ELSEVIER

Journal of Chromatography A, 814 (1998) 97–104

JOURNAL OF CHROMATOGRAPHY A

Temperature effects in high-performance anion-exchange chromatography of oligosaccharides

Eva Landberg*, Arne Lundblad, Peter Påhlsson

Department of Biomedicine and Surgery, Division of Clinical Chemistry, University Hospital, S-581 85 Linköping, Sweden

Received 11 February 1998; received in revised form 4 May 1998; accepted 4 May 1998

Abstract

High-performance anion-exchange chromatography (HPAEC) with pulsed amperometric detection has been widely used for analysis of mono-, oligo- and polysaccharides. Many factors that affect separation of carbohydrates by HPAEC have been evaluated, however effect of temperature has not been carefully studied. In the present study, neutral and sialylated oligosaccharides from human milk and different types of *N*-linked oligosaccharides were analysed by HPAEC at temperatures ranging from 13 to 30°C. *N*-Acetyl neuraminic acid, galacturonic acid and stachyose were also analysed since they have been used as internal standards when analysing various oligosaccharides by HPAEC. All oligosaccharides showed decreased retention times with increased temperature. Even small differences in temperature (i.e. ±5°) resulted in considerable changes in retention times. In addition, individual oligosaccharides showed different relative changes in retention time with increased temperature. By changing the temperature, a switch in elution order of individual oligosaccharides were sometimes found. These results show that retention times relative to an internal standard cannot be used for oligosaccharide identification unless temperature is carefully controlled. Regulation of temperature is also a valuable tool in achieving optimal separation of oligosaccharides by HPAEC. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Temperature effects; Oligosaccharides

1. Introduction

In the last decade, high-performance anion-exchange chromatography (HPAEC) has proven to be a useful tool for carbohydrate analysis. By this chromatographic method separation is accomplished for most carbohydrates. HPAEC has been used to analyse monosaccharides [1–4], sialic acids [5,6], milk oligosaccharides [3,7–10], *N*-linked oligosaccharides [11–15], O-linked oligosaccharides [3,16] and polysaccharides [17,18]. Coupling the chromatographic system to a pulsed amperometric detection (PAD) system gives a sensitivity in the nanomol range. The column is built up of small anion-exchange resin spheres ionically attached to larger cation-exchange resin spheres. The material has a high stability even at high pH, which is a prerequisite for separation of neutral oligosaccharides [19]. Carbohydrates are weak acids with respect to their hydroxyl groups, with pK_a values of 12–14. The highly alkaline conditions used in HPAEC makes it possible for some of these hydroxyl groups to be ionized and thus interact with the anion-exchange matrix.

The order of elution is in part dependent on the size of the oligosaccharides but also on substituents, linkage positions and branching [2,20–22]. Oligo-

*Corresponding author.

0021-9673/98/$19.00 © 1998 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(98)00381-1

saccharides with charged substituents will be retained more efficiently on the column. The separation is also affected by the accessibility of the oligosaccharide oxyanions to the functional groups of the stationary phase, which is in turn dependent on the three dimensional structure of the oligosaccharide. It is therefore not always easy to foresee the retention time of an individual oligosaccharide [23].

Factors affecting separation and retention times of different oligosaccharides by HPAEC are the type of eluent, the pH of the eluent, the pH gradient and counter ion gradient, flow-rate and temperature. Many of these factors have been evaluated, however the effect of temperature on HPAEC has not been studied thoroughly.

2. Experimental

2.1. Materials

Milk oligosaccharides were from BioCarb (Lund, Sweden) and stachyose was from Sigma (St. Louis, MO, USA). A fucosylated sialylated biantennary complex type standard (A2F) was purchased from Oxford Glycosciences (Abdingdon, UK). Desialylation of this compound was performed by mild acid hydrolysis in 2 *M* acetic acid at 80°C for 3 h [24] (yielding NA2F). A nonfucosylated biantennary *N*-glycan (NA2) was obtained from human transferrin (Sigma). *N*-Linked oligosaccharides were released by *N*-glycosidase F digestion, reduced and purified as previously described [25] and desialylated by mild acid hydrolysis. A high-mannose *N*-glycan standard ($Man_5GlcNAc_2$–$Man_9GlcNAc_2$) was from Oxford Glycosciences. A reduced high-mannose *N*-glycan standard ($Man_5GlcNAc_2$-ol–$Man_9GlcNAc_2$-ol) was obtained from ribonuclease B (Sigma). *N*-linked oligosaccharides were released by *N*-glycosidase F digestion, reduced and purified [25]. *N*-Acetyl neuraminic acid was from BioCarb. Structures of the standard oligosaccharides are presented in Table 1.

