Comparison of isocratic retention models for hydrophilic interaction liquid chromatographic separation of native and fluorescently labeled oligosaccharides

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In this work, we have investigated retention of maltooligosaccharides and their fluorescent deriva-tives in hydrophilic interaction liquid chromatography using four different stationary phases. The non-derivatized maltooligosaccharides (maltose to maltoheptaose) and their derivatives with 2-aminobenzoic acid, 2-aminobenzamide, 2-aminopyridine and 8-aminonaphthalene-1,3,6-trisulfonic acid were analyzed on silica gel, aminopropyl silica, amide (carbamoyl-bonded silica) and ZIC-HILIC zwit-terionic sulfobetain bonded phase. The partitioning of the analytes between the bulk mobile phase and adsorbed water-rich layer, polar and ionic interactions of analytes with stationary phase have been eval-uated and compared. The effects of the mobile phase additives (0.1% (v/v) of acetic acid and ammonium acetate in concentration range 5–30 mmol L^{-1}) on retention were described. The suitability of different models for prediction of retention was tested including linear solvent strength model, quadratic model, mixed-mode model, and empirical Neue–Kuss model. The mixed-mode model was extended to the parameter describing the contribution of monomeric glucose unit to the retention of non-derivatized and derivatized maltooligosaccharides, which was used for evaluation of contribution of both, oligosaccharide backbone and end-group to retention.

Keywords:
Hydrophilic interaction liquid chromatography; Retention; Oligosaccharides; Liquid chromatography/mass spectrometry Fluorescent derivatives

1. Introduction

The analysis of saccharides and their conjugates with peptides and proteins is difficult due to the wide range of physico-chemical properties and low concentrations of such compounds presented in biological systems. Native oligosaccharides lack chromophore groups, so a derivatization procedure usually has to be applied prior to the analysis. The separation of biologically important oligosaccharides can be utilized by chromatographic methods based on lectin affinity, porous graphitic carbon, or, because of the strongly polar character of the compounds, by hydrophilic interaction liquid chromatography (HILIC) [1].

The retention of compounds in HILIC, which is sometimes also called aqueous normal-phase liquid chromatography, is generally due to three mechanisms—partitioning of analytes between organic-rich mobile phase and water layer adsorbed on stationary phase, polar and ionic (ion-exchange) interactions. Different stationary phases available for HILIC separations, including bare silica, silica gel phases with chemically bonded polar groups (amino-, nitrile-, diol-, pentafluorophenyl-), carbohydrate based phases, mixed-mode, and zwitterionic phases, may enhance particular type of interaction. The stationary phases used in HILIC have been summarized in several review articles [2,3]. The type of packing together with the composition of mobile phase affects the thickness of the adsorbed diffuse water layer on surface of silica-based stationary phase, which influences the retention of polar analytes [4,5].

The fundamental description of retention in HILIC under isocratic conditions can be provided using several models. Unlike in reversed-phase LC, the linear solvation strength model (i.e. the linear dependency of the logarithm of retention factor on concentration of stronger elution solvent; Eq. (1) [6]) is generally not well suitable for description of retention in HILIC although it is considered a partition mechanism of separation. The quadratic model (Eq. (2)) is more suitable for description of retention for mobile phases used over wide range of concentration of organic modifier [7]:

$$\ln k = \ln k_0 - S \times \varphi \quad (1)$$

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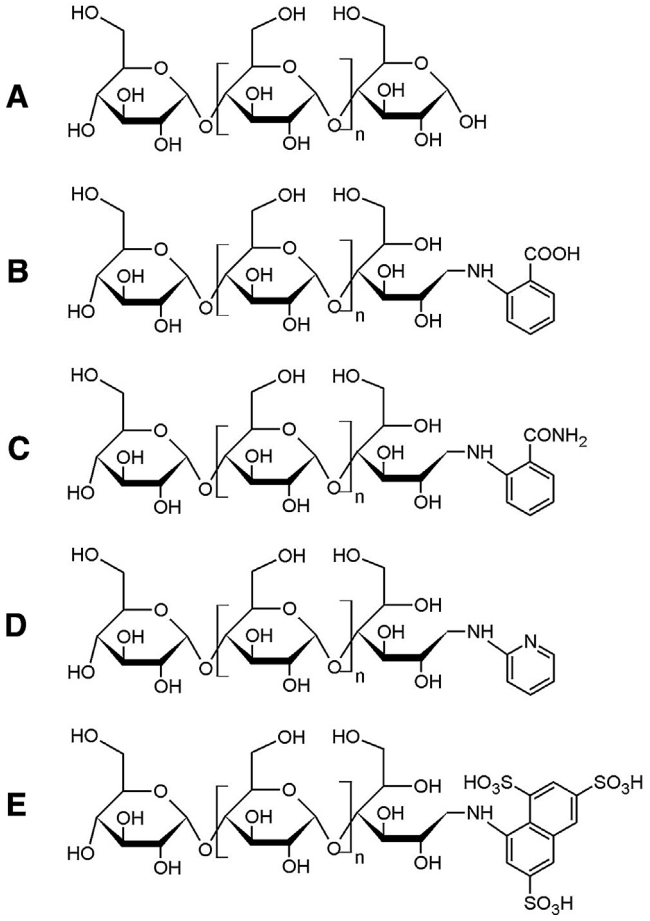

Fig. 1. Structures of non-derivatized maltooligosaccharides (**A**) and their derivatives with 2-aminobenzoic acid (**B**), 2-amino benzamide (**C**), 2-amino pyridine (**D**) and 8-aminonaphthalene-1,3,6-trisulfonic acid (**E**). n = 0–5 in oligomeric series from maltose to maltoheptaose.

$$\ln k = \ln k_0 + S_1 \times \varphi + S_2 \times \varphi^2 \quad (2)$$

where k_0 represents retention factor of an analyte in weaker eluent; φ is volume fraction of stronger eluent in mobile phase and S, S_1 and S_2 are parameters describing elution strength of the eluent used.

For prediction of retention in both isocratic and gradient RP-LC, empirical model introduced by Neue and Kuss can be used, which provide easy analytical solution of integration of gradient equation [8]:

$$\ln k = \ln k_{00} + 2\ln(1 + a \times \varphi) - \frac{B \times \varphi}{1 + a \times \varphi} \quad (3)$$

where k_{00} is the extrapolated intercept (retention factor in pure weak eluent), B is slope of the relationship and a is the coefficient responsible for curvature of the dependency.

