



# Combined effects of mobile phase composition and temperature on the retention of homologous and polar test compounds on polydentate C<sub>8</sub> column

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## ABSTRACT

Combined effects of temperature and mobile phase on the reversed phase chromatographic behavior of alkylbenzenes and simple substituted benzenes were investigated on a Blaze C<sub>8</sub> polydentate silica-based column, showing improved resistance against hydrolytic breakdown at temperatures higher than 60 °C, in comparison to silica-based stationary phases with single attachment sites. For better insight into the retention mechanism on polydentate columns, we determined the enthalpy and entropy of the transfer of the test compounds from the mobile to the stationary phase. The enthalpic contribution dominated the retention at 80% or lower concentrations of methanol in the mobile phase. Entropic effects are more significant in 90% methanol and in acetonitrile–water mobile phases. Anomalies in the effects of mobile phase on the enthalpy of retention of benzene, methylbenzene and polar benzene derivatives were observed, in comparison to regular change in enthalpy and entropy of adsorption with changing concentration of organic solvent and the alkyl length for higher alkylbenzenes. The temperature and the mobile phase effects on the retention are practically independent of each other and – to first approximation – can be described by a simple model equation, which can be used for optimization of separation conditions.

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## 1. Introduction

Even though much less used than in GC, temperature is an important parameter affecting separation efficiency, retention and selectivity in HPLC [1,2]. Using high temperature in HPLC is attractive as it offers faster analysis due to the reduction in mobile phase viscosity and enhanced solute diffusion. Further, it may improve the peak shapes, it needs less organic solvent in the mobile phase to accomplish the analysis and – last but not least – it allows alternative approaches to optimization of separation selectivity, which usually slightly decreases, but may more or less significantly change at elevated temperatures [3]. The beneficial effects of high temperature are especially advantageous in HPLC of large molecules [4,5]. In reversed-phase chromatography, a 4–5 °C increase in temperature causes a decrease in retention corresponding approximately to a 1% increase in concentration of methanol or acetonitrile [6], hence the temperature control is usually less effective a tool for controlling the retention than adjusting the composition of the mobile phase.

In recent years simultaneous optimization of temperature and mobile phase composition, or of the temperature and gradient profile in HPLC has been found useful in method development [7–11]

and high temperature operation is attracting increased popularity in LC separations. The regulation of temperature is very convenient and simple, as it requires only a column thermostat allowing fast enough equilibration over the working temperature range. The operation at elevated temperature is facilitated by efficient pre-heating of the mobile phase to the column temperature.

In addition to the restrictions imposed by low boiling temperatures of some solvents and by the thermal sample instability, the main reason for relatively rare use of temperature as the operation parameter in HPLC is probably limited resistance of many silica-based chemically bonded stationary phases against hydrolysis at the temperatures higher than 60 °C, especially in aqueous mobile phases at pH below 3 or above 8. Hence, high-temperature HPLC applications are still rare and – until recently – have been restricted to stationary phases based on non-silica supports such as graphitized carbon, zirconium oxide based phases or polystyrene/divinylbenzene copolymers [4,12], which unfortunately usually show lower separation efficiency (HETP) than the silica-based column materials. Recently introduced silica-based polydentate reversed-phase materials with multiple-site bonded group attachment to the silica gel support show high efficiency and can be used even at temperatures over 100 °C [11]. It is believed that the polydentate bonded ligands may shield the surface of the silica gel support and protect it against hydrolysis at increased temperature.

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The type of the support and the chemistry of chemically bonded moieties have significant influence on the chromatographic properties of HPLC columns. However, in spite of the strong role of mobile phase in HPLC, simultaneous effects of the mobile phase and temperature on the retention mechanism have been relatively rarely studied in systematic manner [7–10]. The objective of the present study is the investigation of such combined effects on the retention of simple test compounds differing in polarities on a polydentate column with extended temperature stability, to elucidate possible differences in the retention mechanism at elevated temperatures. In the frame of this study, we focused attention on thermodynamic data and on the characteristics of various descriptors characterizing non-polar and specific polar contributions to the retention of homologous alkylbenzenes and benzene derivatives with various polar substituents.

## 2. Theoretical

### 2.1. The retention in homologous series

In reversed-phase HPLC with binary aqueous–organic mobile phases, the effect of the volume fraction of the organic solvent in the mobile phase,  $\varphi$ , on the logarithm of the retention factor,  $k = (t_R - t_0)/t_0$ , can be described – to first approximation – by a simplified equation (Eq. (1)) often used in practice for method development and optimization [13,14]:

$$\ln k = a - m\varphi \quad (1)$$

$t_R$  is the solute retention time,  $t_0$  is the column hold-up time,  $a$  is the logarithm of the retention factor extrapolated to pure water as the mobile phase and  $m$  is the parameter characterizing the effect of the solvent on the retention (the solvent strength parameter).

In homologous or oligomer series, the parameters  $a$  and  $m$  of Eq. (1) regularly increase with increasing number of repeat monomer units,  $n$  [15–18].

$$a = a_0 + a_1 n \quad (2)$$

$$m = m_0 + m_1 n \quad (3)$$

Introducing Eqs. (2) and (3) characterizing the effect of the number of repeat methylene units in the homologous series on the parameters of Eq. (1), Eq. (4) was derived to describe the retention in a homologous or oligomer series in binary mobile phases with varying composition [18]:

$$\ln k = a_0 + a_1 n - (m_0 + m_1 n)\varphi = \ln \beta + n \ln \alpha \quad (4)$$

Here,  $\ln \alpha = (a_1 - m_1\varphi)$  is a measure of the repeat group (e.g., methylene) selectivity, i.e., the contribution of a methylene group to the energy of retention in a homologous series (in terms of  $\ln k$ ) and  $\ln \beta = (a_0 - m_0\varphi)$  characterizes the contribution of the non-repeat part of the molecule (the end-group) to the retention (such as, e.g., the phenyl group in the alkylbenzene series). The experimental parameters  $a_1$ ,  $m_1$  of Eq. (4) for homologous alkylbenzenes, can be used as a measure of the contributions of water and of the organic solvent, respectively, on the reversed-phase lipophilic (methylene) selectivity, and the parameters  $a_0$ ,  $m_0$ , to characterize the contributions of water and of the organic solvent in the mobile phase on the end-group (phenyl) contributions to the  $\ln k$  in binary aqueous–organic mobile phases [18].

### 2.2. Temperature effects – the enthalpy and entropy of retention

The retention in reversed-phase liquid chromatography usually decreases at elevated temperatures, presumably by reducing the difference in cohesive energy and hydrogen-bonding interactions

between the mobile and the stationary phases. Other polar interactions are believed to be less affected by temperature variation in reversed-phase liquid chromatography, which therefore has the largest effect on the retention of compounds that differ in size and hydrogen-bonding basicity. Consequently, significant temperature effects on separation selectivity can be expected when the relative retention of two solutes is sensitive to conformation changes of the stationary phase at varying temperature, especially for partly ionized solutes or solutes significantly differing in the sizes or shapes of molecules, leading to differences in the entropy of retention [9].

