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Characterization of various reversed-phase columns using the linear free energy relationship
I. Evaluation based on retention factors

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Abstract

Nine prepacked narrow-pore and six wide-pore reversed-phase columns containing various ligands (C_{18}, C_8, C_4, CN) and obtained from different manufacturers were investigated. Retention factors of 34 solutes of widely different type were determined under isocratic conditions using an acetonitrile–water (30:70) mobile phase. Retention data were evaluated by principal component analysis (PCA) in order to compare column characteristics. Based on the same datasheet, stationary phase properties were compared in the frame of the linear free energy relationship (LFER) using solvation parameters of the solutes studied. The fitting coefficients of the LFER-based regression equations are characteristic of the individual stationary phases and represent the extent of the various molecular interactions contributing to the retention process. Column characterization furnished by PCA and LFER is discussed in detail.

Keywords: Linear free energy relationships; Stationary phases, LC; Retention behaviour; Principal component analysis; Column characetristics; Retention factors

1. Introduction

Notwithstanding the rapid development of other chromatographic techniques (ion chromatography, chiral separations etc.) reversed-phase high-performance liquid chromatography (RP-HPLC) continues to dominate the field and accounts for approximately 60% of the HPLC separations performed [1]. Since the introduction of RP stationary phases in the early 1970s a large variety of RP packings have been commercialized by various manufacturers [2,3]. According to recent estimates over 500 different RP columns are commercially available. In addition to the narrow-pore (NP, 6–15 nm) silica supports generally used for the separations of small molecules, in the past decade a new family of RP-packings has been developed for the separation of biopolymers using wide-pore (WP, 30–400 nm) silica supports, shorter ligands and improved bonding chemistry [4].

Despite the widespread use of RP-HPLC, the characterization and comparison of various RP stationary phases have been manifold and controversial. Numerous reports can be found in the literature on testing and characterizing RP phases by chromatographic methods using different sets of test compounds.

Since the first report of Goldberg [5] in 1982

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several hundreds of studies have been published using different test mixtures and a variety of mobile phase compositions to evaluate chromatographic characteristics such as hydrophobicity, polarity, silanol activity, acidic or basic properties and steric selectivity of the RP columns [6–24]. The most widely known and referred testing procedures were reported by Engelhardt and co-workers [2,7–10], Tanaka and co-workers [11,12], Jandera [13–15], Sander and Wise [16–18] and Claessens and co-workers [19–21]. The above testing procedures have been developed for the characterization of narrow-pore stationary phases with fixed, generally high organic modifier concentrations and cannot be applied to wide-pore packings because of inadequate retention due to their lower surface area and lower carbon content. Although many wide-pore stationary phases are commercially available they have rarely been compared and critically evaluated [22]. Notwithstanding their increasing applications, there is no generally accepted testing procedure for characterizing and comparing different wide-pore RP packings.

In our laboratory a number of wide-pore RP columns containing various ligands and obtained from different manufacturers were investigated with numerous test solutes. Evaluation of the columns was accomplished by single parametric methods selecting different descriptors [23] as well as with the aid of multivariate statistical techniques [24]. In an earlier study we characterized and compared RP columns of different pore size and ligand, using gradient elution technique under standardized gradient conditions [6].

2. Theoretical

Several years ago Kamlet and Taft and their co-workers derived general equations for the correlation of solute effects in various distribution processes as solubility, distribution between solvents, distribution between gases and condensed phases as well as within condensed phases [25–31]. Since several of the descriptors were calculated from UV–visible shift measurements, the equations have often been referred as solvatochromic or linear solvation energy relationships (LSERs). A general form of the LSER equation in terms of solute parameters is given by

$$\begin{aligned} \mathrm{SP} &= \mathrm{SP}_0 + m(V/100) + s(\pi^* + \mathrm{d}\delta) \\ &= b\beta_\mathrm{m} + a\alpha_\mathrm{m} \end{aligned} \quad (1)$$

where SP denote solvent-dependent properties, V is a measure of solute volume, π^* is dipolarity/polarizability, δ is a polarizability correction parameter, β_m is hydrogen-bond acceptor (HBA) basicity and α_m is hydrogen-bond donor (HBD) acidity and SP_0, m, s, b, a are the regression coefficients.

The LSER approach has been applied extensively to the study of HPLC [32–38] and GC separations [39–43] with generally good results. Based on this model, a free energy related term in a phase transfer process can be correlated with various fundamental solute descriptor properties. In HPLC the logarithmic retention factor (or other retention characteristic of the system as $\log k_\mathrm{w}\ S$) are separated into several molecular interaction terms and can be written as

$$\begin{aligned} \log k = (\log k)_0 &+ m(\delta_\mathrm{m}^2 - \delta_\mathrm{s}^2)V_2/100 \\ &+ s(\pi_\mathrm{m}^* - \pi_\mathrm{s}^*) + a(\beta_\mathrm{m} + \beta_\mathrm{s})\alpha_2 \\ &+ b(\alpha_\mathrm{m} + \alpha_\mathrm{s})\beta_2 \end{aligned} \quad (2)$$

where $(\log k)_0$ is an independent term, m, s, a and b are the regression coefficients, $V_2/100$ is the ''normalized'' volume of the solute, δ is the hildebrand solubility parameter, π^*, α and β are the Kamlet–Taft solvatochromic parameters given in Eq. (1). The subscripts s and m denote the stationary and mobile phase, respectively.

Eq. (2) can be simplified in two different ways [38]: when a system with a fixed mobile phase composition and a fixed column stationary phase is considered, the equation becomes

$$\log k = (\log k)_0 + m_1 V_2/100 + s_1\pi_2^* + a_1\alpha_2 + b_1\beta_2 \quad (3)$$

where the coefficients m_1, s_1, a_1 and b_1 depend on the stationary and mobile phase solubility (δ_2), polarity (π^*), and HBA basicity (β) and HBD acidity (α). Eq. (3) allows the correlation of the retention of different solutes in the same column and mobile phase with solute properties. Using the same mobile phase composition and solutes for different stationary phases, the regression coefficients will characterize the individual stationary phases as regards to their contributions to the various molecular interactions.

For a given solute on a fixed column but with different mobile phase composition Eq. (2) can be rewritten as

$$\log k = (\log k)_s + m_2\delta_m^2 + s_2\pi_m^* + a_2\beta_2 + b_2\alpha_m \quad (4)$$

where $(\log k)_s$ depends on $(\log k)_0$ and on the parameters of the stationary phase, m_2, s_2, a_2 and b_2 depend on the solute parameters.