The description of retention based on localized surface adsorption used in normal-phase chromatography can be usually applied in HILIC over a limited mobile phase composition range [9]:

$$\ln k = \ln k_0 - n \ln \varphi_{H_2O} \quad (4)$$

where n is the ratio of cross-sectional areas occupied by analyte molecules and by water molecules.

Mechanism of retention (adsorption vs. partitioning) using Eqs. (1) and (4) was compared by McCalley [10]. The studies determining relative importance of both mechanisms were inconclusive as the retention of compounds in HILIC is generally multiparametric process yielding deviations from linearity for both equations [11].

Similarly to RP-LC, the retention of analytes can exhibit non-linear behavior over a wide range of concentration of stronger eluent in mobile phase. This non-linearity can be attributed to the interactions between solutes and solvent, which are not considered in aforementioned equation (Eq. (4)). Therefore, several mixed-mode models were recently introduced, which allows precise characterization of retention in dependency on wide concentration range of solvents in mobile phases [12,13]:

$$\ln k = a + b \ln \varphi_{H_2O} + c\varphi_{H_2O} \quad (5)$$

where a is parameter related to interaction energy between solutes and stationary and mobile phase, b is coefficient related to the direct analyte-stationary phase interaction and c is coefficient related to the interaction energy between solutes and solvents.

Some of the stationary phases used in HILIC can exhibit different retention behavior in low and high-water concentration range in mobile phase. In order to better describe the retention in low-water concentration mobile phases, Jandera and Hájek have introduced a four parameter equation, which can be successfully applied for description of dual HILIC-RP retention mechanism [14]:

$$\ln k = a_2 + m_{RP} \times \varphi_{H_2O} - m_{HILIC} \times \ln\left[1 + b_2 \times \varphi_{H_2O}\right] \quad (6)$$

where the parameters m_{RP} and m_{HILIC} have similar meaning as parameters b and c of Eq. (5) and parameter b_2 corrects the retention in very low water concentration region.

Both native and fluorescently labeled oligosaccharides can be successfully separated in HILIC using various stationary phases. Separation of neutral oligosaccharides has been reported on cyclodextrin bonded phases [15]. Retention of neutral saccharides is usually controlled by partitioning mechanism, but ionic carbohydrates are affected by electrostatic attractive or repulsive interactions, which weakened with increasing concentration of buffer in mobile phase and thus increased ionic strength [16]. When acidic additives to the mobile phase are used, splitting of the peaks can be observed corresponding to the separation of α- and β-anomers [16,17]. For separation of derivatives of oligosaccharides, bonded amide phases provide better selectivity in comparison to the bare silica stationary phase [18,19]. In this work, we have investigated retention of native maltooligosaccharides and their fluorescent derivatives using four different silica-based stationary phases including neutral (silica, amide), charged (amino) and zwitterionic sulfobetaine (ZIC-HILIC) phases. Retention of four types of commonly used derivatives of oligosaccharides with 2-aminobenzoic acid (2-AA), 2-aminobenzamide (2-AB), 2-aminopyridine (2-AP) and 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) was studied under isocratic conditions and evaluated using above mentioned retention models. The effects of concentration of water in mobile phase and addition of ionic compounds on retention and on contributions of oligosaccharide structural units to the retention were assessed.

2. Experimental

2.1. Materials and reagents

The standards of maltooligosaccharides (maltose to maltoheptaose) as well as acetonitrile, acetic acid, ammonium acetate used for preparation of mobile phases and acenaphthene used for the determination of columns hold-up volume were purchased from Sigma–Aldrich (St. Louis, MI, USA). Deionized ultra-pure water with resistivity 18.2 MΩ cm^{-1} used for preparation of mobile phases was purified using Milli-Q Reference system (Merck Millipore, Billerica, MA, USA). Chemicals used for derivatization of oligosaccharides, 2-aminobenzoic acid, 2-aminobenzamide, 2-aminopyridine, 8-aminonaphthalene-1,3,6-trisulfonic acid, dimethylsulfoxide, and sodium cyanoborohydride

were also obtained from Sigma–Aldrich. All chemicals and standards were of highest available purity.

The retention of native non-derivatized and labeled oligosaccharides (Fig. 1) was studied using four commercially available columns. The list of the columns together with their properties and determined hold-up volumes is shown in Table 1.

2.2. Equipment

All separations of native and labeled oligosaccharides were performed using a Shimadzu modular liquid chromatograph consisting of two LC-20ADXR pumps connected with 180 μL mixer and SIL-20ADXR autosampler (all Shimadzu, Kyoto, Japan). The columns were placed into the LCO102 column thermostat (ECOM, Prague, Czech Republic). For LC/UV experiments, a Shimadzu SPD-20A UV detector equipped with semi-micro flow cell (5 mm optical path; 2.5 μL cell volume) was used. For LC/MS experiments, the column outlet was connected through a grounded stainless steel zero dead volume union to a Turbo V source of AB Sciex QTrap 4500 mass spectrometer operated in ESI mode (AB Sciex, Framingham, MA, USA).

2.3. Methods

The hold-up volumes of the columns were determined by injecting of 100 ppm acenaphthene solution in 50, 75 and 90% (v/v) of acetonitrile/water mixture with UV detection at 254 nm. The extra-column contribution of the system determined by replacing column with zero dead volume union and by injection of acenaphthene was 21.9 μL. The mobile phases consisted of mixtures of acetonitrile and water (30–85% of acetonitrile in water, v/v) with addition of 0.1% acetic acid, and ammonium acetate (5, 10, 20 and 30 mmol L^{-1}). The isocratic retention data were measured at flow rate of mobile phase 0.05 mL min^{-1} and column temperature 30 °C. The injection volume of the samples was 0.5 μL. Both, column dead volumes and retention volumes determined under isocratic conditions were corrected for extra-column contributions.

