SCIENTIFIC PAPERS
OF THE UNIVERSITY OF PARDUBICE
Series A
Faculty of Chemical Technology
20 (2014)

UTILIZATION OF HYDROSILATED SILICA-BASED STATIONARY PHASES FOR SEPARATION OF ANTIOXIDANTS

Jan SOUKUP, Petr JANÁS and Pavel JANDERA[1]
Department of Analytical Chemistry,
The University of Pardubice, CZ–532 10 Pardubice

Received March 11, 2014

We studied the influence of mobile phase composition on the retention of phenolic acids and flavonoid compounds on five different hydrosilated silica-based stationary phases in buffered aqueous acetonitrile. Cogent UDC cholesterol™, Cogent bidentate C18™ and Cogent Phenyl hydride™ columns show significant dual reversed-phase/normal-phase retention behaviour, while Cogent Diamond hydride™ and Cogent Silica-C™ columns provide very low retention in the reversed-phase mode. The effect of the aqueous acetate buffer concentration on retention factors of phenolic acids and flavonoid compounds over the full mobile phase composition range, including both aqueous normal-phase (ANP) and reversed-phase (RP) mechanisms, can be described by a four-parameter equation for dual-retention mechanism. At increasing temperature, the retention factors and peak widths decrease in the aqueous normal-phase range. In agreement with van't Hoff model, linear ln k versus 1/T plots were observed, showing a single

[1] To whom correspondence should be addressed.

retention mechanism. From among the stationary phases tested, Cogent UDC cholesterol™ column has high temperature stability (up to 100 °C) and provides the most selective and efficient separations of flavones in the ANP mode and Cogent Diamond hydride™ is the most selective and effective for separation of phenolic acids.

Introduction

Hydrosilated silica gel (silica-C) as a material of HPLC separation was introduced more than two decades ago. However, its interesting chromatographic properties have only recently found increasing use. It is prepared *via* hydrosilation process, in which up to 95 % of silanols on the surface of silicagel are replaced by significantly less polar Si-H groups [1]. It can be used in normal-phase liquid chromatography with organic mobile phases [2]; however, its main application range is in aqueous normal phase LC. The aqueous normal-phase LC mode (ANP) employs polar stationary phases in the combination with mobile phases containing high concentrations of organic solvents (usually acetonitrile) in water, often with a buffer additive [3]. In the past years, ANP technique has attracted attention as a perspective complementary alternative to reversed-phase HPLC for separations of polar compounds, which still represent a challenging problem [4-10]. Particularly interesting are ANP applications for the analysis of peptides and of the biopolymers [11-13] and in pharmaceutical [14] or metabolite analysis [15-17]. 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. In the ANP range, polar compounds may be retained due to combined adsorption on the adsorbent surface and partition into the diffuse adsorbed aqueous layer. Ion-exchange interactions with charged functional groups may contribute to the retention of ionic or partly ionized samples, so that the resulting ANP mechanism may be quite complex. Hydrosilated silica gel surface shows less attraction for water molecules as compared with the ordinary silica gel type B. Due to its more hydrophobic surface, hydrosilated silica is believed to form less dense adsorbed water layer at its surface than other, more polar stationary phases employed for ANP separations [18-24]. The hydride surface of hydrosilated silica is slightly hydrophobic and can retain some weakly polar compounds in highly aqueous mobile phases in the reversed-phase mode (RP), even though much less strongly than common C18 or C8 alkylsilica stationary phases. To increase the retention of hydrophilic compounds under reversed-phase conditions, the hydrophobicity of the silica hydride surface was enhanced by chemical modification introducing low-polarity bonded groups, which also provide some new selectivity properties for separations of polar compounds. Figure 1 shows schematically the surface structure of hydrosilated silica and

ordinary silica (A) and of hydrosilated silica modified with bidentate C18 (B) and cholesterol (C) groups. Silica hydride modified with undecanoic acid (UDA silica) is also commercially available. Diamond hydride stationary phases, which contain ca 2.5 % carbon, show improved ANP separation selectivity for mono-, di- and tri-phosphate nucleotides, due to enhanced ion-interaction (ion repulsion) properties with respect to the Diamond hydride column [24].

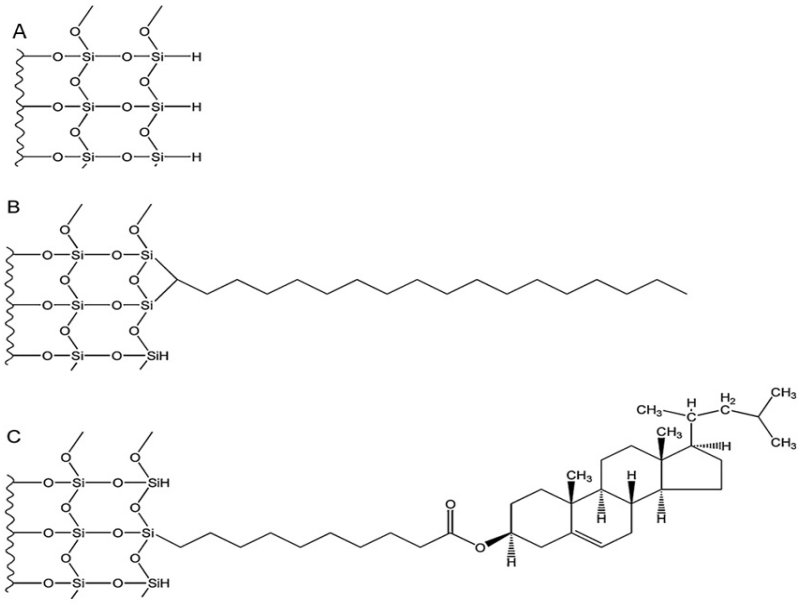

Fig. 1 Structures of hydrosilated silica-based stationary phases: A – hydroslated silica, B – C18 bidentate, C – UDC cholesterol

Silica hydride columns modified with non-polar moieties show some features of dual reversed-phase/normal-phase retention mechanism and can be used either in highly aqueous mobile phases in the RP mode, or for separations in the aqueous normal-phase (ANP) mode in buffered mobile phases containing more than 50-70 % acetonitrile [25], unlike the un-modified silica hydride column, which shows very low hydrophobic selectivity and retention under RP conditions [26].

In normal-phase chromatography (NP), the retention increases with increasing sample polarity. The mobile phase affects significantly the retention in liquid chromatography and in classical non-aqueous adsorption NP chromatography the retention decreases as the concentration of a polar solvent with high elution strength increases in binary organic mobile phases. Likely, at high concentrations of the organic solvent in the ANP mobile phase range, the retention on polar stationary phases decreases at increasing concentration of water as the more polar solvent in aqueous-organic mobile phases. On the same polar column, the retention may decrease for more polar samples and at increasing concentrations of organic solvent in highly aqueous binary mobile phases, showing typical RP behavior. Consequently, the graphs displaying the effects of

the composition of aqueous-organic mobile phases on the retention show characteristic "U shape". Assuming additivity of the ANP and RP contributions to the retention, the effect of the volume fraction of water (or of an aqueous buffer), φ_{water}, on the retention factors, k, in the full composition range of aqueous-organic mobile phases can be described, to first approximation, by Eq. (1) [27,28]

$$\log k = a_1 + m_{RP}\varphi_{water} - m_{ANP}\log\varphi_{water} \quad (1)$$

The parameter m_{ANP} characterizes the effect of the aqueous component on the rate of decreasing ANP contribution to the retention in highly organic mobile phases, while the parameter m_{RP} describes its effect on the rate of increasing contribution of the RP mechanism to the retention in the aqueous-rich mobile phases; a_1 is an empirical constant and has no exact physical meaning. The parameters a_1, m_{RP} and m_{ANP} can be determined by non-linear regression of the experimental retention factors measured at varying volume fractions of water (or of aqueous buffer) in the mobile phase [29]. Equation (1) applies in ANP systems only if the sample is very strongly retained in 100% acetonitrile, otherwise Eq. (2) often offers better approach to the description of the dual ANP/RP retention mechanism [30]

$$\log k = a_2 + m_{RP}\varphi_{water} - m_{ANP}\log(1 + b\varphi_{water}) \quad (2)$$

m_{RP} and m_{ANP} have similar meaning as in Eq. (1); the parameter b is the correction term for limited ANP retention in mobile phases with very low concentrations of water.

The potential role of temperature effects in HPLC method development has not been yet fully recognized, obviously because of a limited temperature stability of the ordinary stationary phases chemically bonded on the silica gel type B support (often only up to 60 °C). However, bare silica and some stationary phases bonded on hydrosilated silica surface show enhanced temperature stability range. For these columns, the control of temperature has some advantages, as it can be easily adjusted in the instrument equipped with a thermostatted column compartment. In most RP and ANP separation systems an increase in temperature causes a decrease in retention. Solvent viscosity decreases at higher temperature, causing diffusion coefficients to increase, which often improves the efficiency of separation (column plate number) and peak shape [31]. Further, the column backpressure decreases at increased temperature, so that higher flow rates can be used for faster separations [32]. Temperature often affects chromatographic selectivity, especially for ionizable compounds such as weakly acidic phenolic compounds, as the ionization equilibria usually can be shifted by a change in temperature.

The effects of thermodynamic temperature, T (in Kelvin) on the sample

retention factors, k, is described by van't Hoff equation (Eq. (3)) [33-36]

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

According to Eq. (3), the log k versus $1/T$ plots should be linear, the 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 change in the standard partial molar entropy connected with 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. Possible deviations of the experimental data from the linear Eq. (3) may indicate changing retention mechanism in the investigated temperature range [36-40].

Hearn and Zhao [41] observed non-linear log k versus $1/T$ plots for several polypeptides in acetonitrile-water mobile phases on an alkylsilica stationary phase and explained it by temperature effects on changing heat capacity, so that the entropy of retention, ΔS^0, depends on temperature. The intercept term, A_i, includes the contribution of the column phase ratio, which is assumed to be independent of temperature; however, Guillarme *et al*. [42] attributed some deviations from the linearity of the log k versus $1/T$ experimental plots to possible changes in the phase ratio caused by the temperature effects on the system backpressure.