The solute's solvatochromic properties were derived from solvent solvatochromic measurement of the absorption bands for a series of indicator compounds [25–29]. Difficulties arose due to the lack of solvatochromic parameters for less common solutes and because a huge number of solute parameters had to be estimated from a very small solvent database. In addition, as the solvent parameters were derived from UV–visible shifts, they are not thermodynamic parameters [44]. Abraham and co-workers introduced new solute parameters derived from equilibrium measurements on the solutes themselves such as GC data, water–solvent partition coefficients and data relating to the molecular structure [45–47]. Chromatographic retention data have been correlated through the LFER or solvation equation:

$$\log k = c + rR_2 + s\pi_2^* + a\sum\alpha_2^H + b\sum\beta_2^H + vV_x \quad (5)$$

where c is the intercept, R_2 is an excess molar refraction, π_2^* is the solute dipolarity/polarizability, $\Sigma\alpha_2^H$ is the solute overall or effective HBA acidity, $\Sigma\beta_2^H$ is the solute overall or effective HBA basicity. V_x is the McGowan characteristic volume. The coefficients in Eq. (5) are determined by multivariate regression analysis and serve to characterize the phase investigated. The r is a measure of the propensity of the phase to interact with solute n and π-electron pairs; s measures the phase dipolarity/polarizability; a is a measure of the phase HB basicity; b is a measure of the phase HB acidity; v is a measure of phase hydrophobicity. If Eq. (5) is applied to the distribution between two phases, the coefficients will refer to differences between the phases concerned. The methodology is in principle is the same as that used in the LSER based Kamlet system but the above solute descriptors (solvation parameters) are thermodynamic Gibbs energy related quantities. They are the correct parameters to be used in the LFER equation to describe Gibbs energy related data such as chromatographic retention [44]. It should be noted that not all the terms in Eq. (5) are statistically significant in any given case. For this reason various forms of Eq. (5) can be found in the literature. Another problem is that Kamlet solvatochromic parameters and Abraham solvation parameters are often intermittently used in publications. The advantage of the solvation parameters is, that a huge database is available containing more than 2000 chemical compounds [45–48].

In the last years LFERs have been used to characterize and compare various RP stationary phases [46,49–52]. The coefficients c, r, s, a, b and v in Eq. (5) are characteristic of the phase system, i.e., a particular RP-HPLC column with a given mobile phase composition. If different columns are studied with the same mobile phase, the coefficients will characterize the various columns i.e., the contribution of the stationary phases to the individual molecular interactions.

In this study, we compared and evaluated specific retention properties of 15 different RP-HPLC columns based on isocratic retention data, by using a chemometric method and by constructing LFER equations. In a subsequent paper we will focus on selectivity differences and investigate the possible use of the LFER approach for characterizing selectivity variations in our column set.

3. Experimental

Retention data were measured on a Merck–Hitachi LiChrograph consisting of an L-6200 programmable pump, a Rheodyne 7215 injector with 10 μl loop and a L-4250 UV–Vis detector operating at 220 nm. Data acquisition was performed by the D-7000 HPLC System Manager software.

The RP-HPLC columns investigated in this study, nine narrow-pore and six wide-pore phases, are listed separately in Table 1, together with their main characteristics as provided by the manufacturers.

Test solutes were of analytical grade and were purchased from different manufacturers. They were selected to cover a wide range of chemical properties. List of the 34 solutes and corresponding

Table 1
Characteristics of the columns

Column	Manufacturer	Dimensions (mm×mm I.D.)	Ligand type	Particle size (μm)	Pore size (nm)	Surface area (m^2/g)	% C approx.	Abbreviation
LiChrospher 100 RP-18e	Merck (Darmstadt, Germany)	125×4.0	C_{18}	5.0	10	350	21.6	M-C_{18}e
LiChrospher 100 RP-18	Merck	125×4.0	C_{18}	5.0	10	350	21.0	N-C_{18}
Purospher RP-18e	Merck	125×4.0	C_{18}	5.0	12	350	18.0	M-PURe
Purospher	Merck	250×4.0	C_{18}	5.0	8	500	18.5	M-PUR
LiChropsher PAH	Merck	250×3.0	C_{18}	5.0	15	200	20.0	N-PAH
SymmetryShield RP-C_{18}	Waters (Milford, MA, USA)	150×3.9	C_{18}	5.0	10	340	n.a.	SYM-C_{18}
SymmetryShield RP-C_8	Waters	150×3.9	C_8	5.0	10	340	15.0	SYM-C_8
LiChrosorb RP-select B	Merck	125×4.0	C_8	5.0	6	300	11.4	M-RP-B
LiChrospher 100 RP-8	Merck	125×4.0	C_8	5.0	10	350	12.5	M-C_8
Aquapore OD-300	Applied Biosystems (San Jose, CA, USA)	100×4.6	C_{18}	7.0	30	100	n.a.	A-C_{18}
Synchropak RP-C_{18}	Synchrom (Linden, IN, USA)	100×4.6	C_{18}	6.5	30	n.a.	7.5	S-C_{18}
Aquapore Butyl	Applied Biosystems	100×4.6	C_4	7.0	30	100	n.a.	A-C_4
Synchropak RP-C_4	Synchrom	100×4.6	C_4	6.5	30	n.a.	7.5	S-C_4
Zorbax SB 300 C_8	Rockland Technologies (Newport, DE, USA)	150×4.6	C_8	5.0	30	45	1.5	Z-C_8
Zorbax SB 300 CN	Rockland Technologies	150×4.6	CN	5.0	30	45	1.2	Z-CN

n.a. = Not available.

solvation parameters [45–48] pertaining to Eq. (5) are shown in Table 2.

On all the columns, retention time of test solutes were measured in duplicate, using the same premixed acetonitrile–water (30:70, v/v) mobile phase. Acetonitrile and water were of chromatographic grade obtained from Merck (Darmstadt, Germany). For calculation of retention factors, column dead time was determined by injecting 0.05 m*M* sodium nitrate solution. Reproducibility of sequential mea-

Table 2
Test solutes and solvation parameters

Compound	Abbreviation	R_2	π_2^*	$\Sigma\alpha_2^H$	$\Sigma\beta_2^H$	V_x
Ethylbenzene	EB	0.613	0.50	0	0.15	0.9982
Toluene	T	0.601	0.52	0	0.14	0.8573
Bromobenzene	BrB	0.882	0.73	0	0.09	0.8914
Chlorobenzene	CB	0.718	0.65	0	0.07	0.8388
Caffeine	CAF	1.500	1.60	0	1.33	1.3632
Dimethyl phtalate	PDM	0.780	1.41	0	0.88	1.4288
Pyridine	PYR	0.631	0.84	0	0.52	0.6753
Acetophenone	AP	0.818	1.01	0	0.48	1.0139
Methylbenzoate	MBO	0.733	0.85	0	0.46	1.0726
Ethylbenzoate	EBO	0.689	0.85	0	0.46	1.2135
Benzyl cyanide	BC	0.751	1.15	0	0.45	1.012
N,N-Dimethylaniline	DMA	0.957	0.84	0	0.42	1.098
Anisole	AN	0.708	0.75	0	0.29	0.916
o-Nitrotoluene	ONT	0.866	1.11	0	0.27	1.032
Nitrobenzene	NB	0.871	1.11	0	0.26	0.891
Hydroquinone	HQ	1.000	1.000	1.16	0.6	0.834
p-Nitrophenol	PNP	1.070	1.72	0.82	0.26	0.9493
Methylparaben	MP	0.9	1.37	0.69	0.45	1.1313
Ethylparaben	EP	0.86	1.35	0.69	0.45	1.2722
Propylparaben	PP	0.86	1.35	0.69	0.45	1.4131
Butylparaben	BP	0.86	1.35	0.69	0.45	1.554
β-Naphtol	BNA	1.520	1.08	0.61	0.40	1.144
α-Naphtol	ANA	1.520	1.05	0.60	0.37	1.144
Phenol	P	0.805	0.89	0.60	0.30	0.7751
p-Cresol	PCR	0.820	0.87	0.57	0.32	0.916
o-Cresol	OCR	0.840	0.86	0.52	0.31	0.916
p-Ethylphenol	PEP	0.800	0.90	0.55	0.36	1.0569
3,5-Dimethylphenol	DP35	0.82	0.84	0.57	0.36	1.0569
2,6-Dimethylphenol	DP26	0.860	0.79	0.39	0.39	1.0569
p-Nitroaniline	PNA	1.220	1.91	0.42	0.38	0.991
Benzyl alcohol	BA	0.803	0.87	0.33	0.56	0.916
Aniline	A	0.955	0.96	0.26	0.5	0.8162
o-Toluidine	OT	0.966	0.92	0.23	0.59	0.9571
α-Naphtylamine	NA	1.670	1.26	0.2	0.57	1.185

surements were excellent, with an average deviation of 1% in k retention factors. It was established by Engelhardt et al. [2] that through the addition of salt or using buffer solutions the silanophilic interactions can be reduced and bad columns can be made to look good. In order to evaluate and compare the different packing materials without modification of surface properties water was used without any additive for pH and ionic strength adjustment. Sample mixtures were prepared using the mobile phase to approx. 2 mg/ml concentration, which corresponded to 0.008–0.015 mg/g stationary phase. It was described earlier, that retention of basic solutes is independent of the sample size if the linear capacity range of 0.1 mg sample per g stationary phase is not exceeded [9].

