Our results are in agreement with those of Blom et al. [22], with uronic acids being significantly more retained than NeuAc on HPAEC. However, the latter method requires that a split gradient is used to separate neutral and acidic species by HPAEC, whereas separation can be achieved in one gradient on PGC–HPLC. The increased sensitivity of HPAEC is also an advantage for monosaccharide analyses where desalting and further analysis is less of a priority.

Recently HPAEC has been used to profile sulphated oligosaccharide alditols from a cystic fibrosis patient [6], although the large salt concentrations required may hinder further analysis of minor oligosaccharides present. Chondroitin sulphate disaccharides have also been profiled by HPAEC, although at the high pH required for separation and detection the disaccharides are unstable due to peeling reactions [7], thus they must be reduced to alditols prior to analysis. Oligosaccharides prepared from hyaluronan analysed by HPAEC at pH 6.5 [8] showed minimal peeling, but at this pH pulsed amperometry is no longer a suitable detection method and UV proved unsuitable due to the high absorbance of acetate [8]. Reductive amination procedures have been used extensively to introduce chromophores for HPLC detection [16–19,23], with 2-aminopyridine (PA) being the most common [23]. More recently, a 2-dimensional (2D) sugar mapping technique has been developed for N-linked PA oligosaccharides on reversed-phase and normal-phase HPLC columns and capillary electrophoresis [24–27]. PGC-based chromatographies will prove to be a useful additional column for 2D sugar mapping and preparation of pure oligosaccharides, including the monitoring of derivatisation efficiency, with 2-AB labels for reagent array analysis method (RAAM) sequencing [21], mass spectrometry and NMR.

Acknowledgements

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References

[1] E.F. Hounsell, in C.K. Lim (Editor), HPLC of Small Molecules, IRL Press, Oxford, 1986.
[2] M.J. Davies, K.D. Smith, R.A. Carruthers, W. Chai, A.M. Lawson and E.F. Hounsell, J. Chromatogr., 646 (1993) 317–327.

[3] T. Hayase, M. Sheykhanazari, V.P. Bhavanandan, A.V. Savage and Y.C. Lee, Anal. Biochem., 211 (1993) 72–80.
[4] B.J. Campbell, M.J. Davies, J.M. Rhodes and E.F. Hounsell, J. Chromatogr., 622 (1993) 137–146.
[5] K.O. Lloyd and A. Savage, Glycoconjugate J., 8 (1991) 493–498.
[6] J.-M. Lo-Guidice, J.-M. Wieruszeski, J. Lemoine, A. Verbert, P. Rousel and G. Lamblin, J. Biol. Chem., 269 (1994) 18794–18813.
[7] S. Shibata, R.J. Midura and V.C. Hascall, J. Biol. Chem., 267 (1992) 6548–6555.
[8] S. Holmbeck and L. Lerner, Carbohydr. Res., 239 (1993) 239–244.
[9] P. Scudder, P.W. Tang, E.F. Hounsell, A.M. Lawson, H. Mehmet and T. Feizi, Eur. J. Biochem., 157 (1986) 365–373.
[10] W. Chai, E.F. Hounsell, C.J. Bauer and A.M. Lawson, Carbohydr. Res., 269 (1995) 139–156.
[11] R.J. Linhardt, K.G. Rice, Y.S. Kim, D.L. Lohse, H.-M. Wang and D. Loganathan, Biochem. J., 254 (1988) 781–787.
[12] R.J. Linhardt, K.N. Gu, D. Loganathan and S.R. Carter, Anal. Biochem., 181 (1989) 288–296.
[13] J. Kříž, E. Adamcova, J.H. Knox and J. Hora, J. Chromatogr. A, 663 (1994) 151–161.
[14] M.J. Davies, K.D. Smith, A.M. Harbin and E.F. Hounsell, J. Chromatogr., 609 (1992) 125–131.
[15] M. Stefansson and B. Lu, Chromatographia, 35 (1993) 61–66.
[16] N.O. Maness, E.T. Miranda and A.J. Mort, J. Chromatogr., 587 (1991) 177–183.
[17] R. Zhang, Z. Zhang, G. Liu, Y. Hidaka and Y. Shimonishi, J. Chromatogr., 646 (1993) 45–52.
[18] K.R. Anumula, Anal. Biochem., 220 (1994) 275–283.
[19] J.C. Bigge, T.P. Patel and R.B Parekh, Proc. 2nd International Glycobiology Symp., 1994.
[20] T.P. Patel, J.C. Bigge and T.B. Parekh, Proc. 2nd International Glycobiology Symp., 1994.
[21] C.J. Edge, T.W. Rademacher, M.R. Wormald, R.B. Parekh, T.D. Butters, D.R. Wing and R.A. Dwek, Proc. Natl. Acad. Sci. USA, 89 (1992) 6338–6342.
[22] H.J. Blom, H.C. Andersson, D.M. Krasnewich and W.A. Gahl, J. Chromatogr., 533 (1990) 11–21.
[23] S. Hase, T. Ibeki and T. Ikenaka, J. Biochem., 95 (1984) 197–203.
[24] N. Tomiya, Y.C. Lee, T. Yoshida, Y. Wada, J. Awaya, M. Kurono and N. Takahashi, Anal Biochem., 193 (1991) 90–100.
[25] N. Takahashi, Y. Wada, J. Awaya, M. Kurono and N. Tomiya, Anal. Biochem., 208 (1993) 96–109.
[26] S.Suzuki, K. Kakehi and S. Honda, Anal. Biochem., 205 (1992) 227–236.
[27] A. Kondo, M. Kiso, A. Hasegawa and I. Kato, Anal. Biochem., 219 (1994) 21–25.