From the experimental data set measured over a sufficiently broad temperature range, one may calculate the enthalpy of the retention and selectivity, $-\Delta H^0$, from the slope, B_i, and the entropy, ΔS^0, from the intercept, A_i, of the log k versus $1/T$ plots. For the calculation of the entropy, the numerical value of the phase ratio in the column should be known, which may not be easy to determine because of difficulties with clear definition of the boundary between the region occupied by the stationary and by the mobile phase in the column [43]. For this purpose, a simplified convention (even though not exact) can be accepted, defining the volume of the stationary phase, V_S, as the part of the total column volume, V_C, into which non-retained compounds cannot penetrate. With this convention, the phase ratio can be calculated from the total column porosity, $\varepsilon_T = V_M/V_C$ [44,45]

$$\frac{V_S}{V_M} = \frac{V_C - V_M}{V_M} = \frac{1 - \varepsilon_T}{\varepsilon_T} \quad (4)$$

ANP methods are suitable for the separation of phenolic acids and flavonoid compounds in plants, fruit and vegetables [29,30]. The interest in the analysis of phenolic acids and flavonoids is continuously increasing, due to the protection antioxidant role of many phenolic compounds in human body against cancer and coronary heart diseases. The objective of the present work was to investigate the effects of mobile phase and temperature on the retention, selectivity and resolution of phenolic acids and flavonoid compounds and to compare possibilities of their separation on various hydrosilated silica-based columns in the ANP and in the reversed-phase (RP) modes.

Experimental

All the experiments were measured using an HPLC setup including a high pressure pump (ECOM, Prague, the Czech Republic) connected with a variable UV detector from the same manufacturer. The columns were placed in a thermostatted column compartment and the detection wavelength was set to 280 nm, the UV absorption maximum for phenolic acids and flavonoid compounds.

Table I Characteristics of Cogent C silica columns

Column	L mm	Id mm	V_M ml	ε_T	H mm	T_{max} °C	pH range
Cogent Silica-C™ 75×4.6 mm	75	4.6	1.01	0.8	0	60	2.0-7.0
Cogent Diamond hydrideTM 100×4.6 mm	100	4.6	1.22	0.7	0	60	2.5-7.0
Cogent UDC cholesterol™ 75×4.6 mm	75	4.6	0.8	0.6	0	100	2.0-8.0
Cogent bidentate C18™ 75×4.6 mm	75	4.6	0.8	0.6	0	80	2.0-9.2
Cogent Phenyl hydride™ 150×4.6 mm	150	4.6	1.58	0.6	0	80	1.0-8.0

Materials and Reagents

The characteristics of the silica hydride-based columns (all from MicroSolv, Eatontown, NJ, USA) are listed in Table I. Figure 1 shows the structures of Cogent Silica-C™, Cogent UDC cholesterol™ hydride and Cogent bidentate C18™.

Table II Names, numbers and acronyms of the phenolic acids studied

Compound	IUPAC name	No.	Abb.
Salicylic acid	2-Hydroxybenzoic acid	1	SAL
Coumaric acid	(E)-3-(4-Hydroxyphenyl)-2-propenoic acid	2	COU
p-Hydroxybenzoic acid	4-Hydroxybenzoic acid	3	PHB
Ferulic acid	(E)-3-(4-Hydroxy-3-methoxy-phenyl)prop-2-enoic acid	4	FER
Vanillic acid	4-Hydroxy-3-methoxybenzoic acid	5	VAN
Sinapic acid	3-(4-Hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid	6	SIN
Syringic acid	4-Hydroxy-3,5-dimethoxybenzoic acid	7	SYR
4-Hydroxy-phenylacetic acid	2-(4-Hydroxyphenyl)acetic acid	8	HPA
Protocatechuic acid	3,4-Dihydroxybenzoic acid	9	PRO
Caffeic acid	3-(3,4-Dihydroxyphenyl)-2-propenoic acid	10	CAF
Gallic acid	3,4,5-Trihydroxybenzoic acid	11	GAL
Chlorogenic acid	(1*S*,3*R*,4*R*,5*R*)-3-{[(2*Z*)-3-(3,4-Dihydroxyphenyl)prop-2-enoyl]oxy}-1,4,5-trihydroxycyclohexanecarboxylic acid	12	CLG

The standards of phenolic acids (Table II) and flavonoid compounds (Table III and Fig. 2) were purchased from Sigma Aldrich in the best available purity. Acetonitrile (LiChrosolv grade), ammonium 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).

Methods

The mobile phases were prepared by mixing appropriate volumes of 10 mmol l^{-1} solution of ammonium acetate (with pH adjusted to 3.26 by formic acid) in water with 10 mmol l^{-1} solution of ammonium acetate in acetonitrile. The stock solutions of phenolic acid standards were prepared in 95% aqueous acetonitrile and working solutions were obtained by diluting the stock solutions in the mobile phase. The

Table III Names, numbers and acronyms of the flavonoid compounds

Compound	IUPAC name	No.	Abb.
7-Hydroxyflavone	7-Hydroxy-2-phenylchromen-4-one	1	HFL
Flavone	2-Phenylchromen-4-one	2	FLA
Apigenin	5,7-Dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-	3	API
Biochanin A	5,7-Dihydroxy-3-(4-methoxyphenyl)chromen-4-one	4	BIA
Vanillin	4-Hydroxy-3-methoxybenzaldehyde	5	VIN
4-Hydroxy-coumarin	2-Hydroxychromen-4-one	6	HCO
Hesperidin	(2*S*)-5-Hydroxy-2-(3-hydroxy-4-methoxyphenyl)-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-[[(2*R*,3*R*,4*R*,5*R*,6*S*)-3,4,5-Trihydroxy-6-methyloxan-2-yl]oxymethyl]oxan-2-yl]oxy-2,3-dihydrochromen-4-one	7	HES
Esculin	7-Hydroxy-6-{[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy- 6-(hydroxymethyl)-2-tetrahydropyranyl]oxy}-2-chromenone	8	ESC
Naringin	7-[[2-O-(6-Deoxy-α-L-mannopyranosyl)-β-D-glucopyranosyl]]oxy]-2,3-dihydro-5-hydroxy-2-(4-hydroxyphenyl)-4H-1-benzopyran-4-one	9	NAR
Hesperetin	(S)-2,3-Dihydro-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-1-benzopyran-4-one	10	HPR
Naringenin	5,7-Dihydroxy-2-(4-hydroxyphenyl)chroman-4-one	11	NRG
Scopoletin	7-Hydroxy-6-methoxychromen-2-one	12	SCO

column hold-up volume, V_M, was determined as the elution volume of toluene in the pure acetonitrile as the mobile phase. Before each new series of experiments, the columns were equilibrated by flushing with 30 column hold-up volumes of the fresh mobile phase and the separation temperature was adjusted at 40 °C. The retention times, t_R, were measured over the full composition range of the mobile phases containing 10 mmol l^{-1} ammonium acetate in aqueous acetonitrile. The measurements were repeated in triplicate and arithmetic means of the experimental retention times, t_R, and the appropriate column hold-up time, t_M, were used to calculate the retention factors, $k = t_R/t_M - 1$. The Adstat 1.25 software (Trilobyte Statistical Software, Pardubice, the Czech Republic) was utilized for the determination of the parameters of Eq. (2) by non-linear regression of the experimental data sets.

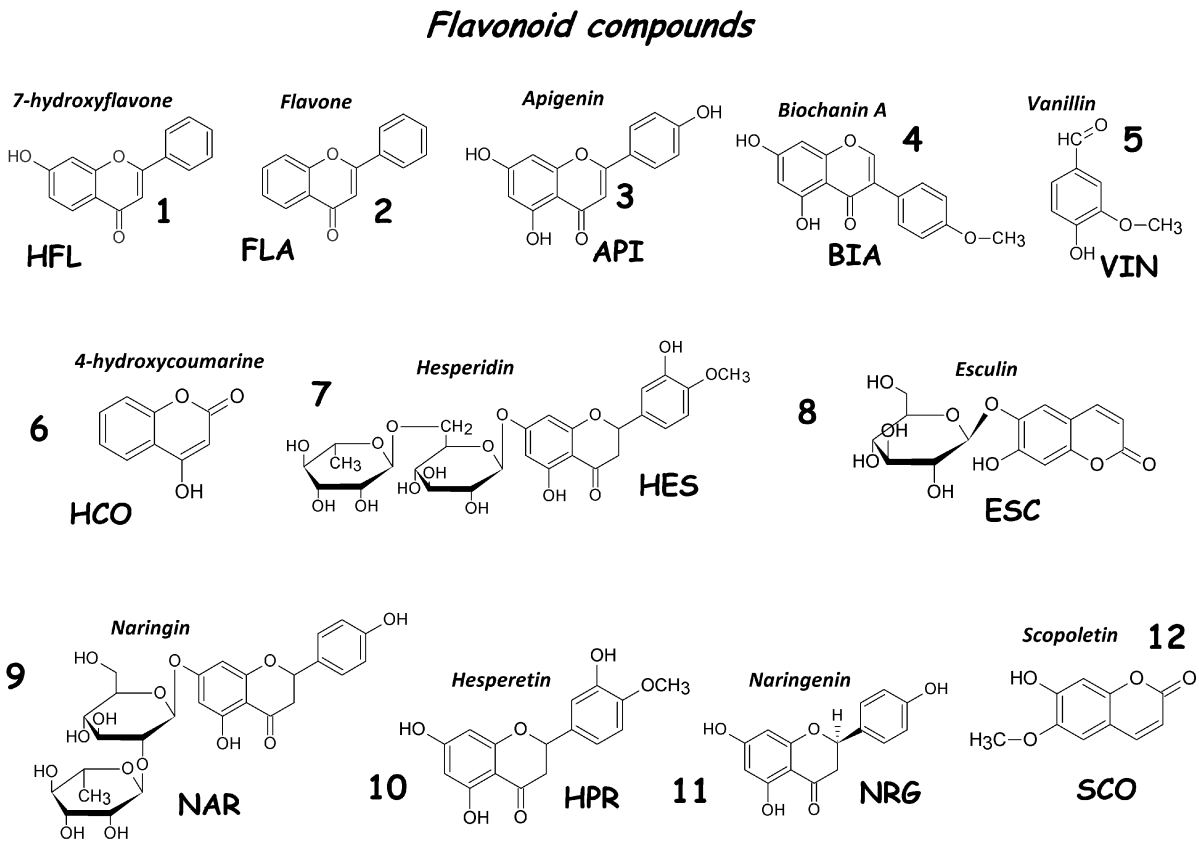

Fig. 2 Structures of flavonoid compounds tested

Results and Discussion

Effect of Mobile Phase Composition on Retention of Phenolic Acids and Flavonoid Compounds in ANP and RP Modes

The effect of the mobile phase composition was investigated in order to elucidate the retention mechanism of the phenolic acids and flavonoid compounds on five silica hydride based columns. The column dimensions and other characteristics are listed in Table I.

