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Prediction of Gradient Retention Data for Hydrophilic Interaction Liquid Chromatographic Separation of Native and Fluorescently Labeled Oligosaccharides

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Highlights:

- Gradient retention times of maltooligosaccharides were predicted
- Prediction accuracy was compared for isocratic and gradient input data
- 15 gradient profiles with different steepnesses were compared
- Four types of derivatization agents were studied
- Method development was speeded-up using oligomeric mixed-mode model

Abstract

In this work, we have investigated the predictive properties of mixed-mode retention model and oligomeric mixed-mode model, taking into account the contribution of monomeric units to the retention, in hydrophilic interaction liquid chromatography. The gradient retention times of native maltooligosaccharides and their fluorescent derivatives were predicted in the oligomeric series with

number of monomeric glucose units in the range from two to seven. The maltooligosaccharides were separated on a packed column with carbamoyl-bonded silica stationary phase and 15 gradient profiles with different initial and final mobile phase composition were used with the gradient times 5; 7.5 and 10 min. The predicted gradient retention times were compared for calculations based on isocratic retention data and gradient retention data, which provided better accuracy of the results. By comparing two different mobile phase additives, the more accurate retention times were predicted in mobile phases containing ammonium acetate. The acidic derivatives, prepared by reaction of an oligosaccharide with 2-aminobenzoic acid or 8-aminonaphthalene-1,3,6-trisulfonic acid, provided more accurate predictions of the retention data in comparison to native oligosaccharides or their neutral derivatives. The oligomeric mixed-mode model allowed prediction of gradient retention times using only one gradient profile, which significantly speeded-up the method development.

Keywords: Hydrophilic interaction liquid chromatography; Gradient elution; Prediction; Oligomers; Mixed-mode retention model

1 Introduction

Hydrophilic interaction liquid chromatography (HILIC) is a technique suitable for the separation of polar or ionic compounds, which are not retained in the reversed-phase liquid chromatography system (RP) and on the other hand are too strongly retained in the normal-phase (NP) liquid chromatography system; or which have poor solubility in apolar mobile phases used in the NP-LC [1]. Different stationary phases used in HILIC as well as recent progress in understanding of the retention mechanism were thoroughly described in several review articles [2-4].

Although HILIC is used in many practical applications both, in the isocratic and in the gradient elution mode, for the separations of polar compounds such as nucleosides [5], glycans [6,7], peptides [8], amino acids [9,10] and other compounds, the retention mechanism is still not fully understood [11,12]. The retention in HILIC is more complicated compared to the retention in RP system due to many types of interactions involved in the separation process. It is generally accepted that both, partitioning and adsorption, contributes to the retention in HILIC [13]. The proportion of each mechanism depends on physico-chemical properties of a stationary phase, the type and composition of a mobile phase, buffer concentration and type of an analyte [14]. The contribution of adsorption and partitioning in HILIC mechanism was extensively studied by Gritti et al [15] using combination of different models of molecular dynamics simulations for determination of an analyte concentration in rigid and diffuse water layer. The results suggested that it is difficult to generalize the importance of partitioning and adsorption contribution to the retention, which depends on analyte properties and experimental parameters used. The multiparametric process of retention can be characterized by several mixed-mode models [16,17]. The models describe non-linearity of retention of an analyte in a wide range of mobile phase composition due to additional interactions between solutes and solvent [18]. One of the commonly used mixed-mode models is described by the following equation:

$$\ln k = a + b \ln \varphi_{H_2O} + c\, \varphi_{H_2O} \qquad (1)$$

where k is the retention factor of an analyte measured under isocratic conditions, a is the parameter related to the interaction energy between solutes and stationary and mobile phase, b is the coefficient related to the direct analyte-stationary phase interaction and c is the coefficient related to the interaction energy between solutes and solvents. Another suitable retention model frequently used for the description of retention in HILIC is the Neue-Kuss model [5,19,20], expressed by the following equation:

$$\ln k = \ln k_w + 2\ln(1 + S_2 \cdot \varphi) - \frac{S_1 \cdot \varphi}{1 + S_2 \cdot \varphi} \quad (2)$$

where k_w is the extrapolated intercept (i.e. retention factor in pure weak eluent), S_1 is slope of the relationship and S_2 is the coefficient responsible for curvature of the dependency.

The retention volume under gradient conditions can be calculated from general gradient equation [21,22]:

$$\int_0^{t_R - t_0} \frac{d_t}{t_0 \cdot k} = 1 \quad (3)$$

where t_R is the retention time of a solute under gradient conditions, t_0 is the column dead time and k is the solute retention factor, referring to the actual composition of mobile phase during the gradient run. When employing mixed-mode model (Eq. (1)), it is not possible to obtain the analytical solution of the Eq. (3) and it has to be solved by a numerical integration. On the other hand, the integration of the Neue-Kuss model can be solved analytically and therefore it can be preferred among the other retention models [23], although it provides slightly less accurate predictions in HILIC in comparison to the mixed-mode model [20]. It is also worth mentioning that the Neue-Kuss model was developed for simplifying of the prediction process and its parameters are therefore not directly related to the physico-chemical parameters.