The distribution constant  $K = e^{-(\Delta G/RT)}$  depends on the change of the Gibbs energy connected with the solute retention,  $\Delta G^0$ , and is related to the corresponding changes of entropy,  $\Delta S^0$ , and enthalpy,  $\Delta H^0$ , of the transfer of the solute from the mobile to the stationary phase:

$$\Delta G^0 = -RT \ln K = \Delta H^0 - T\Delta S^0 \quad (5)$$

If a single retention mechanism controls the retention over a broad temperature range, the effect of temperature, on the retention factor,  $k$ , can be described by van't Hoff equation [19]:

$$\begin{aligned} \ln k &= \ln K + \ln \frac{V_S}{V_M} = -\frac{\Delta G^0}{RT} + \ln \frac{V_S}{V_M} = \frac{\Delta S^0}{R} + \ln \frac{V_S}{V_M} - \frac{\Delta H^0}{RT} \\ &= A_i + \left(\frac{B_i}{T}\right) \end{aligned} \quad (6)$$

In such a case, the  $\ln k$  versus  $1/T$  plots are linear, the slope parameter  $B_i$  being proportional to the standard partial molar enthalpy of transfer of the solute  $i$  from the mobile phase to the stationary phase,  $-\Delta H^0$ ; the parameter  $A_i$  includes the standard partial molar entropy of the transfer of the solute  $i$  from the mobile phase to the stationary phase,  $\Delta S^0$ , and the phase ratio (the ratio of the volumes of the stationary,  $V_S$ , and of the mobile,  $V_M$ , phases) in the chromatographic system.  $R$  is the gas constant and  $T$  is the thermodynamic temperature (in Kelvin) [19–22]. Hence, by plotting  $\ln k$  versus  $1/T$  over a sufficiently broad temperature range, one may calculate the enthalpic and the entropic contributions to chromatographic retention and selectivity,  $-\Delta H^0$  from the slope and  $\Delta S^0$  from the intercept of the plot. Linearity or non-linearity of van't Hoff plots may provide information on whether or not the retention mechanism(s) may change over the studied temperature range [19,22–27]. As  $\ln k$  increase in linear manner with  $-\Delta H^0$  (Eq. (7)), the slope of the plot of  $\ln k$  versus  $\Delta H^0$ , measured at several different temperatures, should be the same for various compounds, if a single mechanism controls the retention, such as in a homologous series [27].

The calculation of the entropic contribution to the retention from the intercept term,  $A_i$ , of Eq. (6) requires the numerical value of the phase ratio in the column to be known, which is not straightforward. The first problem is that it is generally impossible to determine correctly the boundary between the region occupied by the stationary and the mobile phase in the column, without adopting some convention [28]. The volume of the mobile phase in the column,  $V_M$ , may not correspond exactly to the column hold-up volume necessary for the calculation of the retention factor,  $k$ , which is usually determined from the elution time of a non-retained marker compound,  $t_0$ . The determination of the volume of the stationary phase,  $V_S$ , is even more complicated and controversial issue. Rigorous considerations relate  $V_S$  to the surface area of the stationary phase, to the carbon content, the density or the mass of the stationary phase in the column. Neither quantity is directly measurable with a packed column, and calculations based on the data published by manufacturers (not always available) may not be reliable; further, batch measurements are time consuming and rarely possible, as few HPLC column manufacturers supply bulk materials. Hence, it seems that – even though theoretically not rigorous – the most

practical way to overcome this problem is to accept the convention relating the sample concentration in the stationary phase to the space in the column non-occupied by the bulk mobile phase, i.e., the difference of the bulk volume of the column and the column occupied by the mobile phase, even though it is clear that the adsorbed sample is accumulated only in a relative narrow surface layer on (or close to) the adsorbent surface inside the pores. Then the volume of the stationary phase can be calculated from the difference of the geometrical inner volume of the empty column,  $V_C$ , and the column hold-up volume,  $V_0$ :  $V_S = V_C - V_0$ . This simplified convention is often accepted – even though not explicitly stated – when calculating the adsorption isotherms in liquid media. Anyway, this is the reason why the phase ratio is usually included in the entropic term when presenting the liquid chromatography thermodynamic data. Assuming that the phase volume ratio,  $V_S/V_M$ , in the column is approximately independent of the mobile phase composition and including the whole part of the column inaccessible to the marker compound as the stationary phase, the phase volume ratio can be, to first approximation, estimated as:

$$\frac{V_S}{V_M} = \frac{1 - \varepsilon_T}{\varepsilon_T} \quad (7)$$

$\varepsilon_T$  is the total column porosity.

Deviations of the retention and/or selectivity from the van't Hoff rule (Eq. (6)) occasionally observed at elevated temperatures are attributed to secondary retention mechanisms, reduced ionization, changes in the sample solubility in the mobile phase at higher temperatures and – less often – to the temperature effects on the phase ratio [21,29–32]. In reversed-phase chromatography, the experimentally observed increase in  $\ln k$  with  $-\Delta H^0$  is often much greater than expected from the slope of Eq. (6),  $(1/RT)$ , which is attributed to changes in the entropy of retention, caused by structural modifications of the sample molecules connected with the changes in the retention enthalpy.

Linear relationships between  $-\Delta H^0$  and  $\Delta S^0$  (Eq. (8)) were found for reversible adsorption of solutes to alkyl bonded stationary phases [27], referred to as enthalpy–entropy compensation plots, which represent an example of a linear free energy relationship, a useful tool for establishing the similarity of the retention mechanism for different solutes:

$$\Delta H^0 = T_C \Delta S^0 + \Delta G_\beta^0 \quad (8)$$

$T_C$  is so-called compensation temperature, at which the enthalpic and the entropic contribution to the energy of retention  $\Delta G_\beta^0$  compensate each other when the process is governed by a single retention mechanism. Assuming such common retention mechanism giving rise to the entropy–enthalpy compensation (a constant  $\Delta G_\beta^0$ ) in a homologous series or a series of other structurally related compounds, the combination of Eqs. (6) and (8) yields Eq. (9), relating the logarithms of the retention factors of the individual solutes,  $k_T$ , measured at a constant temperature,  $T$ , to the enthalpy of retention,  $-\Delta H^0$ , which is independent of  $T$ . In such a series, the compensation temperature,  $T_C$ , can be calculated from the slope of Eq. (9) [27]:

$$\ln k_T = -\frac{\Delta H^0}{R} \left( \frac{1}{T} - \frac{1}{T_C} \right) - \frac{\Delta G_\beta^0}{RT_C} + \ln \frac{V_S}{V_M} \quad (9)$$

Finally, the combination of Eqs. (1) and (6) yields Eq. (10), which can be used for the description of simultaneous effects of the mobile phase and temperature on the retention in reversed-phase systems [33]:

$$\ln k = A_1 + \frac{A_2}{T} + A_3\varphi + \frac{A_4\varphi}{T} \quad (10)$$

**Table 1**

Analytes tested and their interaction (polarity) indices,  $I_x$  [18,34].

Analyte	Symbol	$I_x$
Uracil, $V_M$ marker	UR	
Benzene	BE	–
Toluene	MB	–
Ethylbenzene	EB	–
Propylbenzene	PB	–
Butylbenzene	BB	–
Amylbenzene	AB	–
Hexylbenzene	HB	–
Benzonitrile	BN	5.32
Nitrobenzene	NB	4.49
Anisole	ANS	3.85
<i>p</i> -Dichlorobenzene	DCB	1.05
3-Chlorotoluene	CT	2.20
Acetophenone	ACE	5.60
2-Bromonitrobenzene	BNB	3.44

### 3. Experimental

#### 3.1. Equipment

A liquid chromatograph was assembled from an LC-10AD pump (Shimadzu, Kyoto, Japan), a UV detector (LCP 2564, ECOM, Prague, Czech Republic) operated at 254 nm and a model 7125 manual sample valve injector with a 10- $\mu$ L loop (Rheodyne, Berkeley, CA, USA). A personal computer with a CSW chromatographic data station was used to collect the detector data. The column was placed in a column thermostat (ECOM, Prague, Czech Republic), which enables keeping the pre-set constant temperature within  $\pm 0.1$  °C temperatures up to 95 °C.

#### 3.2. Materials and reagents

A Blaze C<sub>8</sub> (100 mm  $\times$  4.6 mm, 3  $\mu$ m) polydentate silica-based column was purchased from Selerity Technologies Inc. (Salt Lake City, UT, USA). Methanol and acetonitrile of HPLC grade (Lichrosolv) were obtained from Merck (Darmstadt, Germany), distilled water was purified using a Milli-Q water purification equipment (Millipore Intertech, Bedford, MA, USA). The mobile phases were filtered using a Millipore (Bedford, MA, USA) 0.45- $\mu$ m filter and degassed in ultrasonic bath before use.