Principal component analysis and multivariate linear regression analysis were performed with Statistica 5.0 for Windows software (StatSoft, USA).

4. Results and discussion

The aim of present study was to provide a comparison of RP-HPLC phases of different pore sizes and ligands. Column characterization was achieved by means of two different approaches: (1) principal component analysis (PCA) of retention data

and (2) constructing LFER equations using the same database. In the latter case, we were curious to see if the LFER model is applicable for evaluating and comparing RP phases of such diversity.

Factor analysis and related data reduction techniques (PCA [53–56], correspondence factor analysis [57,58], target factor analysis [59]) have routinely been applied to detect similarities among HPLC packing materials. The usual procedure of above listed methods is to set up a database that may contain different chromatographic indices of test solutes, such as retention factor, selectivity, asymmetry, number of theoretical plates, or mixed. Subsequently performed analysis groups test compounds into abstract factors with different weights, while the column set is characterized upon calculating the corresponding scores on the factors previously extracted.

4.1. Principal component analysis

In this paper we applied PCA for characterizing our column set comprising 15 RP-HPLC columns. Since the above multivariate techniques, among them PCA, have been described elsewhere, for terminology and mathematical details the reader is referred to the literature [59,60]. In Table 3 the input data matrix is shown, consisting the decimal logarithm of 34 retention factors measured on all the 15 columns.

Correlation matrix for test substances (not shown) confirmed high degree of intercorrelation, thus the application of PCA toward reducing data dimensionality seemed logical. Before proceeding to calculation, an outlier, HQ had to be eliminated from subsequent analysis due to the large relative differences in the solute's retention factor on the various columns. The principal component (PC) extraction was followed by VARIMAX rotation to yield an easier interpretable PC structure. Table 4 shows the first three extracted PCs representing 93.7% of the original variance in the retention data matrix. Additional factors were found of minor importance and were not evaluated. The so-called factor loadings listed in Table 4 can be regarded as correlation coefficients between the retention of test solutes and the respective PC. The real meaning of a PC may be explored by examining the high-loading compounds it contains. Nevertheless, the user needs an a priori knowledge of the chemical character of his test substances.

Other general characteristics of most factor analytical techniques can be seen in Table 4: each solute contributes with smaller or larger extent to each factor (here, PC). As a consequence, the estimation of special molecular forces that should be attributed to certain PC is rather arbitrary. Thus, with PCA, individual interactions governing retention process on a HPLC column is difficult to unravel.

In our case, PC1 extracted more than 40% of original variance and was the most important factor in differentiating among the column set. Strongly retained test solutes with pronounced hydrophobic character (aromatic hydrocarbons, halogenated aromatics, esters, nitro-compounds such as T, EB, CB, BRB, EBO, NB etc.) displayed the highest loadings on this PC. Thus, hydrophobicity still remained the main distinguishing property of the phases, despite the great variability of columns' surface chemistry.

PC2 with 33.5% variance was found of commensurable importance to PC1. Taking into consideration that more than the half of 34 test compounds were carrying phenolic OH group, and all were grouped into this PC with high loading, the relatively high importance of these acidic solutes is not that astonishing. The most acidic solutes were MP, P, PNP, ANA and PNA as indicated by higher (>0.6) loadings.

The third PC accounted for a much smaller proportion (19.5%) of variance and included basic compounds like CAF, PYR, A with considerable weights. DMA as a sterically hindered tercier amine was recognized as predominantly hydrophobic compound and was classified into PC1, a phenomenon that was described earlier [57].

For column characterization, the score matrix was calculated based on the extracted PC structure. Instead of numerical values, a graphical presentation of columns in the subspace of PCs is given (Fig. 1a,b). In Fig. 1a the scattering of columns along the horizontal axis indicates hydrophobic property of stationary phases. Phases that possess large carbon content (M-PURe, M-C_{18}e, M-C_{18} and M-PAH) or large surface area (M-PUR) scored high on this axis. Narrow-pore C_8 materials were identified as having lower hydrophobic retentive properties than NP-C_{18} ones. Hydrophobicity for WP packings decreased

Table 3
Log k values in acetonitrile–water (30:70, v/v)