2.2. High-performance anion-exchange chromatography

The system used for HPAEC consisted of a gradient pump, PAD-2 detector with gold electrode, autosampler (AS3500), a CarboPac PA-100 guard column (50×4 mm) and a CarboPac PA-100 (250×4 mm) column from Dionex (Sunnyvale, CA, USA). The gradient programs used were as described in Table 2. Gradient 1 was used for separation of neutral milk oligosaccharides. Sialylated milk oligosaccharides were separated using gradient 2, and *N*-linked oligosacchaides using gradient 3.

Eluants were filtered and degassed before use and constantly held under helium pressure. For all analyses a flow-rate of 1.0 ml/min was used and 25 μl of sample was injected. Each run was directly preceeded by a similar run where water was injected. The column was thermostated by circulating water in a jacket. Temperatures ranging from 13–40°C were used.

2.3. Theory

The retention of a substance under isocratic conditions was expressed from the following expression:

$$k' = (V_r - V_0)/V_0 \quad (1)$$

where k' is the capacity factor, V_r is the retention volume of the analyte and V_0 the retention volume of an unretained molecule. The distribution of the analyte between the mobile phase and the stationary phase is determined by the standard free energy change ΔG°, associated with the transfer of the analyte from the mobile to the stationary phase. The corresponding equilibrium constant, *K*, is given by:

$$K = e^{-\Delta G^\circ/RT} \quad (2)$$

and is related to k':

$$k' = K\phi \quad (3)$$

where Φ is the volume ratio of the stationary phase and the mobile phase in the column, which is believed to be independent of temperature [26]. This gives:

$$\ln k' = -\Delta H^0/RT + \Delta S^0/R + \ln \phi \quad (4)$$

where ΔH^0 and ΔS^0 are the standard enthalpy and entropy changes for analyte transfer from the mobile phase to the stationary phase. When enthalpy and

Table 1

Peak	Trivial name (abbreviated)	Oligosaccharide structure
S	Stachyose	Galα(1-6)Galα(1-6)Glcα(1-2)βFru
G	GalU	Galacturonic acid
N	Neu5Ac	5-Ac-neuraminic acid
1	LNDI	Fucα(1-2)Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glc | Fucα(1-4)
2	3FL	Galβ(1-4)Glc | Fucα(1-3)
3	LNFIII	Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc | Fucα(1-3)
4	LNFII	Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glc | Fucα(1-4)
5	Lactose	Galβ(1-4)Glc
6	LNFI	Fucα(1-2)Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glc
7	LNnT	Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc
8	LNT	Galβ(1-3)GlcNAcβ(1-3)Galβ(1-4)Glc
9	LSTc	Neu5Acα(2-6)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)Glc
10	6SL	Neu5Acα(2-6)Galβ(1-4)Glc
11	3SL	Neu5Acα(2-3)Galβ(1-4)Glc
12	NA2	Galβ(1-4)GlcNAcβ(1-2)Manα(1-6) | Manβ(1-4)GlcNAcβ(1-4)GlcNAcβ-ol | Galβ(1-4)GlcNAcβ(1-2)Manα(1-3)
13	NA2F	Galβ(1-4)GlcNAcβ(1-2)Manα(1-6) | Manβ(1-4)GlcNAcβ(1-4)GlcNAcβ-ol | | Galβ(1-4)GlcNAcβ(1-2)Manα(1-3) Fucα(1-6)

entropy changes are temperature invariant, plots of ln k' versus $1/T$ (Van 't Hoff plots) are linear [27–29].

3. Results

3.1. Effect of temperature on HPAEC separation of neutral milk oligosaccharides

A mixture of neutral milk oligosaccharides were separated by HPAEC at different temperatures. At low temperature (13°C) sufficient separation was achieved for all oligosaccharides except for LNDI and 3FL (Fig. 1, peak 1 and 2 respectively, Table 1). As temperature was increased, separation of these two milk oligosaccharides was improved, however at higher temperature (25 and 30°C) 3FL coeluted with LNFIII instead. Stachyose was added to the oligosaccharide mixture since it has previously been used as an internal standard when analysing milk oligosaccharides by HPAEC [8]. Stachyose was separated from the other milk oligosaccharides at 13, 15, 25 and 30°C, but comigrated with LNnT at 20°C (Fig. 1, peaks S and 7 respectively, Table 1). Increasing the temperature from 15 to 25°C resulted in a shift of elution order of stachyose and LNnT. In general, retention time was decreased with increased temperature, however this effect was more pronounced for 3FL and stachyose. This experiment shows that small changes in temperature greatly affects retention times of milk oligosaccharides, but also that altering temperature can be useful to achieve separation.

