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Solvent effects on the retention of oligosaccharides in porous graphitic carbon liquid chromatography

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ABSTRACT

Porous graphitic carbon (PGC) is known as well suited adsorbent for liquid chromatography of carbohydrates. In this work we report on systematic investigations of solvent effects on the retention mechanism of fluorescence labeled malto-oligosaccharides on PGC. The adsorption mechanism was found to depend on the type of organic modifier used in the mobile phase. Positive adsorption enthalpies and entropies, which have already been reported in the literature, were solely produced using acetonitrile. Both alternative solvents (tetrahydrofuran, 2-propanol) yielded in contrast negative enthalpies. As plausible retention mechanism for oligosaccharides on PGC applying acetonitrile as mobile phase component we propose the formation of a dense and highly ordered solvation layer of the PGC surface with the linear acetonitrile molecules. Adsorption of analyte molecules requires a displacement of numerous acetonitrile molecules, which explains the positive enthalpy and entropy values measured. The interplay of enthalpic and entropic contributions to the overall adsorption phenomena results in strongly temperature dependent chromatographic selectivity values.

1. Introduction

Porous graphitic carbon (PGC) was introduced in the 1980s as a novel stationary phase for liquid chromatography. Aside its advantageous physical and chemical properties it also exhibits increased selectivities for methylene groups of aromatic hydrocarbons compared to conventional reversed phases [1]. Further investigations with substituted aromatic hydrocarbons showed additional interactions of induced dipoles on the PGC surface with polar moieties causing an increase of retention [2]. This effect was termed "polar retention effect on graphite" (PREG). Exploiting this phenomenon, separations of very polar analytes, even of inorganic ions can be accomplished on this material [3]. Hence, PGC proved especially useful for analysis of very polar molecules, which are not, or only poorly retained in reversed-phase chromatography, e.g. oligosaccharides [4]. These groups otherwise represent challenges for separation sciences.

Native oligosaccharides are not retained in reversed-phase chromatography. Even when derivatized with a fluorescent dye retention is quite low limiting the fraction of organic solvents in the mobile phase below ten percent. Such a low organic content compromises sensitivity of detection by mass spectrometry, which is often necessary to identify analytes. Alternative chromatography methods for oligosaccharide analysis, e.g. high-pH anion exchange chromatography (HPAEC) or hydrophilic interaction liquid chromatography (HILIC), usually exhibit low selectivities for isomers, while PGC has demonstrated to offer remarkable selectivities for isomeric oligosaccharides, e.g. for complex protein glycans [5–9].

Despite its frequent use in this field the retention mechanism of carbohydrates on PGC is not fully understood. General aspects of the retention properties of PGC were reviewed recently [10,11] discussing hydrophobic interactions as well as the polar retention effect. Another review focusing on oligosaccharide analysis on PGC briefly summarizes findings concerning the retention principle for oligosaccharides [4]. But due to the limited data available the parameters determining the retention properties of free and fluorescence labeled oligosaccharides are still unclear. Very recently a paper was published investigating the retention properties of PGC for certain linear oligosaccharides derived from cell wall polysaccharides [12], demonstrating the influence of monosaccharide composition, charges and acetylation of hydroxide groups on the retention.

An interesting observation in PGC chromatography is the increase of retention of oligosaccharides with higher temperature [13], this is in contrast to results derived for other analytes, as e.g. for aromatic hydrocarbons [14]. A study on glucosaminoglycan disaccharides reported positive and negative adsorption enthalpies depending on the pH [15].

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Herein we report on investigations of solvent effects on the retention of fluorescently labeled malto-oligosaccharides (maltotriose – Mal3, maltotetraose – Mal4, maltopentaose – Mal5, maltohexaose – Mal6, maltoheptaose – Mal7) on PGC. These compounds were selected as simple model system consisting of homogeneous, linear chains containing analytes composed of only glucose units as building blocks.

The analytes are labeled with 2-aminobenzamide (2AB) by reductive amination for sensitive detection by fluorescence, which is a common strategy for the analysis of protein glycans [16]. The interaction of PGC with the oligosaccharides in different organic modifiers (acetonitrile – MeCN, propionitrile – EtCN, 2-propanol – 2-POH, tetrahydrofuran – THF) is specified by van't-Hoff plots. Hence, the logarithm of the retention factor k is plotted against the inverse temperature ($1/T$). The slopes of these plots correspond to $-\Delta H/R$ and the ordinate intercepts correspond to $\Delta S/R - \ln(\beta)$. Hence, if the phase ratio β is known, van't-Hoff plots yield information about enthalpic and entropic contributions to the free energy of adsorption of the analytes on PGC. The phase ratio of Hypercarb® was reported in literature [17].