V_0[a]:	0.71	0.70	0.70	1.49	0.96	0.85	0.85	0.72	0.71	1.05	0.85	1.20	1.05	0.86	1.12
Column: Compound	M-C_{18}e	M-C_{18}	M-PURe	M-PUR	M-PAH	SYM-C_{18}	SYM-C_8	M-RP-B	M-C_8	A-C_{18}	S-C_{18}	Z-C_8	A-C_4	S-C_4	Z-CN
EB	1.954	1.933	1.994	1.651	1.761	1.805	1.741	1.720	1.694	1.360	1.233	0.950	0.966	0.535	
T	1.635	1.620	1.673	1.351	1.445	1.501	1.457	1.434	1.419	1.058	0.950	0.708	0.716	0.675	0.369
BrB	1.792	1.778	1.806	1.480	1.557	1.643	1.609	1.547	1.545	1.182	1.064	0.813	0.788	0.723	0.502
CB	1.696	1.685	1.710	1.360	1.449	1.546	1.523	1.467	1.469	1.089	0.979	0.736	0.728	0.666	0.464
CAF	−0.019	0.041	−0.041	−0.347	0.057	−0.187	−0.093	0.099	0.159	−0.286	−0.108	−0.226	−0.197	−0.301	−0.456
PDM	1.037	1.043	1.028	0.721	0.886	0.872	0.967	0.946	0.978	0.521	0.505	0.379	0.383	0.330	0.199
PYR	0.264	0.494	0.301	0.151	0.374	0.054	0.134	0.427	0.463	−0.017	0.142	−0.046	0.000	−0.005	−0.062
AP	0.896	0.929	0.933	0.663	0.801	0.799	0.852	0.865	0.881	0.420	0.438	0.288	0.295	0.246	0.121
MBO	1.209	1.233	1.254	0.974	1.083	1.103	1.129	1.126	1.130	0.695	0.675	0.483	0.480	0.436	0.243
EBO	1.533	1.547	1.578	1.282	1.388	1.407	1.414	1.405	1.402	0.994	0.941	0.717	0.702	0.664	0.385
BC	0.999	1.011	1.018	0.724	0.872	0.903	1.002	0.960	0.983	0.526	0.503	0.376	0.404	0.348	0.071
DMA	1.493	1.512	1.508	1.201	1.332	1.330	1.374	1.298	1.306	0.908	0.840	0.612	0.593	0.541	0.305
AN	1.201	1.208	1.212	1.003	1.074	1.157	1.171	1.124	1.145	0.741	0.675	0.486	0.501	0.450	0.245
ONT	1.463	1.468	1.476	1.144	1.275	1.330	1.306	1.316	1.337	0.908	0.840	0.655	0.638	0.575	0.305
NB	1.181	1.235	1.181	0.894	1.021	1.063	1.123	1.072	1.104	0.647	0.602	0.467	0.449	0.388	0.252
HQ	0.031	0.077	0.012	−0.444	−0.023	−0.044	0.130	0.006	0.120	−0.219	−0.181	−0.210	−0.150	−0.218	−0.168
PNP	0.602	0.664	0.597	0.750	0.400	0.709	0.880	0.585	0.622	0.253	0.262	0.167	0.135	0.060	0.008
MP	0.671	0.714	0.664	0.425	0.559	0.687	0.820	0.639	0.699	0.225	0.232	0.174	0.171	0.111	0.025
EP	0.951	1.005	0.940	0.732	0.816	0.961	1.084	0.888	0.936	0.466	0.449	0.351	0.338	0.283	0.186
PP	1.279	1.329	1.267	1.059	1.134	1.287	1.384	1.171	1.212	0.762	0.720	0.585	0.551	0.492	0.335
BP	1.626	1.624	1.614	1.382	1.466	1.616	1.696	1.467	1.495	1.089	1.019	0.736	0.788	0.666	0.412
BNA	1.264	1.271	1.304	1.003	1.112	1.304	1.350	1.145	1.145	0.706	0.644	0.492	0.520	0.459	0.338
ANA	1.302	1.305	1.304	1.098	1.163	1.437	1.475	1.208	1.228	0.796	0.721	0.562	0.579	0.533	0.381
P	0.591	0.601	0.597	0.332	0.501	0.606	0.697	0.585	0.622	0.155	0.188	0.070	0.135	0.060	0.008
PCR	0.864	0.897	0.851	0.583	0.753	0.844	0.917	0.778	0.829	0.378	0.374	0.279	0.297	0.224	0.121
OCR	0.917	0.946	0.907	0.625	0.796	0.908	0.974	0.821	0.870	0.419	0.374	0.279	0.297	0.224	0.121
PEP	1.161	1.204	1.147	0.902	1.021	1.132	1.187	1.048	1.084	0.647	0.602	0.467	0.449	0.388	0.252
DP35	1.123	1.153	1.110	0.837	0.995	1.097	1.148	1.002	1.045	0.607	0.596	0.471	0.455	0.379	0.236
DP26	1.188	1.217	1.181	0.884	1.055	1.148	1.181	1.052	1.092	0.655	0.596	0.471	0.455	0.379	0.236
PNA	0.758	0.759	0.746	0.452	0.592	0.740	0.912	0.717	0.765	0.278	0.296	0.195	0.213	0.152	0.144
BA	0.443	0.475	0.450	0.203	0.400	0.369	0.446	0.467	0.503	0.038	0.116	−0.008	0.042	−0.036	−0.049
A	0.542	0.583	0.557	0.297	0.493	0.459	0.546	0.560	0.586	0.111	0.176	0.039	0.094	0.019	−0.180
OT	0.773	0.816	0.796	0.551	0.716	0.692	0.750	0.755	0.770	0.307	0.344	0.179	0.219	0.152	−0.012
NA	1.212	1.240	1.215	0.955	1.095	1.152	1.202	1.093	1.118	0.680	0.664	0.483	0.474	0.414	0.305

[a] V_0 = Column void volume in ml.

considerably by decreasing ligand length. But there were only slight differences in hydrophobicity between C_4 (A-C_4 and S-C_4) and C_8 (Z-C_8) columns, while Z-CN column represented a pronounced lack of hydrophobic character.

In Fig. 1b the horizontal axis indicates column HBA basicity (the complimentary column property for the stronger retention of acidic phenolic compounds), while PC3 measures column HBD acidity. SYM-C_8 and SYM-C_{18} phases both have embedded carbamate moiety in their hydrocarbonaceous ligands. The revealed above average HBA basicity of these packing materials is a consequence of this special functionality. The greater basicity of SYM-C_8 over the SYM-C_{18} column is due to the enhanced accessibility of carbamate groups in the shorter alkyl chain media. The other specialty column, M-PUR was endcapped with hydrophilic amino-group containing silyl derivate that increases column HBA basicity, a phenomenon which was detected by relatively high scores on PC2. Non-endcapped narrow-pore columns (M-C_8, M-RP-B, M-C_{18} and also M-PAH) scored high on HBD acidity, and relatively high on HBA basicity axis, indicating a greater

Table 4
PCA loadings of test solutes (loadings higher than 0.65 in bold)

Compound	PC1	PC2	PC3
EB	**0.796**	0.442	0.355
T	**0.776**	0.471	0.359
BrB	**0.769**	0.487	0.345
CB	0.754	0.502	0.357
CAF	0.269	0.284	**0.905**
PDM	0.636	0.556	0.467
PYR	0.378	0.245	**0.805**
AP	0.642	0.553	0.465
MBO	**0.705**	0.518	0.418
EBO	**0.745**	0.483	0.398
BC	0.643	0.556	0.459
DMA	**0.743**	0.496	0.381
AN	**0.702**	0.545	0.381
ONT	**0.732**	0.487	0.419
NB	**0.665**	0.560	0.422
PNP	0.570	**0.696**	0.145
MP	0.541	**0.724**	0.402
EP	0.584	**0.688**	0.363
PP	0.648	0.637	0.343
BP	**0.706**	0.573	0.331
BNA	0.629	**0.655**	0.312
ANA	0.632	**0.688**	0.277
P	0.507	**0.713**	0.431
PCR	0.592	**0.658**	0.408
OCR	0.604	**0.659**	0.385
PEP	0.656	0.611	0.375
DP35	0.648	0.616	0.392
DP26	0.665	0.597	0.384
PNA	0.486	**0.753**	0.394
BA	0.491	0.615	0.557
A	0.577	0.553	0.554
OT	0.634	0.547	0.481
NA	**0.672**	0.585	0.385
Explained variance	13.522	11.062	6.388
% of total	40.9	33.5	19.4

amount of unsubstituted silanol groups that can enter H-bond donor and H-bond acceptor interactions, as well. Highly hydrophobic, endcapped Merck columns (M-C_{18}e, M-PURe) were well balanced in terms of HBD and HBA properties, located close to the origin of the PC2–PC3 plane. The outstanding basicity of Z-CN was a consequence of its HB acceptor cyano ligand and low phase coverage. Among the other WP media, S-C_{18} column displayed significantly greater acidity than A-C_{18}, while practically no difference were detected in their HBA basicity.

The projection of columns on PC axes reflected reasonably well differences of the columns studied, such as between wide-pore and narrow-pore materials, between C_{18}, C_8 and shorter alkyl chain columns, or differences between columns of special surface chemistry.

4.2. Linear free energy relationships

Contrary to the majority of multivariate methods adopted for characterization of stationary phases, the LFER method is based on a thermodynamically derived solvation parameter model. The unique advantage of the LFER approach relies in its ability to measure independently the contribution of individual molecular interactions to the retention process. This is achieved by constructing LFER regression equations in the general form of Eq. (5), using multivariate linear regression analysis. As it was set out in the Theoretical part, regression coefficients will characterize the difference of certain interactions between the stationary and mobile phase. If the same mobile phase is used with different columns, regression coefficients can be directly applied for characterization of stationary phases.