Table 2
Different gradient programs for HPAEC

Program	Time (min)	0.2 *M* NaOH (%)	0.1 *M* NaOH/ 0.5 *M* NaAc (%)	Water (%)
1	0	15	0	85
	20	15	0	85
	34	50	0	50
	49	47	6	47
	56	30	40	30
	61	30	40	30
	65	15	0	85
	70	15	0	85
2	0	48	4	48
	8	48	4	48
	30	42	16	42
	55	30	40	30
	60	30	40	30
	65	48	4	48
	70	48	4	48
3	0	50	0	50
	70	30	40	30
	80	0	100	0
	85	50	0	50
	95	50	0	50

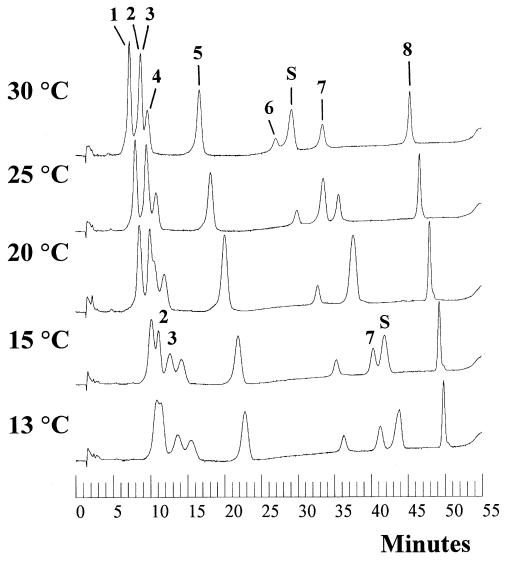

Fig. 1. Neutral milk oligosaccharides analysed by HPAEC–PAD at different temperatures. Gradient program 1 (Table 2) was used. The individual oligosaccharide structures are listed in Table 1.

Optimal separation of the milk oligosaccharides was accomplished at 15°C.

3.2. Effect of temperature on HPAEC separation of sialylated milk oligosaccharides

Sialylated milk oligosaccharides were also subjected to HPAEC-analysis at different temperatures (Fig. 2, Table 1). Galacturonic acid was added to the mixture since it has previously been used as an internal standard in the analysis of milk oligosaccharides [8]. At low temperature (13–15°C) there was poor separation of LSTc and 6SL, but separation was improved as temperature was increased. Galacturonic acid showed a small change in retention time in response to temperature differences compared to the sialylated milk oligosaccharides.

3.3. Effect of temperature on HPAEC of reduced and unreduced high mannose type oligosaccharides

A high mannose type *N*-glycan standard, consisting of five to nine mannose residues linked to the chitobiose core ($Man_5GlcNAc_2$–$Man_9GlcNAc_2$),

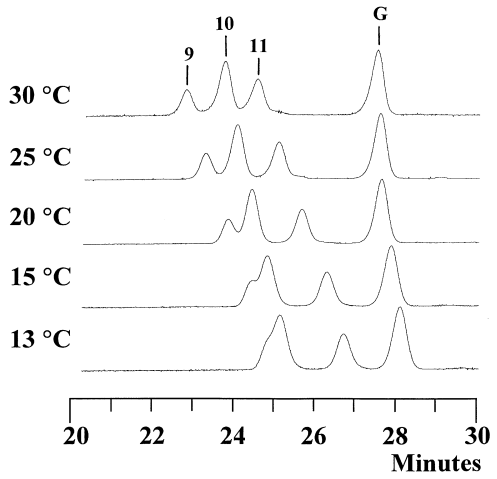

Fig. 2. Sialylated milk oligosaccharides analysed by HPAEC–PAD at different temperatures. Gradient program 2 (Table 2) was used. The individual oligosaccharide structures are listed in Table 1.