The satisfactory accuracy of prediction of the gradient retention times is dependent not only on the retention model used, but also on the set of scouting experiments, providing input data for the calculations. For HILIC separation of amino acids, two gradient scouting runs were enough for the precise predictions of retention times even with the curved gradient profiles [9]. Contrary, three isocratic and one gradient scouting run was necessary for the prediction of gradient data for the model set of nine compounds, covering broad range of the physico-chemical properties. Due to the non-linear retention behavior, the predictive elution window shifting and stretching approach provided tool for the fast optimization of separation of pharmaceutically relevant mixtures [20]. Based on the published studies, it is evident that

the prediction of gradient retention data in HILIC is more difficult and usually much less accurate than in the RP-LC [20,24].

In this work, we have investigated possibilities of the prediction of retention times of native maltooligosaccharides and their fluorescent derivatives using the mixed-mode model and recently introduced oligomeric mixed-mode model [14], which is characterized by the following equation:

$$\ln k = a_2 + b_2 \ln \varphi_{H_2O} + c_2 \varphi_{H_2O} + d\, n_{Dp} \qquad (4)$$

where the parameters a_2, b_2, c_2 have similar meaning as in Eq. (1) for retention of monomeric unit (glucose) and parameter d describes contribution of this unit to the retention, according to their number in molecule of oligosaccharide, n_{Dp}. Experimental retention data were measured by applying 15 linear gradients with different steepness and initial or final mobile phase composition. The predicted retention times were calculated using isocratic and gradient retention times as input data. The predictive errors of both models were compared.

2 Experimental

2.1 Materials and reagents

Acetonitrile, acetic acid, ammonium acetate, and the standards of maltooligosaccharides (maltose to maltoheptaose), were purchased from Sigma-Aldrich (St. Louis, MI, USA). De-ionized ultra-pure water used for preparation of mobile phases and stock solutions was purified using Milli-Q Reference system (Merck Millipore, Billerica, MA, USA). Derivatization agents, 2-aminobenzoic acid, 2-aminobenzamide, 2-aminopyridine, 8-aminonaphthalene-1,3,6-trisulfonic acid, dimethylsulfoxide, and sodium cyanoborohydride were also obtained from Sigma-Aldrich. The derivatives of oligosaccharides were prepared by the slightly modified procedure originally published by Jackson [25], described elsewhere

[14]. Before separation, the derivatives were diluted 500times with 50 % (v/v) of acetonitrile/water mixture.

Both, isocratic and gradient retention data of native non-derivatized and labelled oligosaccharides were measured on TSKgel Amide-80 column (100 mm x 1.0 mm i.d.) packed with 5 µm particles. The hold-up volume of this column corrected for extra-column contributions of the LC system, $V_{m,corr}$, was 43.7 µL.

2.2 Equipment

Gradient separations were performed on Eksigent microLC 200 system, consisted of binary gradient pump, CTC PAL autosampler, thermostated column and sample compartments (SCIEX, Framingham, MA, USA). The column outlet was directly connected to a Turbo V source of AB Sciex QTrap 4500 mass spectrometer operated in ESI mode (SCIEX, Framingham, MA, USA). For determination of gradient dwell volume, the column inlet capillary was connected to the UV detector Saphire 600 CE (ECOM, Prague, Czech Republic).

2.3 Methods

The mobile phases consisted of mixtures of acetonitrile and water with addition of 0.1 % (v/v) acetic acid or 30 mmol·L^{-1} ammonium acetate. The pH of the mobile phase measured at 25°C was 3.24 for acetic acid solution and 6.69 for ammonium acetate water solution. Linear gradient profiles used are summarized in **Table 1**, where φ_0 is initial mobile phase composition, φ_G is final mobile phase composition, t_G and V_G is the gradient time and volume, respectively, and B is the gradient steepness. The gradient steepness is defined by following equation:

$$B = \frac{\Delta\varphi}{V_G} = \frac{\varphi_G - \varphi_0}{V_G} \quad (5)$$

All retention data were measured at the 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. The gradient dwell volume was determined using acetone in the mobile phase and by measurement of absorbance at 230 nm. The determined value of 1.07 μL was considered negligible in comparison with retention volumes of the compounds and hence it was neglected throughout the calculations. Retention data from isocratic separations of native maltooligosaccharides and their fluorescent derivatives corrected for extra-column contribution of LC system used were obtained from our recently published work [14].

