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Effects of the operation parameters on Hydrophilic Interaction Liquid Chromatography separation of phenolic acids on zwitterionic monolithic capillary columns

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ABSTRACT

Strongly polar phenolic acids are weakly retained and often poorly separated in reversed-phase (RP) liquid chromatography. We prepared zwitterionic polymethacrylate monolithic columns for micro-HPLC by *in situ* co-polymerization in fused-silica capillaries. The capillary monolithic columns prepared under optimized polymerization conditions show some similarities with the conventional particulate commercial ZIC-HILIC silica-based columns, however have higher retention and better separation selectivity under reversed-phase conditions, so that they can be employed for dual-mode HILIC-RP separations of phenolic acids on a single column. The capillary polymethacrylate monolithic sulfobetaine columns show excellent thermal stability and improved performance at temperatures 60–80 °C. The effects of the operation conditions on separation were investigated, including the type and the concentration of the organic solvent in the aqueous-organic mobile phase (acetonitrile and methanol), the ionic strength of the acetate buffer and temperature. While the retention in the RP mode decreases at higher temperatures in mobile phases with relatively low concentrations of acetonitrile, it is almost independent of temperature at HILIC conditions in highly organic mobile phases. The best separation efficiency can be achieved using relatively high acetate buffer ionic strength (20–30 mmol L^{-1}) and gradient elution with alternately increasing (HILIC mode) and decreasing (RP mode) concentration of aqueous buffer in aqueous acetonitrile. Applications of the monolithic sulfobetaine capillary columns in alternating HILIC-RP modes are demonstrated on the analysis of phenolic acids in a beer sample.

1. Introduction

The interest in separation of strongly polar compounds of natural or synthetic origin is continuously increasing. Separation of many polar compounds in HPLC is difficult, as such compounds are too weakly retained in LC reversed-phase (RP) LC, but too strongly in traditional non-aqueous normal-phase (NP) LC (known also as adsorption LC). Separation of many polar compounds considerably improves in the hydrophilic interaction (HILIC) mode [1], which employs polar stationary phases common in non-aqueous (adsorption) normal-phase chromatography in combination with the RP mobile phases. HILIC is essentially aqueous normal-phase chromatography on polar columns such as silica gel (usually with decreased surface silanol concentration) [2–4], hydrosilated silica [5–7], silica gel with chemically bonded aminopropyl- [8,9], amido- [10], cyclodextrin- [11], carbamate [12], diol [13], polyethylene glycol [14], poly(2-hydroxyethyl aspartamide) [1], etc., organic-polymer materials such as TSKgel Amide [12], or ion-exchangers, such as poly(2-sulfoethyl aspartamide) [15], polyCAT A [16], etc. The performance of various types of stationary phases used for HILIC applications was reviewed recently [17].

More recently, zwitterionic sufoalkylbetaine stationary phases (ZIC HILIC) have been introduced for HILIC separations of inorganic salts, small organic ionic compounds and proteins and other samples [18–20]. The active layer grafted on wide-pore silica gel or polymer support contains both strongly acidic sulfonic acid groups and strongly basic quaternary ammonium groups in 1:1 molar ratio, separated by a short alkyl spacer. A low net negative surface charge of the bonded layer is attributed to larger distance of the sulfonic groups from the silica gel surface. This charge is only little affected by pH. Even though the retention mechanism on the sulfobetaine columns obviously includes both HILIC and ion-exchange mechanism, the ion-exchange interactions of the zwitterionic stationary phases are assumed to be weak in comparison to ion-exchangers [21] and the chromatographic properties of zwitterionic materials significantly differ from other HILIC phases.

Stationary phases bonded on silica gel have limited chemical and thermal stability with respect to organic-polymer station-

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ary phases. In addition to particulate organic-polymer adsorbents, which often show lower separation efficiency than silica-based columns, monolithic columns were developed, containing a continuous block of highly porous material that contains open through pores inside the monolith, forming a highly interconnected network of flow-through channels due to which monolithic columns generally show low flow resistance and excellent flow-through properties.

Even though organic-polymer monolithic columns are commonly employed for separations of biopolymers and other compounds in reversed-phase mode [22,23], only few HILIC applications for polar compounds in the HILIC mode have been reported so far [24–28], probably due to the lack of commercially available polar monomers for the preparation of polar organic-polymer monoliths and limited solubility of very polar monomers in most commonly used porogens, which requires polymerization in new porogen solvent mixtures with relatively high concentrations of water [25].

Holdšvendová et al. [24] used capillary hydroxymethyl methacrylate-based monolithic columns for separation of mixed-sequence oligonucleotides in the HILIC mode using the gradient of acetonitrile in 100 mM aqueous triethylamine acetate. Jiang et al. prepared a porous monolithic poly-(SPE-*co*-EDMA) capillary column by thermo-initiated co-polymerization of *N*,*N*-dimethyl-*N*-methacryloxyethyl-*N*-(3-sulfopropyl)ammonium betaine (MEDSA) and ethylene dimethacrylate (EDMA) cross-linker, which showed good selectivity for neutral, basic and acidic polar analytes in the HILIC mode [25]. Later, we used similar approach to prepare a zwitterionic polymethacrylate monolithic columns for micro-HPLC (MON-MEDSA-EDMA). Because of poor solubility of MEDSA in non-aqueous porogen solvents, we employed mixtures of 1-propanol, 1,4-butanediol and water as porogen solvent mixtures and we optimized porosity, permeability and efficiency of the sulfobetaine monolithic columns by adjusting the composition of the polymerization mixture [26].

In aqueous-organic mobile phases, water is preferentially adsorbed on the surface of silica and other polar adsorbents; consequently, a diffuse water-rich layer forms on the adsorbent surface. Polar compounds may be retained due to combined adsorption on the adsorbent surface, partition into the diffuse adsorbed layer, and ion-exchange interactions with charged functional groups, which may contribute to the retention of ionic or partly ionized samples. Hence, the resulting HILIC mechanism may be quite complex, however the common feature of HILIC separations is that, like in adsorption NP chromatography, the retention increases with sample polarity and decreases with increasing content of more polar solvent in the mobile phase—in this case water.