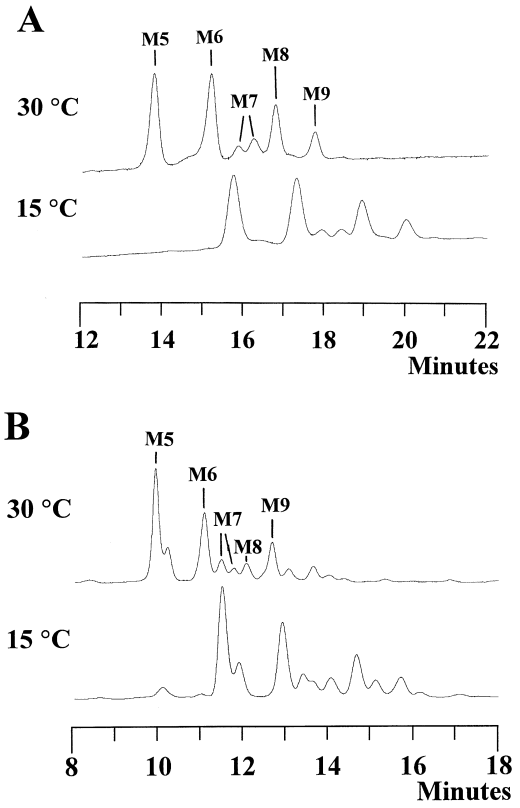

Fig. 3. *N*-Linked high mannose type oligosaccharides analysed by HPAEC–PAD at 15 and 30°C. Gradient program 3 (Table 2) was used. A: non reduced *N*-glycans $Man_5GlcNAc_2$–$Man_9GlcNAc_2$ (M5–M9). B: reduced *N*-glycans $Man_5GlcNAc_2$-ol–$Man_9GlcNAc_2$-ol (M5–M9).

was analysed at 15 and 30°C (Fig. 3A). When column temperature was increased from 15 to 30°C, retention time of all high mannose type oligosaccharides in the mixture decreased by approximately 2.0 min. A mixture of reduced high mannose oligosaccharides ($Man_5GlcNAc_2$-ol–$Man_9GlcNAc_2$-ol) was also analysed by HPAEC–PAD at different temperatures (Fig. 3B). The reduced oligosaccharides also showed decreased retention times with increasing temperature. However, temperature did not affect the smaller oligosaccharides with five and six mannose residues to the same degree. When temperature was increased from 15 to 30°C the retention time of these compounds only decreased 1.5 and 1.8 min respectively, compared to a decrease of 2.0 min for $Man_7GlcNAc_2$ to $Man_9GlcNAc_2$ (Fig. 3).

3.4. Effect of temperature on HPAEC of complex type oligosaccharides

Two reduced complex type *N*-glycans (NA2 and NA2F, Table 1), only differing in the internal fucose substitution, were analysed together with Neu5Ac. The nonfucosylated biantennary *N*-glycan (NA2) showed the largest change in retention time when subjected to different column temperatures during HPAEC (Fig. 4). When temperature was increased from 15 to 30°C the retention time of NA2 decreased 2.6 min, whereas the retention time of NA2F only decreased 2.0 min. Retention time of Neu5Ac only changed 1.2 min, when temperature was increased from 15 to 30°C (Fig. 4).

3.5. Temperature and pH dependence of HPAEC of oligosaccharides

There is a reverse relationship between temperature and pH of a solution. Thus, an increase in temperature will lower the pH of a NaOH solution. To analyse whether an increase in NaOH concentration could generate the same effects on retention times of oligosaccharides as a decrease in temperature, a mixture of lactose, LNFI, stachyose and

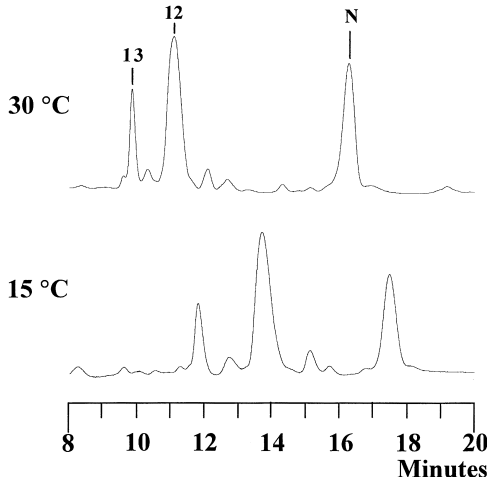

Fig. 4. *N*-Linked complex type oligosaccharides analysed by HPAEC–PAD at different temperatures. Gradient program 3 (Table 2) was used. The individual oligosaccharide structures are listed in Table 1.