All tested columns can be used for aqueous normal-phase separations of flavonoid compounds and of less polar phenolic acids with a single phenolic –OH group in mobile phases containing more than 85 % acetonitrile. Protocatechuic, caffeic, gallic and chlorogenic acids with 2 or 3 phenolic groups (Nos. 9, 10, 11 and 12 in Table II) are too strongly retained in the ANP mode and show very asymmetric peaks. The peak symmetry did not improve significantly when changing the pH of the mobile phase, or when varying the ammonium acetate buffer ionic strength in between 5 mmol l^{-1} and 20 mmol l^{-1}.

The retention factors, *k*, of all phenolic acids and flavones increase at decreasing concentration of the aqueous ammonium acetate buffer in the organic-

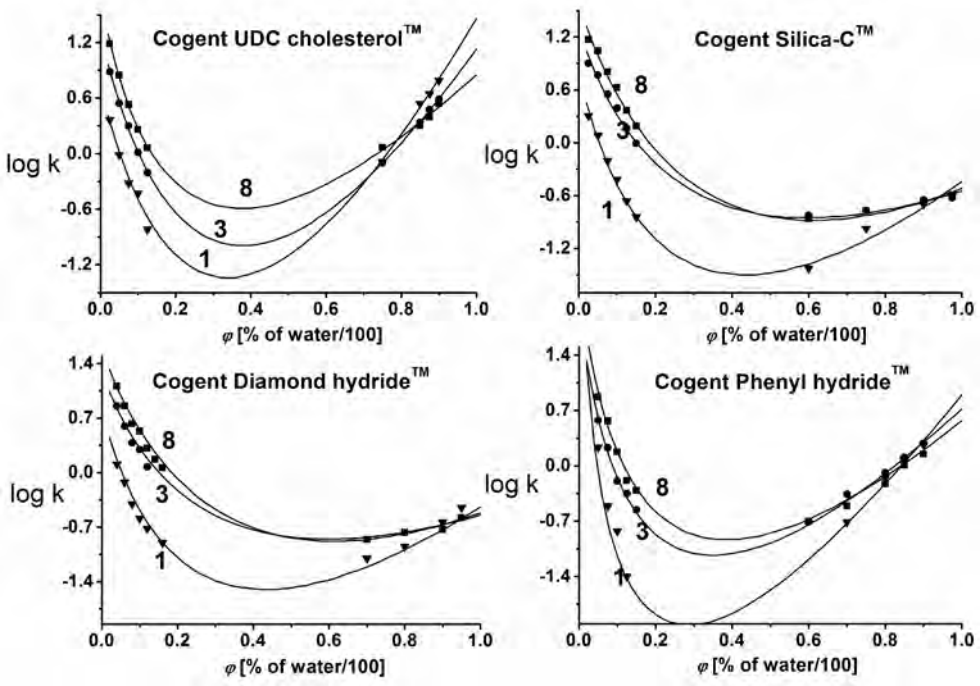

Fig. 3 Effect of volume fraction of aqueous buffer (10 mmol l^{-1} ammonium acetate, pH 3.26), φ, on retention factors, *k*, of flavonoid compounds. Temperature 40 °C; flow rate, F_m = 0.5 ml min^{-1}; sample volume 10 µl. Numbers of flavonoid compounds are as in Table III

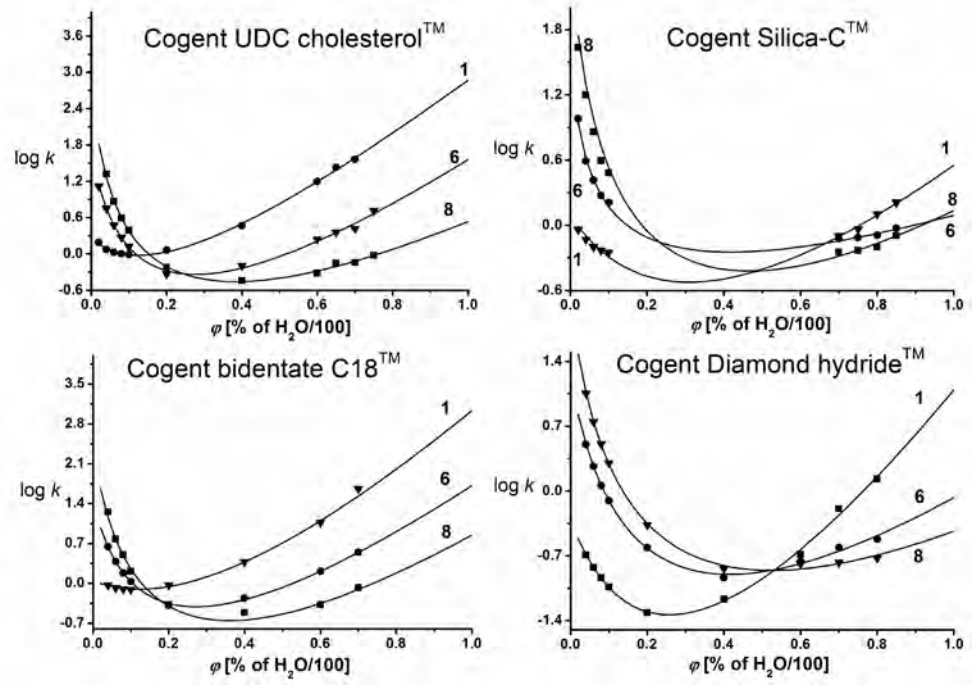

Fig. 4 Effect of volume fraction of aqueous buffer (10 mmol l^{-1} ammonium acetate, pH 3.26), φ, on retention factors, *k*, of phenolic acids. Temperature 40 °C; flow rate, F_m = 0.5 ml min^{-1}; sample volume 10 µl. Numbers of phenolic acids are as in Table II

rich ANP mobile phases and increase in highly aqueous mobile phases (RP mode). The experimental data show characteristic U-shape plots of retention factors versus the concentration of aqueous buffer in the mobile phase, φ, on all five hydrosilated silica columns: Cogent Silica-C™, Cogent Diamond hydride™, Cogent UDC cholesterol™, Cogent bidentate C18™ and Cogent Phenyl hydride™. Some examples are shown in Figs 3 and 4. In the dual ANP/RP retention behaviour, the reversed-phase retention mechanism predominates in the mobile phases with more than 60 % water, where the ANP contributions to the retention are negligible. In the mobile phases with high volume fraction of organic solvent, the aqueous normal-phase mechanism largely controls the retention. In the intermediate mobile phase range from 20 % to 55 %, neither of these two mechanisms dominates due to compensation of solvophobic and polar interactions which leads to very low retention.

On all five columns (Cogent Silica-C™, Cogent Diamond hydride™, Cogent UDC cholesterol™, Cogent bidentate C18™ and Cogent Phenyl hydride), the experimental data (points) agree with the values calculated using the best-fit parameters of Eq. (2) (a_2, m_{RP}, m_{ANP} and b) listed in Tables IV-VI.

Table IV Best-fit parameters a, m_{RP}, m_{ANP} and b of Eq. (2) and coefficients of determination, R^2, of phenolic acids

A – Cogent Silica-C™					
Phenolic acid	a_2	m_{RP}	m_{ANP}	b	R^2
SAL	0.80	6.16	8.17	7.06	0.9924
COU	0.97	5.22	8.12	6.32	0.9950
PHB	1.29	3.89	6.91	5.74	0.9906
FER	1.34	5.21	9.50	4.41	0.9898
VAN	1.36	4.05	7.17	5.62	0.9911
SIN	1.40	5.10	9.31	4.51	0.9878
SYR	1.50	4.19	7.56	5.52	0.9940
HPA	1.56	5.39	10.77	3.94	0.9927
B – Cogent bidentate C18™					
Phenolic acid	a_2	m_{RP}	m_{ANP}	b	R^2
SAL	0.72	13.12	18.35	3.72	0.9924

Phenolic acid	a_2	m_{RP}	m_{ANP}	b	R^2
COU	1.01	10.15	19.27	3.14	0.9922
PHB	1.28	11.14	16.01	4.09	0.9981
FER	1.26	23.86	57.72	1.51	0.9994
VAN	1.30	13.31	22.29	2.96	0.9990
SIN	1.30	29.86	88.07	11.35	0.9996
SYR	1.47	18.40	37.26	2.11	0.9992
HPA	1.71	6.43	7.10	9.61	0.9943

C – Cogent UDC cholesterol™

Phenolic acid	a_2	m_{RP}	m_{ANP}	b	R^2
SAL	3.46	8.33	5.73	78.42	0.9883
COU	1.14	6.94	6.15	11.42	0.9755
PHB	2.05	7.10	6.68	17.32	0.9843
FER	2.12	7.29	6.24	18.82	0.9831
VAN	2.09	6.30	5.75	20.00	0.9866
SIN	1.97	8.33	7.82	12.56	0.9814
SYR	2.01	7.62	7.70	12.40	0.9856
HPA	2.31	6.63	6.87	15.59	0.9904

D – Cogent Diamond hydride™

Phenolic acid	a_2	m_{RP}	m_{ANP}	b	R^2
SAL	11.11	6.35	18.91	0.74	0.9421
COU	8.74	–6.70	2.19	14.5	0.9901
PHB	9.15	–7.34	2.23	9.18	0.9815
FER	1.21	6.34	10.21	4.12	0.9912
VAN	1.45	5.12	7.28	5.51	0.9929

Phenolic acid	a_2	m_{RP}	m_{ANP}	b	R^2
SIN	1.51	5.01	8.91	4.01	0.9817
SYR	1.65	3.99	7.21	6.06	0.9728
HPA	1.78	4.79	10.21	3.21	0.9953

Table V Best-fit parameters a, m_{RP}, m_{ANP} and b of Eq. (2) and coefficients of determination, R^2, of flavonoid compounds

A – Cogent Silica-C™					
Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
HFL	0.31	3.00	3.21	7.31	0.9826
FLA	0.06	17.18	68.33	0.73	0.9763
API	0.20	2.58	2.86	6.88	0.9911
BIA	0.09	4.55	6.04	3.75	0.9736
VIN	0.09	0.99	0.99	10.63	0.9940
HCO	1.74	1.57	1.54	124.16	0.9856
HES	2.95	1.91	2.19	154.45	0.9699
ESC	2.43	3.25	3.74	29.40	0.9923
NAR	2.92	2.40	2.65	94.98	0.9843
HPR	0.35	1.82	1.34	30.01	0.9901
NRG	0.77	5.46	3.46	41.83	0.9912
SCO	0.29	0.97	0.80	28.55	0.9878
B – Cogent Diamond hydride™					
Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
HFL	–0.26	2.66	2.78	7.94	0.9862
FLA	–0.52	5.39	5.27	4.77	0.9935
API	–0.19	4.54	4.48	7.08	0.9910
BIA	–0.32	7.90	8.01	5.47	0.9875

Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
VIN	–0.46	5.11	10.15	1.97	0.9965
HCO	1.24	4.13	4.62	14.04	0.9716
HES	1.99	3.65	4.25	22.88	0.9914
ESC	2.06	3.29	4.34	20.46	0.9823
NAR	1.83	4.50	5.81	12.77	0.9717
HPR	–0.28	2.08	1.76	13.90	0.9821
NRG	–0.34	2.41	2.22	9.74	0.9844
SCO	–0.14	2.19	1.83	15.49	0.9942

C – Cogent UDC cholesterol™

Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
HFL	0.76	4.36	2.26	18.75	0.9852
FLA	0.20	8.34	16.12	1.28	0.9923
API	0.55	5.15	3.12	11.74	0.9933
BIA	0.27	5.71	3.15	8.75	0.9899
VIN	0.09	2.21	1.16	16.58	0.9799
HCO	1.71	5.48	4.01	24.46	0.9865
HES	2.42	7.02	6.26	17.79	0.9963
ESC	2.79	4.11	3.85	44.29	0.9785
NAR	2.11	5.59	5.08	19.35	0.9932
HPR	0.12	4.75	2.94	8.44	0.9971
NRG	0.16	4.62	2.69	10.48	0.9975
SCO	0.31	2.87	1.66	17.02	0.9913

D – Cogent bidentate C18™

Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
HFL	0.45	5.81	4.31	6.90	0.9903

Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
FLA	0.23	9.73	19.93	1.22	0.9922
API	0.30	7.12	6.15	5.48	0.9715
BIA	0.06	8.55	10.09	2.56	0.9878
VIN	-0.03	3.55	3.96	3.34	0.9913
HCO	1.46	6.17	4.67	17.49	0.9917
HES	2.75	6.15	4.79	38.43	0.9855
ESC	2.39	5.88	5.51	21.23	0.9721
NAR	2.42	6.17	4.67	21.11	0.9941
HPR	0.09	5.00	2.95	9.64	0.9909
NRG	0.11	5.90	4.02	8.05	0.9813
SCO	0.14	2.91	1.95	10.25	0.9904

Table VI Best-fit parameters a, m_{RP}, m_{ANP} and b of Eq. (2) and coefficients of determination, R^2, on Cogent Phenyl hydride™ column

Phenolic acids	a_2	m_{RP}	m_{ANP}	b	R^2
SAL	0.42	5.12	4.72	10.11	0.9867
COU	0.92	6.07	4.87	13.64	0.9763
PHB	1.52	4.32	2.83	58.26	0.9911
FER	1.10	6.61	5.47	12.84	0.9736
VAN	1.27	5.29	4.12	21.38	0.9940
SIN	1.19	6.93	5.76	12.92	0.9856
SYR	1.39	6.01	5.03	16.84	0.9699
HPA	1.55	4.94	4.01	25.57	0.9923
Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
HFL	0.76	4.36	2.26	18.75	0.9852
FLA	0.20	8.34	16.12	1.28	0.9923

Flavonoids	a_2	m_{RP}	m_{ANP}	b	R^2
API	0.55	5.15	3.12	11.74	0.9933
BIA	0.27	5.71	3.15	8.75	0.9899
VIN	0.09	2.21	1.16	16.58	0.9799
HCO	1.71	5.48	4.01	24.46	0.9865
HES	2.42	7.02	6.26	17.79	0.9963
ESC	2.79	4.11	3.85	44.29	0.9785
NAR	2.11	5.59	5.08	19.35	0.9932
HPR	0.12	4.75	2.94	8.44	0.9971
NRG	0.16	4.62	2.69	10.48	0.9975
SCO	0.31	2.87	1.66	17.02	0.9913

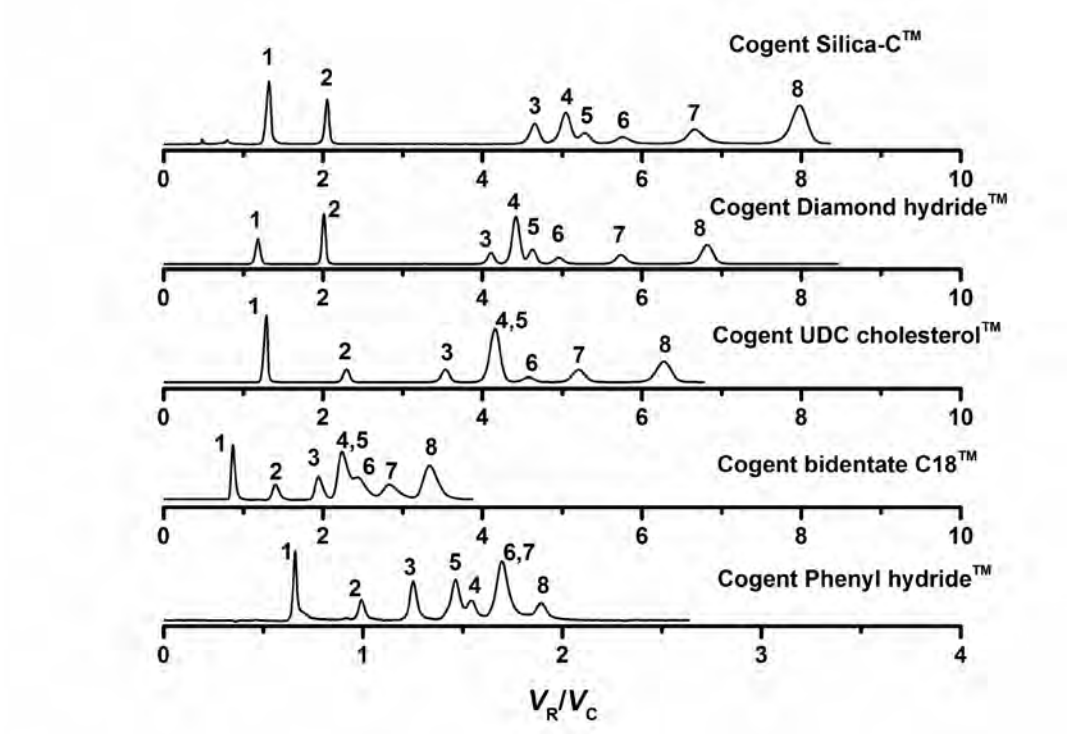

Fig. 5 Separation of phenolic acids in ANP mode on columns tested. Experimental conditions: mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26). Temperature 40 °C; flow rate, F_m = 0.5 ml min^{-1}; sample volume 10 µl. Numbers of peaks are as in Table II

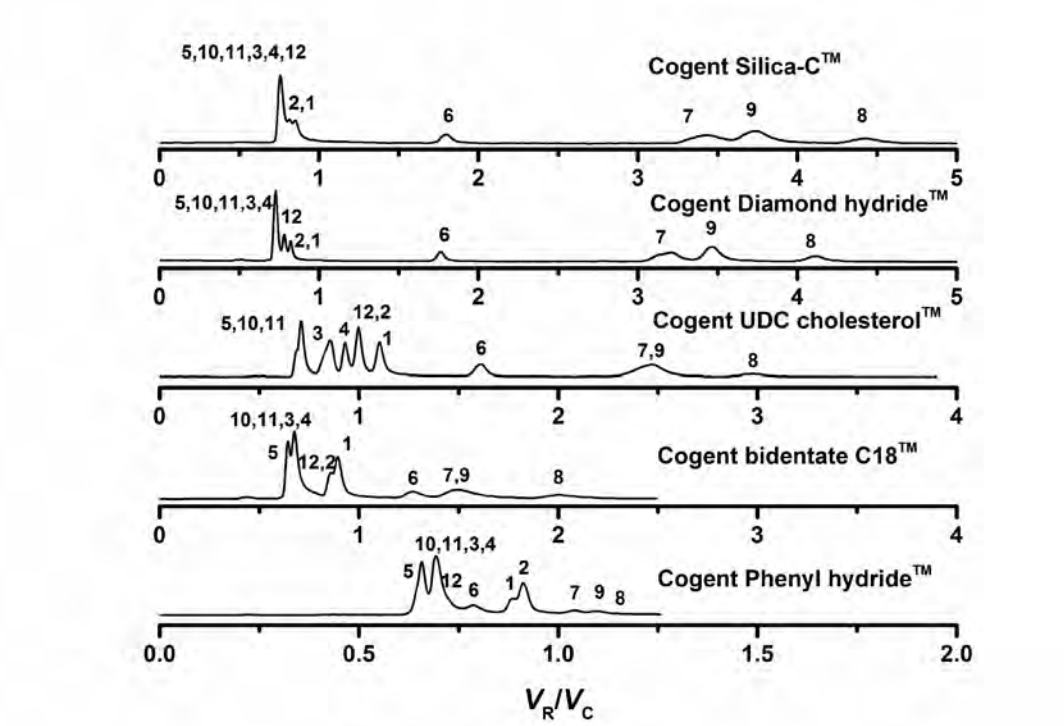

Fig. 6 Separation of flavonoids in ANP mode on columns tested. Experimental conditions: mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26). Temperature 40 °C; flow rate, F_m = 0.5 ml·min^{-1}; sample volume 10 µl. Numbers of peaks are as in Table III

The four-parameter Eq. (2) fits better the experimental retention data than the three-parameter Eq. (1) over the broad composition range of the mobile phase, including the low φ ANP range and the high φ RP range (full lines in Figs 3 and 4). High values of the multiple correlation coefficients, R^2, in Tables IV-VI demonstrate a good validity of Eq. (2) to describe the dual ANP/RP retention model for phenolic acids and flavonoid compounds on hydrosilated silica columns.

Figures 5 and 6 show ANP separations of eight phenolic acids and twelve flavonoid compounds on Cogent Silica-C™, Cogent Diamond hydride™, Cogent UDC cholesterol™ Cogent bidentate C18™ and Cogent Phenyl hydride™ columns in 95% acetonitrile containing 10 mmol l^{-1} ammonium acetate. For better comparison sake, the elution volumes, V_R, are normalized with respect to the column volume, V_C. The elution order of phenolic acids and flavonoids is similar on all the columns tested, except Cogent Phenyl hydride™. The retention decreases with decreasing polarity of the stationary phase surface: Cogent Silica-C™ > Cogent Diamond hydride™ > Cogent UDC cholesterol™ > Cogent bidentate C18™ > Cogent Phenyl hydride™. Cogent Silica-C™ and Cogent Diamond hydride™ columns provide better ANP separation of the phenolic acids tested than the columns with hydrophobic surface modification at 40 °C in buffered mobile phase containing 5 % aqueous component in acetonitrile (Fig. 5). However, Cogent UDC cholesterol™ column fits better for separation of flavonoid compounds under the same chromatographic conditions (Fig. 6).