On some moderately and even on strongly polar stationary phases, the retention decreases as the concentration of the organic solvent (methanol or acetonitrile) increases in highly aqueous binary mobile phases, whereas it decreases with increasing concentration of water in mobile phases with high concentrations of organic solvent. The plots of the retention times (or retention factors) *versus* the composition of binary aqueous-organic mobile phases show "U-shape" with retention minima at the "U-turn" composition of the aqueous-organic mobile phase, depending on the polarity of sample and on the type of stationary phase [7,14,30]. This behavior is attributed to the combination of the solvophobic interactions with alkyl chains and other non-polar moieties in the stationary phase in water-rich mobile phases (RP retention mechanism), and interactions with the polar groups in the stationary phase controlling the retention at high concentrations of the organic solvent, usually acetonitrile (aqueous normal-phase mechanism).

The U-shape of the experimental plots of k *versus* the volume fraction of an aqueous buffer, φ(BUF), can be described by Eq. (1) over the broad range of aqueous-organic mobile phase composition (HILIC and RP, at $\varphi(\mathrm{BUF}) > 0.02$) [29]:

$$\log k = a_1 + m_1 \cdot \varphi(\mathrm{BUF}) - m_2 \cdot \log \varphi(\mathrm{BUF}) \quad (1)$$

The parameters a_1, m_1 and m_2 of Eq. (1) can be determined by non-linear regression of the experimental retention factors measured at varying volume fractions of water in the mobile phase. The parameter m_1 characterizes the effect of increasing concentration of water in the mobile phase on the contribution of RP mechanism to the retention, whereas the parameter m_2 is a measure of the opposite effect, characteristic for the HILIC contribution to the retention in highly organic mobile phases, a_1 is an empirical constant.

Capillary monolithic polymethacrylate zwitterionic sulfobetaine columns show distinct dual RP-HILIC retention mechanism of polar compounds. In a recent study we showed that the elution order of phenolic acids is similar on the MON-MEDSA-EDMA columns as on silica-based sulfobetaine ZIC-HILIC columns, with some differences in selectivity; however the reversed-phase retention is significantly enhanced on the new monolithic columns in comparison to the ZIC-HILIC columns. The retention and HILIC mobile phase range increase in the order: PEG < Hydride silica < Luna HILIC < DIOL < zwitterionic columns. On the other hand, the RP retention shows almost inverse order: ZIC HILIC < DIOL < LUNA HILIC < PEG columns. The MON-MEDSA-EDMA monolithic columns show the RP behavior over a broader mobile phase range than the commercial ZIC-HILIC column [31].

Monolithic columns based on organic polymers are well known to provide excellent separation of proteins and other biopolymers, whereas they usually show rather low efficiencies for the separations of low-molecular samples [22]. This agrees with our previous work, where we compared the performance of our capillary monolithic polymethacrylate sulfobetaine columns with other columns for separation of proteins alkylbenzenes and some phenolic compounds [29,31]. However, in this earlier work, the separation conditions were selected empirically. In present work, we investigated in detail the effects of the operation conditions in HILIC chromatography of phenolic acids and flavone compounds on the capillary MON-MEDSA-EDMA columns in the HILIC and RP modes. We focused attention on the molar concentration and volume fraction of the buffer in aqueous-organic mobile phase and especially on the temperature effects on possible improvement of the selectivity and efficiency of separation. To our best knowledge, the temperature effects on HILIC separation have not been studied in systematic manner so far.

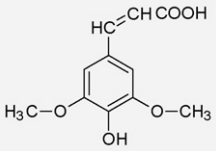
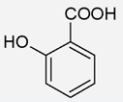
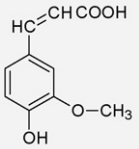
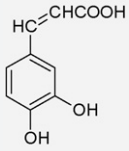
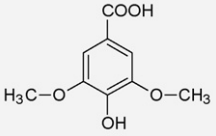
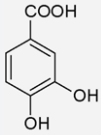
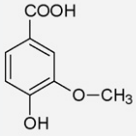
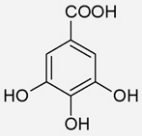
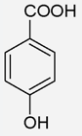
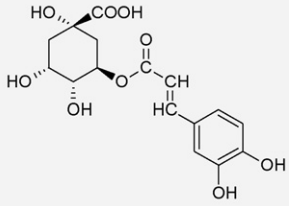
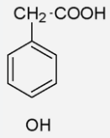
2. Experimental

2.1. Materials

Ethylene dimethacrylate (EDMA), [2-(methacryloyloxy)ethyl]-dimethyl-(3-sulfopropyl)-ammonium hydroxide (MEDSA), 3-(trimethoxysilyl)propyl methacrylate, 1-propanol, and uracil were purchased from Sigma–Aldrich (Milwaukee, WI, USA). 1,4-butanediol, hydrochloric acid, toluene, acetic acid, phenol and 2,2′-azo-bis-isobutyronitrile (AIBN) were obtained from Fluka (Buchs, Switzerland). Thiourea was purchased from Lachema (Brno, Czech Republic). Gallic (GAL), protocatechuic (PRO), 4-hydroxybenzoic (PHB), 4-hydroxyphenylacetic (HPA), vanillic (VAC), ferulic (FER), syringic (SYR), sinapic (SIN), salicylic (SAL), and chlorogenic (CHL) acids were obtained from Fluka (Buchs, Switzerland). Caffeic acid (CAF) was purchased from Sigma–Aldrich (St. Louis, MI, USA). The structures are shown in Table 1.

Acetonitrile and methanol for HPLC (LiChrosolv grade) were obtained from Merck (Darmstadt, Germany), ammonium acetate

Table 1
Structures and short names of phenolic acids.

Compound	Short name	Structure	Compound	Short name	Structure
Sinapic acid	SIN		Salicylic acid	SAL	
Ferulic acid	FER		Caffeic acid	CAF	
Syringic acid	SYR		Protocatechuic acid	PRO	
Vanillic acid	VAC		Gallic acid	GAL	
p-hydroxybenzoic acid	PHB		Chlorogenic acid	CHL	
4-hydroxyphenyl-acetic acid	HPA				

(TraceSelect, 99,995%) from Fluka (Buchs, Switzerland) and formic acid (98%) from Riedel-de Haën (Seelze, Germany). Distilled water was purified in a Demiwa 5ROI station (Watek, Ledeč nad Sázavou, Czech Republic). The beer sample (Pilsner Urquell, Plzen, Czech Republic) was obtained in a local supermarket.

2.2. Instrumentation

An 1100 Liquid Chromatograph (Agilent, Palo Alto, CA, USA) equipped with a micro-flow binary pump, a degasser, an autosampler, a diode-array UV detector with a micro flow cell and a thermostatted column compartment and a ChemStation for LC 3D was used for all LC separations. The diode-array UV detector wavelengths were set to 220, 254, 280, 300 and 320 nm.