In this study, the same acetonitrile–water (30:70) mobile phase was used throughout the experiments, and LFER regression equations were calculated using log k values of Table 2 as dependent variable, and solvation parameters of Table 3 as independent variable set.

The resulting regression coefficients and the corresponding statistical descriptors are summarized in Table 5. For clarity of subsequent considerations, a graphical presentation of the coefficients, together with a 95% confidence interval is provided in Fig. 2 parts a–f. The goodness-of-fit of the equations were in most cases good ($R>0.98$), the validity of regression hypothesis were proved for all columns by the Fisher F-test [$F_{\text{crit }99\%}$ (5, 28)=4.30]. These statistical indicator values confirm that the LFER model adequately describes retention even when applied to a wide variety of RP columns of different pore size and ligand. The somewhat poorer fit observed for Z-CN column, may be due to its small retentive power in the acetonitrile–water (30:70) mobile-phase and the associated higher level of error in measuring k retention factors.

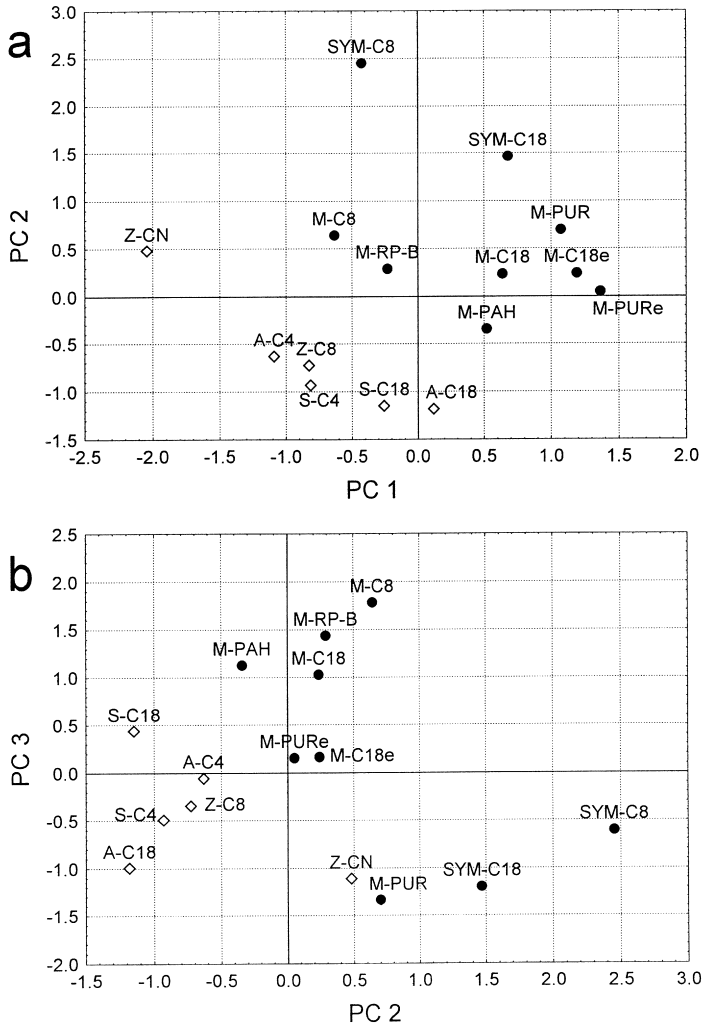

Fig. 1. Score plots, column characterization obtained by PCA. (a) PC1 vs. PC2; (b) PC2 vs. PC3. Symbols denote: (◇) WP columns; (●) NP columns.

The numerical range and size of coefficients were in good agreement with values previously reported for octadecyl and cyano columns when using the same mobile phase [42]. Since coefficients account for differences in particular interaction involved in the stationary phase and mobile phase, respectively, a positive sign would indicate that the respective molecular interaction is stronger in the stationary than in the mobile phase. This is the case for *v* and *r* coefficients, where the *v* measures the combination of cavity formation and dispersive interactions, while *r* arises from n- and π-electron interactions. An increase in solute size (V_x) and/or excess molar refractivity (R_2) leads to increased retention on all the columns studied. However, if we compare the magnitude of these two coefficients, it is obviously seen that cavity formation together with dispersion is far more critical term that affect retention than the electron-involved type interactions. On the other side, coefficients with negative sign (*a*, *b* and *s*) correspond to molecular forces that act favorably in the mobile phase, and therefore, decrease solute retention. HB donor and HB acceptor interactions and dipolar-type forces fall into this category.

Table 5
Phase system coefficients of LFER equations and regression statistics

Column	*v*	*r*	*b*	*a*	*s*	*c*	*n*[a]	*R*[b]	*F*[c]	S.D.[d]
M-C_{18}e	1.954	0.303	−1.949	−0.587	−0.481	0.241	34	0.986	194	0.087
M-C_{18}	1.838	0.260	−1.841	−0.568	−0.453	0.349	34	0.986	198	0.082
M-PURe	1.950	0.310	−1.965	−0.618	−0.501	0.283	34	0.987	204	0.086
M-PUR	1.886	0.270	−2.060	−0.596	−0.330	−0.023	34	0.984	169	0.092
M-PAH	1.758	0.279	−1.608	−0.547	−0.517	0.224	34	0.988	222	0.072
SYM-C_{18}	2.009	0.388	−2.095	−0.432	−0.450	0.021	34	0.990	301	0.070
SYM-C_8	1.888	0.331	−1.990	−0.340	−0.319	0.052	34	0.989	249	0.071
M-RP-B	1.628	0.234	−1.603	−0.539	−0.374	0.287	34	0.990	277	0.060
M-C_8	1.575	0.211	−1.534	−0.485	−0.352	0.323	34	0.989	268	0.058
A-C_{18}	1.621	0.228	−1.594	−0.478	−0.404	−0.095	34	0.982	149	0.082
S-C_{18}	1.379	0.185	−1.298	−0.446	−0.339	−0.023	34	0.980	138	0.072
Z-C_8	1.215	10.52	−1.166	−0.339	−0.266	−0.143	34	0.984	174	0.054
A-C_4	1.147	0.149	−1.086	−0.312	−0.287	−0.081	34	0.982	152	0.055
S-C_4	1.126	0.144	−1.095	−0.331	−0.287	−0.105	34	0.981	140	0.058
Z-CN	0.830	0.150	−0.929	−0.175	−0.187	−0.179	32[e]	0.960	65	0.065

[a] Number of test solutes.
[b] Pearson *R* correlation coefficient of the regression.
[c] Fischer *F*-test.
[d] Standard deviation.
[e] For Z-CN column, log *k* of CAf and HQ were not considered because of inadequate retention.

Among them, HBA-basicity of solutes is of major importance, indicated by the relatively large *b* coefficients of the LFER equations computed.

In the following, a term-by-term analysis of the regression coefficients will be provided in order to evaluate and compare stationary phase properties on the basis of the LFER model.