The Cogent Silica hydride™ and Cogent Diamond hydride™ columns retain the

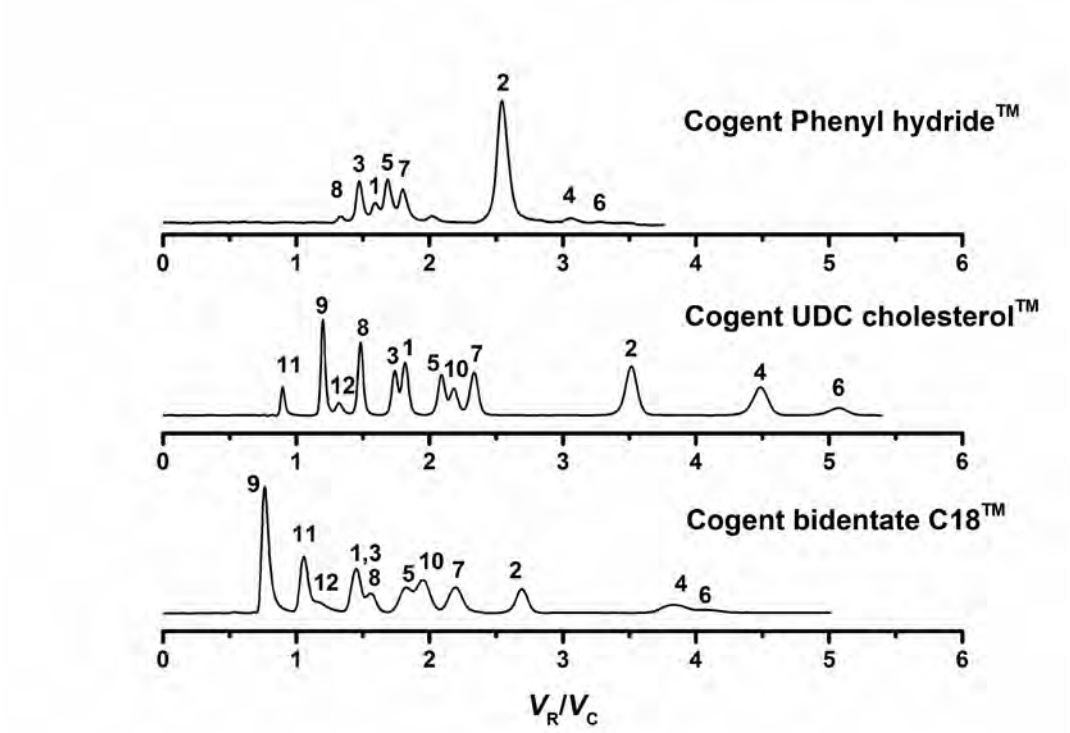

Fig. 7 Separation of phenolic acids in the RP mode on columns tested. Experimental conditions: mobile phase – 10 mM ammonium acetate in 85/15 water/acetonitrile (water acidified with formic acid to pH 3.26). Temperature 40 °C; flow rate, F_m = 0.5 ml min^{-1}; sample volume 10 µl. Numbers of peaks are as in Table II

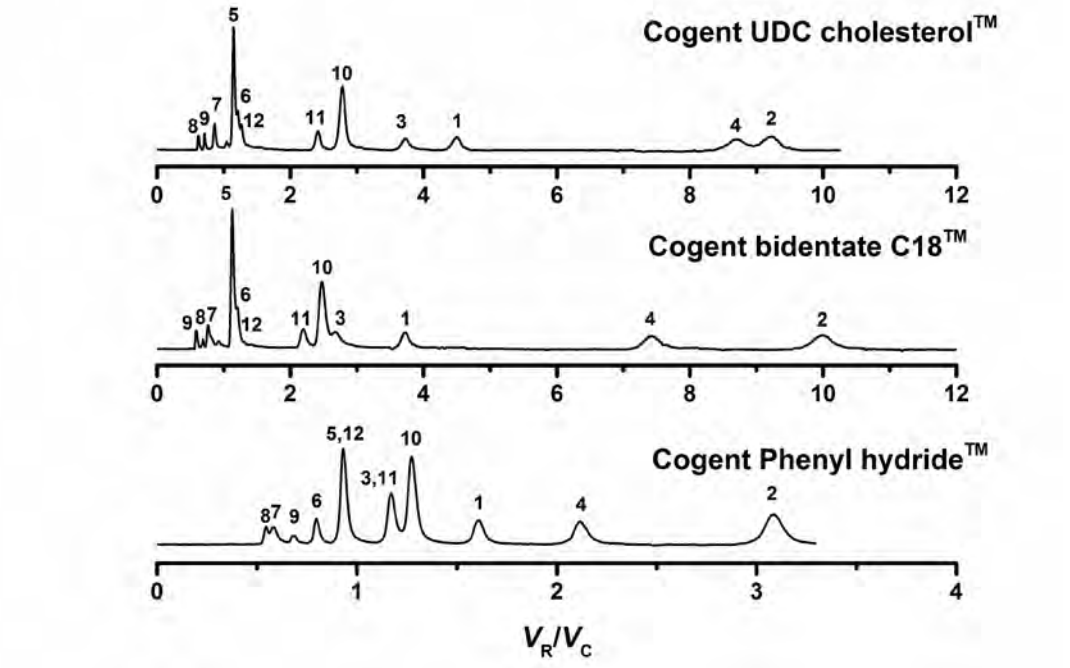

Fig. 8 RP separation of flavonoid compounds. Experimental conditions: mobile phase – 10 mM ammonium acetate in 65/35 water/acetonitrile (water acidified with formic acid to pH 3.26); flow rate, F_m = 0.5 ml min^{-1}; sample volume 10 µl. Numbers of peaks are as in Table III

tested phenolic acids and flavonoid compounds very weakly in highly aqueous mobile phases and are not suitable for their separations under reversed-phase conditions. Hydrosilated silica materials modified with non-polar groups such as octadecyl (Cogent bidentate C18™), cholesteryl (Cogent UDC cholesterol™) or phenyl (Cogent Phenyl hydride™) are more hydrophobic and show enhanced retention in the reversed-phase mode and enable reversed-phase separations of phenolic acids and flavonoid compounds in buffered mobile phases containing less than 30 % acetonitrile. Examples of separation of phenolic acids and flavonoid compounds are shown in Figs 7 and 8. The elution order of both phenolic acids and flavonoid compounds in RP mode is almost reversed in comparison to their elution order in ANP mode. From among five columns tested, Cogent UDC cholesterol™ column provides the best resolution and selectivity of phenolic acids and flavonoid compounds in RP mode.

Influence of Temperature on Retention of Phenolic Acids and Flavonoid Compounds in ANP Mode

In many cases, the influence of temperature on the separation process is an unjustly underestimated parameter and deserves more attention. We investigated the ANP retention in the temperature range from 35 °C up to the limits of column thermal stability given in Table I. The retention data at temperatures lower than 35 °C may be less accurate when employing an air circulated thermostat without external cooling facility and, therefore, were not included. As show the chromatograms on Cogent UDC cholesterol™ column in Fig. 9, the increasing temperature up to 60 °C improves the peak widths and decreases the retention times of flavonoid compounds, but using the temperatures higher than 60 °C may cause drastic decrease in resolution. By changing temperature, even the selectivity may be significantly changed, as show chromatograms in Fig. 10, where syringic acid (peak 7) and 4-hydroxyphenylacetic acid (peak 8) are separated at 40 °C, co-elute at 55 °C and have reversed elution order at 60 °C. At the high temperature column stability limits (100 °C for the Cogent UDC cholesterol™ column and 80 °C for Cogent bidentate C18™ column and Cogent Phenyl hydride™ column), most phenolic acids and flavonoid compounds were too weakly retained and could not be separated even at very low concentration of aqueous component (2 %) in the mobile phase.

The best-fit regression parameters of Eq. (3), i.e., the intercepts, A_i, the slopes, B_i, and the correlation coefficients, R^2, of the experimental ln k of phenolic acids versus $1/T$ data plots, measured under ANP conditions in acetonitrile containing 5 % 10 mmol l^{-1} ammonium acetate buffer are presented in Tables VII, VIII and XI.

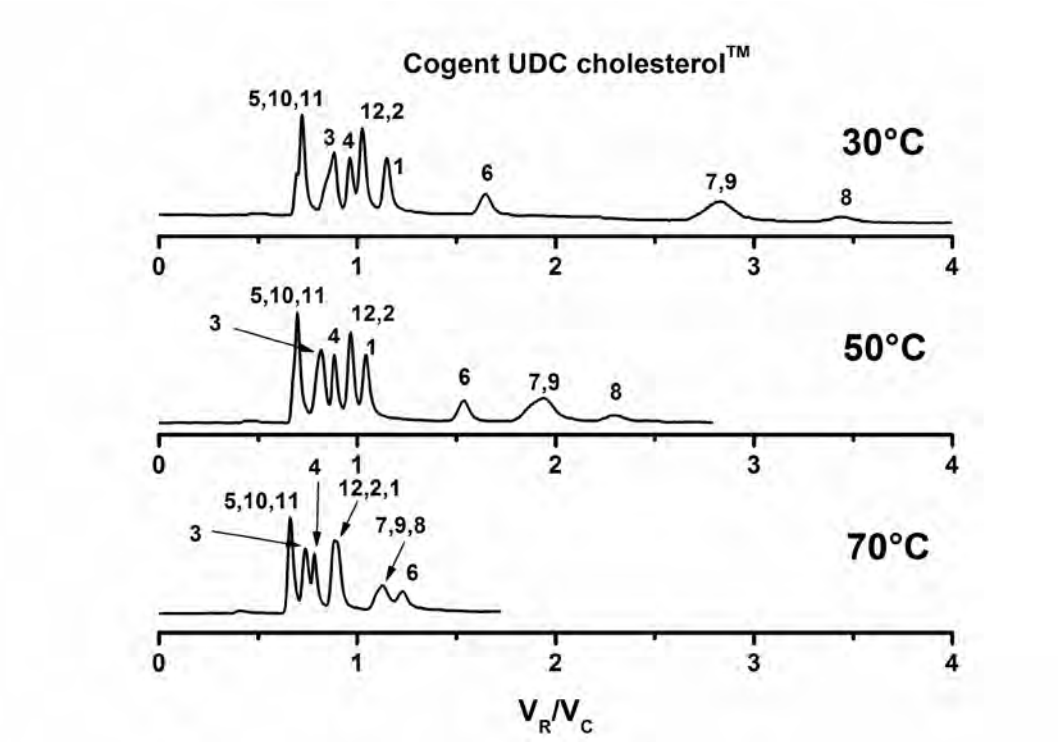

Fig. 9 ANP separation of flavonoid compounds on cholesterol hydride column at 30, 50 and 70 °C. Experimental conditions: mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26); flow rate, F_m = 0.5 ml min^{-1}; sample volume 10 µl. Numbers of peaks are as in Table III