2. Experimental

2.1. Chemicals and reagents

All chemicals used were analytical grade or better. Solvents used for chromatography were at least HPLC grade. Malto-oligosaccharides, 2-aminobenzamide (2AB), acetic acid, tetrahydrofuran (THF), dimethyl sulfoxide (DMSO), $NaBH_4$, $Na[BH_3CN]$ and formic acid were ordered from Sigma–Aldrich (Vienna, Austria). Acetonitrile (MeCN), propionitrile (EtCN) and 2-propanol (2-POH) were from Merck (Darmstadt, Germany). Ammonia solution was from AppliChem (Darmstadt, Germany). Water was prepared by a Milli-Q® system (Millipore MA, USA). PD MiniTrap™ G10 columns were ordered from GE Healthcare (Vienna, Austria).

2.2. Sample preparation

Oligosaccharides were derivatized with 2-aminobenzamide (2AB) to enable sensitive fluorescence detection. The labeling solution consisted of 50 mg/mL 2AB and 63 mg/mL $Na[BH_3(CN)]$ in DMSO/acetic acid at a ratio of 7:3. 15 μL of this solution were added to 9 μL of a standard solution of malto-oligosaccharide (1 mg/mL). The mixture was incubated at 37 °C over night. Excess label was depleted by application to PD MiniTrap™ G10 gel filtration columns.

For the control experiment employing non-labeled oligosaccharide alditols the corresponding standards were reduced by a 1% $NaBH_4$ solution at room temperature for 4 h. Excess reagent was neutralized with acetic acid. Reduced malto-oligosaccharide alditols are referred to as Mal3 (maltotriose), Mal4 (maltotetraose), Mal5 (maltopentaose), Mal6 (maltohexaose) and Mal7 (maltoheptaose).

2.3. Instrumentation

Fluorescence chromatograms were recorded on an Agilent 1200 SL system, consisting of a binary pump, a vacuum degasser, an autosampler, a column thermostat and a fluorescence detector. Non-fluorescent labeled oligosaccharide alditols were chromatographed on an Agilent 1100 system with an analogous setup, but with a corona charged aerosol detector (CAD).

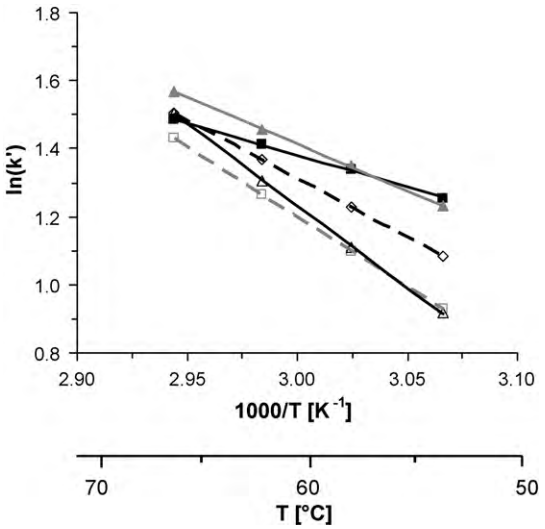

Fig. 1. Van't-Hoff plots of 2AB-labeled malto-oligosaccharides (maltotriose ■, maltotetraose ▲, maltopentaose ◊, maltohexaose □ and maltoheptaose △) in 35% acetonitrile. The second abscissa shows the corresponding temperature.

2.4. Chromatographic conditions

The retention study experiments were conducted on PGC columns (Hypercarb®, Thermo Electron Corporation). For experiments with EtCN a 100 mm × 3 mm (i.d.) column with 3 μm particles was used. All other experiments were performed on a 150 mm × 4.6 mm (i.d.) column with 5 μm particles.

Mobile phase A was 50 mM ammonium formate at pH 3.8. Mobile phase B additionally contained the respective organic modifier. The fraction of organic solvent was adjusted to achieve adequate retention times for all analytes in the studied temperature range. For 2AB-labeled malto-oligosaccharides a fraction of 35% MeCN, 17.5% 2-POH, 10% THF and 9.5% EtCN were applied, respectively. Since EtCN is only slightly soluble in water, 0.1% HCOOH was added to the mobile phases instead of a buffer to avoid separation of aqueous and organic phase in this case.

The temperature of the column was measured using an external thermometer with an accuracy of 0.1 °C.

2AB-labeled oligosaccharides were detected by fluorescence spectrometry with excitation at 250 nm and an emission wave length of 428 nm.