The V_x McGowan volume is one of the most significant solvation parameter that affects retention. The main bulk phase property complimentary to solute size is cohesiveness of the environment (mobile phase or the stationary interphase) in which solute molecules partition into. In this respect, independent studies [49,61,62] support the view that alkyl bonded stationary phase is far less cohesive than the water-rich mobile phase. Hence, greater amount of free energy is required to create solute size cavity in the mobile phase compared to that in the stationary phase. It is expected then, that columns of longer alkyl chain or more excessively covered surface can be identified in the frame of LFER approach by their greater (positive) *v* coefficients. In fact, narrow-pore C_{18} materials had notably greater *v* coefficients (Fig. 2a) than did WP phases, indicating that cavity formation is facilitated by highly covered hydrocarbonaceous interphase (average *v* for NP-C_{18} is 1.899, for NP-C_8 is 1.697 and for the WP columns 1.220). Confidence intervals for *v* showed no vital difference in the ease of cavity formation for the different NP octadecyl phases and the same holds true for the group of wide-pore Z-C_8, A-C_4 and S-C_4 packings. Creating solute size cavity was more difficult for M-PAH than for its C_{18} counterparts (indicated by somewhat smaller *v*), which is presumably due to the rigidity of the polymeric ligand environment of this column. Encapped columns like M-C_{18}e, M-PURe and also the SymmetryShield materials (SYM-C_{18} and SYM-C_8) showed notably higher *v* values than non-endcapped columns, primarily as a consequence of their dense hydrophobic surfaces, indicating a higher coverage of packing surface. Among WP materials, A-C_{18} resulted in unusually large *v* coefficient, which made this column more similar to NP-C_8 packings. As expected, Z-CN with the smallest carbon content scored the lowest on the *v* parameter scale.

The coefficient *r* (Fig. 2b) in Eq. (5) refers to the difference between the solvated bonded and mobile phase to interact with solute n- and π-electrons. The positive *r* obtained for all columns indicates that electron-involved interactions are slightly stronger in the stationary than in the mobile phase. The sig-

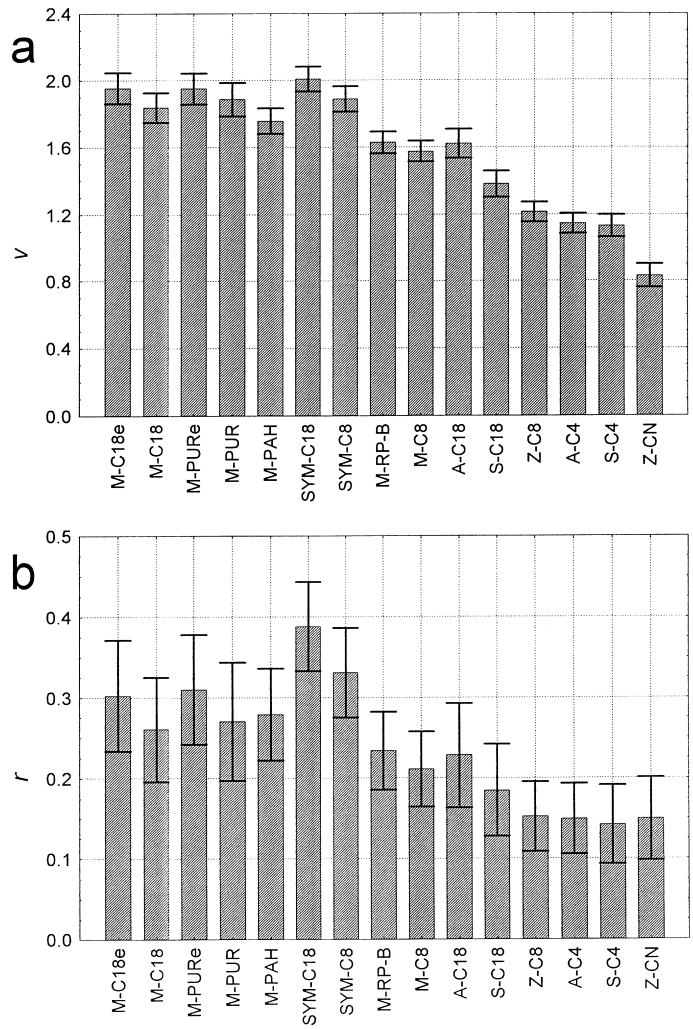

Fig. 2. Regression coefficients of columns, obtained by LFER equations. Error bars indicate 95% confidence interval. (a) *v* coefficient; (b) *r* coefficient; (c) *b* coefficient; (d) *a* coefficient; (e) *s* coefficient; (f) *c* intercept.

nificantly large *r* constants obtained for SymmetryShield columns suggest that solutes capable of donating n- and π-electrons are longer retained on these specialty columns (see for example ANA or BNA as solutes having large R_2 parameter). Similar observation can be made for Z-CN column where the *r* regression coefficient, unlike any other, was comparable to *r* coefficients obtained for the group of Z-C_8, A-C_4 and S-C_4 columns. These examples of enhanced ability of the phases to enter electron-involved interactions may be the consequence of the special surface chemistry, namely, the π-electron-rich carbamate on the SYM columns, and nitrile functional groups on Z-CN, respectively. For the rest of the column set, *r* coefficient was slightly but significantly larger on NP vs. WP packings. It is assumed that NP phases through their high surface area and bonding density possess stronger ability to engage in such electron-involved interactions. Moreover, larger positive *r* values on NP over WP columns indicate, that n–π electron involved interactions should mainly originate from solute–bonded alkyl ligand complexes.

The *b* coefficient (Fig. 2c) of LFER equations

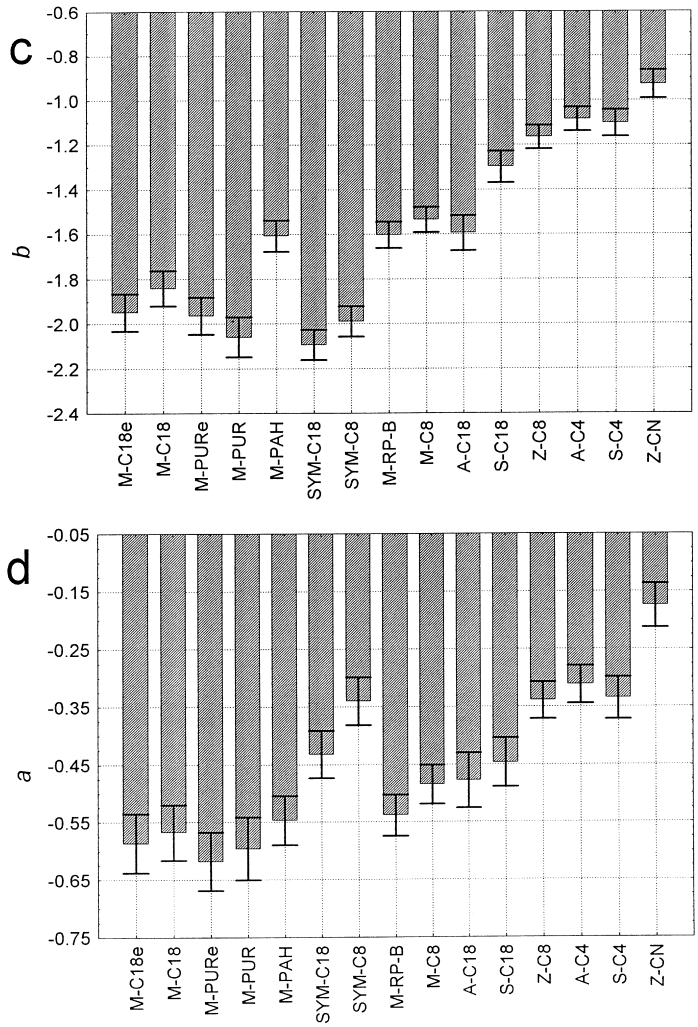

Fig. 2. (*continued*)

