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Retention dependence on temperature in reversed-phase and hydrophilic interaction liquid chromatography

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The retention mechanism in reversed-phase liquid chromatography (RPLC) and in hydrophilic interaction liquid chromatography (HILIC) have been investigated by examining the temperature dependence of retention of the chosen phenolic acids on two columns modified by hydrosilated silica – hydrosilated silica column, Diamond hydride column, cholesterol column and C_{18} bidentate column. The linear dependences of retention on temperature were observed in the HILIC as well as in the RPLC on all columns tested. Van't Hoff equation was applied to describe the retention mechanism thoroughly, and its slopes and intercepts were used for calculations of thermodynamic constants for the transfer of a solute from the mobile phase to the stationary phase (ΔH^0 and ΔS^0).

Key words: phenolic acids, HILIC, van't Hoff equation, hydrosilated silica

INTRODUCTION

Nowadays, the interest in separation of strongly polar compounds of natural or synthetic origin is rapidly increasing. Separation of many polar compounds in HPLC is difficult because those compounds are too weakly retained in LC reversed-phase RPLC, but too strongly in a non-aqueous normal-phase NPLC. These problems can be overcome via hydrophilic interaction (HILIC) mode [1], which utilizes polar stationary phases common in NPLC in combination with aqueous–organic mobile phases, typical to RPLC. Molecules of water from the aqueous-organic mobile phase are attracted and consequently adsorbed onto the surface of the polar stationary phase to form a diffuse water-rich layer. The retention of polar compounds in HILIC systems is caused by the combined effect of partition into the diffuse water-rich layer, adsorption on the surface and ion-exchange interactions with charge bearing functional groups, which obviously contribute to the retention of ionic compounds. 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 the increasing content of more polar solvent in the mobile phase – in this case water [2].

Temperature also has a strong effect on the retention mechanism especially in case of weak acids or bases, where the ionization equilibrium is temperature dependent [3]. This has been reported frequently in the literature, yet its potential role in the method development is still underexploited. The relationship between retention factor, k, and thermodynamic temperature, T (in Kelvin), is described by van't Hoff equation [4] (Eq. 1.):

$$\ln k = \ln K + \ln \frac{V_S}{V_M} = \frac{-\Delta G^0}{RT} + \ln \frac{V_S}{V_M} = \\ = \frac{\Delta S^0}{R} + \ln \frac{V_S}{V_M} - \frac{\Delta H^0}{RT} = A_i + \left(\frac{B_i}{T} \right), \quad (1)$$

In such a case, the dependence of ln k on $1/T$ plots is expected to be linear. The parameter B_i is proportional to

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the standard partial molar enthalpy of transfer of the solute *i* from the mobile phase to the stationary phase, – ΔH^0 and the parameter A_i involves 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) in the chromatographic system. The V_M is not identical to the column hold-up volume, necessary for the calculation of the retention factor, *k*. *R* is the gas constant and *T* is the thermodynamic temperature (in Kelvins) [5–7]. By plotting ln *k* versus 1/*T* over a sufficiently broad temperature range, the enthalpic and the entropic contributions to retention and selectivity, – H^0 from the slope and S^0 from the intercept of the plot, may be calculated. Van't Hoff plots can provide the information on whether or not the retention mechanisms change over the studied temperature range [8–11]. The calculation of the entropic contribution to the retention from the intercept, A_i, of Eq. (1) requires the numerical value of the phase ratio in the column to be known, which seems to be a serious problem because of the impossibility to determine correctly the boundary between the region occupied by the stationary and the mobile phase in the column, without adopting some convention [12]. The determination of the volume of the stationary phase, V_S, is even a more complicated problem. Fortunately, it can be smartly resolved by using Eq. (2), where ε_T is the total column porosity which can be easily calculated from Eq. (3) [13].

$$\frac{V_S}{V_M} = \frac{1-\varepsilon_T}{\varepsilon_T}, \quad (2)$$

$$\varepsilon_T = \frac{V_M}{V_C}, \quad (3)$$

Here, V_M corresponds to the column hold-up volume and V_C is assigned to inner volume of the empty column [14].