For LC/MS analyses, the derivatives and native oligosaccharides were detected in the Enhanced MS mode. The mass spectra were recorded in *m/z* range 300–1700 with the following source conditions: source temperature 400 °C, curtain gas 30, GS1 (nebulizer gas pressure) – 30 psi, GS2 (auxiliary gas pressure) – 0 psi, scan rate 10 000 Da/s. The ANTS and 2-AA derivatives were detected in negative ion mode; native oligosaccharides, 2-AB and 2-AP derivatives were detected in positive ion mode. The parameters in positive/negative ion mode were as follows: entrance potential 10/−10 V, ion spray voltage 4500/−4500 V, declustering potential 135/−135 V, collision energy 10/−10 V.

2.3.1. Preparation of labeled oligosaccharides

The derivatives of oligosaccharides were prepared according to the slightly modified procedure originally published by Jackson [20]. Thus a stock solution containing 70 mg mL^{-1} of derivatization agent (2-AA, 2-AB, 2-AP, ANTS) in acetic acid/water mixture (3:17, v/v) and a stock solution of 0.8 mol L^{-1} of sodium cyanoborohydride in dimethylsulfoxide were prepared. Approximately 1 mg of each oligosaccharide standard was placed in Eppendorf tube and 15 μL of derivatization agent solution and 15 μL of cyanoborohydride solution were added. The reaction mixture was placed in 40 °C forced air oven for 15 hours. After the reaction, the labeled oligosaccharides were diluted 500times with 50 % of acetonitrile/water mixture (v/v) and subjected to LC/MS experiments.

2.3.2. Evaluation of retention data

For each oligosaccharide or oligosaccharide derivative, the reconstructed ion chromatogram was created and exported in ASCII format from Analyst software (version 1.6.2; AB Sciex, Framingham, MA, USA). The data were deconvoluted by non-linear regression with Gaussian function using STATISTICA data analysis system (version 12; StatSoft, Tulsa, OK, USA). The determined retention times and hold-up times (volumes) were corrected for extra-column contribution of the system and the retention factors were calculated. The retention data were fitted with the retention models using STATISTICA software and the regression parameters together with the standard deviations and regression statistics were obtained.

3. Results and discussion

3.1. Influence of mobile phase composition

Maltooligosaccharides studied in this work (maltose to maltoheptaose) are strongly polar compounds with neutral character in a wide range of pH. They exhibit similar acid-base properties ($pK_a = 12.39 \pm 0.20$), but differ significantly in their polarity. The logarithms of octanol/water partition coefficients, log P_{ow}, are in range of -4.83 ± 0.61 for maltose to -14.21 ± 1.06 for maltoheptaose [21]. In HILIC, the retention of non-derivatized maltooligosaccharides is dependent on the number of monomeric glucose units and increases with respect to the decreasing log P_{ow} values (thus with increasing polarity of the compounds). The example of their retention on neutral amide-type stationary phase is shown in Fig. 2, expressed as dependence between the retention factor, *k*, and the volume concentration of water in the mobile phase with addition of 0.1% (v/v) of acetic acid, φ_{H_2O}(Fig. 2**A**), natural logarithm of the retention factor, ln *k*, vs. φ_{H_2O} (Fig. 2**B**), or as ln *k* vs. natural logarithm of the volume concentration of water in the mobile phase, ln φ_{H_2O} (Fig. 2**C**). The semilogarithmic and logarithmic plots were fitted with Eqs. (1)–(5) and the regression parameters were calculated. The detailed results of regression for retention of non-derivatized maltooligosaccharides on amide-type column are presented in supplementary information (Table S1) together with the coefficient of determination and adjusted coefficient of determination. The regression characteristics mean error of prediction (MEP), and Akaike information criterion (AIC) were used for evaluation of suitability of the model in fitting the retention data and thus in description of particular interactions considered by the model (Table 2). Both, MEP and AIC criterions are suitable for evaluation of the quality, by which the selected model fits the given experimental data set, relative to each of the other models used. The MEP measures the quality of a predictor, i.e. the squared distance between the prediction by the model used and experimentally determined value of the retention factor. The AIC criterion is based on information theory [22] and it describes the loss of information for specific model, which is used to represent the process generating the set of experimental data (in our case the retention of compounds in HILIC). The preferred model from given set of candidates is than the one with minimum AIC and MEP values. For both linear models (linear solvation strength and adsorption model; Eqs. (1) and (4)), the goodness-of-fit decreases with increasing number of glucose units, which is illustrated by increasing mean error of prediction and Akaike information criterion. The non-linear models better describe the retention behavior of oligosaccharides in the tested range of concentration of water/acetonitrile in mobile phase, however, the application of Neue-Kuss model (Eq. (3)) led to the statistical insignificance of some of the regression parameters (mostly parameter *B*, which is the slope of the dependency; Table S1), probably due to the low number of isocratic experiments in the low- and high-water concentration region. As the least-square fitting of semilogarithmic scale of dependency may produce errors at high *k*-values [23], we have tested also the regression of the data in arithmetic scale, but with no improvement of the significance of

Table 1
Column types, dimensions, manufacturers and hold-up volumes corrected for extra-column contributions, $V_{M,corr}$, determined using acenaphthene as non-retained marker with the standard deviation of five consecutive measurements in parentheses.

Column no.	Trade name, manufacturer	Dimensions (column length x inner diameter) [mm]	Particle size [μm]	Pore size [nm]	$V_{M,corr}$ [μL]
1	Ascentis Si, Sigma–Aldrich, St. Louis, MO, USA	100 × 1.0	3.0	10.0	55.7 (0.25)
2	Luna NH_2, Phenomenex, Torrance, CA, USA	100 × 1.0	3.0	9.6	58.0 (0.61)
3	TSKgel Amide-80, Tosoh Bioscience, Stuttgart, Germany	100 × 1.0	5.0	8.0	43.7 (0.51)
4	SeQuant® ZIC®-HILIC, MERCK, Darmstadt, Germany	150 × 1.0	3.5	10.0	63.1 (0.63)

Table 2
Regression characteristics (mean error of prediction, MEP, and Akaike information criterion, AIC) of retention models for non-derivatized maltooligosaccharides on amide-type stationary phase (TSKgel Amide-80 column) using mobile phase with addition of 0.1% (v/v) of acetic acid calculated with Eqs. (1)–(5).