2.3. In situ preparation of MON-MEDSA-EDMA capillary columns

Polyimide-coated 320 μm i.d. fused-silica capillaries (J & W, Folsom, CA, USA) were used to prepare monolithic polymethacrylate HILIC columns. Three capillary columns with monolithic MON-MEDSA-EDMA stationary phases were prepared in silica capillaries by *in situ* (co)polymerization of MEDSA as the monomer (19.98%) and EDMA as the cross-linker (15.0%) in porogen solvent mixture, containing 1-propanol (25.13%), 1,4-butanediol (24.97%) and water (14.91%). Optimization of the polymerization mixture was described earlier [29]. Using thermal initiation with 1% AIBN, the polymerization reaction was performed at 60 °C for 20 h. Then, both ends of the capillaries were cut and the monolithic column was washed with mobile phase before use. The properties of the capillary columns are given in Table 2.

2.4. Chromatographic methods

For isocratic separations of phenolic acids on MON-MEDSA-EDMA capillary columns, mixtures of acetonitrile and aqueous buffer containing 0.01 M CH_3COONH_4 adjusted with formic acid to pH 3.17 (before dilution with acetonitrile) were used as the mobile phases. The same components of the mobile phase were used in gradient elution. This buffer provided more stable gradient baseline and more reproducible retention times than acetate/acetic acid, or formate/formic acid buffers. In the HILIC mode, gradients were run with increasing concentrations of aqueous buffer (solvent B) in acetonitrile (solvent A), whereas in the RP mode the concentration of acetonitrile (in this case solvent B) increased in the buffer used as the solvent A. Before use, all mobile phases were filtered over a Millipore 0.45 μm filter and degassed by ultrasonication.

Sample solutions were prepared in the mobile phase at concentrations yielding adequate detector response. The column hold-up volumes were determined from the elution volumes of uracil as non-retained marker in the RP-mode (in 50% acetonitrile) and of

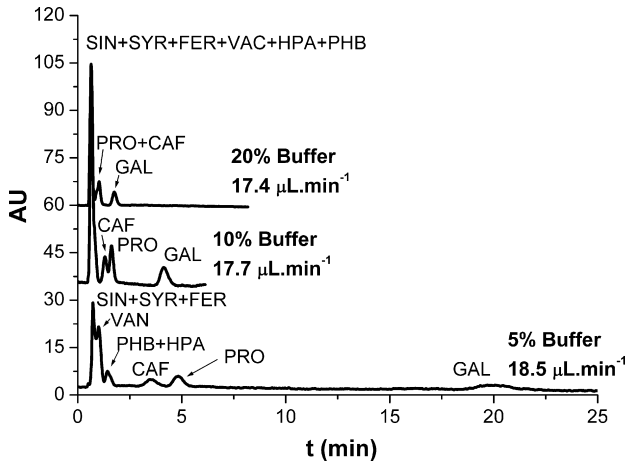


Fig. 1. Isocratic separation of phenolic acids on a MON-MEDSA-EDMA A column under HILIC conditions in mobile phases containing various concentrations of 20 mmol L^{-1} aqueous ammonium acetate buffer (pH 3.17); in acetonitrile. Temperature: 25 °C; injection volume: 20 nL; UV detection: 220 nm; the compounds are as in Table 1.

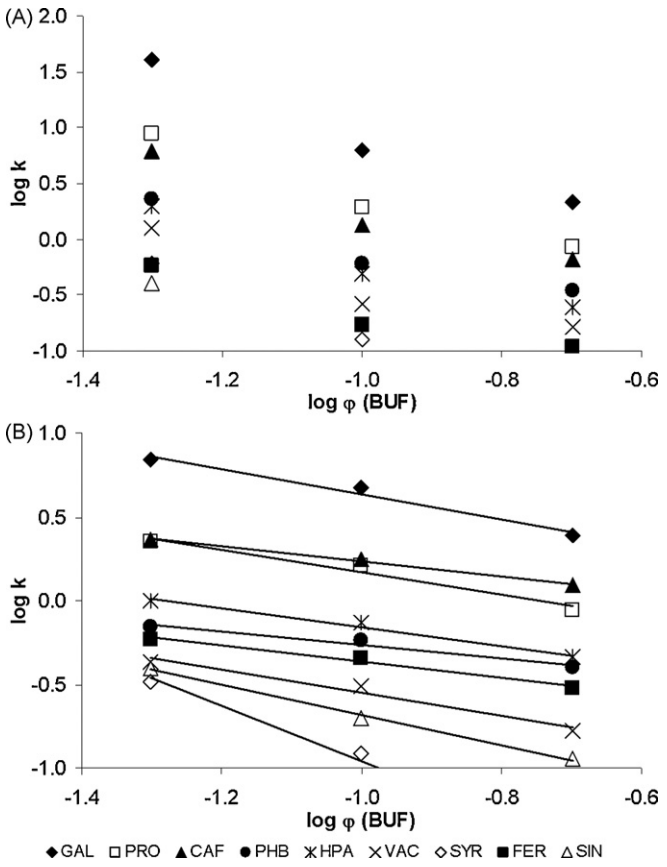


Fig. 2. Effects of the volume fraction of the acetate buffer (20 mmol L^{-1}, pH 3.17) in aqueous acetonitrile (A) and methanol (B) on the retention factors, *k*, of phenolic acids under HILIC conditions. Column: MON-MEDSA-EDMA A; temperature: 25 °C.

toluene in HILIC-mode (in 98% acetonitrile)—Table 2. Sample volumes of 20 nL were injected and the flow-rate of the mobile phase was adjusted in the range between 3 μL min^{-1} and 20 μL min^{-1}. The actual flow-rate was checked using a stop-watch and a calibrated 100 μL microburette, connected to the detector outlet. The time necessary for re-equilibration of the column to the initial conditions after the end of the gradient was 10 min at the flow-rate 5 μL min^{-1}, both in the HILIC and in the RP modes, which is comparable to other HILIC systems tested earlier [30]. To this time, we actually added additional 6 min corresponding to the instrumental gradient delay time. All separations were performed at specified temperature setting in the range 30–80 °C. The experimental retention times, t_R, of sample compounds and the column hold-up volumes, t_0, were used to calculate the retention factors, $k = (t_R/t_0 - 1)$.