MATERIALS AND METHODS

Equipment

All the experiments were measured on Hewlett Packard 1090 (Palo Alto, CA, USA) with a binary gradient pump, a thermostated column compartment and an autosampler. Instrumentation was also equipped with an UV diode array detector. The system was controlled by HPchem software programme.

Materials and reagents

The characteristics of the Cogent hydrosilated silica gel columns (all from MicroSolv, Eatontown, NJ, USA), are listed in Table 1.

The standards of phenolic acids were purchased from Sigma Aldrich in the best available purity; the structures are shown in Fig. 1. Acetonitrile (LiChrosolv grade), amonium acetate and formic acid (both reagent grade) were obtained from Merck, Darmstadt, Germany. Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Water for preparation of mobile phase was purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA). ACN, LiChrosolv was obtained from Merck. All phenolic acids were purchased from Sigma-Aldrich in excellent purity. The phenolic acids are properly described in Fig. 1.

Method

The mobile phases were prepared by mixing the appropriate volumes of 10 mmol/L solution of CH_3COONH_4 in water (with pH adjusted to 3.26 by addition of a few drops of HCOOH) with 10 mmol/L solution of CH_3COONH_4 in acetonitrile. The stock solutions of phenolic acid standards were prepared in 95% aqueous acetonitrile and working solutions were obtained by the appropriate dilution of the stock solutions in the mobile phase. The column hold-up volume, *VM*, was determined as the elution volume of toluene in the HILIC mode (*VM, T*) and of uracil in the RP mode (*VM, U*) as the non-retained markers. Before each new series of experiments, the columns were equilibrated by flushing with 20 column hold-up volumes of the fresh mobile phase and the separation temperature was adjusted. The retention times, *tR*, were measured over the full composition range of the mobile phases containing 10 mmol/L ammonium acetate in aqueous acetonitrile. The measurements were repeated in triplicate and arithmetic means of the experimental retention times, *tR*, and the appropriate column hold-up time, *tM, T*, or *tM, U*, were

Table 1. **Characteristics of columns. A – silica hydride, B – UDC cholesterol, C – C18 bidentate column, D – Diamond hydride. *L* – Column length, *I. d.* – internal dimension; V_M – hold-up volume measured with toluene in 95% acetonitrile ($V_{M,T}$ – HILIC mode) and with uracil in 15% acetonitrile ($V_{M,U}$ – RP mode), V_S/V_M – phase ratio in HILIC and in RP mode**

column	L [mm]	*Id* [mm]	$V_{M,T}$ [mL] (HILIC)	$V_{M,U}$ [mL] (RP)	t_{MAX} [°C]	V_S/V_M (HILIC)	V_S/V_M (RP)
A	76	4.6	0.57	0.59	100	1.17	1.13
B	76	4.6	0.54	0.54	100	1.33	1.33
C	76	4.6	0.52	0.59	80	1.38	1.13
D	100	4.6	1.04	1.04	60	0.60	0.60