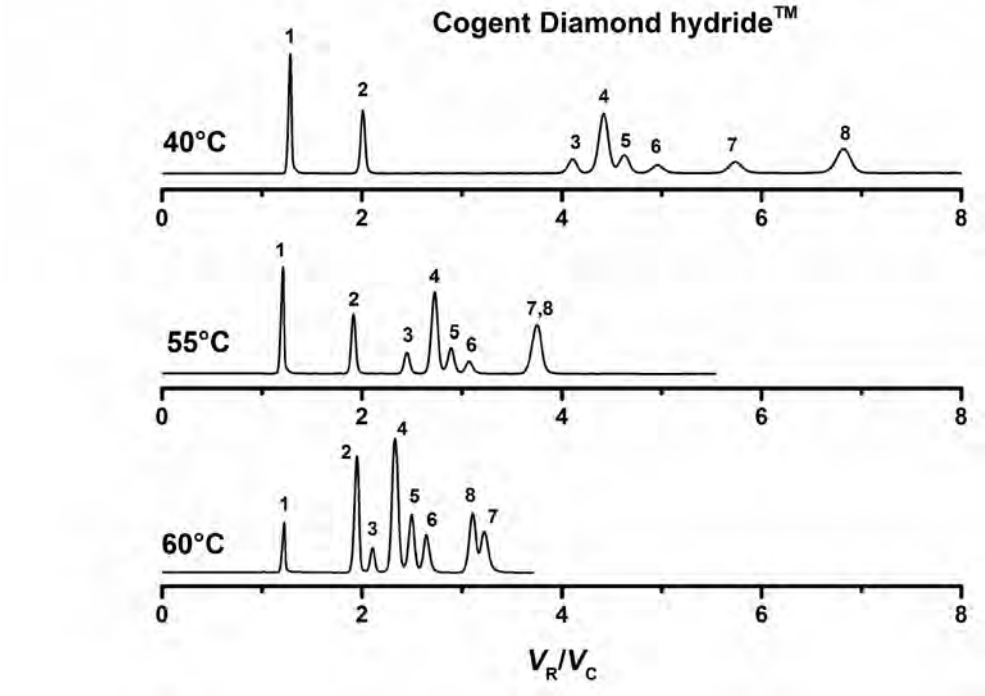

Fig. 10 ANP separation of phenolic acids on Diamond hydride column at 40, 55 and 60 °C. Experimental conditions: mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26); flow rate, F_m = 0.5 ml min^{-1}; sample volume 10 µl. Numbers of peaks are as in Table II

Table VII Best-fit parameters of phenolic acids A_i and B_i of Eq. (3) and coefficients of determination, R^2. Experimental conditions: ANP mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26); Cogent Silica-C™ column temperature 35-60 °C; Cogent Diamond hydride™ column temeprature 35-60 °C

Cogent Silica-C™		A_i	B_i	R^2	
1	SAL	–0.92	496.6	0.9721	
2	COU	–1.11	762.1	0.9632	
3	PHB	–10.66	4057.9	0.9892	
4	FER	–10.18	3938.4	0.9934	
5	VAN	–10.09	3928.1	0.9917	
6	SIN	–10.38	4049.4	0.9914	
7	SYR	–9.66	3877.1	0.9894	
8	HPA	–11.89	4627.4	0.9919	
Cogent Silica-C™		$-\Delta H^0$ kJ mol^{-1}	ΔS^0 J mol^{-1} K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
1	SAL	4.13	–9.07	1.59	1.37
2	COU	6.34	–10.65	2.43	2.10
3	PHB	33.74	–90.05	12.96	11.17
4	FER	32.74	–86.06	12.58	10.85
5	VAN	32.66	–85.31	12.54	10.82
6	SIN	33.67	–87.72	12.93	11.15
7	SYR	32.23	–81.74	12.38	10.68
8	HPA	38.47	–100.28	14.78	12.74
Cogent Diamond hydride™		A_i	B_i	R^2	
1	SAL	–0.83	567.3	0.9952	
2	COU	0.06	472.1	0.9661	
3	PHB	–10.38	3997.2	0.9988	
4	FER	–9.52	3752.3	0.9988	

Cogent Diamond hydride™		A_i	B_i	R^2
5	VAN	–9.06	3623.9	0.9989
6	SIN	–9.16	3680.8	0.9986
7	SYR	–8.12	3405.8	0.9946
8	HPA	–11.22	4430.3	0.9991

Cogent Diamond hydride™		$-\Delta H^0$ kJ mol^{-1}	ΔS^0 J mol^{-1} K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
1	SAL	4.72	–2.63	1.81	1.56
2	COU	3.93	4.77	1.51	1.30
3	PHB	33.23	–82.02	12.76	11.01
4	FER	31.20	–74.87	11.98	10.33
5	VAN	30.13	–71.05	11.57	9.98
6	SIN	30.60	–71.88	11.75	10.14
7	SYR	28.32	–63.23	10.88	9.38
8	HPA	36.83	–89.01	14.15	12.20

Table VIII Best-fit parameters of phenolic acids A_i and B_i of Eq. (3) and coefficients of determination, R^2. Experimental conditions: ANP mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26); Cogent UDC cholesterol™ column temperature 35-100 °C; Cogent bidentate C18™ column temperature 35-80 °C

Cogent UDC cholesterol™		A_i	B_i, K	R^2
1	SAL	–5.80	2006.4	0.9903
2	COU	–6.43	2459.9	0.9907
3	PHB	–13.41	4804.4	0.9962
4	FER	–13.16	4788.1	0.9969
5	VAN	–13.03	4747.3	0.9968
6	SIN	–13.39	4894.6	0.9966
7	SYR	–12.78	4754.5	0.9963

Cogent UDC cholesterol™		A_i	B_i, K	R^2	
8	HPA	–15.20	5574.5	0.9972	
Cogent UDC cholesterol™		$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
1	SAL	16.68	–30.05	6.41	5.52
2	COU	20.45	–34.29	7.86	6.77
3	PHB	39.94	–85.67	15.34	13.23
4	FER	39.81	–70.46	15.29	13.18
5	VAN	39.47	–76.19	15.16	13.07
6	SIN	40.69	–80.02	15.63	13.48
7	SYR	39.53	–76.52	15.18	13.09
8	HPA	46.35	–94.07	17.80	15.35
Cogent bidentate C18™		A_i	B_i, K	R^2	
1	SAL	–3.28	1014.4	0.9644	
2	COU	–3.79	1454.8	0.9744	
3	PHB	–9.97	3525.8	0.9981	
4	FER	–8.14	2986.4	0.9915	
5	VAN	–8.83	3231.3	0.9849	
6	SIN	–9.29	3407.1	0.9836	
7	SYR	–8.87	3334.9	0.9939	
8	HPA	–10.98	4053.2	0.9900	
Cogent bidentate C18™		$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
1	SAL	8.43	–11.72	3.24	2.79
2	COU	12.10	–15.96	4.65	4.01
3	PHB	29.31	–67.34	11.26	9.71
4	FER	24.83	–52.12	9.54	8.22
5	VAN	26.87	–57.86	10.32	8.90

Cogent bidentate C18™		$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
6	SIN	28.33	–61.68	10.88	9.38
7	SYR	27.73	–58.19	10.65	9.18
8	HPA	33.70	–75.73	12.94	11.16

The best-fit parameters of Eq. (3) for flavonoid compounds are listed in Tables IX-XI. High coefficients of determination, R^2, demonstrate a good linearity in agreement with Eq. (3) to the experimental retention data for all the phenolic acids and flavonoid compounds on the columns tested under ANP conditions, suggesting that a single retention mechanism controls the retention over a broad temperature range.

Table IX Best-fit parameters of flavonoid compounds A_i and B_i of Eq. (2) and coefficients of determination, R^2. Experimental conditions: ANP mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26). Cogent Silica-C™ column temperature 35-60 °C; Cogent Diamond hydride™ column temeprature 35-60 °C

Cogent Silica-C™	A_i	B_i, K	R^2
HFL	–1.83	541.6	0.9930
FLA	–3.86	1082.3	0.9932
API	–2.83	817.4	0.9922
BIA	–3.22	886.2	0.9916
VIN	–3.81	1117.5	0.9934
HCO	–0.18	385.5	0.9990
HES	–8.94	3374.8	0.9931
ESC	–8.96	3465.4	0.9819
NAR	–8.22	3166.4	0.9846
HPR	–3.142	887.1	0.9968
NRG	–3.41	964.4	0.9941
SCO	–3.29	974.9	0.9928

Cogent Silica–C™	$-\Delta H^0$, kJ	ΔS^0 ,J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
HFL	4.50	–16.64	1.73	1.49
FLA	9.00	–33.54	3.46	2.98
API	6.80	–24.91	2.61	2.25
BIA	7.37	–28.23	2.83	2.44
VIN	9.29	–33.11	3.57	3.08
HCO	3.21	–2.88	1.23	1.06
HES	28.06	–75.75	10.78	9.29
ESC	28.81	–75.91	11.07	9.54
NAR	26.33	–69.74	10.11	8.72
HPR	7.38	–27.54	2.83	2.44
NRG	8.02	–29.78	3.08	2.66
SCO	8.11	–28.75	3.11	2.68

Cogent Diamond hydride™	A_i	B_i, K	R^2
HFL	–3.23	648.5	0.9946
FLA	–6.06	1281.7	0.9922
API	–4.37	921.0	0.9941
BIA	–8.12	1916.8	0.9893
VIN	–6.45	1524.5	0.9954
HCO	–0.74	422.6	0.9881
HES	–8.75	3191.4	0.9900
ESC	–7.81	2983.2	0.9990
NAR	–7.86	2936.9	0.9975
HPR	–6.81	1599.8	0.9889
NRG	–5.59	1220.2	0.9970
SCO	–6.95	1784.8	0.9901

Cogent Diamond hydride™	$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
HFL	5.39	–22.56	2.07	1.79
FLA	10.66	–46.06	4.09	3.53
API	7.66	–32.07	2.94	2.54
BIA	15.94	–63.19	6.12	5.28
VIN	12.68	–49.36	4.87	4.20
HCO	3.51	–1.89	1.35	1.16
HES	26.53	–68.49	10.19	8.79
ESC	24.80	–60.68	9.53	8.21
NAR	24.42	–61.06	9.38	8.09
HPR	13.30	–52.31	5.11	4.41
NRG	10.15	–42.21	3.90	3.36
SCO	14.84	–53.46	5.70	4.91

Table X Best-fit parameters of flavonoid compounds A_i and B_i of Eq. (2) and coefficients of determination, R^2. Experimental conditions: ANP mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26). Cogent UDC cholesterol™ column temperature 35-100 °C; Cogent bidentate C18™ column temeprature 35-80 °C.