3. Results and discussion

3.1. Acetonitrile as organic solvent

Typically, MeCN is used as organic modifier in the running buffer for PGC chromatography of free oligosaccharides [4,5,18–20]. Other solvents are only rarely applied [8,21]. Methanol being polar protic, exhibits a low elution strength and is therefore not suitable for higher or charged oligosaccharides. Higher alcohols act as stronger eluents, so 2-POH is occasionally utilized, sometimes mixed with MeCN to reduce the viscosity [8,22]. THF, being polar non-protic, is described as a very strong eluent on PGC and is therefore employed as an alternative solvent in this study.

Applying MeCN as organic modifier the slopes of the van't-Hoff plots of the labeled oligosaccharides are negative, corresponding to positive adsorption enthalpies, as illustrated in Fig. 1. The linearity of the plots was excellent ($R^2 > 0.9995$) in the investigated temperature range. Usually adsorption enthalpies in HPLC are negative, because retention is driven by a gain of energy. But using MeCN as component in a hydro-organic mobile phase positive adsorp-

Table 1
Adsorption enthalpies and entropies of 2AB-labeled malto-oligosaccharides applying different mobile phases. Values and confidence intervals (95%) are given.

	Solvent	Mal3	Mal4	Mal5	Mal6	Mal7
ΔH (kJ/mol)	35% MeCN	15.7 ± 0.882	22.8 ± 0.581	28.6 ± 0.405	34.2 ± 1.42	40.1 ± 2.32
	17.5% 2-POH	−21.0 ± 4.27	−20.1 ± 4.85	−19.6 ± 5.14	−20.0 ± 5.03	−19.9 ± 5.70
	10% THF	−35.0 ± 9.53	−36.4 ± 9.76	−37.3 ± 9.01	−38.7 ± 8.50	−41.3 ± 7.62
	20% MeCN/5% THF[a]	−4.10 ± 2.62	−2.58 ± 3.32	−1.97 ± 3.34	−2.00 ± 3.86	−2.57 ± 5.01
	9.5% EtCN[a]	1.78 ± 5.26	2.58 ± 6.16	3.13 ± 3.70	3.42 ± 4.33	3.40 ± 3.38
ΔS (J/mol K)	35% MeCN	64.1 ± 2.65	85.9 ± 1.75	102 ± 1.22	118 ± 4.28	136 ± 6.99
	17.5% 2-POH	−47.5 ± 13.0	−45.1 ± 14.7	−44.5 ± 15.6	−46.1 ± 15.3	−45.7 ± 17.3
	10% THF	−88.7 ± 28.7	−93.5 ± 29.3	−97.6 ± 27.1	−102 ± 25.6	−110 ± 22.9
	20% MeCN/5% THF[a]	4.22 ± 8.43	8.70 ± 10.7	9.80 ± 10.8	9.12 ± 12.4	7.88 ± 16.1
	9.5% EtCN[a]	20.3 ± 16.5	22.3 ± 19.3	22.6 ± 11.6	22.3 ± 13.6	22.0 ± 10.6

[a] Applied model is not significant.

tion enthalpies have been reported [13], which is affirmed by our results (Table 1). Additional glucose units cause a steady increase of the adsorption enthalpy for this series of analytes. The highest enthalpy value measured (Mal7) was 156% higher than the value corresponding to Mal3.

Since the adsorption of the labeled oligosaccharides is endothermic the retention is driven by an increase of the entropy of the system. Similar to the enthalpy values, the gain of entropy increases with the number of glucose units of the analyte, as shown in Table 1. Thus Mal7 exhibits a 105% higher adsorption entropy than Mal3.

An additional glucose unit results in both, an increase of the adsorption enthalpy, represented by a steeper graph in the van't-Hoff plot, and an increased adsorption entropy, represented by a larger intercept of the ordinate. Hence, selectivities and even elution orders of oligosaccharides may highly depend on the column temperature, as illustrated by the van't-Hoff plots in Fig. 1.

Concluding from the van't-Hoff data, we propose that the virtually linear MeCN molecules form an ordered layer on the PGC surface. Adsorption of big analyte molecules, e.g. an oligosaccharide, premises the displacement of a high number of MeCN molecules requiring an accordingly high amount of energy, which significantly exceeds the energy gain by the interaction of the labeled carbohydrate with PGC. Thus, the increase of entropy generated by desorbed MeCN molecules causes negative changes of free enthalpy, and thus retention. This hypothesis explains the positive adsorption enthalpies and entropy values for oligosaccharides on the MeCN–PGC system, whereas aromatic small molecules, as used in several studies, do not comply with this model.