Compound	Parameter	Model				
		LSS, Eq. (1)	Quadratic, Eq. (2)	Neue–Kuss, Eq. (3)	Adsorption, Eq. (4)	Mixed-mode, Eq. (5)
Maltose	MEP ($\cdot 10^3$)	8.38	2.12	2.59	2.21	2.47
	AIC	−59.17	−75.83	−38.10	−74.59	−74.16
Maltotriose	MEP ($\cdot 10^3$)	12.15	2.56	1.37	2.55	1.86
	AIC	−54.37	−72.21	−42.70	−72.44	−75.90
Maltotetraose	MEP ($\cdot 10^3$)	23.92	2.97	2.47	6.99	1.93
	AIC	−46.19	−70.25	−39.39	−60.77	−75.08
Maltopentaose	MEP ($\cdot 10^3$)	0.39	1.30	3.66	0.13	0.73
	AIC	−40.24	−79.84	−47.23	−52.95	−86.05
Maltohexaose	MEP ($\cdot 10^3$)	40.63	0.85	2.37	12.19	0.81
	AIC	−40.24	−84.25	−38.52	−54.81	−84.87
Maltoheptaose	MEP ($\cdot 10^3$)	53.11	0.99	1.14	18.04	1.17
	AIC	−36.99	−82.35	−35.39	−50.00	−80.79

regression parameters. As the retention of maltooligosaccharides was not studied in mobile phase with very low concentration of water, Eq. (6) was not used for evaluation of the experimental data. Based on the regression characteristics shown in Table 2 and Table S1, it is evident that *R*-squared and adjusted *R*-squared parameters alone are not sufficient for distinguishing of the suitability of particular model for description of retention. Significant differences were however observed using other two criterions, AIC and MEP. The values of MEP were significantly lower for non-linear models in comparison to linear models. For most of the oligosaccharides, the AIC criterion was lowest for mixed-mode model. According to the regression characteristics presented in Table 2, the mixed mode non-linear model was thus found the best in describing the experimental retention data among the models used, which is generally in agreement with published studies [24].

The regression parameters determined for maltooligosaccharides are dependent on the number of glucose units in the molecules and changes mostly linearly (Table S1). This suggests that the retention of oligomeric series can be described altogether with any of the Equations, which can be extended with the term accounting for structural contribution of oligomeric unit can be added. We have verified this possibility by extending the mixed-mode model (Eq. (5)) to following equation:

$$\ln k = a_2 + b_2 \times \ln \varphi_{H_2O} + c_2 \times \varphi_{H_2O} + d \times n_{Dp} \quad (7)$$

where the a_2, b_2 and c_2 parameters have the similar meaning like in Eq. (5) for glucose (monomeric unit) and parameter d is contribution of the oligomeric unit to the retention. Applying Eq. (7) on retention of non-derivatized maltooligosaccharides, the regression characteristics were obtained as follows: coefficient of determination 0.9776, Akaike information criterion −273.58 and mean error of prediction 0.0225. The values are comparable with those for models fitted separately (Table 2) for each oligosaccharide. The Eq. (7) was further used for evaluation of the contributions of glucose units for derivatives of oligosaccharides.

3.2. Influence of the type of derivatization agent on retention of oligosaccharides

The native maltooligosaccharides do not contain chromophore groups in their structures and the derivatization reactions are usually applied prior to their analyses. The selection of derivatization agent also influences the retention in HILIC. We have compared retention of four maltooligosaccharide derivatives, prepared by reductive amination with 2-aminobenzoic acid, 2-aminobenzamide, 2-aminopyridine and 8-aminonaphthalene-1,3,6-trisulfonic acid on amide-type stationary phase (TSKgel Amide-80 column). The amide-type stationary phase provided the highest retention of native oligosaccharides (as shown in Section 3.4) and thus was selected for the comparison of retention of derivatives. The influence of different end-group in the molecule of derivatized maltopentaose on retention behavior is shown in Fig. 3 in mobile phase containing 0.1% (v/v) of acetic acid (Fig. 3**A**) and 30 mmol L^{-1} ammonium acetate (Fig. 3**B**). The experimental dependencies were fitted with mixed-mode isocratic retention model (Eq. (5)) and the calculated regression parameters are shown in Table 3. In the mobile phase containing acetic acid, the retention of non-derivatized maltopentaose and its ionic derivatives (2-AA, 2-AB) is similar as well as the regression parameters for these derivatives; however, the 2-AP derivative is generally more strongly retained on amide-type stationary phase. The shift in retention for derivatization with 2-AP can be possibly attributed to the protonation of nitrogen in pyridine structure in acidic mobile phase, yielding increased ion-exchange interactions with stationary phase. The hypothesis is supported also by increased retention of 2-AP derivatives with respect to the non-derivatized oligosaccharides on silica gel stationary phase, while the zwitterionic phase and aminopropyl silica gel phase shown comparable retention of both classes of compounds (data not shown). The ANTS derivatives, which are strong acids, provided steeper dependency of logarithm of retention factor on volume fraction of water in mobile phase than other derivatives (Fig. 3**A**). The retention of derivatives in mobile phase containing acetic acid is also influenced by changing ionic strength of the mobile phase, which is very low using acidic additives in solutions with high concentration of acetonitrile [25].