Alkylbenzenes and non-homologous simple benzene derivatives used as the test standards were purchased from Merck (Darmstadt, Germany). The interaction indices of the benzene derivatives from the literature [18,34] are listed in Table 1.

#### 3.3. Methods

The column hold-up volume,  $V_0$ , was determined using uracil as non-retained marker. In all experiments, the columns were first equilibrated with approximately 20 column hold-up volumes,  $V_0$ , of the mobile phase. The samples were dissolved in the mobile phase and 10  $\mu$ L sample volumes were injected in each experiment. The retention times,  $t_R$ , were measured at three various concentrations of acetonitrile and methanol in water at various temperatures and converted to the retention volumes,  $V_R$ . Arithmetic means of three repeated measurements were used to calculate the retention factors,  $k = (V_R/V_0 - 1)$ . The best-fit parameters of Eqs. (1), (4)–(10) were determined by linear, multi-linear and non-linear regression of the experimental data using the Adstat software (Trilobyte, Prague, Czech Republic). All logarithmic data in this work are presented as natural logarithms,  $\ln(x)$ .

**Table 2**

Parameters of Eq. (4):  $a_0$ ,  $a_1$ ,  $m_0$ ,  $m_1$ , determined by regression analysis of the experimental retention data of alkylbenzenes.

Parameter	ACN			MeOH		
	40 °C	60 °C	80 °C	40 °C	60 °C	80 °C
$a_0$	1.61	1.46	1.51	1.80	1.87	1.70
$a_1$	0.33	0.32	0.33	0.53	0.48	0.46
$R^2$ (Eq. (2))	0.9999	0.9999	0.9999	1.0000	0.9989	0.9999
$m_0$	2.42	2.29	2.41	2.44	2.60	2.42
$m_1$	0.27	0.25	0.27	0.47	0.41	0.40
$R^2$ (Eq. (3))	0.9994	0.9993	0.9992	0.9999	0.9971	0.9997

ACN, 50–70% acetonitrile/water; MeOH, 70–90% methanol/water;  $R^2$ , correlation coefficient;  $n = 18$  at each temperature.

#### 4. Results and discussion

The retention of alkylbenzenes and non-homologous benzene derivatives was investigated in the temperature range of 40–80 °C in binary mobile phases containing 50–70% acetonitrile and 70–90% methanol in water. The retention data were not measured at lower temperatures, which can be affected by significant deviations, if the setting is not at least 10 °C above the ambient, when using an air-circulated thermostat without external cooling facility. In this work, the average column hold-up volume determined using uracil as the marker compound at all temperatures tested was  $1.07 \pm 0.01$  mL in mobile phases containing methanol in water, corresponding to the total column porosity  $\varepsilon_T = 0.64$ ; and  $0.93 \pm 0.01$  mL in mobile phases containing acetonitrile in water ( $\varepsilon_T = 0.56$ ). The differences in porosity can be probably attributed to stronger adsorption of acetonitrile in the stationary phase in comparison to methanol.

##### 4.1. Homologous alkylbenzenes

The parameters  $a_0$ ,  $a_1$ ,  $m_0$ ,  $m_1$  of Eq. (4) for alkylbenzene series determined in aqueous–organic mobile phases with varying concentrations of ACN and MeOH in water are listed in Table 2. The correlation coefficients  $R^2$  close to 1 prove the validity of Eq. (4) for the column tested in the temperature range between 40 and 80 °C, both in acetonitrile–water and in methanol–water mobile phases.

The data in Table 2 show that temperature has only minor effect on the parameters of Eq. (4).

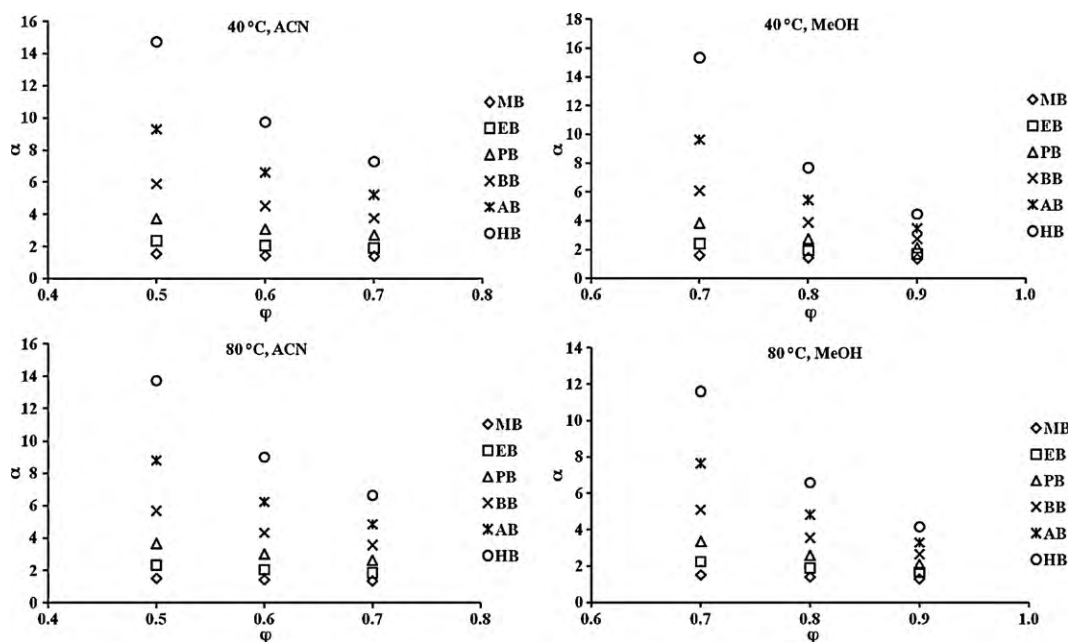
The selectivity (relative retention),  $\alpha$ , in the alkylbenzene series, related to benzene, decreases with increasing concentration of organic solvent in the mobile phase (Fig. 1). At the same concentration (70%) and temperature, methanol provides higher methylene selectivity in the homologous alkylbenzene series than acetonitrile; the homologous selectivity is comparable in 70% acetonitrile and in 80% methanol. The temperature increase from 40 to 80 °C causes a minor decrease in the relative retention in acetonitrile–water mobile phases and a more significant decrease in methanol–water mobile phases.

Table 3 shows the results of the regression of the experimental retention–temperature dependences for the analytes tested: the best-fit values of the intercepts,  $A_i$ , the slopes,  $B_i$ , of Eq. (6) and the correlation coefficients,  $R^2$ . Linear  $\ln k$  versus  $1/T$  plots with correlation coefficients close to 1 were observed for alkylbenzenes in 70% acetonitrile and in 70% methanol, demonstrating the validity of the van't Hoff model, suggesting that a single retention mechanism controls their retention over the experimental temperature range 40–80 °C. The correlation coefficients decrease and the linearity of the experimental plots impairs to some extent in mobile phases with higher concentrations of methanol (80–90%), but even more significantly at lower concentrations of acetonitrile (60% and 50%), which may indicate more or less significant change in the retention mechanism when varying the experimental temperature. Possibly, secondary interactions contribute to the retention controlled primarily by the hydrophobic effect at lower acetonitrile concentrations and at high methanol concentrations.

Eq. (6) can be re-written as Eq. (11):

$$\ln k = c + d \left( \frac{-\Delta H^0}{RT} \right) \quad (11)$$

In agreement with the van't Hoff model, the experimental values of  $d$  are close to 1, in between 1.10 and 1.12 for all alkylbenzenes in all mobile phases tested (Table 3). The intercepts ( $c$ ) of Eq. (11) are almost independent of the size of alkylbenzenes in 70% methanol, (except for benzene) and in 80% methanol, (except for benzene and toluene), so that the experimental data for all alkylbenzenes fit the



**Fig. 1.** Effects of the temperature,  $T$ , and volume fraction of the organic solvent,  $\phi$ , on the relative retention of homologous alkylbenzenes, related to benzene ( $\alpha = k_i/k_{BE}$ ).