3. Results and discussion

3.1. Mobile phase effects on HILIC separations

We investigated the effects of the following main factors controlling the effects of aqueous-organic mobile phases on the retention of phenolic acids on the capillary MON-MEDSA-EDMA columns in the HILIC mode: (a) is the volume fraction of the aqueous buffer in the mobile phase and (b) is the ionic strength (molar concentration) of ammonium acetate in the aqueous buffer component of the mobile phase and (c) is the operation temperature. Table 3 shows the retention factors, *k*, of phenolic acids in the mobile phases at the temperatures tested.

Fig. 1 shows the separation of phenolic acids in mobile phases containing various volume fractions of aqueous ammonium acetate buffer in aqueous-organic mobile phases containing acetonitrile

Increasing volume fraction of acetate buffer (20 mmol L^{-1}) in the mobile phase strongly speeds up the elution of phenolic acids and the separation improves at decreasing proportion of the aqueous buffer (Table 3). This agrees with the behavior generally observed in normal-phase systems, as aqueous buffers are more polar and hence have higher elution strength than any organic solvent under HILIC conditions. Fig. 2 illustrates the effects of the volume fraction, φ(BUF), of the aqueous buffer in the high-organic (HILIC) range of aqueous-organic mobile phase on the retention. If we neglect the contribution of solvophobic mechanism to the retention in highly organic mobile phases, Eq. (1) is simplified to Eq. (2), which can be used – to first approximation – to estimate the retention in the HILIC mobile phase range for compounds not very strongly retained in pure organic solvent [29]:

$$\log k = \log k_B - m \cdot \log \varphi(\mathrm{BUF}) \quad (2)$$

Of course, Eq. (2) cannot be used in mobile phases with higher concentrations of the aqueous components. For compounds more strongly retained in pure organic solvent, positive deviations of retention from Eq. (2) can be expected [14]. This explains signifi-

Table 2
Characteristics of the capillary MON-MEDSA-EDMA columns.

Column	*L* [mm]	V_M^{TO} [μL] (HILIC)	V_M^{UR} [μL] (RP)	ε_T^{TO} (HILIC)	ε_T^{UR} (RP)	$K_f \times 10^{10}$[cm^2]
A	149	7.6	10.0	0.64	0.84	6.34
B	173	9.1	11.4	0.66	0.82	6.14
C	181	11.0	11.8	0.64	0.81	6.40

L—Column length, i.d. = 320 μm; V_M—hold-up volume measured with toluene in 98% acetonitrile (HILIC mode) and with uracil in 50% acetonitrile (RP mode), ε_T—total porosity in the HILIC and in the RP modes, K_f—permeability.

Table 3
Retention factors, k, of phenolic acids on the capillary MON-MEDSA-EDMA columns in mobile phases with different concentrations of ammonium acetate in water (pH 3.17, adjusted by formic acid) at various temperatures (T, °C).

c (NH_4Ac)	φ(BUF)	T	k								
[mmol L^{-1}]	in ACN	[°C]	SIN	FER	SYR	VAC	HPA	PHB	CAF	PRO	GAL
10	0.05	25	0.400	0.580	0.610	1.250	1.970	2.300	6.170	8.780	40.230
	0.10	25	0.054	0.171	0.126	0.261	0.486	0.604	1.333	1.919	6.288
	0.20	25	0.036	0.109	0.073	0.164	0.245	0.345	0.655	0.864	2.155
10	0.10	25	0.168	0.306	0.246	0.385	0.572	0.730	1.615	2.283	7.652
20	0.10	25	0.165	0.323	0.230	0.412	0.607	0.770	1.783	2.402	8.516
30	0.10	25	0.206	0.308	0.310	0.442	0.664	0.773	1.648	2.259	9.247
40	0.10	25	0.231	0.337	0.316	0.479	0.729	0.868	1.934	2.710	9.201
50	0.10	25	0.214	0.317	0.290	0.449	0.693	0.837	1.931	2.643	9.177
20	0.10	30	0.363	0.432	0.462	0.634	0.931	1.064	2.110	2.849	–
		40	0.435	0.561	0.531	0.698	0.969	1.128	2.381	3.281	–
		50	0.415	0.546	0.526	0.689	0.990	1.160	2.518	3.542	–
		60	0.400	0.525	0.508	0.678	0.989	1.165	2.525	3.560	–
		70	0.361	0.494	0.479	0.664	0.988	1.191	2.592	3.711	–
		80	0.352	0.481	0.477	0.653	0.988	1.211	2.739	3.799	–
20	0.85	30	5.625	8.581	2.472	3.860	2.472	6.130	12.107	3.860	2.894
		40	4.301	6.481	2.040	3.113	2.040	4.782	8.940	3.113	2.719
		50	3.241	4.773	1.623	2.419	1.623	3.671	6.459	2.419	2.157
		60	2.677	3.851	1.399	2.049	1.399	3.041	5.100	2.049	1.843
		70	2.144	3.075	1.187	1.715	1.187	2.507	4.022	1.715	1.614
		80	1.783	2.492	0.077	1.441	0.077	2.096	3.219	1.441	1.356
c (NH_4Ac)	φ(BUF)	T	k								
[mmol L^{-1}]	in MeOH	[°C]	SIN	FER	SYR	VAC	HPA	PHB	CAF	PRO	GAL
10	0.05	25	0.397	0.588	0.610	0.326	1.000	0.697	2.309	2.247	6.980
	0.10	25	0.200	0.453	0.126	0.123	0.735	0.579	1.769	1.619	4.724
	0.20	25	0.113	0.300	0.073	0.033	0.459	0.398	1.241	3.918	2.479

c, mmol L^{-1}: molar concentration of ammonium acetate in the aqueous component of the mobile phase; φ(BUF) volume fraction of the aqueous buffer in acetonitrile (ACN) and methanol (MeOH).
Short names of the acids are as in Table 1.

cant deviations of the log k *versus* log φ(BUF) plots with acetonitrile as the organic solvent in Fig. 2A, in comparison with aqueous methanol, where the phenolic acids are less retained (Fig. 2B), so that the retention can be described by Eq. (2) to first approximation, even though the correlation coefficients are not very high, see Table 4 showing best-fit parameters of Eq. (2) found by linear regression of the experimental data measured in methanol-buffer mobile phases.