LNnT was analysed by HPAEC at a fixed temperature with different NaOH concentrations or at a fixed NaOH concentration at different temperatures (Fig. 5A and B). An increased NaOH concentration resulted in decreased retention times, whereas a decrease in temperature resulted in increased retention times of the oligosaccharides (Fig. 5). An increase in NaOH concentration will increase the ionic strength of the solution, which explains the decreased retention times. This experiment clearly shows that the effects of temperature on oligosaccharide chromatography cannot be achieved only by changing the NaOH concentration of the eluant.

Increased temperature usually results in decreased retention times for most analytes in ion-exchange chromatography [34]. A strict temperature dependence according to Eq. (4) would predict that a plot of ln k' versus $1/T$ (Van 't Hoff plot) of a substance generates a linear relationship. Van 't Hoff plots of lactose, LNFI, stachyose and LNnT analysed by HPAEC at a fixed NaOH concentration at different temperatures were found to be linear for all oligosaccharides with a strong dependence of k' on temperature (Fig. 6). This further indicates that the effects on retention times are primarily an effect of temperature and not of other associated changes in the system.

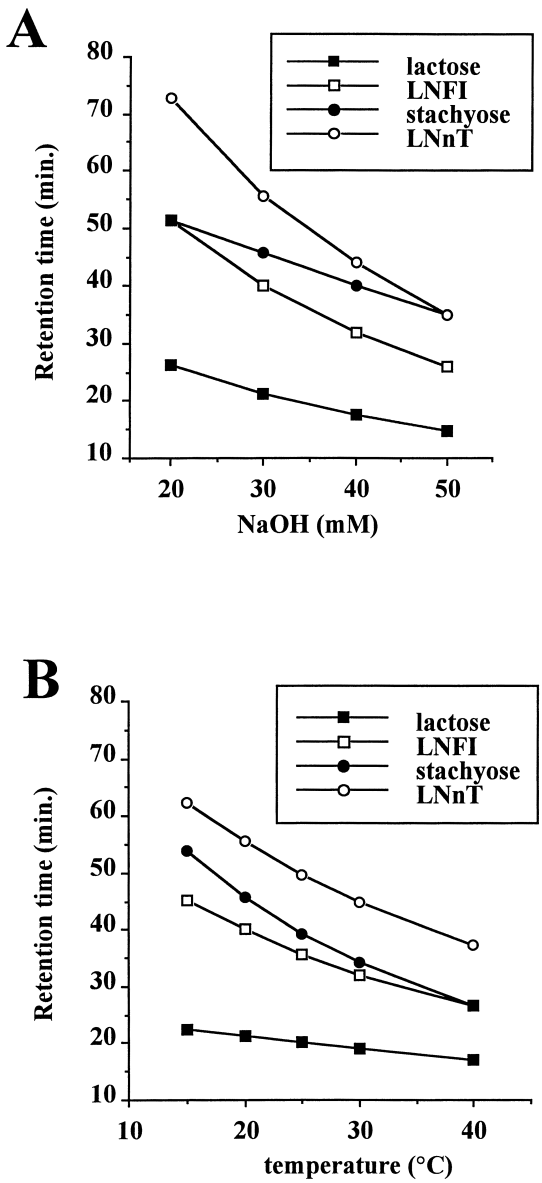

Fig. 5. Effect on retention time on HPAEC with varying NaOH concentration and temperature. Lactose, LNFI, stachyose and LNnT were analysed. Isocratic eluton with varying concentrations of NaOH (20–50 m*M*) was used with a fixed temperature of 20°C (A). In (B) temperature was varied between 15 and 40°C with a fixed NaOH concentration of 30 m*M*.