**Table 3**  
Parameters  $A_i$ ,  $B_i$  of Eq. (6) and parameters  $c$ ,  $d$  of Eq. (11) for homologous alkylbenzenes in A: acetonitrile/water (ACN) and B: methanol/water (MeOH) mobile phases.

	50% ACN					60% ACN					70% ACN				
	$B_i$	$A_i$	$R^2$	$c$	$d$	$B_i$	$A_i$	$R^2$	$c$	$d$	$B_i$	$A_i$	$R^2$	$c$	$d$
(A)															
BE	571.1	-0.87	0.9093	-1.08	1.12	581.6	-1.48	0.9312	-1.69	1.11	557.9	-1.95	0.9755	-2.12	1.10
MB	603.9	-0.55	0.8620	-0.77	1.12	591.0	-1.16	0.8969	-1.37	1.12	608.4	-1.79	0.9991	-1.99	1.10
EB	590.0	-0.09	0.8435	-0.31	1.12	609.8	-0.86	0.8845	-1.08	1.12	594.9	-1.45	0.9961	-1.64	1.11
PB	623.6	0.27	0.8281	0.03	1.12	638.6	-0.56	0.8680	-0.79	1.12	631.2	-1.21	0.9949	-1.42	1.11
BB	659.2	0.61	0.8255	0.36	1.12	684.6	-0.32	0.8731	-0.57	1.12	684.9	-1.05	0.9954	-1.27	1.11
AB	702.2	0.92	0.8258	0.66	1.12	724.0	-0.06	0.8769	-0.33	1.12	731.7	-0.87	0.9975	-1.11	1.11
HB	748.1	1.23	0.8300	0.95	1.12	778.0	0.15	0.8880	-0.13	1.12	791.4	-0.72	0.9985	-0.98	1.11
BN	632.5	-1.58	0.9438	-1.80	1.11	650.7	-2.20	0.9853	-2.42	1.11	674.2	-2.82	0.9932	-3.03	1.10
NB	708.0	-1.54	0.9743	-1.49	1.11	645.8	-1.94	0.9710	-2.16	1.11	670.7	-2.60	0.9985	-2.81	1.10
ANS	596.0	-1.03	0.9030	-1.24	1.12	575.0	-1.55	0.9444	-1.75	1.11	619.6	-2.25	0.9987	-2.44	1.10
DCB	647.1	-0.21	0.8662	-0.45	1.12	614.8	-0.82	0.9143	-1.04	1.12	628.3	-1.49	0.9999	-1.69	1.10
CT	646.3	-0.22	0.8768	-0.46	1.12	598.9	-0.79	0.9108	-1.00	1.12	622.2	-1.49	0.9999	-1.69	1.10
ACE	533.2	-1.37	0.9645	-1.56	1.11	529.9	-1.89	0.9571	-2.08	1.11	641.8	-2.75	0.9830	-2.94	1.10
BNB	671.2	-1.01	0.9053	-1.25	1.12	653.0	-1.62	0.9503	-1.85	1.11	683.2	-2.38	0.7888	-2.64	1.12
	70% MeOH					80% MeOH					90% MeOH				
	$B_i$	$A_i$	$R^2$	$c$	$d$	$B_i$	$A_i$	$R^2$	$c$	$d$	$B_i$	$A_i$	$R^2$	$c$	$d$
(B)															
BE	544.3	-1.48	0.9692	-1.64	1.10	573.9	-2.02	0.9989	-2.33	1.10	528.3	-2.62	0.9991	-2.79	1.10
MB	636.5	-1.33	1.0000	-1.53	1.10	586.4	-1.86	0.9915	-2.05	1.11	602.9	-2.58	0.9962	-2.78	1.11
EB	739.4	-1.23	0.9999	-1.47	1.10	637.8	-1.71	0.9717	-1.93	1.11	557.9	-2.22	0.9537	-2.42	1.11
PB	868.8	-1.19	0.9996	-1.47	1.10	715.3	-1.61	0.9720	-1.86	1.11	564.9	-2.02	0.8819	-2.22	1.12
BB	994.4	-1.12	0.9995	-1.45	1.10	792.0	-1.52	0.9616	-1.79	1.11	612.8	-1.92	0.8781	-2.14	1.12
AB	1117.4	-1.06	0.9995	-1.42	1.10	873.2	-1.44	0.9572	-1.74	1.11	662.8	-1.83	0.9288	-2.07	1.11
HB	1242.3	-0.99	0.9995	-1.40	1.10	964.2	-1.38	0.9596	-1.72	1.11	705.9	-1.71	0.9877	-1.95	1.11
BN	743.1	-2.77	0.8915	-2.98	1.09	719.2	-3.25	0.8787	-3.46	1.09	796.1	-4.13	0.9979	-4.38	1.10
NB	826.7	-2.66	0.9536	-2.91	1.10	674.2	-2.80	0.9394	-3.00	1.09	557.9	-3.07	0.9741	-3.26	1.11
ANS	737.5	-2.11	0.9966	-2.34	1.10	584.0	-2.27	1.0000	-2.45	1.10	557.9	-2.80	0.8512	-3.00	1.12
DCB	869.6	-1.59	0.9985	-1.87	1.11	613.8	-1.65	0.9879	-1.86	1.11	576.3	-2.32	0.8649	-2.54	1.12
CT	891.7	-1.62	0.9977	-1.91	1.11	649.4	-1.73	0.9874	-1.95	1.11	557.9	-2.24	0.8646	-2.44	1.12
ACE	858.7	-2.95	0.9745	-3.21	1.10	557.9	-2.63	0.8952	-2.79	1.09	628.3	-3.40	0.9752	-3.61	1.11
BNB	824.1	-2.33	1.0000	-2.59	1.10	633.6	-2.48	0.978	-2.68	1.10	670.7	-3.30	0.9886	-3.53	1.11

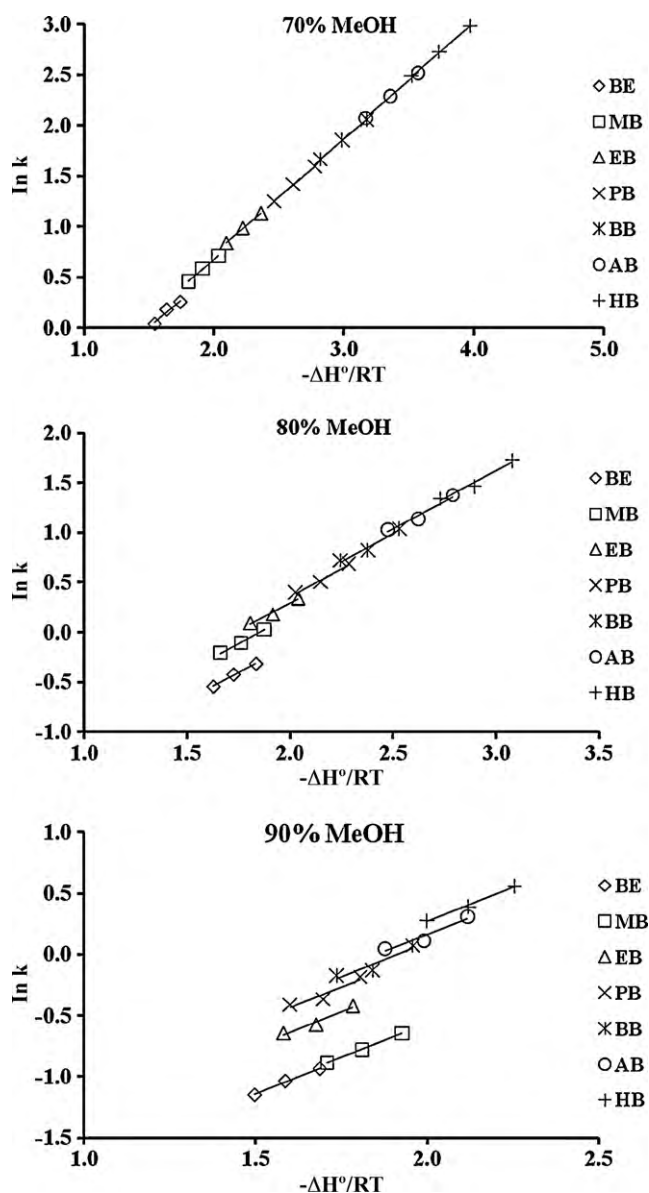
Eq. (6): slope  $B_i = -\Delta H^0/R$  [ $\text{J mol}^{-1} \text{K}^{-1}$ ]; intercept  $A_i = \Delta S^0/R + \ln V_S/V_M$  [ $\text{J mol}^{-1} \text{K}^{-1}$ ];  $R^2$ , correlation coefficient; Eq. (11): slope  $d$ , intercept  $c$ .