The detection conditions of oligosaccharides were as follows. The mass spectra were recorded in *Enhanced MS* mode in *m/z* range 300 – 1700. The ESI source conditions used: source temperature 400 °C, curtain gas 30 psi, 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 using entrance potential 10 V, ion spray voltage 4500 V, declustering potential 135 V and collision energy 10 V. For negative ion mode, the voltages were set to the same value, but with negative potential.

2.3.1 Evaluation of retention data

The gradient retention times of native and derivatized maltooligosaccharides were calculated by numerical integration of Eq. (3), based on both isocratic retention models used, i.e. mixed-mode model (Eq. (1)) and oligomeric mixed-mode model (Eq. (4)). The value of the definite integral was estimated by using midpoint rule. Thus, the value of integral was calculated by the summation of the areas of all rectangles plotted between time zero and t_R-t_0 value divided into the subintervals of equal width (in our case, the width Δt = 0.001 min was selected). The height of each rectangle is than given by the value of integrated function at the

midpoint of the subinterval (Eq. (3)). The iteration of retention time is used, until the value of the integral is equal to one with the actual value of time equal to the reduced retention time of the compound t_R-t_0.

For prediction of the gradient retention times based on the gradient input data, two approaches were used according to the retention model. In the first approach, the experimentally determined retention times for each maltooligosaccharide measured for three gradient profiles (gradients Nos. 7, 8 and 9 in **Table 1**) were used for the iterations of parameters of mixed-mode model (Eq. (1)). The best combination of parameters of retention model was searched, yielding integrals of Eq. (3) for all three gradient profiles equal to one for specific oligosaccharide.

The second approach utilized only one gradient profile (gradient No. 3 in **Table 1**), however, retention times of all oligomers were used at once and the parameters of the oligomeric mixed-mode retention model (Eq. (4)) were iterated. The best combination of parameters was found at the point, where integrals of Eq. (3) were equal to one for all oligosaccharides in the series. The relative deviations between predicted retention times, $t_{R,pred}$, and experimental retention times, $t_{R,exp}$, were calculated by following equation:

$$Dev(\%) = \frac{(t_{R,pred} - t_{R,exp})}{t_{R,exp}} \cdot 100 \quad (6)$$

For numerical integration and iteration of parameters of retention models, proprietary software was developed in the laboratory using Python programming language with built-in mathematical libraries (version 2.5.4., Python Software Foundation). The numerical integration was performed according to the aforementioned procedure. During the iteration procedure, at first the sum of residuals was evaluated between the experimental retention times and times calculated using numerical integration with initial estimates of retention model parameters. Then, the new value of the parameters was searched (differing in pre-set

value of 0.001), yielding lower sum of residuals. The procedure was repeated until the minimum for sum of residuals or maximum pre-set number of iterations was reached.

For comparison of the experimental and calculated retention data, the simulation of the chromatograms was performed in Microsoft Excel software. Based on the calculated retention data, the Gaussian peak profiles were considered with the constant peak width throughout the whole chromatograms.

3 Results and discussion

The computer-assisted method development gradient liquid chromatography usually utilizes the application of isocratic retention models, which describes the retention of compounds in a wide concentration range of mobile phase. In HILIC separations of native and derivatized maltooligosaccharides, published in our recent work [14], we have shown, that a column with carbamoyl-bonded silica stationary phase in combination with acetonitrile/water mobile phase with addition of either 30 $mmol \cdot L^{-1}$ ammonium acetate or acetic acid provide sufficiently selectivity for all the derivatization agents used. The construction of isocratic retention models was however highly time-consuming. To speed up the process and to verify the predictive ability of the models used, we have compared experimental gradient retention times with calculated values based on both, isocratic and gradient input data.

3.1 Prediction based on mixed-mode model

Figure 1 shows the correlation between experimentally obtained and predicted reduced retention times for the mobile phase containing 0.1 % (v/v) of acetic acid. Predictions were done based on isocratic and gradient retention data of native maltooligosaccharides. These data were fitted with the mixed-mode model (Eq. (1)) and the parameters *a*, *b* and *c* were used for predictions of reduced retention times, t_R', calculated as a difference of the compound

retention time and the column dead time, $t_R' = t_R - t_0$. In **Figure 1**, the gradients of four different steepnesses are compared, B = 0.7, 1.1, 1.73 and 2.2 (gradients Nos. 6, 9, 11, 13 in **Table 1**). The steeper gradient profile was used the better correlations between predicted and experimental retention times were achieved for isocratic input data. For gradient input data (Fig. 1C) the better correlation was achieved for the gradients with steepness close to the gradient scouting run. Shorter and steeper gradients may exhibit higher contribution of non-thermodynamic sources affecting the gradient profile [26], which was however not observed in this case.