measures difference in HBD acidity between stationary and mobile phase (the complimentary property to $\Sigma\beta_2^H$ solute HBA basicity). As it was described earlier, mobile phases rich in water have strong HBD acidity [49]. Reversed-phase packing materials exhibit considerably smaller HBD acidity originating from water molecules sorbed in the interphase region and/or accessible acidic silanol sites. Thus, large negative *b* values can be obtained for the less acidic stationary phases, whereas smaller negative constants reveal increasing column acidity. The WP columns investigated in this study were found significantly stronger HBD acidic media than the narrow-pore materials, due to their much lower carbon content and more accessible silica surface. Relative lack of acidity was determined for SYM-C_{18}, SYM-C_8 and M-PUR columns, which had in fact basic functionality inserted in their ligands. In general, longer alkyl ligands (consider the M-C_{18} and M-C_8 pair) or higher carbon load (for the pairs of M-C_{18}e and M-C_{18}) resulted in decreased HBD acidic property of the stationary phases. It was demonstrated earlier [61,62] that sorption of mobile phase constituents (water and organic modifier) is more extensive into bonded phases of lower carbon content. The relatively larger amount of strong HBD acidic mobile

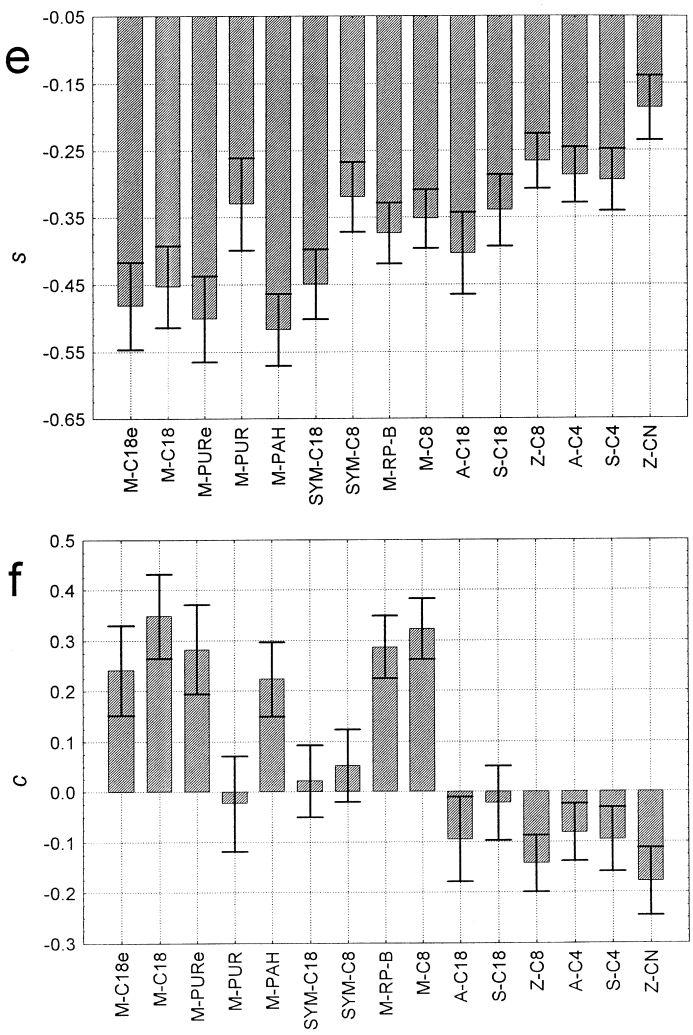

Fig. 2. (*continued*)

phase sorbed was here proved by the general tendency of decreasing *b* coefficients with carbon load. For the polymeric type M-PAH column, in spite of its large carbon content, unusually higher acidity (in the HBD sense) was detected. Here, the different bonding chemistry seems to play a decisive role in affecting bonded phase HB donor acidity.

Column HBA basicity was measured through the value of the *a* coefficient (Fig. 2d). Since the difference in stationary and mobile phase basicity is less pronounced [49], the constants obtained are remarkably smaller than that of for HBD acidity. Columns of lowest HBA basicity were M-PURe and M-C_{18}e. SymmetryShield columns exhibited stronger HBA activity than any of the other NP packings, as a consequence of the pronounced basic character of the carbamate functionality built into the ligands. Similarly to the above case (*b* coefficient), column HBA basicity usually increased with decreasing ligand length and density, though some subtle differences can be perceived. Thus, the two WP-C_{18} materials A-C_{18} and S-C_{18}, that were very similar with respect to HBA basic properties (see above), but concerning the *a* coefficient, S-C_{18} had significantly stronger HBD acidity. To explain this, we may assume that the silica bound HBA basic surface groups have

similar character on these packings, but due to the differences in the base silica and the bonding density, there are far more acidic sites present on S-C_{18} column than on A-C_{18}.

Difference between stationary and mobile phase dipolarity/polarizability is measured through the *s* constant (Fig. 2e) in Eq. (5). Five columns of highest carbon load (M-C_{18}e, M-C_{18}, M-PURe, M-PAH, SYM-C_{18}) displayed the largest negative *s* values that means diminished ability to interact with solute's polarizable functional groups. This finding supports the assumption, that dipolar type interactions are essentially involved when solute have better access to uncovered silica surface. The most dipolar/polarizable column was Z-CN, which is interpreted by the chemical nature of the nitrile head group of the ligand and its most accessible surface. Surprisingly, neither of the other columns have shown essential differences in the *s* coefficients determined. For instance, there were no noticeable differences in *s* between NP-C_8 and WP packings, which has not been found for any of the other coefficients considered.

The *c* intercept (Fig. 2f) in LFER equations represents a part of the retention factors that could not be accounted for by the solvation parameters. Theoretically, it should reflect variations in phase ratio, but due to the difficulties to exactly measure this column and mobile phase dependent property, alteration of *c* coefficients on different packings cannot be adequately evaluated. Yet it is worth noting that NP columns of greater surface area had positive *c* constants, significantly different from zero, unless the bonded phase contained special functionality (M-PUR, SYM-C_{18}, SYM-C_8). For all the WP set the *c* coefficient was found to be negative.

It is apparent when examining numerical values of regression constants, that for all columns investigated cavity formation and stationary phase acidity were the two properties of utmost importance, strongly influencing retention of test solutes. Remaining interactions as n- and π-electron effects, dipolarity/polarizability and the effect of stationary phase basicity were inferior in terms of LFER coefficients, resulting in smaller contributions to the retention.

As was pointed out by Abraham et al. [50], the numerical values of the above discussed regression coefficients depend also on the amount of the stationary phase packed into the column. They proposed to calculate the b/v, a/v, s/v and r/v values, which would reflect the strength of secondary interactions relative to that of cavity formation. The coefficient ratios are given in Table 6. Very small column to column deviation of ratios were found for columns with similar ligands and base silica, for instance for the M-C_{18}e, M-C_{18}, M-PURe triplet, for the M-C_8 and M-RP-B octyl phases or for S-C_4 and A-C_4 WP materials, indicating similar retention mechanism for these columns. However, our results clearly indicate that significant differences between columns' coefficient ratios can be found for different types of columns which would be worth for further investigation in order to get more insight into the retention mechanism involved.