Fig. 3 and the data in Table 3 illustrate the effect of increasing molar concentration (ionic strength) of the acetate buffer (at a constant 10% buffer concentration in the mobile phase) on the retention of phenolic acids on the capillary MON-MEDSA-EDMA column under HILIC conditions. At buffer concentration increasing from 10 mmol L^{-1} to 30 mmol L^{-1}, the retention slightly increases and the resolution of phenolic acids improves; further increase in buffer molarity to 50 mmol L^{-1} has little effect on the separation. This suggests that ion-exchange mechanism does not contribute significantly to the HILIC retention of phenolic acids; otherwise the retention would decrease proportionally to the molar concentration of the buffer.

Table 4
Best-fit parameters a and m of Eq. (2) and correlation coefficients, R^2, for phenolic acids on the capillary MON-MEDSA-EDMA column B in the composition range 5–20% of 20 mmol L^{-1} aqueous acetate buffer (pH 3.17) in methanol.

Compound	$a = \log K_0$	m	R^2
SIN	−1.58 ± 0.05	0.09 ± 0.05	0.997
FER	−0.85 ± 0.06	0.49 ± 0.06	0.984
SYR	−2.61 ± 0.15	1.65 ± 0.14	0.993
VAC	−1.23 ± 0.12	0.68 ± 0.11	0.973
HPA	−0.72 ± 0.07	0.56 ± 0.07	0.985
PHB	−0.67 ± 0.08	0.40 ± 0.08	0.963
CAF	−0.21 ± 0.04	0.45 ± 0.04	0.993
PRO	−0.50 ± 0.12	0.67 ± 0.11	0.972
GAL	−0.11 ± 0.11	0.75 ± 0.11	0.981

Short names of the acids are as in Table 1.

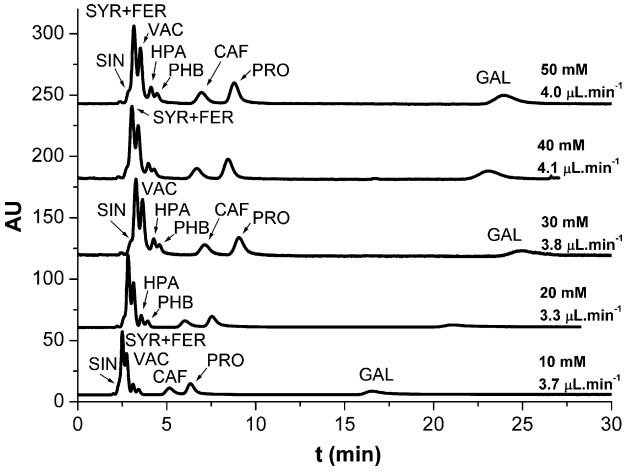


Fig. 3. Separation of phenolic acids using mobile phase with various molar concentrations of ammonium acetate (before dilution with acetonitrile) on a MON-MEDSA-EDMA B column. Isocratic elution, 10% aqueous ammonium acetate (pH 3.17) + 90% Acetonitrile; Temp.: 25 °C; Inj. vol.: 20 nL; UV-detection: 220 nm; the compounds are as in Table 1.

3.2. Temperature effects on HILIC and RP separations

Unlike silica-based columns, the monolithic sulfobetaine columns show excellent stability at temperatures higher than 60 °C. Fig. 4 compares isocratic separation of phenolic acids on the capil-

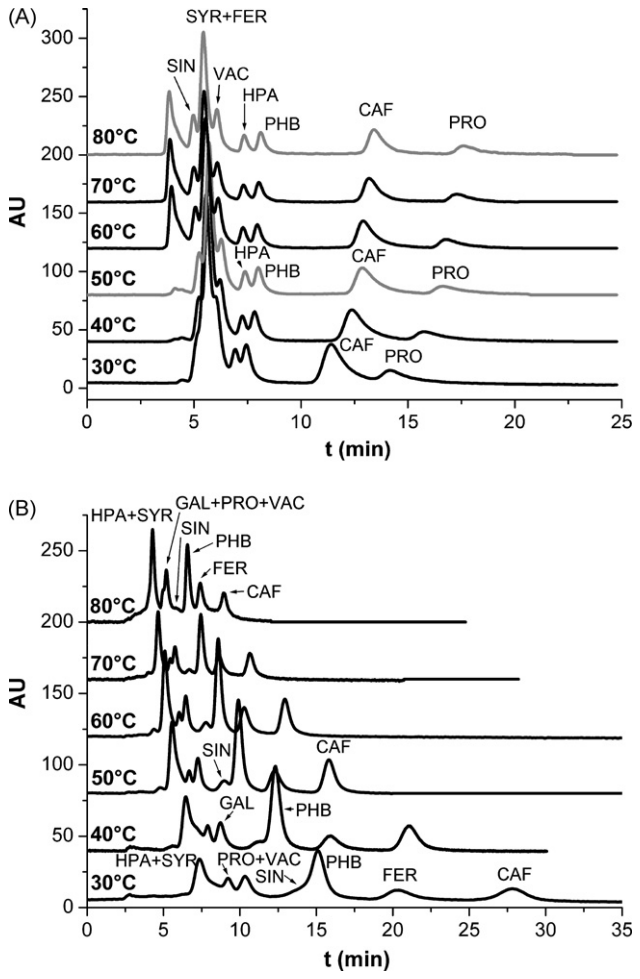


Fig. 4. Separation of phenolic acids at various temperatures (30–80 °C) on a MON-MEDSA-EDMA C column. Isocratic elution; Flow-rate: 5 μL min^{-1}, UV-detection: 280 nm. A: HILIC conditions—10% 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17) + 90% acetonitrile; Flow-rate: 3 μL min^{-1}. B: RP conditions—85% 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17) + 15% acetonitrile. The compounds are as in Table 1.