same plot (Fig. 2). The differences in the values of  $c$  are more significant in 90% methanol (Fig. 2) and in all acetonitrile–water mobile phases (Fig. 3). As the intercept term  $c$  includes the entropic contributions to retention, the plots in Figs. 2 and 3 illustrate increasing entropic contributions to the retention of higher alkylbenzenes in 90% methanol and all acetonitrile–water mobile phases. This behavior may be possibly attributed to the differences in solvation of the sample alkyl chains in the stationary and in the bulk mobile phases. The experimental results suggest that all alkylbenzenes may be similarly solvated in 70% and 80% methanol bulk mobile phases as when retained in the Blaze  $C_8$  stationary phase; however more significant differences in the solvation of the alkyls are likely between the stationary phase and other bulk mobile phases.

Using linear regression of the experimental plots of  $\ln k$  versus  $1/T$  in the temperature range 40–80 °C, the enthalpic contributions to retention,  $-\Delta H^0$ , were calculated from the slopes  $B_i$  and the entropic contributions,  $\Delta S^0$ , from the intercepts  $A_i$  of the van't Hoff (Eq. (6)). Fig. 4 illustrates the effects of the concentration of the organic solvents in the mobile phase on the enthalpic and entropic contributions to the retention. In agreement with the earlier results of other authors [33], the enthalpic contributions to the retention ( $B_i = -\Delta H^0/RT$ ) regularly increase for higher alkylbenzenes in the mobile phases investigated, except for benzene in aqueous methanol and methylbenzene in aqueous acetonitrile (Fig. 4). The entropic contributions to the retention ( $A_i = \Delta S^0/R + \ln V_S/V_M$ ) are negative for all alkylbenzenes in aqueous methanol and in 70% acetonitrile, corresponding probably to more regular conformation of adsorbed molecules with respect to the bulk solution. The enthalpic contributions to the retention decrease with increasing concentration of methanol, but are not significantly affected by

the concentration of acetonitrile, Fig. 4 indicates that the entropic contributions decrease at higher concentrations of the organic solvent. Interestingly, the effects of the concentration of organic solvent on the entropic contribution are more significant for higher alkylbenzenes in aqueous acetonitrile, but are almost independent of alkyl length in aqueous methanol (except for benzene and toluene). Increasing entropic contributions to the retention of higher alkylbenzenes (Fig. 4) can be probably explained by less regular conformation of adsorbed compounds with longer alkyl chains (probably due to increased freedom of movement in the adsorbed solvent layer) with respect to lower alkylbenzenes. These results demonstrate different effects of the solvation on the retention of alkylbenzenes on the bidentate Blaze  $C_8$  stationary phase in aqueous mobile phases containing acetonitrile and methanol.

In the presence of the entropy–enthalpy compensation behavior, Eq. (9) predicts a linear increase of the logarithms of the retention factors,  $k_T$ , of related compounds with increasing enthalpic contribution to the retention, at a constant temperature [27]. Indeed, the experimental plots of  $C_1$ – $C_5$  alkylbenzenes measured at constant temperatures show excellent linearity, with slight deviations for the first member of the series, toluene, in all mobile phases studied (Fig. 5). According to Eq. (9), the slopes and the intercepts of the plots should not depend on the experimental temperature at a constant composition of the mobile phase. In agreement with theory, the regression parameters of the experimental plots in Fig. 5 measured in various mobile phases were very similar regardless of the experimental temperature, 40, 60 and 80 °C, with the differences less than 2% for the intercepts and less than 5% for the slopes. Interestingly, Fig. 5 shows that the slopes of Eq. (9) increase at higher concentration of methanol, but

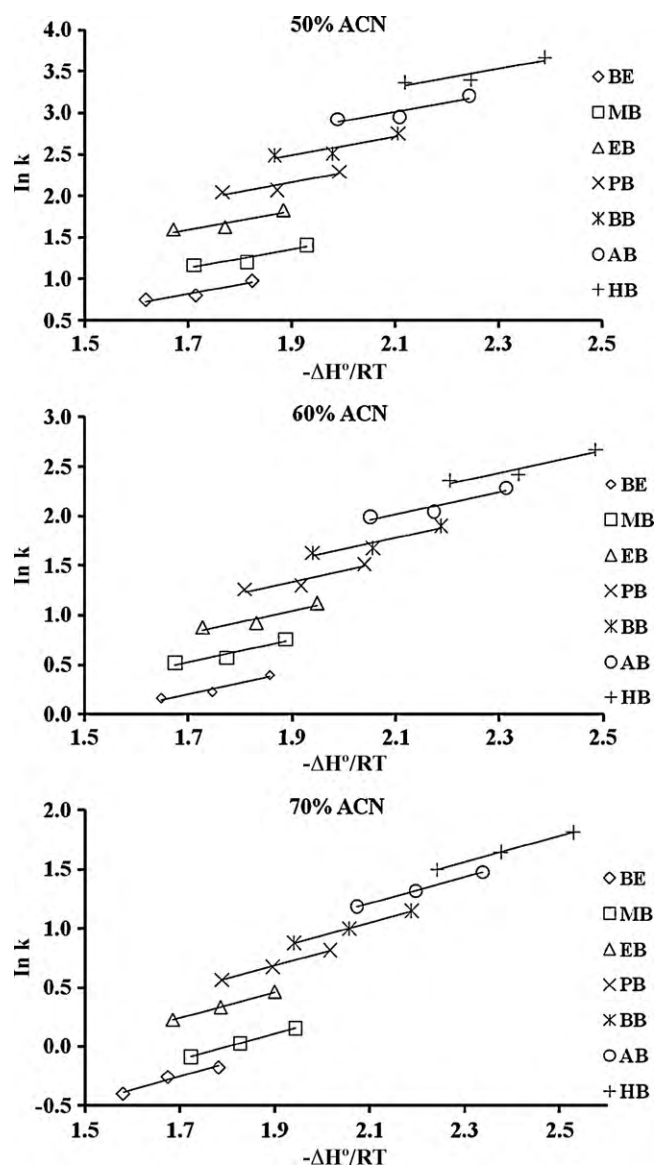


**Fig. 2.** Validity tests of van't Hoff equation (Eq. (6)) for homologous alkylbenzenes in methanol–water mobile phases.  $\ln k$ , natural logarithms of the retention factors;  $-\Delta H^0$  [ $\text{J mol}^{-1}$ ], enthalpic contribution to the retention;  $T$ , thermodynamic temperature, Kelvin,  $R$ , universal gas constant.

decrease at higher concentration of acetonitrile. Generally, the  $\ln k$  versus  $-\Delta H^0$  plots were less affected by varying concentrations of methanol in the mobile phases with respect to varying concentrations of acetonitrile. Using Eq. (9), the compensation temperatures,  $T_C$ , of alkylbenzenes were calculated from the slopes of the plots in Fig. 5 (Table 4). The values of  $T_C$  calculated at various temperatures were in relatively good agreement. All compensation temperatures are deeply below  $0^\circ\text{C}$ , higher in methanol–water than in acetonitrile–water mobile phases.  $T_C$  decreases at increasing concentration of methanol, but increase at increasing concentration of acetonitrile (Table 4).