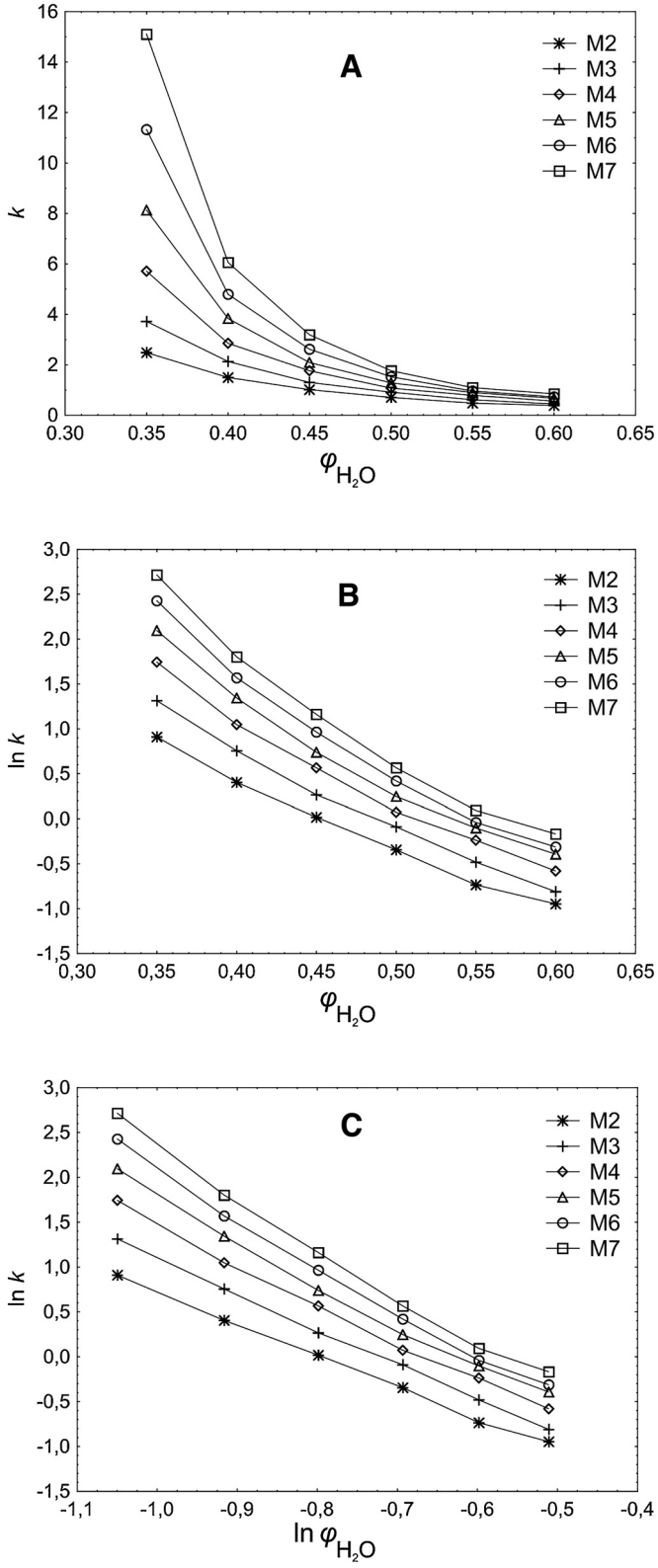

Fig. 2. Retention of non-derivatized maltooligosaccharides in dependency on concentration of water in mobile phase, φ_{H_2O}, expressed in arithmetic **(A)**, semilogarithmic **(B)** and logarithmic scale **(C)**. Column TSKgel Amide-80, mobile phase water with addition of 0.1% (v/v) of acetic acid in mixture with acetonitrile.

In mobile phase containing ammonium acetate, the retention of non-derivatized oligosaccharides is comparable with mobile phase containing acetic acid, but generally decreases with derivatization of the compounds (Fig. 3**B**; Table 3). The retention of 2-AB and 2-AP derivatives is close together for both types of derivatives as shown on both, regression lines and determined regression parameters. As the logarithm of dissociation constant for nitrogen in pyridine is 5.23 [21], the 2-AP derivatives are preferably neutral under such conditions, contrary to the mobile phase containing 0.1% (v/v) of acetic acid. The dependencies are steeper for acidic