By comparing deviations calculated for the predictions based on the parameters obtained from isocratic (**Figure 1B**) and gradient (**Figure 1C**) retention data, it is evident that prediction based on the gradient retention parameters are more accurate (deviations from -4.2 % to -0.75 %). The large errors of the prediction based on isocratic data for very steep gradients can be experienced in HILIC due to the fitting errors of the model used [23]. The **Figure 2** shows the arrangement of the results for the mobile phase containing 30 mmol·L^{-1} ammonium acetate. Also with this mobile phase additive it is confirmed that with increasing steepness of the predicted gradient profile based on the isocratic retention data, the accuracy of prediction is improving. In general, prediction for the separations with mobile phase containing 30 mmol·L^{-1} ammonium acetate are better compared to the predictions for the separation with mobile phase containing 0.1 % (v/v) acetic acid. The relative deviations are between -1.72 % and 2.73 %. The more accurate predictions of retention data can be attributed to the slightly better accuracy of the fitting of isocratic retention model using non-linear regression [14].

Figure 3 shows comparison of the deviations between $t_{R,pred}$ and $t_{R,exp}$ for native and derivatized maltooligosaccharides. Predictions for the separations with the mobile phase containing 30 mmol·L^{-1} ammonium acetate (**Figure 3A**) have deviations between -2 % and

5 %. 2-AB derivatives show the highest deviations between the predicted and experimental retention times. **Figure 3B** shows deviations for separation with the mobile phase containing 30 mmol·L^{-1} ammonium acetate, from -3.93 % to 0.34 %. Also in the case of derivatized maltooligosaccharides, better results were obtained from predictions for the separations with mobile phase containing 30 mmol·L^{-1} ammonium acetate. The less accurate predictions were calculated for 2-AB and 2-AP derivatives, especially for the first and for the second eluting oligomer (maltose and maltotriose).

3.2 Predictions based on extended mixed-mode model

To measure all isocratic retention data, which are needed for construction of the retention model, many hours of experiments are usually required. To speed up this process it is possible to obtain the retention data by using gradient elution. In the case of mixed-mode model, which contains three parameters (*a*, *b* and *c*), it is necessary to run at least three gradients with different steepness (different initial or final mobile phase composition, or gradient time). In the case of oligomeric mixed-mode model, only one gradient profile is sufficient to obtain the parameters a_2, b_2, c_2 and *d*, however, the accuracy of the parameters increases with the repeating analyses. Parameter *d* is describing the contribution of number of glucose units to the retention. The deeper discussion regarding influences of derivatization agents, stationary phases and mobile phases additives on the parameters of oligomeric mixed-mode model was provided in our preceding work [14]. Selected mixture of analytes contained the oligomers with n_{DP} 2 – 7, which is enough for obtaining of all four parameters. **Figure 4** shows comparison between the experimentally measured and simulated gradient separations of 2-AA derivatives. The gradient steepness is *B* = 2.6 (**Figure 4A**; gradient No. 1 in **Table 1**) and B = 1.4 (**Figure 4C**; gradient No. 4 in **Table 1**). The simulations were done based on the isocratic (red chromatogram) and the gradient (blue chromatogram) parameters, a_2, b_2, c_2 and

d. **Figure 4B** shows comparison of deviations between the predicted and experimental retention times for the gradient with B = 2.6 and **Figure 4D** shows deviations for the gradient with B = 1.4. The deviations for gradient separation of 2-AA derivatives with B = 2.6 are in the range between -2.02 % and 2.14 % (from isocratic retention data) and between -1.3 % and 3.37 % (from gradient retention data). Deviations for the gradient separation of 2-AA derivatives with less steep profile (B = 1.4) are in the range from 1.87 % to 11.47 % (from isocratic retention data) and from -3.1 % to 10 % (from gradient retention data). In general, the predictions based on the parameters obtained from the gradient retention data provide better results, similarly to the aforementioned mixed-mode model.

3.3 Comparison of extended and basic mixed-mode model

Isocratic retention data were fitted with the oligomeric mixed-mode model (Eq. (4)) and obtained parameters a_2, b_2, c_2 and d were used for the predictions of gradient retention times and the results were compared with prediction obtained by using the mixed-mode model (Eq. (1)). The **Figure 5** shows comparison of experimentally measured and simulated chromatograms by mixed-mode model (red chromatogram) and extended mixed-mode model (blue chromatogram) for native maltooligosaccharides (A) and 2-AA derivatives (C) and corresponding deviations between predicted and experimental retention times are shown in the **Figure 5B** (native maltologosaccharides) and **Figure 5D** (2-AA derivatives). Deviations between predicted and experimental retention times of native maltooligosacharides are in the range from 0.73 % to 3.64 % (mixed-mode model) and from 2.15 % to 9.32 % (oligomeric mixed-mode model). Deviations for predicted and experimental retention times for 2-AA derivatives are from -0.31 % to 2.48 % (mixed-mode model) and from 4.54 % to 14.66 % (oligomeric mixed-mode model). In overall, the gradient retention times predicted using both models were determined with acceptable accuracy, which indicates, that both models can be

suitable for optimization of gradient separations. Using the oligomeric mixed-mode model, the process is however significantly less time consuming. The retention model can however lose its practical applicability with increasing deviations of predicted data. For example, the error of retention time of 25% was obtained for one oligosaccharide under one gradient profile.