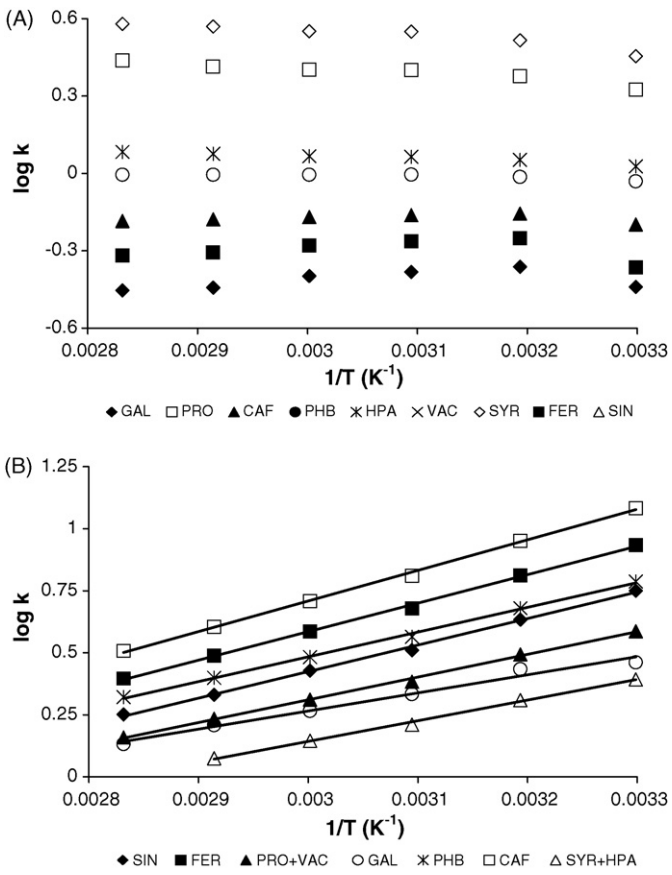


Fig. 5. Temperature effects (30–80 °C) on the retention factors of phenolic acids in the HILIC (A) and in the RP-mode (B) on a MON-MEDSA-EDMA C column. Flow-rate: 3 μL min^{-1}; UV-detection: 280 nm. A: HILIC mode—10% 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17) + 90% acetonitrile. B: RPLC mode—85% 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17) + 15% acetonitrile; Flow-rate: 5 μL min^{-1}. The compounds are as in Table 1.

lary MON-MEDSA-EDMA column under HILIC and RP conditions in the temperature range 30–80 °C. The retention times and retention factors, k, of most phenolic acids do not depend very significantly on temperature, except for caffeic and protocatechuic acids (Fig. 5A, Table 3), but the resolution noticeably improves at increasing temperatures (Fig. 4A). On the other hand, the retention of phenolic acids on the monolithic MON-MEDSA-EDMA column in the RP mode significantly decreases (Fig. 5B) and the resolution impairs (Fig. 4B) at increasing temperature.

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, Eq. (3) [32]:

$$\ln k = \ln K + \ln \frac{V_S}{V_M} = -\frac{\Delta G^0}{RT} + \ln \frac{V_S}{V_M} = \frac{\Delta S^0}{R} + \ln \frac{V_S}{V_M} - \frac{\Delta H^0}{RT} = A_i + \left(\frac{B_i}{T}\right) \quad (3)$$

In such a case, the $\ln k$ *versus* $1/T$ plots should be 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 from the mobile phase to the stationary phase, ΔS^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 Kelvins) [32–35]. Fig. 5B shows that this is obviously the case in the reversed-phase mode separation on the monolithic MON-MEDSA-EDMA column, confirmed by good correlation coefficient of the experimental data fitted to Eq. (3) by linear regression analysis in Table 5. This means that the retention in the RP mode is principally controlled by enthalpic contribution. On the other hand, the retention factors in the HILIC range of mobile phase are (almost) independent of the temperature (Fig. 5A) and the slopes of the van't Hoff plots of $\log k$ *versus* $1/T$, which are proportional to the enthalpic contribution to the retention, are close to zero. This suggests that the retention of phenolic acids in the HILIC mode is probably controlled mainly by entropic contributions, possibly originating in different solvation of samples in the stationary and in the bulk mobile phase.

Table 5
Best-fit parameters A_i and B_i of Eq. (3) in the temperature range 30–80 °C, and correlation coefficients, R^2, for phenolic acids on the capillary MON-MEDSA-EDMA column C under RP conditions.

Compound	A_i	B_i	R^2
SIN	−2.78 ± 0.07	1069.0 ± 22.9	0.998
FER	−2.86 ± 0.07	1149.7 ± 22.6	0.999
SYR	−2.36 ± 0.08	834.1 ± 25.8	0.997
VAC	−2.44 ± 0.06	916.0 ± 20.0	0.998
HPA	−2.36 ± 0.08	834.1 ± 25.8	0.997
PHB	−2.51 ± 0.06	997.8 ± 20.9	0.998
CAF	−2.99 ± 0.07	1232.8 ± 24.0	0.999
PRO	−2.44 ± 0.06	916.0 ± 20.0	0.998
GAL	−1.93 ± 0.15	730.3 ± 48.2	0.983

Short names of the acids are as in Table 1.

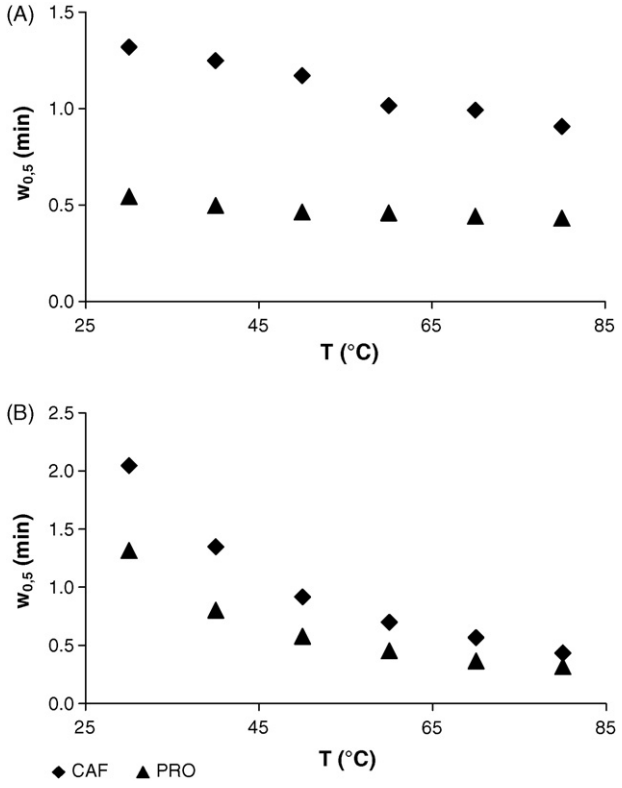


Fig. 6. Temperature effects (30–80 °C) on the peak widths at half height ($w_{0.5}$) of caffeic and p-hydroxybenzoic acid in the HILIC (A) and in the RP (B) modes. Column: MON-MEDSA-EDMA C; UV-detection: 280 nm. A: HILIC—isocratic elution; 10% 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17) + 90% acetonitrile; Flow-rate: 3 μL min^{-1}. B: RPLC—isocratic elution; 85% 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17) + 15% acetonitrile; Flow-rate: 5 μL min^{-1}. The compounds are as in Table 1.