Cogent UDC cholesterol™	A_i	B_i, K	R^2
HFL	–2.77	1003.6	0.9925
FLA	–1.92	690.6	0.9908
API	–3.01	986.0	0.9919
BIA	–2.87	890.2	0.9967
VIN	–2.20	558.4	0.9878
HCO	–2.51	1106.1	0.9881
HES	–7.96	2948.3	0.9821
ESC	–8.70	3256.3	0.9844
NAR	–8.03	2980.1	0.9871

Cogent UDC cholesterol™	A_i	B_i, K	R^2	
HPR	–2.83	775.9	0.9913	
NRG	–2.52	660.3	0.9987	
SCO	–2.37	679.9	0.9930	
Cogent UDC cholesterol™	$-\Delta H^0$, kJ	ΔS^0 ,J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
HFL	8.34	–25.22	3.20	2.76
FLA	5.74	–18.22	2.21	1.90
API	8.20	–27.24	3.15	2.72
BIA	7.40	–26.11	2.84	2.45
VIN	4.64	–20.54	1.78	1.54
HCO	9.20	–23.10	3.53	3.05
HES	24.51	–68.42	9.41	8.12
ESC	27.07	–74.57	10.40	8.97
NAR	24.78	–69.02	9.52	8.21
HPR	6.45	–25.80	2.48	2.14
NRG	5.49	–23.17	2.11	1.82
SCO	5.65	–21.91	2.17	1.87
Cogent bidentate C18™	A_i	B_i, K	R^2	
HFL	–2.89	931.2	0.9960	
FLA	–2.76	914.0	0.9936	
API	–3.42	955.4	0.9815	
BIA	–2.70	713.7	0.9933	
VIN	–2.53	612.5	0.9864	
HCO	–0.89	510.3	0.9873	
HES	–8.07	2831.5	0.9969	
ESC	–7.62	2813.1	0.9972	

Cogent bidentate C18^{TM}	A_i	B_i, K	R^2	
NAR	–7.72	2726.7	0.9950	
HPR	–4.07	1066.1	0.9900	
NRG	–3.57	886.5	0.9862	
SCO	–3.05	821.7	0.9809	
Cogent bidentate C18^{TM}	$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
HFL	7.74	–26.82	2.97	2.56
FLA	7.60	–25.68	2.92	2.52
API	7.94	–31.24	3.05	2.63
BIA	5.93	–25.23	2.28	1.97
VIN	5.09	–23.85	1.96	1.69
HCO	4.24	–10.17	1.63	1.41
HES	23.54	–69.87	9.04	7.80
ESC	23.39	–66.15	8.98	7.75
NAR	22.67	–66.94	8.71	7.51
HPR	8.86	–36.67	3.40	2.94
NRG	7.37	–32.48	2.83	2.44
SCO	6.83	–28.12	2.62	2.26

The van't Hoff plots in Figs 11 and 12 are less steep for less retained analytes (salicylic acid (1) and coumaric acid (3) in Fig. 11 and 7-hydroxyflavone (1) and 4-hydroxycoumarin (6) in Fig. 12) in comparison to more retained phenolic acids and flavonoids (4-hydroxyphenylacetic acid (8) in Fig. 11 and esculin in Fig. 12). To explain these observations, thermodynamic data for the transfer of flavonoids from the mobile phase to the stationary phase were calculated applying Eq. (3) to the parameters of the van't Hoff plots: the standard partial molar entropy, ΔS^0, was calculated from the intercept, A_i, and the slope, B_i, serves for calculation of the standard partial molar enthalpy, ΔH^0. The role of the enthalpic and entropic contributions at a specific experimental temperature can be estimated by comparing the numerical values of B_i/T and A_i, respectively (Tables VII and VIII). The enthalpic contributions, B_i/T, decrease at higher temperatures, and in the case of phenolic acids they are significantly higher than the entropic

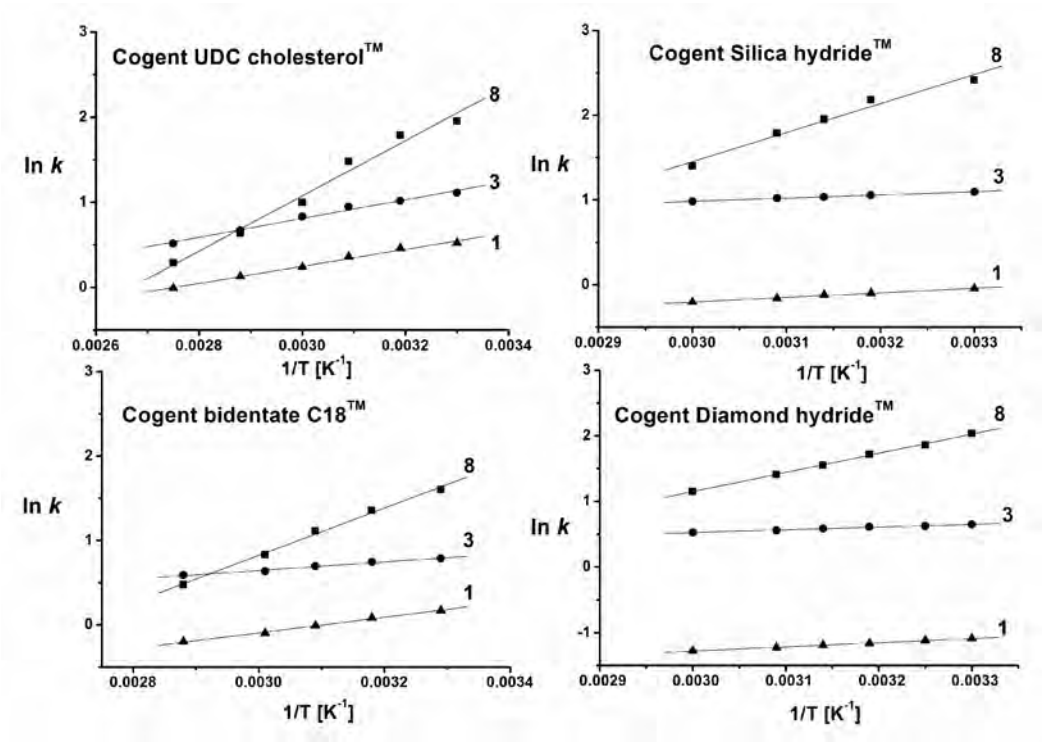

Fig. 11 Temperature effects on retention factors, *k*, of phenolic acids in ANP mode. Experimental conditions: mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26). Numbers of plots of phenolic acids are as in Table II

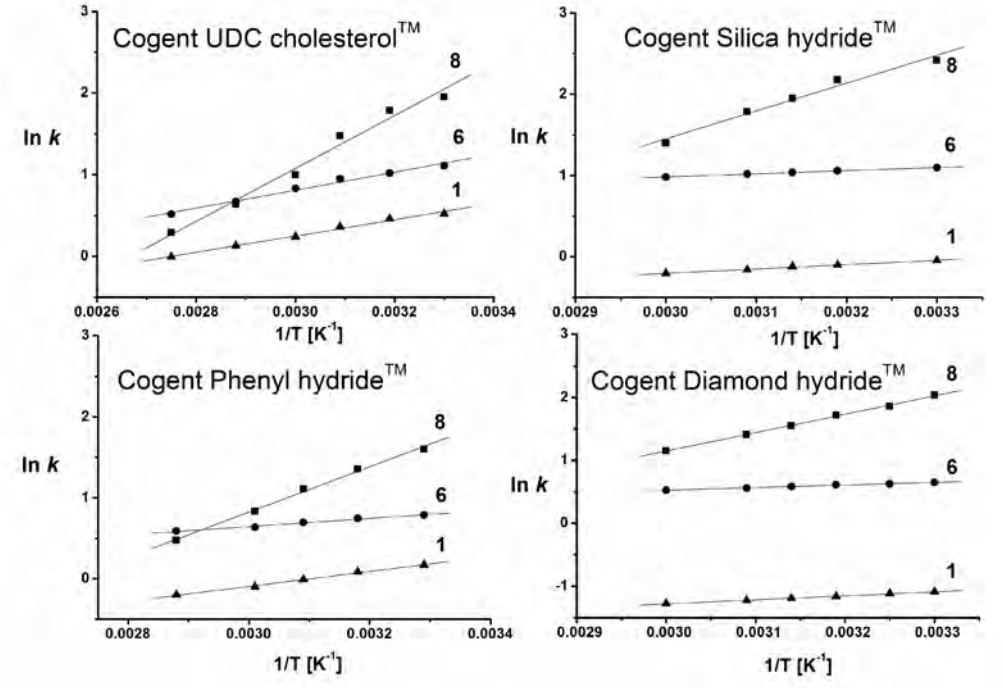

Fig. 12 Temperature effects on retention factors, *k*, of flavonoid compounds in ANP mode. Experimental Conditions: mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26). Numbers of plots of flavonoid compounds are as in Table III

contributions at low (40 °C) as well as at high (90 °C) temperatures on Cogent Silica-C™ and Diamond hydride columns (Table VII). However, the entropic contributions, A_i, are more significant with the modified Cogent UDC cholesterol™ and Cogent bidentate C18™ columns in the ANP mode, and at high temperatures they may be similar to the enthalpic contributions or even slightly higher for weakly retained salicylic (1) and coumaric (2) acids (Table VIII). The data for flavonoid compounds at 40 °C and 90 °C in Tables IX-XI show that the enthalpic contributions, B_i/T, decrease as the temperature increases and are comparable or slightly lower than the entropic contributions for less retained flavonoid aglycones on the five columns tested, while the enthalpic contributions for strongly retained flavone glycosides (hesperidin, naringin and esculin) and 4-hydroxycoumarine are higher in comparison to the entropic ones.