4 Conclusions

Predictions of the gradient retention times of native and derivatized maltooligisaccharides (2-AA, 2-AB, 2-AP a ANTS derivatives) separated in HILIC were done by the two non-linear retention models. The first model was mixed-mode and the second was mixed-mode model extended by parameter *d*. This parameter characterizes the contribution of number of glucose units to the retention. Both models were used for retention time modelling of isocratic and gradient retention data. For prediction and experimental work, several linear gradients with different initial and final mobile phase composition were selected with the gradient times 5, 7.5 or 10 min. The separations were done with two different mobile phase additives, 0.1 % (v/v) of acetic acid and 30 mmol·L^{-1} ammonium acetate.

Predicted retention times were compared with experimentally measured times and the deviations were calculated. More accurate results were obtained with predictions for the separations with mobile phase containing 30 mmol·L^{-1} ammonium acetate, which is in agreement with generally higher retention of the compounds using this additive in mobile phases. Gradient retention data for acidic derivatives of maltooligosaccharides were calculated with higher accuracy than for native compounds and neutral derivatives. The basic mixed-mode model provided the lower deviations between predicted and experimental retention times (maximal deviation 16 % for the mobile phase with 30 mmol·L^{-1} ammonium acetate). Predictions based on parameters obtained from gradient retention data were more

accurate compared to the isocratic retention data. The advantage of extended mixed-mode model is that to obtain parameters a_2, b_2, c_2 and d only one gradient is needed (for the basic mixed-mode model three gradients are needed).

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Figure captions

Fig. 1 Comparison of experimentally determined reduced retention times of native maltooligosaccharides (black lines) and reduced retention times predicted using isocratic (dash lines) and gradient (dotted lines) input data **(A)** in mobile phase with addition of 0.1 % (v/v) acetic acid. Calculated deviations between predicted and experimentally determined retention times obtained from isocratic **(B)** and gradient input data **(C)**. Gradient steepness *B* = 0.7 (gradient No. 6 in Table 1; square symbols), 1.1 (gradient No. 9 in Table 1; diamond symbols), 1.73 (gradient No. 11 in Table 1; triangle symbols) and 2.2 (gradient No. 13 in Table 1; circle symbols).