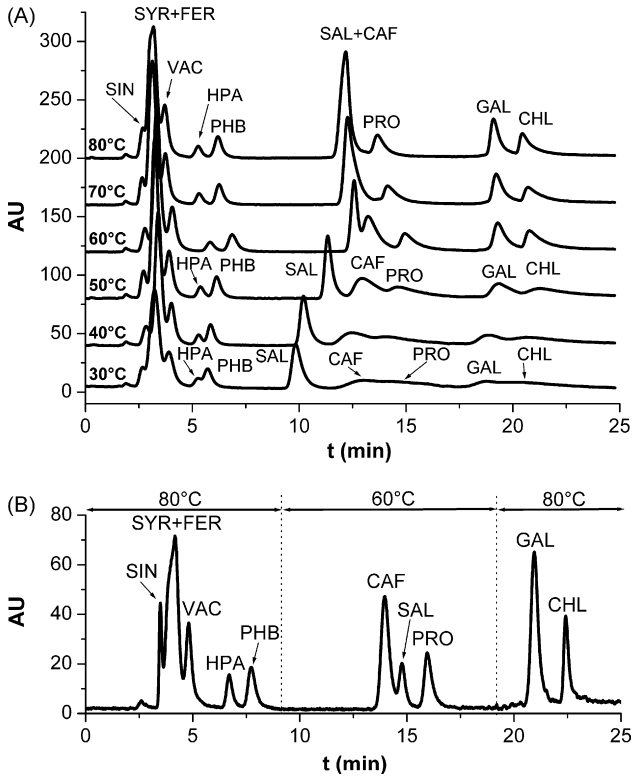


Fig. 7. Gradient elution of phenolic acids in the HILIC mode at different temperatures. Column: MON-MEDSA-EDMA C; Mobile phase A: 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17); Mobile phase B: Acetonitrile; Gradient: 0 min–95% B, 12 min–70% B, 30 min–70% B; Flow-rate: 5 μL min^{-1}; UV-detection: 280 nm. The compounds are as in Table 1.

Generally, the efficiencies of the polymer-based monolithic columns for low-molecular compounds are not very high in the HILIC mode [17]. That is why the number of theoretical plates on the present capillary MON-MEDSA-EDMA column is relatively low in comparison to silica-based columns used in the HILIC mode [14]. As shows Fig. 6, peak widths improve at increased temperature both in the HILIC and in the RP modes. Table 6 illustrates significant increase in column efficiency (the number of theoretical plates, *N*) and decrease in the height equivalent to a theoretical plate, *HETP*, at increasing temperature. Hence high-temperature operation should be preferred for separations on the monolithic MON-MEDSA-EDMA columns, both in isocratic and in gradient HILIC chromatography.

HILIC gradient elution with increasing volume concentration of aqueous buffer (20 mmol L^{-1} ammonium acetate) in acetonitrile improves the resolution of some phenolic acids, such as vanilic, 4-hydroxyphenylacetic and p-hydroxybenzoic acids, or strongly polar gallic and chlorogenic acids. As shows Fig. 7A, the best resolution of phenolic acids is achieved at 80 °C, except for salicylic, caffeic and protocatechuic acids, due to more significant increase in the retention of salicylic acid relatively to the other two acids at increasing temperature and consequent decrease in the separation selectivity in comparison to 60 °C. The separation of phenolic acids significantly improves by using solvent gradient elution combined with the step decrease in temperature from 80 °C to 60 °C in the 9th min and the step increase back to 80 °C in the 19th min (Fig. 7B). To our best knowledge, this is the first example of using combined

Table 6
Efficiency of the on the capillary MON-MEDSA-EDMA column C in HILIC Fm = 5 μL min^{-1}, u = 16.7 μm s^{-1}.

T [°C]	SIN	FER	SYR	VAC	HPA	PHB	CAF	PRO
N/m								
30	3000	3800	4300	5400	7000	6200	2400	2600
40	3400	4800	5100	6600	7900	7900	3200	3800
50	3300	5600	6200	7300	8900	9400	3900	4900
60	3900	5200	6100	7100	10600	9600	5200	6400
70	3700	5200	6400	7300	11100	10600	5700	7500
80	7800	6700	6700	7300	11600	11300	7300	8400
HETP [μm]								
30	337.7	261.0	231.7	186.9	143.2	161.6	416.9	382.9
40	292.7	207.4	195.7	152.1	125.8	126.7	316.0	264.2
50	300.4	177.5	161.6	137.2	111.8	106.7	256.7	202.3
60	257.3	192.6	163.7	140.5	94.2	104.4	192.4	156.8
70	271.2	193.1	155.8	137.9	89.9	94.1	177.0	134.2
80	128.1	148.5	149.3	166.5	86.0	88.4	136.6	119.1

N—number of theoretical plates, HETP—height equivalent to a theoretical plate, short names of the acids are as in Table 1.

Table 7
Parameters of the calibration equation, $A = k \cdot c + q$.

Compound	k	q	r_k	LOD [mg L^{-1}]	LOQ [mg L^{-1}]
PHB	4.906 ± 0.054	−19.393 ± 25.394	0.9996	8.28	19.71
CAF	14.023 ± 0.015	16.508 ± 8.779	0.9999	13.70	19.12
GAL	9.385 ± 0.034	−78.965 ± 40.856	0.9999	40.29	56.48

A = peak areas (mAU min); c = concentration (mg L^{-1}); q = intercept [mAU min]; k = slope, [mAU min L mg^{-1}]; r_k = correlation coefficient; LOD = limits of detection (3× noise level); LOQ = limits of quantification (10× noise level); short names of the acids are as in Table 1.