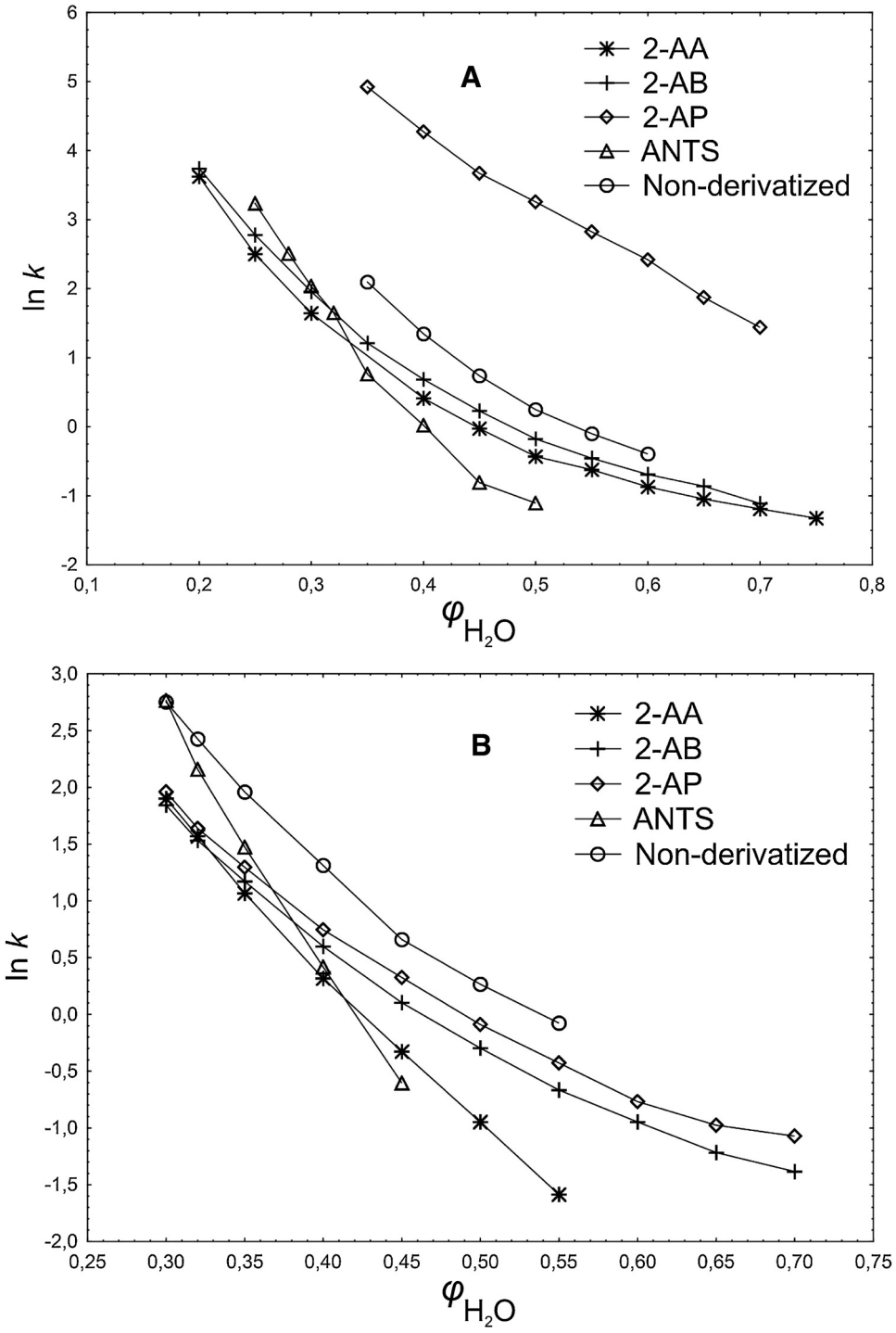

Fig. 3. Effect of derivatization on retention of maltooligosaccharides. Comparison of retention for non-derivatized maltopentaose and different types of derivatives on TSKgel Amide-80 column in mobile phase with addition of 0.1% (v/v) acetic acid **(A)** and 30 mmol L^{-1} ammonium acetate **(B)**.

derivatives (2-AA, ANTS) and changes with dissociation constant of the acids (sulfonic vs. carboxylic acid) indicating, that ionic interactions with stationary phase are stronger for such compounds. As a result, much narrower retention window is observed for separation of ANTS derivatives of maltooligosaccharides. To further study the ionic interactions of derivatives with stationary phases, the retention of maltooligosaccharides was determined in dependency on the concentration of ammonium acetate in mobile phase and the results are discussed in Section 3.3.

Contribution of glucose units and end groups in molecules of maltooligosaccharide derivatives to the retention was verified also using parameter *d* determined from Eq. (7). The results for both mobile phase with addition of 0.1% (v/v) of acetic acid and 30 mmol L^{-1} ammonium acetate, respectively, are shown in Table 3. In agreement with Fig. 3, the contribution of glucose units is similar for non-derivatized oligosaccharides, 2-AA and 2-AB derivatives in mobile phase with acetic acid. For 2-AP derivatives, the ionized end-group containing pyridine strongly influences the retention, thus the contribution of glucose units is less than for

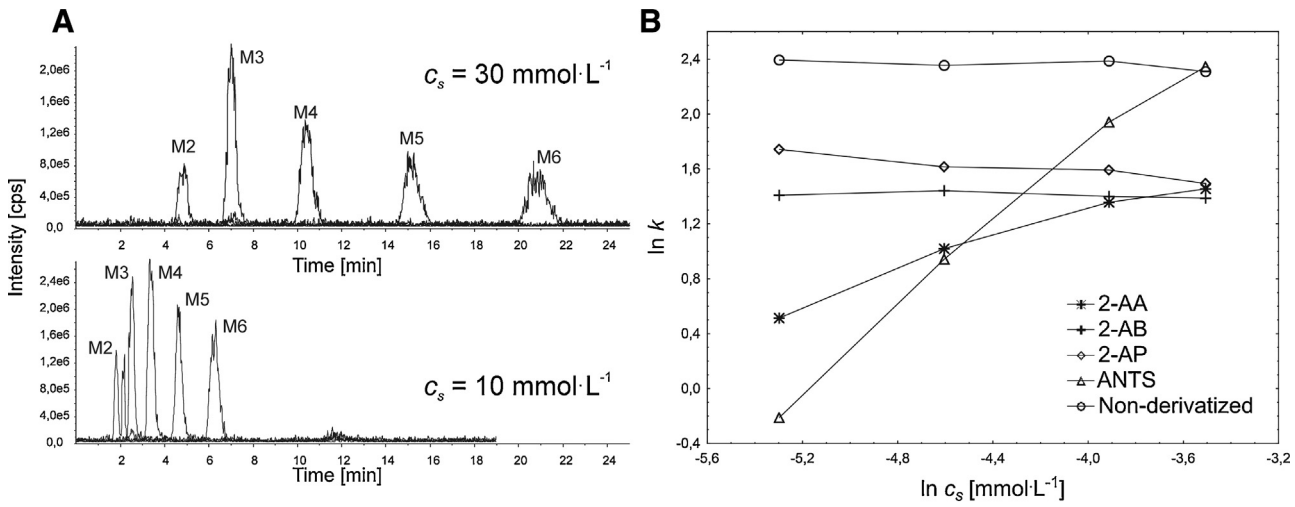

Fig. 4. Effect of the concentration of ammonium acetate on retention of maltooligosaccharides. Reconstructed ion chromatograms for ANTS derivatives of maltooligosaccharides (maltose, M2 to maltohexaose, M6) **(A)** and logarithmic dependency of retention factor, k, on concentration of ammonium acetate, c_s **(B)**; column TSKgel Amide-80, mobile phase containing 30% (v/v) water/acetonitrile mixture.

Table 3
Regression parameters of mixed-mode retention model (Eq. (5)) for non-derivatized maltopentaose and derivatives of maltopentaose on amide-type stationary phase (TSKgel Amide-80 column) using mobile phase with addition of 0.1% (v/v) of acetic acid and 30 mmol L^{-1} ammonium acetate, respectively. Values of parameters are given with the standard deviation in parentheses. Parameter d is determined by fitting the data for set of oligosaccharides using Eq. (7).