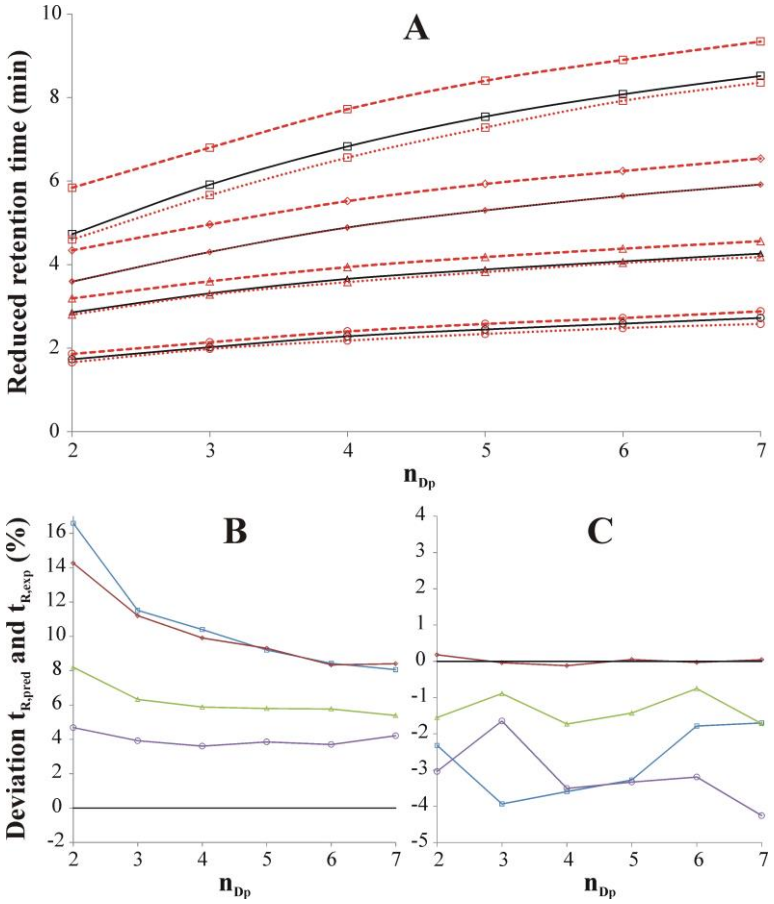

Fig. 2 Comparison of experimentally determined reduced retention times of native maltooligosaccharides (black lines) and reduced retention times predicted using isocratic (dash lines) and gradient (dotted lines) input data **(A)** in mobile phase with addition of 30 mmol·L^{-1} ammonium acetate. Calculated deviations between predicted and experimentally determined retention times using isocratic **(B)** and gradient input retention data **(C)**. Gradient steepness B = 0.7 (gradient No. 6 in Table 1; square symbols), 1.1 (gradient No. 9 in Table 1; diamond symbols), 1.73 (gradient No. 11 in Table 1; triangle symbols) and 2.2 (gradient No. 13 in Table 1; circle symbols).

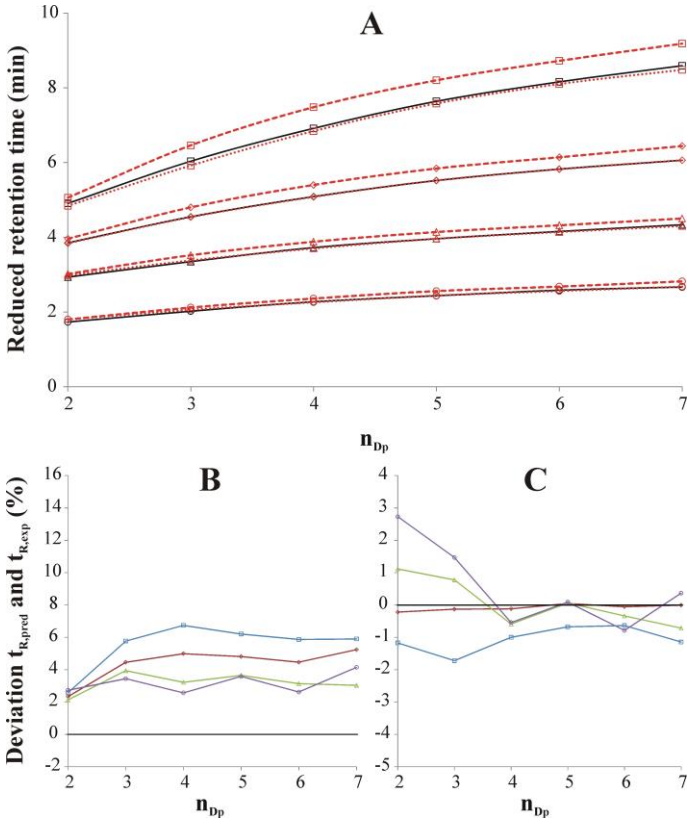

Fig. 3 Comparison of deviations of predicted retention times of native maltooligosaccharides (stars symbols) and maltooligosaccharides derivatized with different agents (ANTS – diamond symbols, 2-AA square symbols, 2-AB triangle symbols, 2-AP cross symbols). Predictions based on mixed-mode retention model (Eq. (1)) using parameters calculated from input data obtained using isocratic experiments **(A)** and gradient experiments **(B)**. Gradient No. 3, mobile phase with addition of 30 mmol·L^{-1} ammonium acetate.