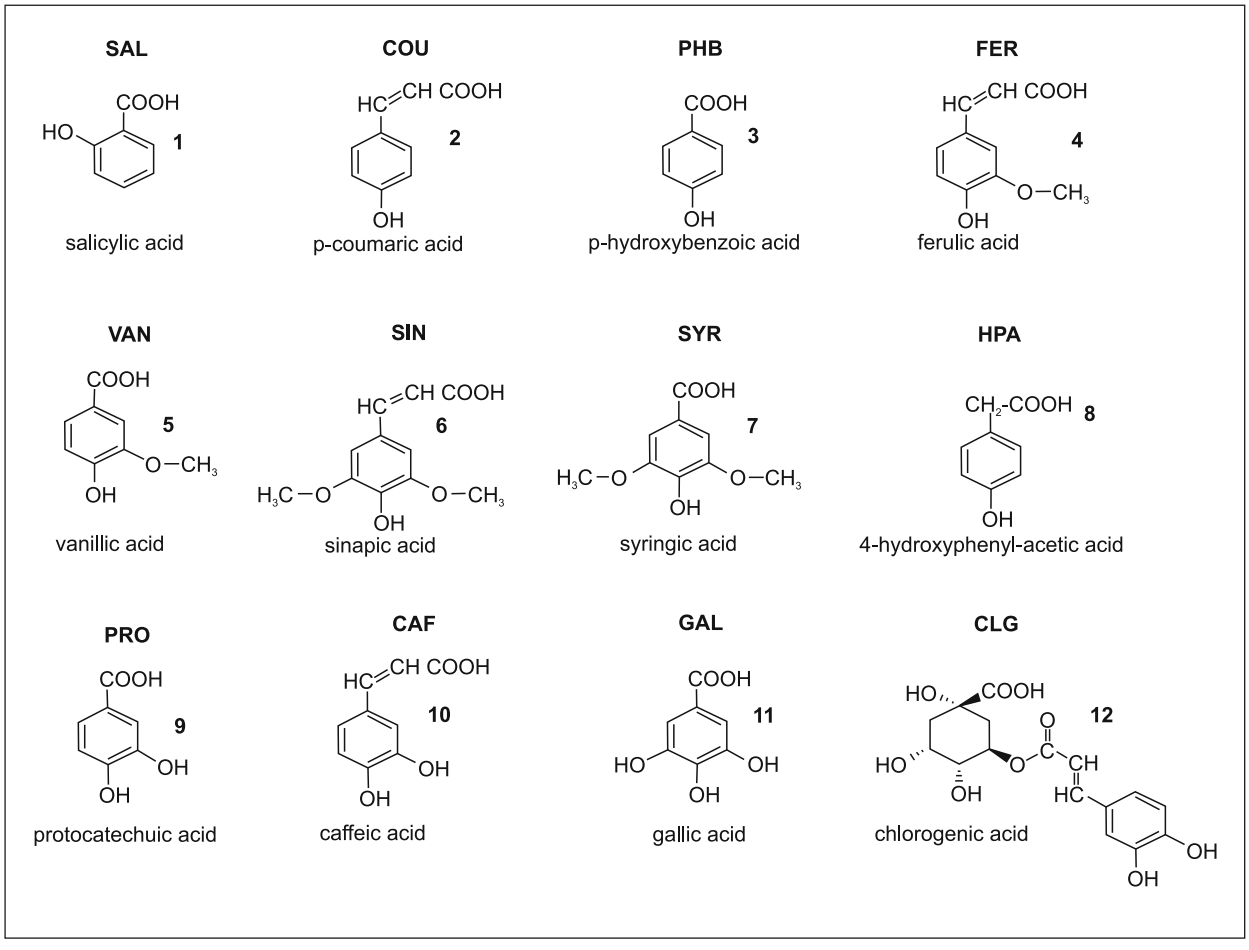

Fig. 1. Structures of the phenolic acid standards

used to calculate the retention factors, $k = tR / tM - 1$. The Adstat 1.25 software (Trilobyte Statistical Software, Pardubice, Czech Republic) was utilized for the determination of the parameters of Eq. by linear regression of the experimental data sets.

RESULTS AND DISCUSSION

Influence of temeperature on HILIC separation process

The retention of phenolic acids in the temperature range from 35 °C up to the column stability limits (60 °C for the Diamond hydride column, 80 °C for the C18 bidentate column and 100 °C for the UDC cholesterol and Silica hydride columns) was investigated in buffered ACN–water mobile phases.

If a single retention mechanism controls the retention over a broad temperature range, the influence of temperature, on the retention factor, k, can be described by van't Hoff equation (Eq. 1). Tables 2 and 3 show the results of the regression of the experimental retention-temperature dependences for the analyte tested in the HILIC mode: the best-fit value of the intercepts, A_i, the slopes, B_i, and the correlation coefficients, R^2. The linear dependence of ln k on $1/T$ under HILIC conditions on all columns tested was observed for all the phenolic acids, shown in Fig. 2.