Mobile phase	Parameter	Derivatization agent				
		Non-derivatized	2-AA	2-AB	2-AP	ANTS
0.1 % (v/v) acetic acid	a	−12.93 (0.53)	−8.80 (0.23)	−6.70 (0.37)	3.51 (1.14)	−14.32 (2.59)
	b	−10.26 (0.30)	−6.80 (0.12)	−5.89 (0.19)	−2.73 (0.67)	−10.68 (1.11)
	c	12.15 (0.64)	7.41 (0.28)	5.05 (0.46)	−4.28 (1.32)	11.41 (4.14)
	R^2	0.9994	0.9995	0.9989	0.9977	0.9949
	Adjusted R^2	0.9993	0.9994	0.9988	0.9973	0.9990
	MEP ($\cdot 10^3$)	0.73	1.59	3.56	4.27	4.73
	AIC	−86.05	−127.94	−113.93	−87.67	−44.38
	d (Eq. (7))	0.230 (0.098)	0.268 (0.032)	0.273 (0.027)	0.153 (0.016)	0.373 (0.019)
30 mmol L^{-1} ammonium acetate	a	−8.14 (0.97)	−2.07 (0.59)	−7.17 (0.54)	−7.23 (0.66)	−7.37 (3.65)
	b	−7.45 (0.51)	−4.21 (0.31)	−6.24 (0.30)	−6.27 (0.37)	−8.30 (1.82)
	c	6.50 (1.23)	−3.66 (0.76)	5.07 (0.65)	5.50 (0.78)	0.34 (4.94)
	R^2	0.9992	0.9998	0.9987	0.9979	0.9993
	Adjusted R^2	0.9991	0.9998	0.9985	0.9977	0.9989
	MEP ($\cdot 10^3$)	1.38	0.62	2.47	3.18	7.43
	AIC	−93.28	−102.24	−122.61	−114.97	−43.52
	d (Eq. (7))	0.265 (0.018)	0.316 (0.013)	0.273 (0.019)	0.287 (0.019)	0.344 (0.012)

non-ionized derivatives. The ANTS derivatives provided highest d in comparison with other derivatives supporting the hypothesis that anionic sulfonic acid groups play less important role in the retention than the rest of the molecules and generally decrease the retention with increasing concentration of water in the mobile phase. In the mobile phase with ammonium acetate, both anionic derivatives (2-AA and ANTS) possessed significantly higher contribution of glucose units to the retention than rest of the compounds.

3.3. Influence of mobile phase additives and their concentration on the retention of oligosaccharides

With increasing concentration of ammonium acetate in mobile phase, the retention of ionic derivatives of oligosaccharides significantly increases. Fig. 4**A** shows separation of ANTS derivatives of maltose to maltohexaose in 30 % (v/v) water/acetonitrile mobile phase containing 10 mmol·L^{-1} and 30 mmol·L^{-1} ammonium acetate. The comparison of the retention in the dependence on the concentration of ammonium acetate is shown in Fig. 4**B**. The non-derivatized oligosaccharides and neutral derivatives (2-AB, 2-AP) are almost unaffected by increasing concentration of ammonium acetate. Thus it can be concluded that the retention of these compounds is not based on ionic-type of interactions (ion-exchange interactions). Contrary, the retention of anionic 2-AA and ANTS derivatives significantly increases within the studied range of concentration of ammonium acetate (5–30 mmol L^{-1}). This effect, which was observed using neutral amide-type and bare silica gel columns can be probably connected to the repulsion of anions from negatively charged stationary phase due to the silanol groups (or residual silanol groups in the amide phase), which would be affected by suppression of dissociation with increasing ionic strength. The positive slope indicates that the mechanism of separation is different from ion-exchange, in which the retention decreases with increasing ionic strength of the buffer. Different slope of dependency for 2-AA and ANTS (Fig. 4**B**) derivatives of oligosaccharides can be then attributed to the number of dissociated anionic groups in the molecule. The influence of ammonium acetate on separation within the studied concentration range is in agreement with observations of retention of phenolic acids on zwitterionic polymethacrylate monolithic microcolumn under HILIC conditions [26].