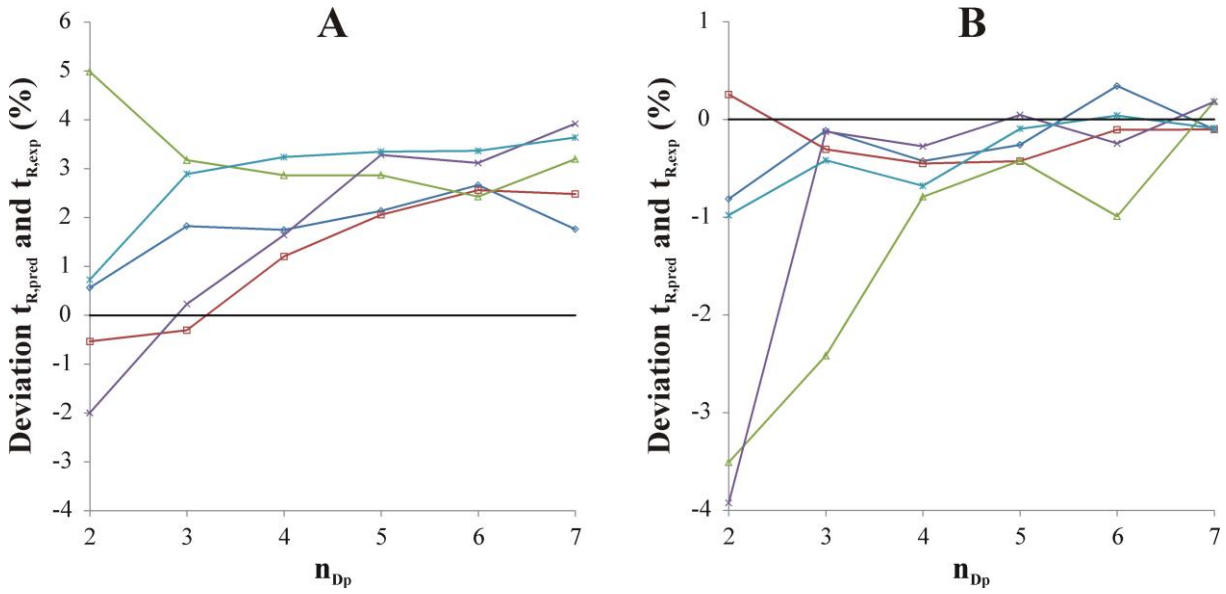

Fig. 4 Separation of 2-AA derivatives (black chromatogram) with gradient profiles No. 1 **(A)** and No. 4 **(C)**. Simulated chromatograms based on retention data predicted using oligomeric mixed-mode model with isocratic (red chromatograms) and gradient input data (blue chromatograms). Deviations of predicted retention times for gradients No. 1 **(B)** and No. 4 **(D)**, calculated from isocratic (red lines) and gradient input data (blue lines). Mobile phase containing 30 mmol·L^{-1} ammonium acetate.

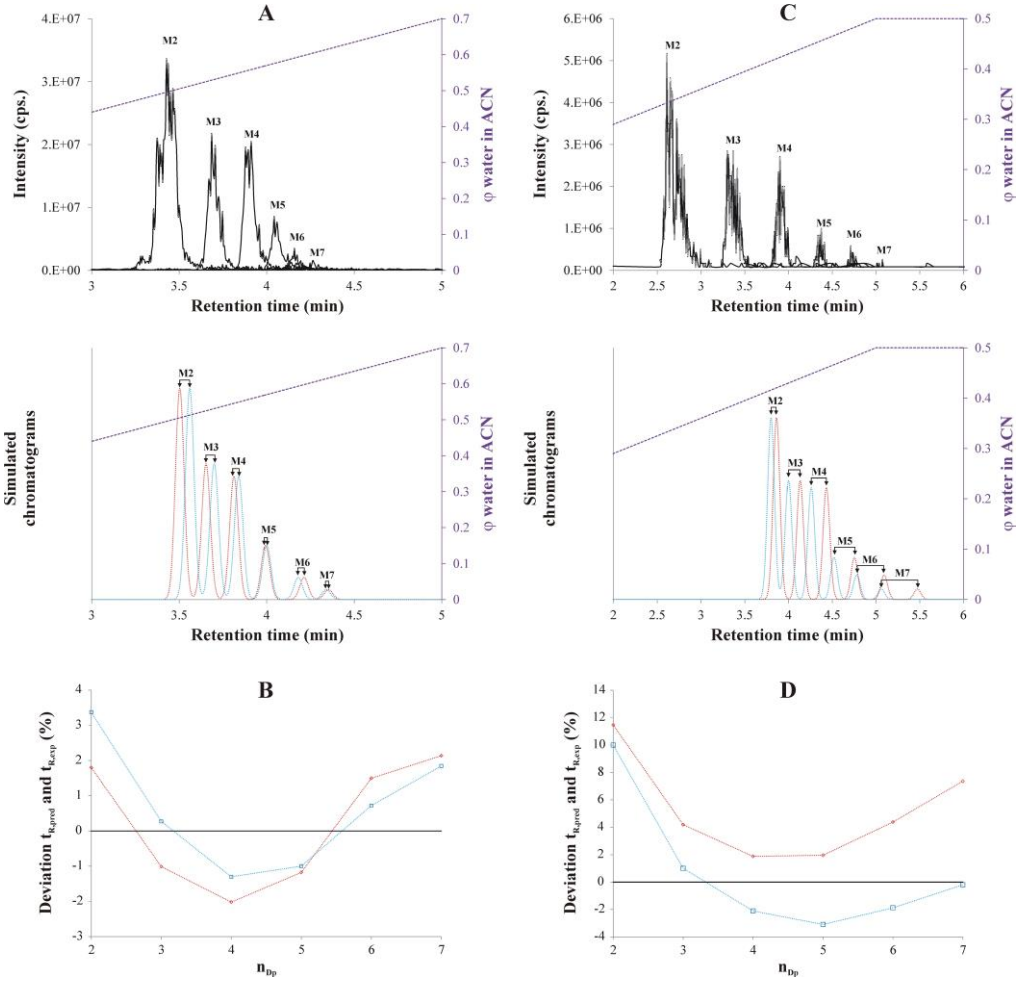

Fig. 5 Separation of native maltooligosaccharides **(A)** and their 2-AA derivatives **(C)** (black chromatograms). Simulated chromatograms based on retention data predicted using mixed-mode model (Eq. (1); red chromatograms) and using oligomeric mixed-mode model (Eq. (4); blue chromatograms). Deviations of predicted retention times of native maltooligosaccharides **(B)** and their 2-AA derivatives **(D)**, calculated for mixed-mode model (red lines) and oligomeric mixed-mode model (blue lines). Gradient profile No. 3, mobile phase with addition of 30 mmol·L^{-1} ammonium acetate.