4. Discussion

In 1983 Rocklin and Pohl described temperature effects on retention times of some mono-, di- and

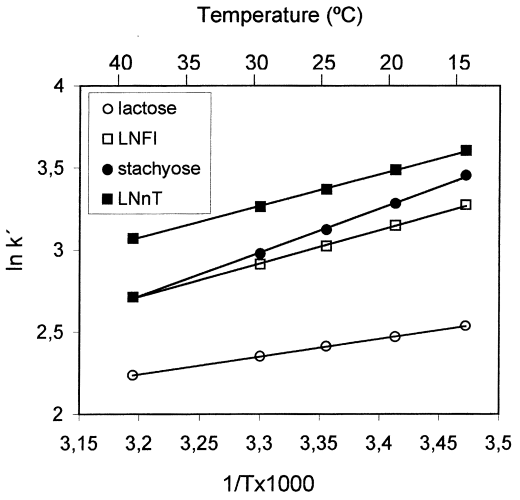

Fig. 6. Van 't Hoff plot of ln k' versus $1/T$ for the oligosaccharides: lactose, LNFI, stachyose and LNnT. Isocratic elution with 30 mM NaOH was used.

tetrasaccharides on HPAEC using a Dionex system equipped with a CarboPac PA-1 column [30]. Retention time of the glycans which were studied generally decreased as temperature was increased. They also reported that there was a shift in elution order of stachyose and maltose when temperature was increased. Since then no studies have been focused on the temperature dependence of HPAEC of carbohydrates. In most studies, analyses of carbohydrates by HPAEC have been performed at ambient temperature.

In the present study, milk oligosaccharides and several *N*-linked oligosaccharides have been analysed by HPAEC using different temperatures. All of the analysed oligosaccharides showed decreased retention time with increased temperature, but the relative change in retention time differed for individual oligosaccharides. It was found that even small differences in temperature (±5°) caused dramatic changes in oligosaccharide elution. Thus, the use of ambient temperature is not satisfactory for HPAEC of oligosaccharides, especially not when retention times are used to verify oligosaccharide structures.

The high sensitivity and resolution of oligosaccharides analysed by HPAEC has prompted several researches to design standard chromatographic conditions for identification of individual oligosaccharides, using a defined gradient program [2,3,7–9,15,23,31,32]. To account for variations in retention times between runs, relative retention time to an internal standard has been suggested to give reliable results [21,33].

In this study, we included three different compounds that all have been used as internal standards. Galacturonic acid and stachyose have earlier been used to quantify milk oligosaccharides [8] and *N*-acetyl neuraminic acid have been used as an internal standard when analysing *N*-linked oligosaccharides [29]. All of these standards showed a marked different response to temperature changes compared to the oligosaccharides which were analysed. Thus, using an internal standard to correct retention times is of no value unless temperature is carefully controlled. On the other hand, when temperature is controlled, retention times of oligosaccharides on HPAEC will be highly reproducible. This study also shows that slightly raising or lowering column temperature can improve separation of oligosaccharides by HPAEC. Temperature is thus an important parameter to be considered for optimizing separation of neutral, sialylated, reduced or non-reduced oligosaccharides. However, temperatures exceeding 45°C are not recommended when analysing oligosaccharides by HPAEC, as degradation and epimerisation then will be an increased problem [30].

An increased temperature generally leads to decreased retention times, which results in reduced k' for all analytes [34]. Van 't Hoff plots of a number of oligosaccharides in this study generated linear relationships, indicating that the enthalpy and entropy changes of each oligosaccharide are invariant of temperature. Thus the effects of temperature on oligosaccharide retention times are primarily due to direct temperature dependence of k'.The slope of the curve in the Van 't Hoff plot thus represents ΔH^0 for individual oligosaccharides (according to Eq. (4)). The differences in ΔH^0 may depend on different three-dimensional structures of the oligosaccharides, which will, together with net charge, affect interactions with the stationary phase.

Temperature is inversely correlated with the pH of a NaOH solution, but we did not find an inverse relationship between changes in retention times for NaOH concentration and temperature. Thus NaOH concentration and temperature will independently

affect the separation of oligosaccharides and both factors must be considered when optimizing the chromatographic conditions.

The anomeric hydroxyl group of carbohydrates is more acidic than the others and therefore interacts more strongly with the anion-exchange matrix. Reduced oligosaccharides will therefore elute earlier than their unreduced counterparts when analysed by HPAEC [2]. This study shows that the temperature effect on retention times of reduced high mannose type *N*-glycans are of the same magnitude as for unreduced oligosaccharides. Thus, the temperature effect on retention times is not greatly affected by removal of interactions caused by the acidic anomeric group. This indicates that the altered k' for a reduced compared to an unreduced oligosaccharide is mainly caused by a change in ΔS^0, rather than ΔH^0.

This study shows the importance of controlling temperature during HPAEC of oligosaccharides, both to accomplish highly reproducible retention times, and to achieve optimal separation of different neutral and sialylated oligosaccharides.