Best-fit parameters of intercepts, A_i, and slopes, B_i, for each phenolic acid standard were determined, thus it enabled the calculation of the standard partial molar entropy of the transfer of the solute from the mobile phase to the stationary phase, ΔS^0, and the standard partial molar enthalpy of transfer of the solute i from the mobile phase to the stationary phase, ΔH^0.

Another acknowledged fact is that the utilization of higher temperatures during the chromatographic process may decrease the peak widths and might also affect the selectivity. It was even observed that in some cases the temperature can influence the separation process in such a measure that some compounds change their elution order which has already been shown in chromatograms (Fig. 3) where peaks 7 and 8 on the Diamond hydride column coelute at 55 °C, but are separated at 40 °C and partially separated but with the reversed order at 60 °C. Increasing the temperature decrease time of the retention, however keeping it higher than 60 °C, it causes loss of the resolution and the selectivity.

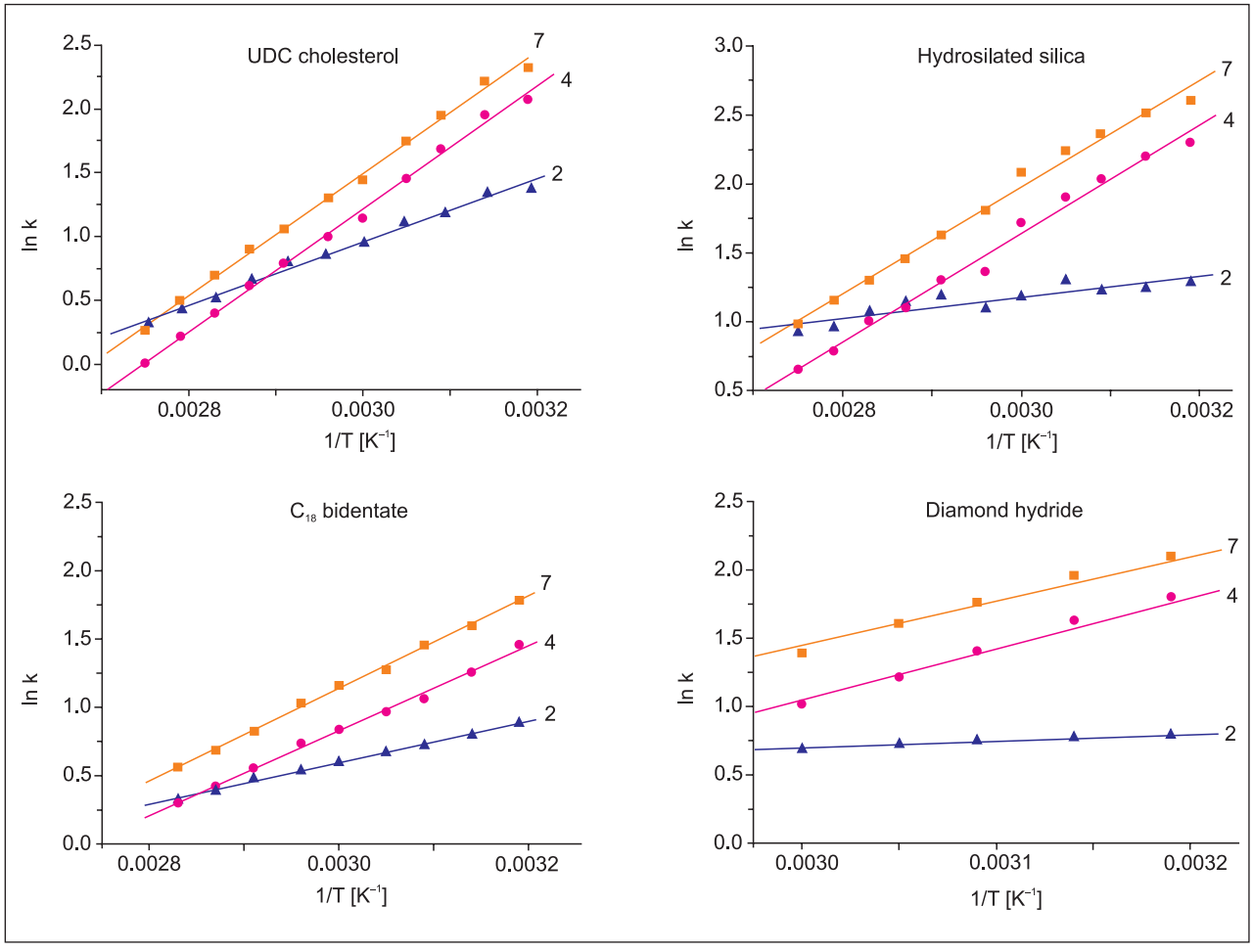

Fig. 2. Temperature effects on the retention factors of phenolic acids in the HILIC mode. Conditions: mobile phase composition – 10 mM NH_4AC in 5 : 95% water:acetonitrile, pH adjusted to 3.26 by HCOOH; F_m = 0.5 mL.min^{-1}; λ = 275 nm; injection volume 10 µL