#### 4.2. Non-homologous simple benzene derivatives

The thermodynamic contributions to the retention of non-homologous substituted benzenes were determined from the best-fit regression values of the slopes,  $B_i$  (enthalpic contribution) and from the intercepts,  $A_i$  (entropic contributions) of the experi-



**Fig. 3.** Validity tests of van't Hoff equation (Eq. (6)) for homologous alkylbenzenes in acetonitrile–water mobile phases.  $\ln k$ , natural logarithms of the retention factors;  $-\Delta H^0$  [ $\text{J mol}^{-1}$ ], enthalpic contribution to the retention;  $T$ , thermodynamic temperature, Kelvin;  $R$ , universal gas constant.

mental  $\ln k$  versus  $1/T$  plots (Eq. (6)), and are listed in Table 3. The correlation coefficients,  $R^2$ , show best and validity of Eq. (6) in 70% acetonitrile (except for BNB) and 70% methanol, like with homologous alkylbenzenes. The deviations from the linearity of the  $\ln k$  versus  $1/T$  plots increase at lower concentrations of acetonitrile and at higher concentrations of methanol.

**Table 4**

Compensation temperatures,  $T_C$  (K) in alkylbenzene homologous series (ethylbenzene–hexylbenzene) in acetonitrile/water (ACN) and methanol/water (MeOH) mobile phase calculated from experimental  $\ln k$  and  $\Delta H^0$  (Fig. 5) using Eq. (9) at three experimental temperatures ( $n = 5$  for each  $T_C$ ).

Experimental temperature ( $^\circ\text{C}$ )	$T_C$ (K)					
	ACN			MeOH		
	50%	60%	70%	70%	80%	90%
40	67.4	81.0	101.6	153.4	136.0	110.9
60	72.4	82.3	103.6	158.0	139.7	113.3
80	73.3	89.7	105.4	162.4	143.1	115.6

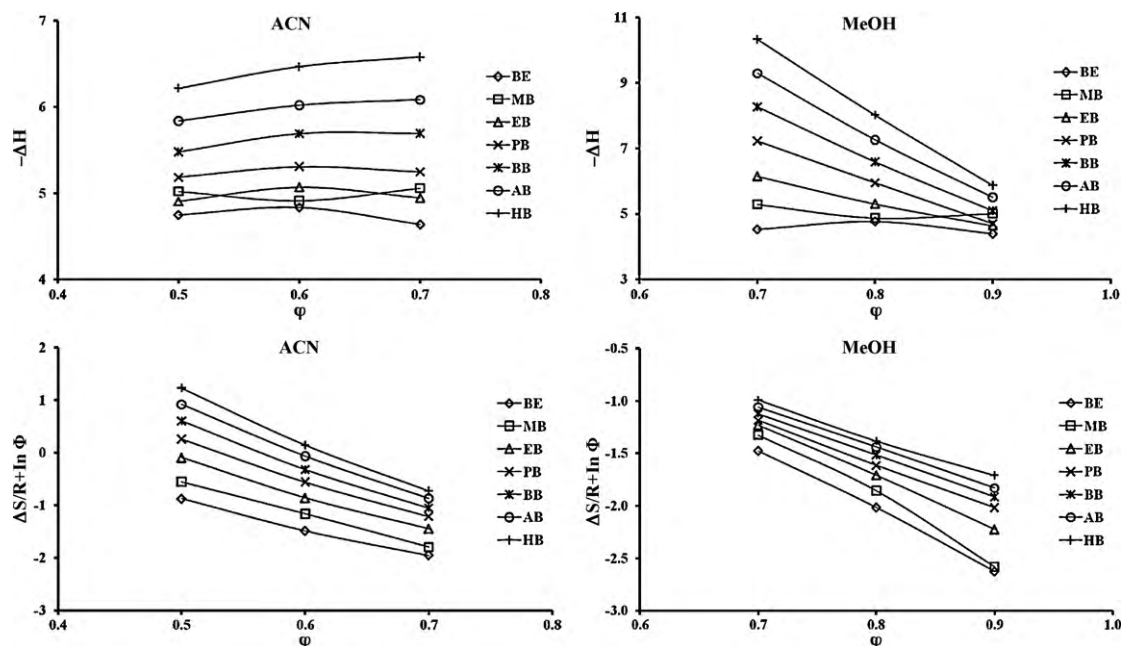


Fig. 4. Effects of the concentration of acetonitrile and methanol,  $\phi$ , in binary aqueous–organic mobile phases on the enthalpic,  $-\Delta H^0$  [J mol<sup>-1</sup>], and on the entropic,  $\Delta S^0/R + \ln \Phi$ , contributions to the retention of homologous alkylbenzenes.

Like with alkylbenzenes, the experimental values of the slope parameters  $d$  of Eq. (11) are in between 1.09 and 1.12, close to the theoretical value of 1 for all tested benzene derivatives

in all mobile phases (Table 3), regardless of the sample polarities. Large differences were found in the intercept parameters,  $c$ , of Eq. (11), depending on the solute polarity. These results

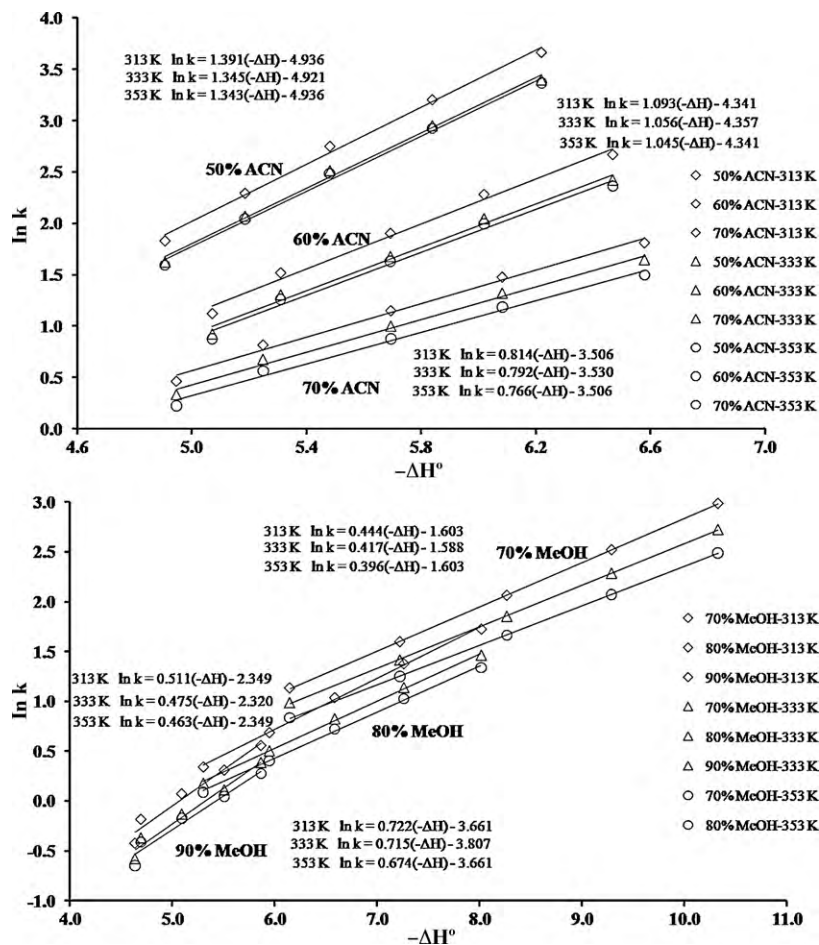
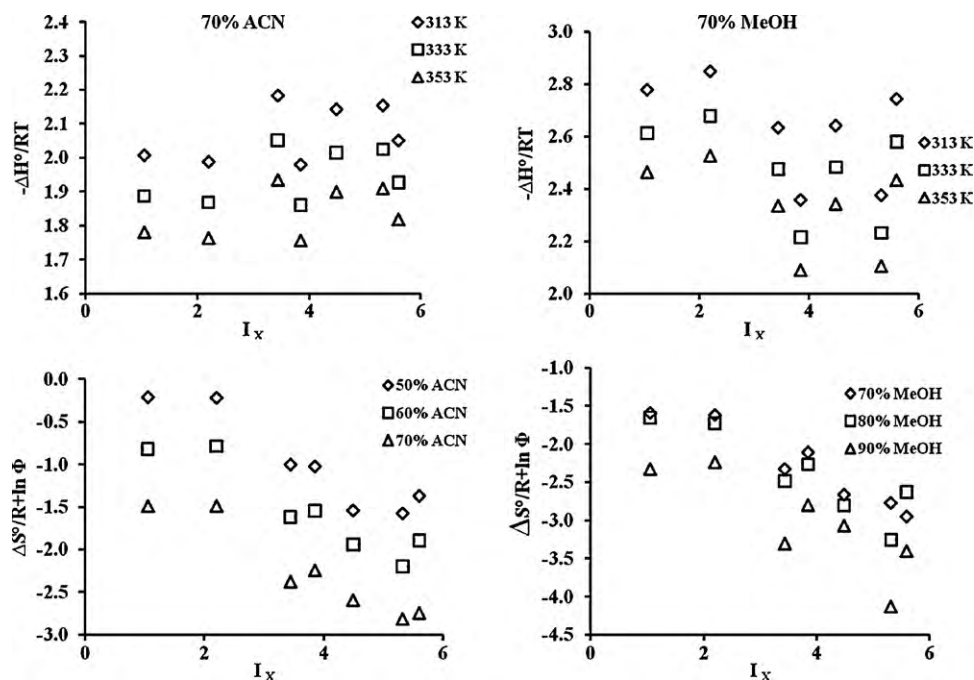