The overall applicability of the LFER model to describe retention in RP-HPLC is illustrated in Fig. 3, where log *k* values calculated by the model was plotted against log *k* measured on nine NP columns. The high correlation coefficients and the low residuals of LFER equations was graphically confirmed by the uniform distribution of data points around the unity-slope line. It is also seen that there was no systematic error in the calculation. Deviation between measured and computed retention factors can be further minimized by improving the accuracy of the LFER solvation parameters. Besides, increasing the number of test solutes when devising regres-

Table 6
Phase system coefficients ratios

Column	b/v	a/v	s/v	r/v
M-C_{18}e	−0.997	−0.300	−0.246	0.155
M-C_{18}	−1.002	−0.309	−0.246	0.141
M-PURe	−1.008	−0.317	−0.257	0.159
M-PUR	−1.092	−0.316	−0.175	0.143
M-PAH	−0.915	−0.311	−0.294	0.159
SYM-C_{18}	−1.043	−0.215	−0.224	0.193
SYM-C_8	−1.054	−0.180	−0.169	0.175
M-RP-B	−0.985	−0.331	−0.230	0.144
M-C_8	−0.974	−0.308	−0.223	0.134
A-C_{18}	−0.983	−0.295	−0.249	0.141
S-C_{18}	−0.941	−0.323	−0.246	0.134
Z-C_8	−0.960	−0.279	−0.219	0.125
A-C_4	−0.947	−0.272	−0.250	0.130
S-C_4	−0.972	−0.294	−0.255	0.128
Z-CN	−1.119	−0.211	−0.225	0.181

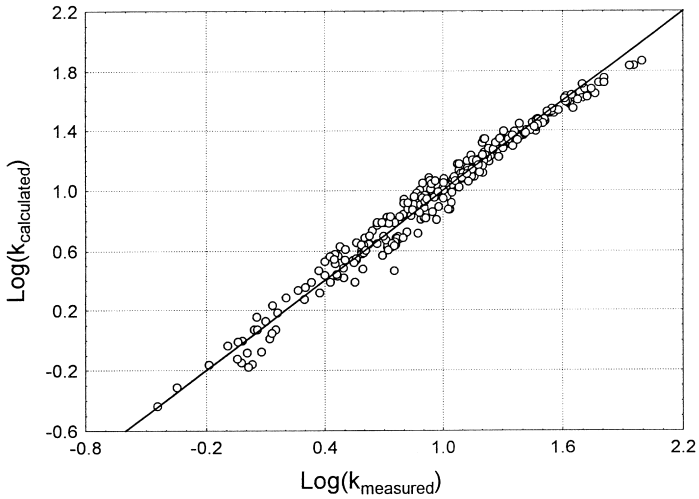

Fig. 3. Comparison of calculated and measured retention data on nine NP columns.

sion lines will result in more exact regression coefficients that would allow finer differentiation between column properties and more accurate retention prediction, as well.

5. Conclusions

5.1. PCA approach

Although the abstract factors called PCs determined by PCA using retention factors for 34 test compounds on 15 different RP-HPLC columns do not have direct physical meaning but the absolute and relative values of loadings (Table 4) can furnish valuable information on the type and extent of the various molecular interactions.

A graphical presentation of the columns in the subspace of PCs (Fig. 1a,b) furnished a grouping of the columns according to their hydrophobic character (PC1), HBA basicity (PC2) and HBD acidity (PC3). The projection of stationary phases on PC axes reflected reasonably well differences of the columns investigated, such as between wide-pore and narrow-pore supports, between C_{18}, C_8 and shorter alkyl chain columns, or differences between columns of special surface chemistry.

Nevertheless, because each solute contributes to smaller or larger extent to each PC extracted, the estimation of the strength of individual molecular forces that should be attributed to a certain PC is rather arbitrary. The classification of columns obtained by PCA does not reflect separately all of the molecular interactions involved.

5.2. LFER approach

The regression coefficients determined by multivariate regression characterize the difference of the individual interactions between the stationary and mobile phase. Since the same mobile phase has been used with different columns, the regression coefficients obtained can be applied to characterize the various stationary phases. The LFER solvation equations provided statistically sound description of retention process on widely different stationary phases investigated, independently of the pore size and type of the ligand. It has been established that the most important molecular properties influencing retention were solute size (V_x) and the hydrogen-bond acceptor (HBA) ability ($\Sigma\beta_2^H$) as indicated by the *v* and *b* coefficients, respectively.

Depending on the type and surface characteristics of the stationary phase, the other types of molecular interactions represented by the regression coefficients *r* (interaction with soulte π- and n-electrons), *a* (HBD acidity), *s* (dipolarity/polraizability) will also

influence the retention process, although to a much smaller extent than in the case of V_x and $\Sigma\beta_2^H$.

Positive v and r coefficients (Fig. 2a,b) indicated that an increase in molecular size or excess molar refractivity contribute to the retention increase in the k retention factor. The negative b, a and s coefficients indicated that HBA, HBD and dipolar properties resulted in a decrease of retention, the HBA property showing the highest effect in this respect.

When comparing WP and NP columns v and r coefficients were significantly higher for the NP column set due to their higher surface area and carbon content. In general, larger alkyl chains and higher surface coverage were reflected in an increase of the above coefficients. Largest negative b values were obtained for the least acidic stationary phases (M-PUR, SYM-C_{18}, SYM-C_8) whereas smaller negative coefficients reveal increasing column acidity (M-C_8, S-C_{18}, Z-C_8, A-C_4, S-C_4, Z-CN).

Since the difference in stationary and mobile phase basicity is less pronounced, the a coefficients obtained are remarkably smaller than b coefficients. Lowest HBA basicity was found for the NP-C_{18} and C_8 columns, with the exeption of SymmetryShield packing materials. The latter is due to the prominent basic character of the carbamate functionality built into the ligands of SYM columns. The s coefficients are in the same range as the a coefficients indicating similar strength when contributing to retention on individual columns. In addition to the diversity in surface area (NP or WP) and in the type of ligands (C_{18}-CN), differences in bonding chemistry such as endcapping (M-C_{18}e, M-PURe), polymeric structure (M-PAH), and specific shielding procedures (SYM-C_{18}, SYM-C_8) exerted also noticeable effects on the molecular interactions and the characteristics of the columns.

Comparing the results of the PCA and LFER evaluation of the column set investigated, it can be established that the principal characteristics of the columns can be ascertained similarly. However, the LFER approach furnishes a more detailed and reliable description on the role and extent of the different molecular interactions. By improving the accuracy of the solvation parameters used in LFER studies, and including an even larger set of test compounds of different types, will further increase the overall accuracy of the model and give more insight into the retention mechanism involved.

6. Symbols

a, b, c, r, s, v	Regression coefficients of Eq. (5)
F	Fischer's F-test on the significance of the regression
HB	Hydrogen bond
HBA	Hydrogen-bond acceptor
HBD	Hydrogen-bond donor
k	Retention factor
LFER	Linear free energy relationship
n	Number of variables used in regression
NP	Narrow-pore
PC	Principal component; abstract variable that is obtained by linear combination of original variables
PCA	Principal component analysis
R	Correlation coefficient (Pearson R)
S.D.	Standard deviation
V_0	Column void volume
WP	Wide-pore
α	Selectivity factor
$\Sigma\alpha_2^H$	LFER solvation parameter for hydrogen-bond donor acidity
$\Sigma\beta_2^H$	LFER solvation parameter for hydrogen-bond acceptor basicity
π_2^*	LFER solvation parameter for dipolarity/polarizability
R_2	LFER solvation parameter for excess molar refractivity
V_x	LFER solvation parameter for McGowan molecular volume

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