Table 2. **Best-fit parameters A_i and B_i of Eq. (1) and correlation coefficients, R^2. HILIC conditions: mobile phase composition – 10 mM NH_4AC in 5 : 95% water:acetonitrile + 4.5 µL of HCOOH; F_m = 0.5 mL.min^{-1}; A – silica hydride column temperature 35–100 °C; B – UDC cholesterol column temeprature 35–100 °C; λ = 275 nm; injection volume 10 µL**

A silica hydride	A_i	B_i	R^2	$-\Delta H^0$ [kJ/mol]	ΔS^0 [J/mol.K]
SAL	–0.92 ± 0.03	496.6 ± 25.4	0.9521	–4.13	–9.07
COU	–1.11 ± 0.04	762.1 ± 11.2	0.9632	–6.34	–10.65
PHB	–10.66 ± 0.25	4057.9 ± 130.7	0.9892	–33.74	–90.05
FER	–10.18 ± 0.12	3938.4 ± 64.1	0.9934	–32.74	–86.06
VAN	–10.09 ± 0.51	3928.1 ± 19.5	0.9917	–32.66	–85.31
SIN	–10.38 ± 0.21	4049.4 ± 22.7	0.9914	–33.67	–87.72
SYR	–9.66 ± 0.23	3877.1 ± 76.7	0.9894	–32.23	–81.74
HPA	–11.89 ± 0.49	4627.4 ± 152.1	0.9919	–38.47	–100.28
B UDC cholesterol	A_i	B_i	R^2	$-\Delta H^0$ [kJ/mol]	ΔS^0 [J/mol.K]
SAL	–5.80 ± 0.22	2006.4 ± 89.1	0.9903	–16.68	–30.05
COU	–6.43 ± 0.12	2459.9 ± 50.7	0.9907	–20.45	–34.29
PHB	–13.41 ± 0.52	4804.4 ± 65.9	0.9962	–39.94	–85.67
FER	–13.16 ± 0.23	4788.1 ± 28.8	0.9969	–39.81	–70.46
VAN	–13.03 ± 0.44	4747.3 ± 199.5	0.9968	–39.47	–76.19
SIN	–13.39 ± 0.35	4894.6 ± 50.4	0.9966	–40.69	–80.02
SYR	–12.78 ± 0.51	4754.5 ± 112.3	0.9963	–39.53	–76.52
HPA	–15.20 ± 0.43	5574.5 ± 78.6	0.9972	–46.35	–94.07

Table 3. **Best-fit parameters A_i and B_i of Eq. (1) and correlation coefficients, R^2. HILIC conditions: mobile phase composition – 10 mM NH_4AC in 5 : 95% water:acetonitrile + 4.5 µL of HCOOH; F_m = 0.5 mL.min^{-1}; C bidentate C_{18} – column temperature 35–80 °C; D – Diamond hydride column temperature 35–60 °C; λ = 275 nm; injection volume 10 µL**