Acknowledgements

We wish to thank Anna-Kristina Granath for excellent technical assistance and Sten Ohlson for critically reading the manuscript. This work was aided by grants from the Swedish Medical Research Council (13X-2) and the County of Östergötland.

References

[1] M.R. Hardy, Methods Enzymol. 179 (1989) 76.
[2] Y.C. Lee, Anal. Biochem. 189 (1990) 151.
[3] G.P. Reddy, C.A. Bush, Anal. Biochem. 198 (1991) 278.
[4] M. Weitzhandler, M. Hardy, J. Chromatogr. 510 (1990) 225.
[5] A.E. Manzi, S. Diaz, A. Varki, Anal. Biochem. 188 (1990) 20.
[6] Y. Zhang, Y. Inoue, S. Inoue, Y.C. Lee, Anal. Biochem. 250 (1997) 245.
[7] C. Kunz, S. Rudloff, A. Hintelmann, G. Pohlentz, H. Egge, J. Chromatogr. B 685 (1996) 211.
[8] S. Thurl, B. Muller-Werner, G. Sawatzki, Anal. Biochem. 235 (1996) 202.
[9] R.R. Townsend, M.R. Hardy, O. Hindsgaul, Y.C. Lee, Anal. Biochem. 174 (1988) 459.
[10] W.T. Wang, Carbohydr. Res. 189 (1989) 1–11.
[11] L.J. Basa, M.W. Spellman, J. Chromatogr. 499 (1990) 205.
[12] G.A. Cooper, J.S. Rohrer, Anal. Biochem. 226 (1995) 182.
[13] G. Pfeiffer, H. Geyer, R. Geyer, I. Kalsner, P. Wendorf, Biomed. Chromatogr. 4 (1990) 193.
[14] J. Rohrer, R.R. Townsend, Glycobiology 5 (1995) 391.
[15] R.R. Townsend, M.R. Hardy, D.A. Cumming, J.P. Carver, B. Bendiak, Anal. Biochem. 182 (1989) 1.
[16] T. Hayase, M. Sheykhanazari, V.P. Bhavanandan, A. Savage, Y.C. Lee, Anal. Biochem. 211 (1993) 72.
[17] K. Koizumi, Y. Kubota, T. Tanimoto, Y. Okada, J. Chromatogr. 464 (1989) 365.
[18] J. van Loo, P. Coussement, L. de Leenheer, H. Hoebregs, G. Smits, Critical Rev. Food Nutr. 35 (1995) 525.
[19] Technical Note 20, Dionex, Sunnyvale, CA, 1993.
[20] M.R. Hardy, R.R. Townsend, Methods Enzymol. 230 (1994) 208.
[21] Y.C. Lee, J. Chromatogr. A 720 (1996) 137.
[22] R.R. Townsend, M.R. Hardy, Glycobiology 1 (1991) 139.
[23] M.R. Hardy, R.R. Townsend, Proc. Natl. Acad. Sci. 85 (1988) 3289.
[24] A. Varki, S. Diaz, Anal. Biochem. 137 (1984) 236.
[25] E. Landberg, P. Påhlsson, A. Lundblad, A. Arnetorp, J.-O. Jeppsson, Biochem. Biophys. Res. Commun. 210 (1995) 267.
[26] W.D. Haidacher, A. Vailaya, C. Horvath, Proc. Natl. Acad. Sci. USA 93 (1996) 2290.
[27] U.R. Melander, B. Chen, C. Horvath, J. Chromatogr. 185 (1979) 99.
[28] V.J. Roush, D.S. Gill, R.C. Willson, J. Chromatogr. A 653 (1993) 207.
[29] T. Takeuchi, M. Kumaki, D. Ishii, J. Chromatogr. 235 (1982) 309.
[30] R.D. Rocklin, C.A. Pohl, J. Liq. Chromatogr. 6 (1983) 1577.
[31] R.R. Townsend, M.R. Hardy, Y.C. Lee, Methods Enzymol. 179 (1989) 65.
[32] E. Watson, A. Bhide, W.C. Kenney, F.-K. Lin, Anal. Biochem. 205 (1992) 90.
[33] P. Hermentin, R. Witzel, J.F.G. Vliegenhart, J.P. Kamerling, M. Nimtz, H.S. Conradt, Anal. Biochem. 203 (1992) 281.
[34] L. R. Snyder, J. J. Kirkland, Introduction to Modern Liquid Chromatography, John Wiley, New York, 1979.