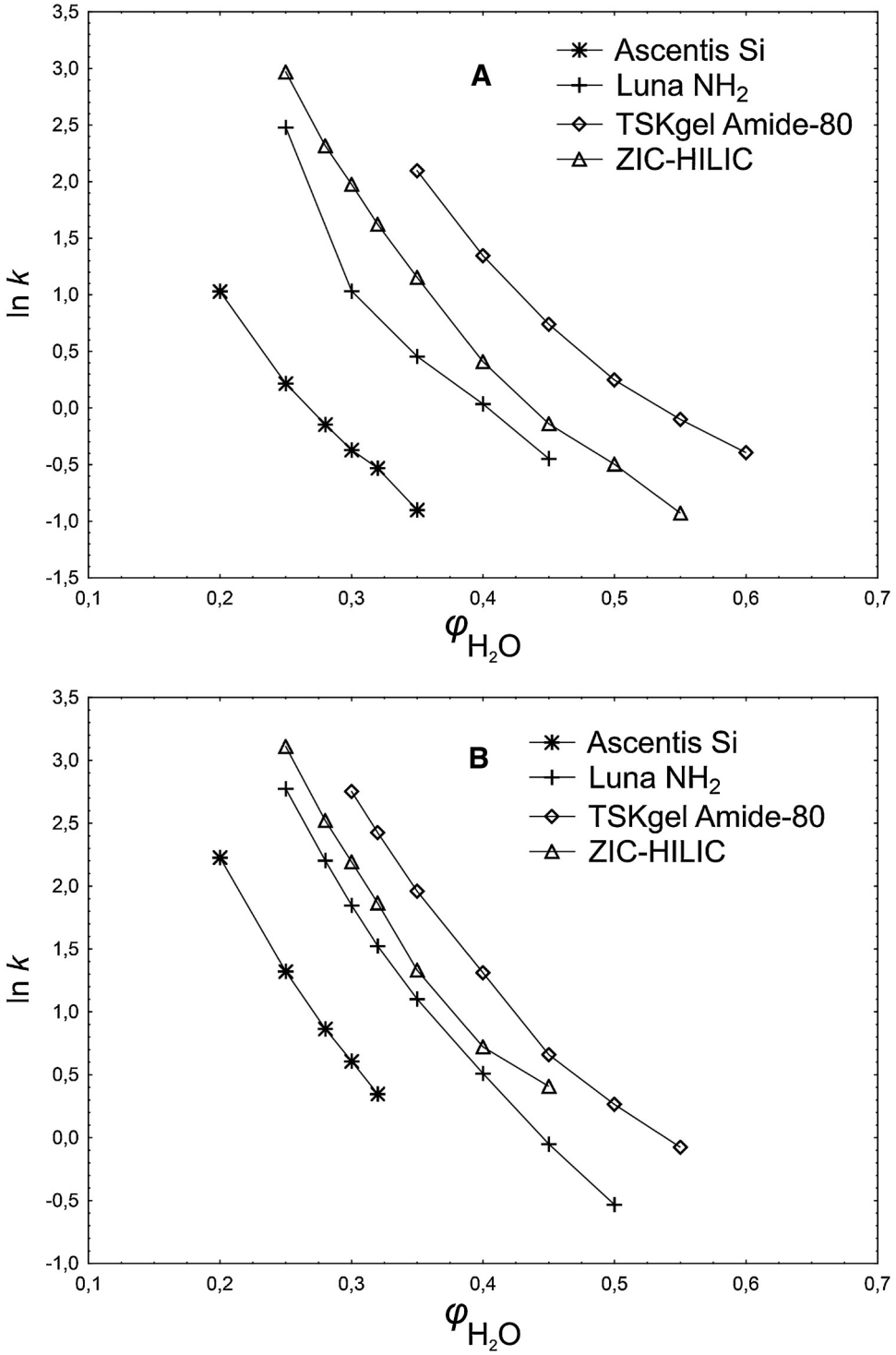

Fig. 5. Comparison of retention of non-derivatized maltooligosaccharides on different columns. Mobile phase with addition of 0.1% (v/v) acetic acid **(A)** and 30 mmol L^{-1} of ammonium acetate **(B)**.

3.4. Comparison of different stationary phases

Due to the character of tested stationary phases, the separation should be predominantly driven by the partitioning mechanism between the aqueous-depleted organic solvent and water-rich layer adsorbed on the surface of stationary phase. Thus, the stationary phases with strong adsorption properties and thicker adsorbed water layer should provide higher retention of native maltooligosaccharides. The effects of type of stationary phase on retention of native non-derivatized oligosaccharides is shown in Fig. 5 in both, mobile phase with addition of 0.1 % (v/v) of acetic acid (Fig. 5**A**) and 30 mmol L^{-1} ammonium acetate (Fig. 5**B**) and the regression parameters after fitting the data with Eq. (5) are shown in Table 4. Among the all columns tested, the highest retention of native oligosaccharides was observed on netural amide-type stationary phase. Compared to other authors, who experienced the higher retention of neutral analytes on zwitterionic column than on amide- or amino- bonded phases [27], supported also by the

Table 4
Comparison of regression parameters of mixed-mode retention model (Eq. (5)) for non-derivatized maltopentaose for different columns using mobile phase with addition of 0.1% (v/v) of acetic acid and 30 mmol L^{-1} ammonium acetate, respectively. Values of parameters are given with the standard deviation in parentheses. Parameter *d* is determined by fitting the data for set of oligosaccharides using Eq. (7).

Mobile phase	Parameter	Column no., stationary phase			
		1, Silica gel	2, Amino propyl silica	3, Amide phase	4, Zwitterionic phase
0.1 % (v/v) acetic acid	*a*	−12.08 (1.52)	−20.18 (2.52)	−14.06 (0.89)	−6.60 (1.16)
	b	−6.62 (0.64)	−12.33 (1.20)	−10.87 (0.50)	−6.35 (0.56)
	c	12.51 (2.50)	22.09 (3.53)	13.53 (1.07)	3.06 (1.62)
	R^2	0.9989	0.9968	0.9994	0.9997
	Adjusted R^2	0.9986	0.9959	0.9993	0.9996
	MEP ($\cdot 10^3$)	1.37	6.15	0.73	1.60
	AIC	−73.16	−51.23	−86.05	−49.91
	d (Eq. (7))	0.229 (0.012)	0.226 (0.031)	0.230 (0.098)	0.245 (0.017)
30 mmol L^{-1} ammonium acetate	*a*	−2.68 (2.38)	−5.95 (0.57)	−8.14 (0.97)	−7.69 (1.23)
	b	−3.30 (0.99)	−5.77 (0.28)	−7.45 (0.51)	−6.83 (0.61)
	c	−2.29 (3.93)	2.85 (0.77)	6.50 (1.23)	5.42 (1.67)
	R^2	0.9978	0.9998	0.9992	0.9993
	Adjusted R^2	0.9972	0.9997	0.9991	0.9990
	MEP ($\cdot 10^3$)	3.04	0.64	1.38	5.91
	AIC	−64.14	−62.71	−93.28	−44.02
	d (Eq. (7))	0.285 (0.017)	0.272 (0.017)	0.265 (0.018)	0.256 (0.017)

stronger adsorption of water on zwitterionic phase [28], the retention of non-derivatized maltooligosaccharides on amide phase is probably influenced also by other types of interactions (such as dipole-dipole interactions). The parameters *d* determined by using Eq. (7) (shown in Table 4) were comparable for all columns confirming that the contribution of glucose units to the retention is approximately constant. For all columns tested, the mobile phase with addition of ammonium acetate provided higher retention and steeper dependencies of retention factors on concentration of water in mobile phase than mobile phase with addition of acetic acid; however, the slopes of the dependencies were comparable for particular mobile phase.

4. Conclusions

In this work, we have studied the retention of non-derivatized maltooligosaccharides and their four derivatives in hydrophilic interaction liquid chromatography. The nature of retention process in HILIC is not straightforward and it is rather multiparametric process, which was confirmed by application of five isocratic retention models on experimental data. Although the goodness-of-fit of the data with particular model cannot be considered as unequivocal evidence for the type of retention, the comparison of regression parameters for compounds in oligomeric series and for derivatives with different end-groups indicate, that the processes contributing to the retention in hydrophilic interaction liquid chromatography are very complex.

Among the studied conditions, the amide-type stationary phase (carbamoyl bonded silica) provided the highest retention of both, non-derivatized maltooligosaccharides and their ionic and non-ionic derivatives. The derivatization with agent containing cationic end-group (2-aminopyridine) provided decreased influence of glucose units on the retention in the acidic mobile phase, while the anionic derivatives enhanced the influence of oligomeric groups and, as a result, much narrower retention window was observed. The ionic derivatives were also significantly influenced by the concentration of ammonium acetate in mobile phase, probably due to the repulsion of anionic carboxy- and sulfo- groups from silanols or residual silanols presented on the surface of stationary phase. Thus the introduction of ionic end-group to the molecule of oligosaccharide can be applied for fine tuning of the separation selectivity, which is especially important for the separation of complex mixtures of saccharides presented in biological matrices.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2016.02.032.

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