C bidentate C_{18}	A_i	B_i	R^2	$-\Delta H^0$ [kJ/mol]	ΔS^0 [J/mol.K]
SAL	–3.28 ± 0.17	1014.4 ± 20.1	0.9644	–8.43	–11.72
COU	–3.79 ± 0.11	1454.8 ± 31.5	0.9444	–12.10	–15.96
PHB	–9.97 ± 0.35	3525.8 ± 83.7	0.9981	–29.31	–67.34
FER	–8.14 ± 0.21	2986.4 ± 105.9	0.9915	–24.83	–52.12
VAN	–8.83 ± 0.41	3231.3 ± 122.6	0.9849	–26.87	–57.86
SIN	–9.29 ± 0.38	3407.1 ± 51.7	0.9836	–28.33	–61.68
SYR	–8.87 ± 0.14	3334.9 ± 41.8	0.9939	–27.73	–58.19
HPA	–10.98 ± 0.46	4053.2 ± 67.8	0.9900	–33.70	–75.73
D Diamond hydride	A_i	B_i	R^2	$-\Delta H^0$ [kJ/mol]	ΔS^0 [J/mol·K]
SAL	–0.83 ± 0.02	567.3 ± 9.1	0.9952	–4.72	–2.63
COU	0.06 ± 0.01	472.1 ± 18.5	0.9661	–3.93	4.77
PHB	–10.38 ± 0.77	3997.2 ± 102.1	0.9988	–33.23	–82.02
FER	–9.52 ± 0.42	3752.3 ± 51.6	0.9988	–31.20	–74.87
VAN	–9.06 ± 0.32	3623.9 ± 147.2	0.9989	–30.13	–71.05
SIN	–9.16 ± 0.21	3680.8 ± 99.5	0.9986	–30.60	–71.88
SYR	–8.12 ± 0.33	3405.8 ± 44.6	0.9946	–28.32	–63.23
HPA	–11.22 ± 0.35	4430.3 ± 87.5	0.9991	–36.83	–89.01

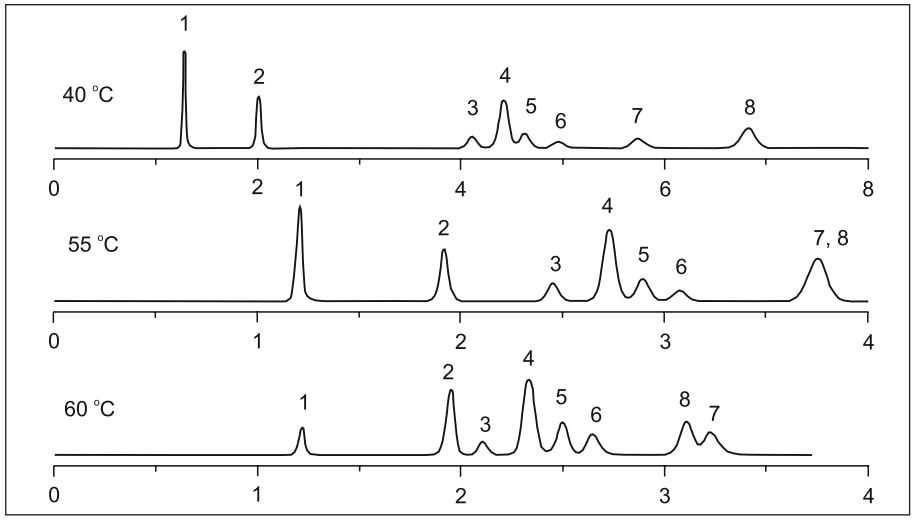

Fig. 3. The HILIC separations at 40, 55, 60 °C on a Diamond hydride column. Isocratic conditions: mobile phase composition – 10 mM NH_4AC in 5 : 95% water:acetonitrile, pH of aqueous part adjusted to 3.26 by HCOOH; F_m = 0.5 mL.min^{-1}; λ = 275 nm; injection volume 10 µL. V_R – retention volume; V_C – volume of empty column