Table XI Best-fit parametersof flavonoid compounds A_i and B_i of Eq. (2) and correlation coefficients, R^2. Experimental conditions: ANP mobile phase – 10 mM ammonium acetate in 5/95 water/acetonitrile (water acidified with formic acid to pH 3.26). Cogent Phenyl hydride™ column temeprature 35-80 °C.

Phenolic acids	A_i	B_i, K	R^2	
SAL	–3.14	1009.4	0.9644	
COU	–3.6	1422.1	0.9744	
PHB	–9.81	3499.2	0.9981	
FER	–7.92	2956.2	0.9915	
VAN	–8.5	3211.1	0.9849	
SIN	–8.95	3396.1	0.9836	
SYR	–8.65	3317.5	0.9939	
HPA	–10.7	4023.2	0.9900	
Phenolic acids	$-\Delta H^0$, kJ	ΔS^0 ,J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
SAL	8.39	–21.55	3.22	2.78
COU	11.82	–25.37	4.54	3.92
PHB	29.09	–77.00	11.17	9.64
FER	24.58	–61.29	9.44	8.14
VAN	26.70	–66.11	10.25	8.84
SIN	28.24	–69.85	10.84	9.35

Phenolic acids	$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
SYR	27.58	–67.36	10.59	9.14
HPA	33.45	–84.40	12.85	11.08
Flavonoid compounds	A_i	B_i, K	R^2	
HFL	–2.821	921.2	0.9912	
FLA	–2.770	911.0	0.9856	
API	–3.520	930.4	0.9819	
BIA	–2.750	725.7	0.9899	
VIN	–2.534	612.5	0.9909	
HCO	–0.889	510.3	0.9928	
HES	–8.297	2852.5	0.9975	
ESC	–7.682	2823.1	0.9947	
NAR	–7.717	2726.7	0.9932	
HPR	–4.100	1080.1	0.9874	
NRG	–3.657	879.5	0.9852	
SCO	–3.080	840.7	0.9880	
Flavonoid compounds	$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
HFL	2.94	2.54	7.66	–18.89
FLA	2.91	2.51	7.57	–18.47
API	2.97	2.56	7.74	–24.71
BIA	2.32	2.00	6.03	–18.30
VIN	1.96	1.69	5.09	–16.51
HCO	1.63	1.41	4.24	–2.83
HES	9.11	7.85	23.72	–64.42
ESC	9.02	7.77	23.47	–59.31
NAR	8.71	7.51	22.67	–59.60

Flavonoid compounds	$-\Delta H^0$, kJ	ΔS^0, J K^{-1}	B_i/T (40 °C)	B_i/T (90 °C)
HPR	3.45	2.97	8.98	–29.53
NRG	2.81	2.42	7.31	–25.84
SCO	2.68	2.32	6.99	–21.05

Conclusion

Hydrosilated silica-based stationary phases provide the retention behaviour which is in accordance with the theory of dual ANP-reversed phase retention mechanism. The dependence of retention of phenolic acids and flavonoid compounds on the composition of buffered aqueous acetonitrile mobile phase can be appropriately described using four-parameter equation, Eq. (2), over the full mobile phase composition range which simultaneously involves both the ANP and the RP retention modes. Cogent Diamond hydride™ and Cogent Silica-C™ columns show very weak retention in the RP mode and can be used only for ANP separations of phenolic acids and flavonoid compounds. On the other hand, the less polar Cogent UDC cholesterol™, Cogent bidentate C18™ and Cogent Phenyl hydride™ stationary phases modified with C18, cholesterol and phenyl hydrophobic ligands provide useful separation in both the ANP and the RP mode, with (almost) reversed elution order and essentially changed separation selectivity. Glycoside flavonoid compounds show enhanced retention in the ANP mode, while they are too weakly retained in the reversed-phase range of mobile phases even in highly aqueous organic mobile phase.

Cogent UDC cholesterol™, Cogent bidentate C18™ and Cogent Phenyl hydride™ provide increased thermal stability with respect to non-hydrosilated silica gel, up to 100 °C or 80 °C, respectively. In the ANP mode, the ln k of phenolic acids and flavonoid compounds show a linear dependence on 1/T, in agreement with the van't Hoff model. The enthalpic contributions to the retention, B_i/T, are higher for strongly retained flavonoid glycosides (hesperidin, naringin, esculin) than the entropic ones, while lower retained flavonoid compounds show higher entropic contributions on all columns tested in the ANP mode. The retention times and bandwidths decrease and the overall separation improves at increasing temperature in the ANP mode up to 60 °C, especially on the Cogent UDC cholesterol™. The separation selectivity and resolution of all flavones strongly decrease above 60 °C, while in the RP mode, the resolution and selectivity do not significantly change over all the studied temperature range, except for the glycoside flavones hesperetin and naringenin on the bidentate stationary phase. From among all the columns tested, the UDC cholesterol column is best suited for the dual mode ANP and RP separations of flavones. The present

results show that both the mobile phase composition and the temperature provide very powerful tools for controlling and optimizing the separation on the silica gel type C columns in the ANP and RP modes.

Acknowledgement

This work was financially supported by the project Enhancement of R&D Pools of Excellence at the University of Pardubice reg. Nr. CZ.1.07/2.3.00/30.0021.

References

[1] Sandoval J.E., Pesek J.J.: Anal. Chem. **61**, 2067 (1989).
[2] Pesek J.J., Matyska M.T., Sharma A.: J. Liq. Chromatogr. Relat. Technol. **31**, 134 (2008).
[3] Alpert A.J.: J. Chromatogr. **449**, 177 (1990).
[4] Hemstrom P., Irgum K.: J. Sep. Sci. **29**, 1784 (2006).
[5] Ikegami T., Tomomatsu K., Takubo H., Horie K., Tanaka N.: J. Chromatogr. A **1184**, 474 (2008).
[6] Dejaegher B., Mangelins D., Vander Heyden Y.: J. Sep. Sci. **31**, 1438 (2008).
[7] Nguyen H.P., Schug K.A.: J. Sep. Sci. **31**, 1465 (2008).
[8] Hsieh Y.: J. Sep. Sci. **31**, 1481 (2008).
[9] Jandera P.: J. Sep. Sci. **31**, 1421 (2008).
[10] Jandera P.: Anal. Acta Chim. **692**, 1 (2011).
[11] Yoshida T.: J. Biochem. Biophys. Met. **60**, 265 (2004).
[12] Tuytten R., Lemiere F., Van Dongen W., Witters E., Esmans E.L., Newton R.P., Dudley E.: Anal. Chem. **80**, 1263 (2008).
[13] Jian W., Edom R.W., Xu Y., Weng N.: J. Sep. Sci. **33**, 681 (2010).
[14] Dejaegher B., Vander Heyden Y.: J. Sep. Sci. **33**, 698 (2010).
[15] Strege M.A.: Anal. Chem. **70**, 2439 (1998).
[16] Iwasaki Y., Ishii Y., Ito R., Saito K., Nakazawa H.: J. Liq. Chromatogr. Relat. Technol. **30**, 2117 (2007).
[17] Spagou K., Tsoukali H., Raikos N., Gika H., Wilson I.D., Theodoridis G.: J. Sep. Sci. **33**, 716 (2010).
[18] Pesek J.J., Matyska M.T.: J. Sep. Sci. **32**, 3999 (2009).
[19] Pesek J.J., Matyska M.T., Dawson G.B., Wilsdorf A., Marc P., Padki M.: J. Chromatogr. A **986**, 253 (2003).
[20] Pesek J.J., Matyska M.T., Yu R.J.: J. Chromatogr. A **947**, 195 (2002).
[21] Pesek J.J., Matyska M.T., Pan X.: J. Chromatogr. A **992**, 57 (2003).
[22] Pesek J.J., Matyska M.T., Fischer S.M., Sana T.R.: J. Chromatogr. A **1204**, 48 (2008).

[22] Pesek J.J., Matyska M.T., Fischer S.M., Sana T.R.: J. Chromatogr. A **1204**, 48 (2008).
[23] Pesek J.J., Matyska M.T., Hearn M.T.W., Boysen R.I.: J. Chromatogr. A **1216**, 1140 (2009).
[24] Matyska M.T., Pesek J.J., Duley J., Zamzami M., Fischer S.M.: J. Sep. Sci. **33**, 930 (2010).
[25] Pesek J.J., Matyska M.T.: J. Sep. Sci. **30**, 637 (2007).
[26] Molikova M., Jandera P.: J. Sep. Sci. **33**, 453 (2010).
[27] Urban J., Škeříková V., Jandera P., Kubíčková R., Pospíšilová M.: J. Sep. Sci. **32**, 2530 (2009).
[28] Jin G., Guo Z., Zhang F., Xue X., Jin Y., Liang X.: Talanta **76**, 522 (2008).
[29] Jandera P., Hájek T., Škeříková V., Soukup J.: J. Sep. Sci. **33**, 841 (2010).
[30] Jandera P., Hájek T.: J. Sep. Sci. **32**, 3603 (2009).
[31] McCalley D.V.: J. Chromatogr. A **902**, 311 (2000).
[32] Vanhoenacker G., Sandra P.: Anal. Bioanal. Chem. **390**, 245 (2008).
[33] Melander W.R., Horvath Cs. in: Cs. Horvath (Ed.), *High-Performance Liquid Chromatography*, vol. 2, Academic Press, NY, 1980, p. 113.
[34] Vigh G., Varga-Puchony Z., J. Chromatogr. **196**, 1 (1980).
[35] Vanhoenacker G., Sandra P.: J. Chromatogr. A **1082**, 193 (2005).
[36] Melander W., Campbell D.E., Horvath C.: J. Chromatogr. **158**, 215 (1978).
[37] Silveston R., Kronberg B.: J. Chromatogr. A **659**, 43 (1994).
[38] Chester T.L., Coym J.W.: J. Chromatogr. A **1003**, 101 (2003).
[39] Kiridena W., Poole C.F., Koziol W.W.: Chromatographia **57**, 703 (2003).
[40] Cole L.A., Dorsey J.G., Dill K.A.: Anal. Chem. **64**, 1324 (1992).
[41] Hearn M.T.W., Zhao G.: Anal. Chem. **71**, 4874 (1999).
[42] Guillarme D., Heinisch S., Rocca J.L.: J. Chromatogr. A **1052**, 39 (2004).
[43] Riedo F., Kovats E.: J. Chromatogr. **239**, 1 (1982).
[44] Jandera P., Colin H., Guiochon G.: Anal. Chem. **54**, 435 (1982).
[45] Jandera P., Krupczyńska K., Vyňuchalová K., Buszewski B.: J. Chromatogr. A **1217**, 6052 (2010).