Fig. 5. Correlations between  $\ln k$  of homologous alkylbenzenes ( $C_1$ – $C_6$ ) and the enthalpy of retention,  $-\Delta H^0$  [J mol<sup>-1</sup>].



**Fig. 6.** Effects of the polarity of substituted benzenes on the enthalpic ( $-\Delta H^\circ$  [J mol<sup>-1</sup>]), and entropic, ( $\Delta S^\circ/R + \ln \Phi$ ), contributions to the retention on a Blaze C<sub>8</sub> column. The values of the interaction indices,  $I_x$ , are in Table 1.

suggest that the differences between non-homologous benzene derivatives are due principally to the entropic effects, decreasing the retention of compounds with more polar substituents (Table 3).

The data in Table 3 suggest some differences between the effects of the concentration of organic solvent,  $\varphi$ , on the enthalpy and entropy of retention of non-homologous compounds in acetonitrile/water and methanol/water mobile phase. Polar benzene derivatives show regular decreases in the entropic contribution to the retention at increasing concentration of the organic solvent, with the exception of chlorotoluene and acetophenone, showing maximum entropic contribution at 80% methanol in the mobile phase. The effects of the solvent concentration on the enthalpic contribution to the retention do not show general trends and may differ from one solute to another.

Fig. 6 illustrates the effect of polarity on the entropic and enthalpic contributions to the retention of simple benzene derivatives. The overall polarity of analytes is characterized by interaction indices,  $I_x$  (Table 1) [18,34]. The data determined in 70% methanol

and in 70% acetonitrile do not show any apparent correlations of enthalpic contributions with solute polarities ( $I_x$ ). On the other hand, the entropic contributions, which are independent of temperature, decrease the retention significantly for more polar compounds with higher  $I_x$ , especially in acetonitrile–water mobile phases, supporting the idea that the presence of polar groups in the sample molecules affects the retention primarily by negative entropic effects. These results are in general agreement with the solvophobic model of retention. Unlike to alkylbenzenes, we could not find a significant trend towards the entropy–enthalpy compensation with the polar benzene derivatives tested, which suggests important effects of the type of polar substituents on the retention mechanism.

#### 4.3. Simultaneous temperature and mobile phase effects on retention

According to the theory [33], simultaneous effects of temperature,  $T$ , and of the volume fraction of the organic solvent,  $\varphi$ , in

**Table 5**

Parameters of Eq. (12) describing the effects of temperature and concentration of the organic solvent on the retention of homologous alkylbenzenes and non-homologous polar test compounds (symbols as in Table 1).

	ACN				MeOH			
	$A_1$	$A_2$	$A_3$	$R^2$	$A_1$	$A_2$	$A_3$	$R^2$
BE	1.91 ± 0.21	570 ± 66	-5.58 ± 0.13	0.9968	2.70 ± 0.10	548 ± 29	-5.98 ± 0.06	0.9995
MB	2.52 ± 0.27	601 ± 82	-6.15 ± 0.16	0.9959	3.50 ± 0.06	610 ± 18	-6.79 ± 0.04	0.9999
EB	3.23 ± 0.29	598 ± 91	-6.71 ± 0.18	0.9957	4.42 ± 0.18	644 ± 52	-7.68 ± 0.10	0.9993
PB	3.86 ± 0.34	630 ± 107	-7.26 ± 0.23	0.9950	5.36 ± 0.27	716 ± 79	-8.71 ± 0.16	0.9989
BB	4.47 ± 0.38	677 ± 118	-7.88 ± 0.24	0.9948	6.23 ± 0.34	800 ± 99	-9.69 ± 0.20	0.9986
AB	5.09 ± 0.42	720 ± 129	-8.50 ± 0.26	0.9946	7.10 ± 0.38	884 ± 111	-10.68 ± 0.22	0.9987
HB	5.70 ± 0.45	772 ± 140	-9.13 ± 0.28	0.9946	7.94 ± 0.43	971 ± 126	-11.63 ± 0.25	0.9986
BN	1.14 ± 0.15	653 ± 45	-5.56 ± 0.09	0.9985	1.39 ± 0.35	753 ± 103	-5.97 ± 0.21	0.9934
NB	1.46 ± 0.13	675 ± 39	-5.82 ± 0.08	0.9990	2.00 ± 0.26	687 ± 78	-6.06 ± 0.16	0.9963
ANS	1.84 ± 0.19	597 ± 58	-5.74 ± 0.12	0.9976	2.52 ± 0.22	627 ± 65	-6.14 ± 0.13	0.9975
DCB	3.16 ± 0.28	630 ± 87	-6.67 ± 0.17	0.9960	4.60 ± 0.28	687 ± 83	-8.07 ± 0.17	0.9976
CT	3.19 ± 0.28	622 ± 86	-6.70 ± 0.17	0.9962	4.61 ± 0.30	700 ± 87	-8.09 ± 0.17	0.9973
ACE	1.14 ± 0.15	568 ± 46	-5.24 ± 0.09	0.9982	1.57 ± 0.28	682 ± 81	-7.70 ± 0.16	0.9954
BNB	2.34 ± 0.34	669 ± 104	-6.68 ± 0.21	0.9944	3.03 ± 0.16	711 ± 46	-7.18 ± 0.09	0.9990

ACN, acetonitrile/water; MeOH, methanol/water;  $R^2$ , non-linear correlation coefficient ( $n=9$  for each analyte and organic solvent).



binary mobile phases on the retention in reversed-phase systems can be described, to first approximation, by Eq. (10). The set of the experimental retention factors of alkylbenzenes and simple non-homologous substituted benzenes was analyzed by multiple non-linear regression with respect to  $\varphi$  and  $1/T$  as variable parameters to find the best-fit parameters of Eq. (10). The results show that the cross-term parameter  $A_4$  is statistically insignificant and can be neglected, meaning that under the conditions of the present study, the parameter  $m$  of Eq. (1) is practically independent of the temperature, even though some earlier work of other authors predicted a decrease in  $m$  at increasing temperature [35,36]:

$$\ln k = A_1 + \frac{A_2}{T} + A_3\varphi \quad (12)$$

We found good fit of the simplified three-parameter (Eq. (12)) to the set of experimental  $k$ , with multiple correlation coefficients in between 0.994 and 0.999, for homologous alkylbenzenes and simple non-homologous substituted benzenes, both in methanol–water and in acetonitrile–water mobile phases (Table 5). The parameters  $A_1$ ,  $A_2$  regularly increase and the parameter  $A_3$  (equivalent to the parameter  $m$  in Eq. (1)) becomes more negative with increasing alkyl length in the homologous alkylbenzene series. The parameter  $A_1$  decreases and the parameter  $A_3$  (equal to  $-m$  in Eq. (1)) becomes less negative for more polar (higher  $I_x$ ) non-homologous substituted benzenes (except for acetophenone in methanol–water mobile phases). There is no obvious systematic effect of the sample polarity on the parameter  $A_2$ . These results suggest: (1) that Eq. (12) can be suitable for prediction of retention on the Blaze C<sub>8</sub> bidentate column when changing operation conditions and (2) that – because of relatively independent effects of the temperature and mobile phase on the retention – the  $T$  and  $\varphi$  can be optimized subsequently, independent of each other.