Influence of temperature on separation process in the reversed-phase mode

The effect of temperature on retention of the phenolic acids was also investigated in the reversed-phase mode on UDC cholesterol and C_{18} bidentate columns. The implementation of van't Hoff equation (Eq. 1) was realized and the linear dependence of ln k on 1/T was observed which was also proved in the HILIC mode. Table 4 contains the results of the regression of the experimental retention-temperature dependences for the compounds tested in the RP mode: the best-fit value of the intercepts, A_i, the slopes, B_i, the correlation coefficients, R^2 as well as the standard partial molar entropy of the transfer of the solute from the mobile phase to the stationary phase, ΔS^0, the standard partial molar enthalpy of the transfer of the solute i from the mobile phase to the stationary phase, ΔH^0. Although, the increase of temperature decreased the retention there were no changes in resolution of sample compounds, described in the chromatograms in Fig. 4.

The usage of higher temperatures in the RP mode is markedly better in comparison to the HILIC mode; indeed, the change of elution order was not observed. The values of enthalpic, B_i, and entropic, A_i, contributions are almost comparable.

Table 4. **Best-fit parameters A_i and B_i of Eq. (1) and correlation coefficients, R^2. RP conditions: mobile phase composition – 10 mM NH_4AC in 85 : 15% water:acetonitrile (pH 3.26); F_m = 0.5 mL.min^{-1}; λ = 275 nm; injection volume 10 µL; B – UDC cholesterol column – measurement in the temperature range 35–100 °C; C – C_{18} bidentate column – temperature 35–80 °C**

B UDC cholesterol	A_i	B_i	R^2	$-\Delta H^0$ [kJ/mol]	ΔS^0 [J/mol·K]
SAL	–5.75 ± 0.09	2257.6 ± 17.5	0.9920	–18.77	–48.69
COU	–5.80 ± 0.03	2374.7 ± 12.6	0.9974	–19.74	–49.11
PHB	–5.13 ± 0.12	1899.9 ± 32.1	0.9859	–15.80	–43.54
FER	–5.15 ± 0.05	2257.6 ± 15.8	0.9983	–18.77	–43.70
VAN	–4.27 ± 0.06	1703.7 ± 24.1	0.9904	–14.16	–36.39
SIN	–4.92 ± 0.02	2227.2 ± 11.1	0.9983	–18.52	–41.79
SYR	–3.15 ± 0.14	1394.3 ± 44.9	0.9753	–11.59	–27.08
HPA	–4.41 ± 0.09	1613.8 ± 26.5	0.9883	–13.42	–37.55
PRO	–4.62 ± 0.21	1593.5 ± 36.2	0.9772	–13.25	–39.30
CAF	–5.60 ± 0.05	2138.3 ± 21.8	0.9923	–17.78	–47.45
GAL	–4.58 ± 0.18	1391.0 ± 41.5	0.9776	–11.56	–38.96
CLG	–5.17 ± 0.05	1797.6 ± 20.5	0.9904	–14.95	–43.87
C–C_{18} bidentate	A_i	B_i	R^2	$-\Delta H^0$ [kJ/mol]	ΔS^0 [J/mol·K]
SAL	–4.42 ± 0.04	1686.9 ± 17.2	0.9934	–14.02	–37.63
COU	–7.61 ± 0.07	2766.8 ± 28.5	0.9978	–23.00	–64.16
PHB	–4.09 ± 0.21	1527.6 ± 68.7	0.9711	–12.70	–34.89
FER	–5.33 ± 0.02	2274.7 ± 19.8	0.9983	–18.91	–45.20
VAN	–4.09 ± 0.06	1610.3 ± 27.1	0.9943	–13.39	–34.89
SIN	–4.88 ± 0.03	2156.7 ± 14.8	0.9982	–17.93	–41.46
SYR	–4.42 ± 0.08	1812.3 ± 21.6	0.9966	–15.07	–37.63
HPA	–4.73 ± 0.02	1718.0 ± 19.5	0.9938	–14.28	–40.21
PRO	–5.20 ± 0.17	1705.2 ± 36.4	0.9887	–14.18	–44.12
CAF	–3.87 ± 0.12	1565.1 ± 60.9	0.9851	–13.01	–33.06
GAL	–6.22 ± 0.28	1854.1 ± 88.3	0.9775	–15.41	–52.60
CLG	–5.33 ± 0.16	1772.9 ± 38.6	0.9845	–14.74	–45.20