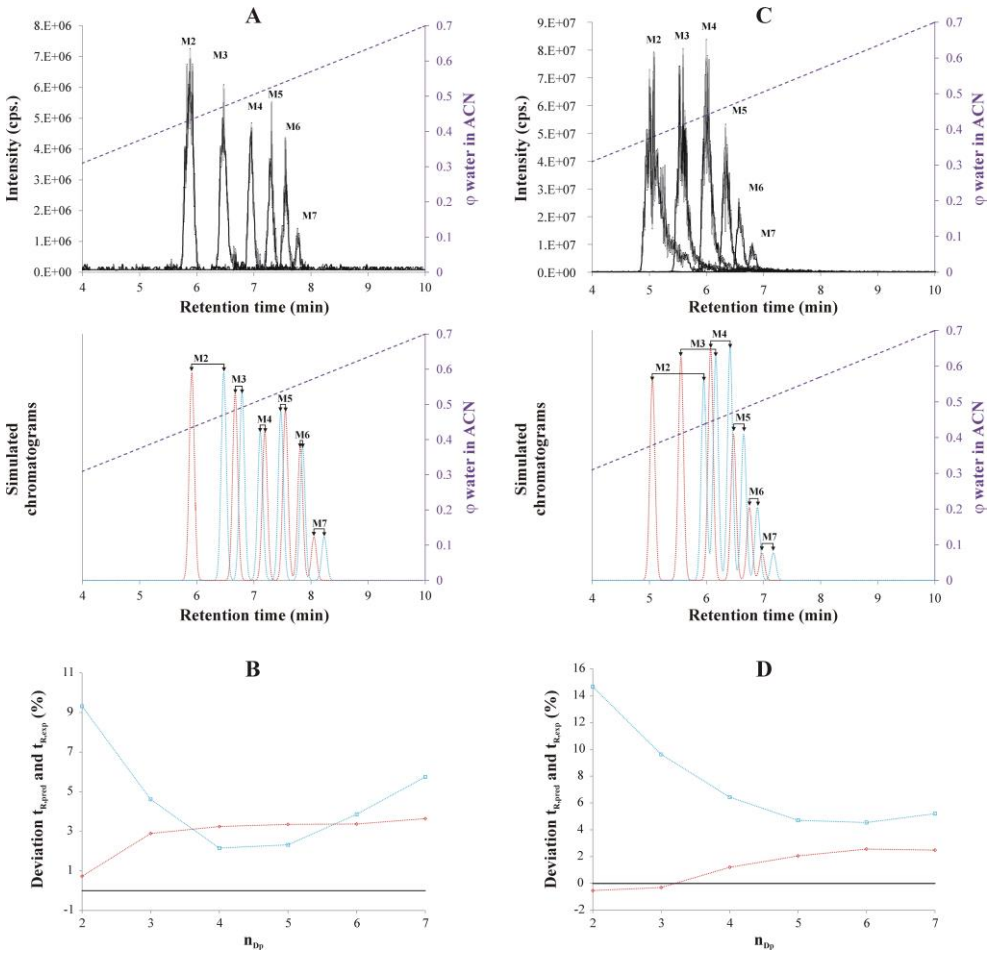

Table 1 Description of linear gradient profiles used; φ_0 – initial concentration of water in mobile phase, φ_G – final concentration of water in mobile phase, t_G – gradient time, V_G – volume of the mobile phase pumped through the column during gradient, B – gradient steepness.

Gradient No.	φ_0	φ_G	t_G ***(min)***	V_G ***(mL)***	B ***(mL^{-1})***
1	0.05	0.7	5.00	0.250	2.60
2			7.50	0.375	1.73
3			10.00	0.500	1.30
4	0.15	0.5	5.00	0.250	1.40
5			7.50	0.375	0.93
6			10.00	0.500	0.70
7	0.15	0,7	5.00	0.250	2.20
8			7.50	0.375	1.47
9			10.00	0.500	1.10
10	0.15	0.8	5.00	0.250	2.60
11			7.50	0.375	1.73
12			10.00	0.500	1.30
13	0.25	0.8	5.00	0.250	2.20
14			7.50	0.375	1.47
15			10.00	0.500	1.10