## 5. Conclusions

Besides the concentration of the organic modifier in the mobile phases, the temperature has important effect upon the retention of both non-polar and polar samples in reversed-phase systems and can therefore be used as very useful tool for controlling the retention and optimizing HPLC conditions with respect to speed, efficiency, and selectivity of separation. Full advantages of temperature control can be utilized with columns sufficiently stable at temperatures higher than 60 °C, such as the bidentate Blaze C<sub>8</sub> column. Investigation of simultaneous effects of temperature and composition of the mobile phase enables measurements of the enthalpic and entropic contributions to the retention, providing thus deeper insight into the retention mechanism [37]. On the Blaze C<sub>8</sub> column, the mechanism of retention of homologous alkylbenzenes does not change in the temperature interval between 40 and 80 °C, where both enthalpic and entropic effects control the retention at 70% or higher concentrations of methanol. Entropic effects arising from the molecular organization of bonded alkyls are the main factors controlling the retention in mobile phases containing 70% or less acetonitrile in water, where probably changing solvation of bonded alkyls may cause entropic expulsion of solute molecules from the bidentate bonded C<sub>8</sub> stationary phase.

The results of the temperature studies of polar benzene derivatives suggest strong effects of the polar substituents on the enthalpic and entropic contributions to the retention. However, it is difficult to formulate conclusions on general trends in the retention mechanism on the basis of the present results, except for decreasing entropic contributions to the retention of polar compounds, which are obviously more significant than the effects of sample polarity on

the enthalpic contributions to the retention. Anyway, to confirm the general validity of these trends, a broader study with a higher variety of compounds in a wider range of experimental conditions will be necessary. Investigation of the temperature effects on LC separations of other types of polar samples, including phenolic acids and flavones, is in progress.

Even though the actual contributions of the enthalpic and entropic effects to the retention mechanism depend on the combined conditions of temperature and mobile phase composition, the results of the present work suggest that the temperature effects and the effects of the composition of aqueous–organic mobile phase on the retention can be formally described as largely independent of each other and can be considered as separate operation variables in the prediction of retention and development of separations on the Blaze C<sub>8</sub> column.

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## References

- [1] G. Vanhoenacker, P. Sandra, J. Chromatogr. A 1082 (2005) 193.
- [2] J.V. Tran, P. Molander, T. Greibrokk, E. Lundanes, J. Sep. Sci. 24 (2001) 930.
- [3] S. Heinisch, J.-L. Rocca, J. Chromatogr. A 1216 (2009) 642.
- [4] T. Greibrokk, T. Andersen, J. Chromatogr. A 1000 (2003) 743.
- [5] W. Lee, D. Cho, B.O. Chun, T. Chang, M. Ree, J. Chromatogr. A 910 (2001) 51.
- [6] T. Andersen, P. Molander, R. Trones, D.R. Hegna, T. Greibrokk, J. Chromatogr. A 918 (2001) 221.
- [7] P.L. Zhu, L.R. Snyder, J.W. Dolan, N.M. Djordjevic, D.W. Hill, L.C. Sander, T.J. Waeghe, J. Chromatogr. A 756 (1996) 21.
- [8] P.L. Zhu, J.W. Dolan, L.R. Snyder, J. Chromatogr. A 756 (1996) 41.
- [9] P.L. Zhu, J.W. Dolan, L.R. Snyder, D.W. Hill, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 756 (1996) 51.
- [10] P.L. Zhu, J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, J.-T. Lin, L.C. Sander, L. Van Heukelem, J. Chromatogr. A 756 (1996) 63.
- [11] G. Vanhoenacker, P. Sandra, Anal. Bioanal. Chem. 390 (2008) 245.
- [12] J. Li, P.W. Carr, Anal. Chem. 69 (1997) 2202.
- [13] L.R. Snyder, J.W. Dolan, J.R. Gant, J. Chromatogr. 165 (1979) 3.
- [14] P. Jandera, J. Churáček, L. Svoboda, J. Chromatogr. 174 (1979) 35.
- [15] A. Tchaplá, S. Heron, H. Cohn, G. Guiochon, Anal. Chem. 60 (1988) 1443.
- [16] P. Jandera, J. Chromatogr. 314 (1984) 13.
- [17] P. Jandera, J. Chromatogr. 449 (1988) 361.
- [18] P. Jandera, in: R.M. Smith (Ed.), Retention and Selectivity in Liquid Chromatography: Prediction, Standardization and Phase Comparison, Elsevier, Amsterdam, 1995, p. 269.
- [19] W.R. Melander, Cs. Horvath, in: Cs. Horvath (Ed.), High-Performance Liquid Chromatography, vol. 2, Academic Press, New York, 1980, p. 113.
- [20] W. Kiridena, C.F. Poole, W.W. Koziol, Chromatographia 57 (2003) 703.
- [21] T.L. Chester, J.W. Coym, J. Chromatogr. A 1003 (2003) 101.
- [22] G. Vigh, Z. Varga-Puchony, J. Chromatogr. 196 (1980) 1.
- [23] S.M. Petrovic, S. Lomic, J. Liq. Chromatogr. 12 (1989) 59.
- [24] R. Silveston, B. Kronberg, J. Chromatogr. 659 (1994) 43.
- [25] B.S. Ludolph, C.Y. Jeng, A.H.T. Chu, S.H. Langer, J. Chromatogr. A 660 (1994) 3.
- [26] R.P.J. Ranatunga, P.W. Carr, Anal. Chem. 72 (2000) 5679.
- [27] W. Melander, D.E. Campbell, C. Horváth, J. Chromatogr. 158 (1978) 215.
- [28] F. Riedo, E. Kováts, J. Chromatogr. 239 (1982) 1.
- [29] D. Bolliet, C.F. Poole, Analyst 123 (1998) 129.
- [30] P. Molander, S.J. Thommesen, I.A. Bruheim, R. Trones, T. Greibrokk, E. Lundanes, T.E. Gundersen, J. High Res. Chromatogr. 22 (1999) 490.
- [31] D.V. McCalley, J. Chromatogr. A 902 (2000) 311.
- [32] H.J. Issaq, S.D. Fox, M. Mahadevan, T.P. Conrads, T.D. Veenstra, J. Liq. Chromatogr. Relat. Technol. 26 (2003) 2255.
- [33] A. Tchaplá, S. Héron, H. Colin, G. Guiochon, Anal. Chem. 60 (1988) 1443.
- [34] P. Jandera, H. Colin, G. Guiochon, Anal. Chem. 54 (1982) 435.
- [35] N. Chen, Y. Zhang, P. Lu, J. Chromatogr. A 603 (1992) 35.
- [36] K. Valkó, L.R. Snyder, J. Glajch, J. Chromatogr. A 656 (1993) 501.
- [37] R. Tijssen, P.J. Schoenmakers, M.R. Boehmer, L.K. Koopal, H.A.H. Billiet, J. Chromatogr. A 656 (1993) 135.