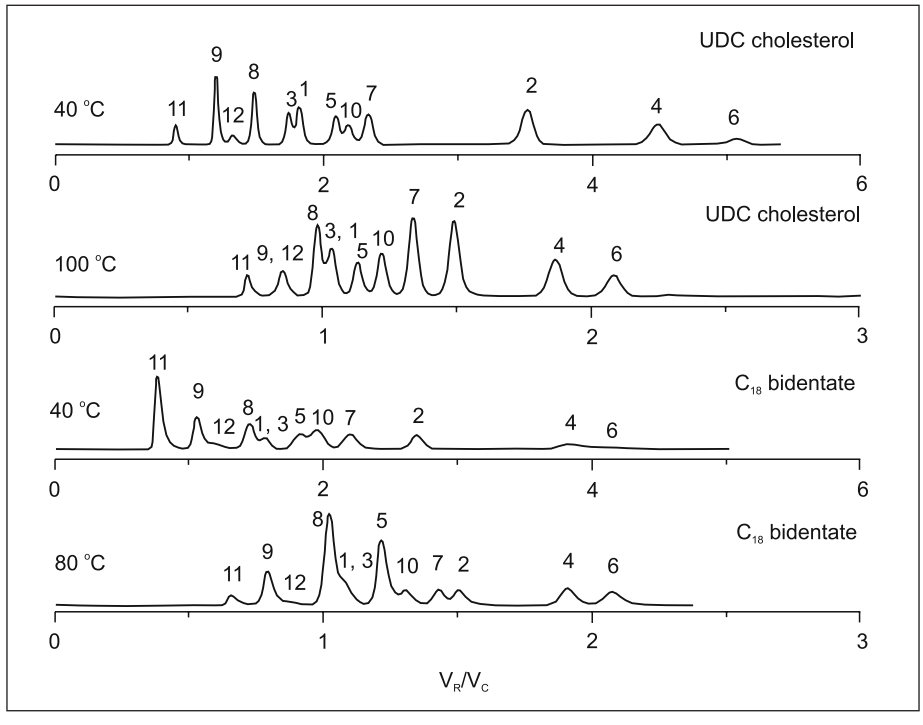

Fig. 4. The separations in the reversed-phase mode. Conditions: mobile phase composition – 10 mM NH_4AC in 85 : 15% water:acetonitrile, pH of aqueous part adjusted to 3.26 by HCOOH; F_m = 0.5 mL. min^{-1}; λ = 275 nm; injection volume 10 µL. V_R – retention volume; V_C – volume of the empty column

CONCLUSIONS

The log *k* of phenolic acids embodies the linear decrease with increasing temperature, in agreement with the van't Hoff model, both in the RP and in the HILIC mode. On the Silica hydride and Diamond hydride columns, the enthalpic contributions to the retention are higher than the entropic contributions in the HILIC mode, but the differences are significantly lower on the C18 bidentate and UDC cholesterol columns, where they are almost comparable at high temperatures, and the entropic effects even may predominate over the enthalpic ones for weakly retained phenolic acids with two or more phenolic -OH groups in the RP mode, or with salicylic and p-coumaric acids in the HILIC mode. From all the columns tested, the UDC cholesterol column fits the best for the dual mode HILIC and RP separations of phenolic acids. Its high thermal stability up to 100 °C is undoubtedly an important advantage in comparison to less stable columns based on the silica B type. The presented results demonstrate the importance of temperature as a complementary tool to the mobile phase composition for the control and optimization of separation on the unmodified and modified hydrosilated silica (type C) columns, showing a dual HILIC / RP retention mechanism.

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