3.2. Comparison to alternative organic solvents

A comparison of MeCN with THF, as well as with the protic solvent 2-POH revealed that these two alternative solvents exhibit similar properties but with clear differences to MeCN (Table 1). Adsorption enthalpies and entropies were negative and the differences in the thermodynamic data of individual analytes are comparably small for these solvents. Since for the organic solvents different fractions were applied the tabled values are not directly comparable between the mobile phases.

In 2-POH variations both enthalpy and entropy values between the malto-oligosaccharides were insignificant, without noticeable trend. The graphs exhibited reasonable linearity ($R^2 > 0.991$). Adsorption is driven by a gain of energy, which is almost independent from the oligosaccharide moiety. We therefore assume that the interaction of the label with the PGC surface dominates the retention of the analytes, while the carbohydrate moiety does not significantly contribute to the overall observed retention characteristics.

In THF the van't-Hoff plots again offered good linearity ($R^2 > 0.992$). According to the data presented in Table 1 tendencies may be assumed within the analyte series, even though the differences in the observed values are not significant. The (negative) values for the adsorption enthalpies and entropies decrease with every elongation of the oligosaccharide chain, as shown in Table 1. The measured adsorption enthalpy of the smallest (Mal3) oligosaccharide is 15% higher than the enthalpy of the biggest oligosaccharide (Mal7), while the measured adsorption entropy is 19% higher. This means although THF is an even stronger eluent than 2-POH, the oligosaccharide moieties weakly interact with the stationary phase and thereby contribute to the retention by increasing the gain of enthalpy of adsorption.

The impact of the column temperature on the selectivity for Mal3 and Mal7 in MeCN, POH and THF is illustrated in Fig. 2. Because of the small differences in adsorption enthalpies observed using 2-POH and THF, respectively, the selectivity is only marginally impacted by the column temperature. By contrast, in MeCN the adsorption enthalpy of Mal7 is more than 150% higher than the enthalpy of Mal3, resulting in changes of the elution orders within the studied temperature range. Significant peak tailing was observed at low temperatures using 2-POH, as well as at high temperatures using MeCN. In both cases the tailing was more pronounced for higher oligosaccharides. The reason for these phenomena are not clear and need further detailed investigation.

3.3. Control experiments

Additional experiment series were carried out to challenge the developed retention model. Firstly, a mixture of MeCN and THF (4:1) was used as organic solvent and the adsorption enthalpies and entropies of the malto-oligosaccharides were determined. According to our retention model the small fraction of THF disturbs the ordered structure of MeCN in proximity to the surface due to its high affinity towards PGC. Thus, significant decreases in enthalpy and entropy values are expected compared to pure MeCN.

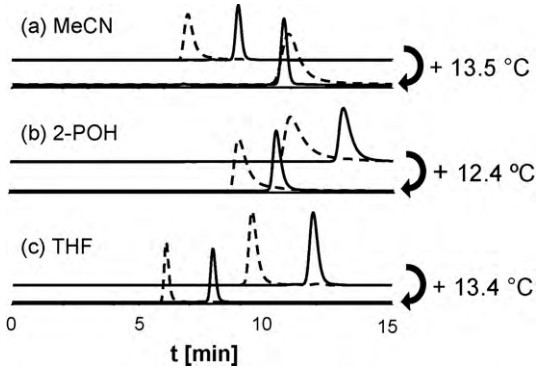

Fig. 2. Overlaid chromatograms of 2AB-labeled maltotriose (solid line) and maltoheptaose (dashed line) at the highest and the lowest temperature of the van't-Hoff plots, respectively. The organic modifiers in the mobile phase were (a) acetonitrile (T_{min} = 53.0 °C, T_{max} = 66.5 °C), (b) 2-propanol (T_{min} = 50.2 °C, T_{max} = 62.6 °C) and (c) tetrahydrofuran (T_{min} = 52.8 °C, T_{max} = 66.2 °C).

Table 2
Adsorption enthalpy and entropy values of reduced malto-oligosaccharides (alditols) on PGC applying 15% acetonitrile in the running buffer.

	Mal3	Mal4	Mal5	Mal6	Mal7
ΔH (kJ/mol)	6.96 ± 1.04	16.2 ± 1.31	23.6 ± 1.88	29.4 ± 2.57	34.0 ± 3.07
ΔS (J/mol K)	17.5 ± 3.09	49.4 ± 3.90	75.8 ± 5.60	97.0 ± 7.65	113 ± 9.16

The van't-Hoff plots exhibited poor linearity for the higher oligosaccharides with R^2-values between 0.897 for Mal3 and 0.337 for Mal7 indicating an alteration of the retention mechanism with the temperature. Hence in this case the linear model for the van't-Hoff plots is not significant. The gained entropy and enthalpy values can only be considered approximations of the average values within the corresponding temperature ranges. This finding may be explained by the displacement of MeCN with THF molecules resulting in a different chemical environment at the PGC surface depending on the temperature. However, the conformational flexibility of the analyte with temperature may also play a role as it is directly connected to the exposure of the hydrophobicity related surface area of the oligomeric analytes. The measured enthalpy values were negative, while adsorption entropies were significantly reduced, but still in the positive range (Table 1).