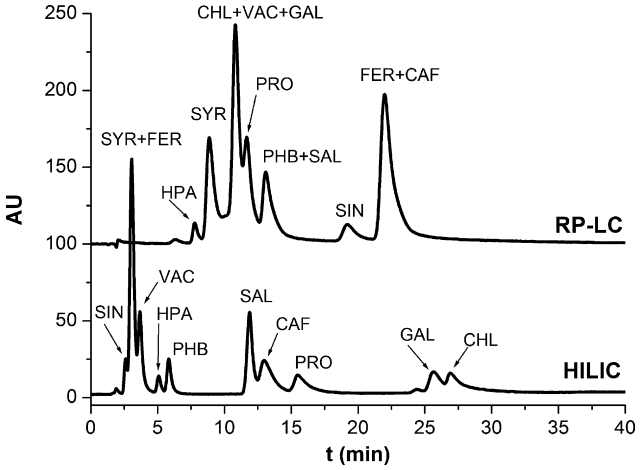


Fig. 8. Comparison of the gradient separation of phenolic acids in the HILIC-mode (A) and in the RP-mode (B). Column: MON-MEDSA-EDMA C; Mobile phase A: 20 mmol L^{-1} aqueous ammonium acetate (pH 3.17); Mobile phase B: acetonitrile; Temperature: 60 °C; flow-rate: 5 μL min^{-1}; UV-detection: 280 nm. HILIC mode: Gradient: 0 min–95% B, 20 min–80% B, 35 min–50%B, 40 min–50% B. RP mode: Gradient: 0 min–5% B, 30 min–15% B, 60 min–50% B. The compounds are as in Table 1.

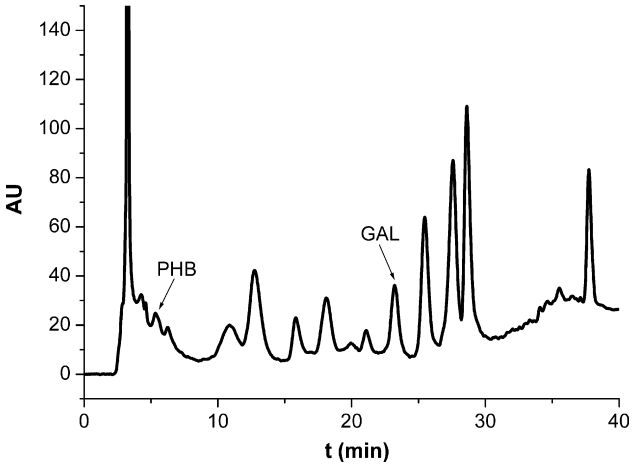


Fig. 9. Separation of 100 nL of a Pilsner Urquell beer sample on a MON-MEDSA-EDMA C column. Conditions are as in Fig. 8.

solvent gradient and programmed temperature conditions in the HILIC separation mode.

3.3. Comparison of separations in the HILIC and in the RP mode

The MON-MEDSA-EDMA capillary column shows roughly inversed retention order in the HILIC and in the RP mode, however with major differences in separation selectivity. Fig. 8 shows that the gradient separation in the HILIC mode (decreasing concentration of acetonitrile in 20 mM acetate buffer) of phenolic acids is better than the reversed-phase gradient separation (increasing concentration of acetonitrile in 20 mM acetate buffer) on the same column. 7 acids of 11 can be partly separated in the RP gradient mode, whereas 10 acids in the HILIC gradient mode at 60 °C and mobile phase flow-rate of 5 μL min^{-1}. The separation selectivity is better in the HILIC mode for gallic/chlorogenic/vanillic/protocatechuic, ferulic/caffeic, or p-hydroxybenzoic/salicylic acids; whereas in the RP mode for sinapic/ferulic/syringic/vanillic acids. Thus complementary selectivities can be used for improved separations on a single MON-MEDSA-EDMA column run subsequently in the HILIC and in the RP modes.

The optimized HILIC gradient conditions were used for separation of polar compounds in beer on a capillary MON-MEDSA-EDMA column in the HILIC mode under the conditions of Fig. 8. Fig. 9 shows an example of separation of a 100 nL sample of non-treated Pilsner Urquell lager beer (Pilsner Urquell Brewery, Czech Republic). Based on the UV spectra, gallic (GAL) and p-hydroxybenzoic (PHB) acids were identified in the sample. Table 7 lists the parameters of calibration curves based on the peak areas recorded at 280 nm, corresponding to the injection of 10 nL of sample standards. As 100 nL of the beer sample was injected, the actual LOQ at S/N = 10 correspond to 5.6 mg L^{-1} GAL and 1.97 mg L^{-1} PHB The concentrations of the acids in the beer sample determined using the calibration curves were close above these limits, 7.04 mg L^{-1} GAL and 2.03 mg L^{-1} PHB. CAF was not detected in concentrations above the LOQ. These results are in agreement with the data obtained previously in the RP-mode, where gallic acid could not be determined due to very low retention [36,37]. The present concentration of gallic acid is slightly higher than the concentration reported elsewhere (5.7 mg L^{-1}) [38]; the difference can be possibly attributed to the differences between various production beer batches. For the identification of other acids, the UV spectra did not provide sufficient evidence and MS spectral information will be necessary.

4. Conclusions

Capillary monolithic polymethacrylate sulfobetaine columns showing dual HILIC-RP retention mechanism are useful for separation of phenolic acids.

The HILIC and the RP modes show complementary selectivities. The stability of the monolithic sulfobetaine column allows rapid switching from the reversed-phase to the HILIC mode and vice versa. Subsequent HILIC mode separation in highly organic mobile phases and RP mode separation in water-rich mobile phases on a single monolithic polymethacrylate sulfobetaine column provides significantly extended information on the sample composition. For this purpose, alternating gradients of decreasing and increasing concentration of acetonitrile in aqueous acetate buffer (20–30 mmol L^{-1}) at 60–80 °C can be recommended.

Monolithic polymethacrylate sulfobetaine columns are stable at high temperatures, which should be used for improved peak shape and separation efficiency. The present study revealed principally different effects of temperature on the retention on our column in the RP and in the HILIC mode. While the retention in the RP mode decreases at increasing temperature as it is usual for the enthalpy-controlled retention mechanism, the retention in the HILIC mode is almost independent of temperature, suggesting primary effects of the entropic contributions to the retention. The

gain in the efficiency at elevated temperature in the HILIC mode can be used without significant sacrificing separation selectivity. Even though the separation efficiency at the ambient or slightly elevated temperature is low, at 60–80 °C the bandwidths are generally acceptable, so that using elevated temperatures is almost must with the monolithic polymethacrylate zwitterionic columns. Moreover, step temperature programming combined with gradient elution can be used for fine tuning the resolution.

The capillary polymethacrylate solfobetaine monolithic columns show excellent long-term stability, we did not observe significant changes in column properties after 450 runs over the period of 20 months. The research of monolithic columns with dual retention mechanism is continuing.

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