In another experiment EtCN instead of MeCN was applied as the organic modifier in the aqueous mobile phase. As a homologue of MeCN it should possess similar chemical properties, but the shape of the molecule is not linear anymore. Therefore adsorbed EtCN molecules may not form such densely packed structured layers on the PGC surface as MeCN. Analogous to the MeCN/THF mixture, adsorption enthalpies and entropies were significantly reduced for the EtCN system compared to the MeCN system (Table 1).

Again the plots generally are not in accordance to a linear model indicating an alteration of the retention mechanism in the studied temperature range. Furthermore the temperature dependencies of the malto-oligosaccharides were low. Both of these facts result in low correlation factors for the van't-Hoff plots (R^2 between 0.180 and 0.661).

Enthalpies do not increase with the glucose units and entropies are also approximately constant for all tested analytes. This may be due to decreased interactions between the carbohydrate moiety and the PGC surface.

As clearly demonstrated by these data, the adsorption mechanism of oligosaccharides on PGC strongly depends on the nature of the organic solvent and thus of the "solvated" PGC surface. Neither THF nor 2-POH produced positive adsorption enthalpies. Therefore, we assume that positive enthalpy and entropy values are specific for MeCN. The properties of EtCN lie between MeCN and THF or 2-POH, which also complies with the proposed retention model.

Experiments with (non-labeled) oligosaccharide alditols affirmed that the effects were not caused by the fluorescence label, as these compounds also exhibit positive values and a steady increase by the elongation of the glucose chain using MeCN (Table 2). Furthermore these data demonstrate that ordered layers are also formed at MeCN fractions in the range of 15%. Anyhow, the aromatic moiety of the labeled oligosaccharides was found to interact strongly with the PGC surface, as the organic fraction has to be more than doubled to achieve elution of the labeled oligosaccharides. Relative differences between the oligosaccharide alditols were found to exceed those of the labeled derivatives indicating strong interactions between the label and the stationary phase causing a retention increment.

4. Conclusion

For the analysis of oligosaccharides by PGC-HPLC acetonitrile (MeCN) is typically applied as component of a hydro-organic mobile phase. Compared to 2-propanol (2-POH) and tetrahydrofuran (THF), which are described in literature as alternative solvents with sufficient elution strength, MeCN offers unique properties as organic solvent. According to the developed retention model the virtually linearly shaped MeCN molecules form in the first place a dense and highly ordered layer on the PGC surface representing thus its "solvation" status. According to this model analyte molecules adsorbing on the surface displace a number of these solvent molecules, which necessitates a high amount of energy, proportional to the interaction area. This explains the positive enthalpy values measured as well as the increments of adsorption enthalpy with every glucose unit. The liberated MeCN molecules increase the entropy of the system, resulting in negative free energy values and therefore strong retention of these highly polar compounds.

Addition of a stronger eluent disturbs the structured arrangement of MeCN molecules, reducing the contribution of the entropy to the adsorption. A similar effect was observed when replacing MeCN with EtCN. Even though EtCN possesses similar chemical properties as MeCN, its carbon chain is not linear, which may somewhat hinder the formation of very ordered layers. Entropy and enthalpy values are lower than for MeCN, but entropy significantly contributes to adsorption.

The aromatic fluorescence label significantly increases the overall observed retention, as demonstrated by the comparison of 2AB-labeled malto-oligosaccharides with the corresponding reduced oligosaccharides. For the elution of the alditols a fraction of 15% MeCN was found sufficient, whereas labeled oligosaccharides were eluted in 35% MeCN. Nonetheless, qualitatively the data were quite similar, characterizing also the retention of the alditols as entropy driven with enthalpy and entropy increments for every glucose unit.

The column temperature was identified as important factor impacting the selectivity of PGC at least with MeCN in the running buffer. With 2-POH and THF only minor variations of selectivities for malto-oligosaccharides with the temperature were observed.

The robustness of the PGC chromatographic system using MeCN as organic modifier is quite sensitive to the column temperature, so special care is needed to keep it constant. However, also other effects seem to influence the retentivity and selectivity of PGC chromatographic systems and of the PGC surface, respectively, but which are subject of additional investigation.

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