Investigations on the chromatographic behaviour of zwitterionic stationary phases used in hydrophilic interaction chromatography

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ABSTRACT

Two commercial stationary phases possessing a sulfobetaine zwitterionic bonded ligand (ZIC-HILIC and Nucleodur HILIC) were compared under hydrophilic interaction chromatographic (HILIC) conditions. First of all, the separation of 12 model compounds chosen among neurotransmitters and presenting a diversity of ionization states (anionic, cationic and zwitterionic) was studied under varied operating conditions. The effects of the percentage of acetonitrile, ammonium acetate concentration and temperature of the mobile phase were compared on the two columns. Secondly, a generally applicable retention model was established, based on chromatographic retention data ($\log k$) acquired for 76 model compounds. The chosen compounds are small molecules presenting a wide diversity of molecular structures and are relevant to biomedical and pharmaceutical studies. To account for their retention behaviour, a modified version of the solvation parameter model was designed: two additional molecular descriptors were introduced, to account for ionic interactions with anionic and cationic species. The retention equations obtained allow a rationalization of the interactions contributing to retention and separation in the HILIC systems considered.

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1. Introduction

The mechanism of retention in hydrophilic interaction chromatography (HILIC) appears to be complex. The primary retention mechanism in HILIC is supposed to be partition of the analyte between the organic-rich mobile phase and the immobilized aqueous layer at the surface of the stationary phase, as per the speculation by Alpert [1]. Any polar stationary phase which can retain water could be used in the HILIC mode. Interaction between the solute and the functional groups of the stationary phase (dipole–dipole, hydrogen bonding and electrostatic interactions) also occurs, as evidenced by the different separations observed when the stationary phase is varied [2–5]. Both partition and adsorption mechanisms are thus believed to contribute to the overall retention of analytes in HILIC conditions.

Among the existing HILIC stationary phases, phases with zwitterionic ligands bonded on silica (ZIC-HILIC) or polymer substrates (pZIC-HILIC) were first introduced by SeQuant/Merck. Macherey-Nagel has recently introduced an identical sulfobetaine-bonded silica phase (Nucleodur HILIC). Nesterenko et al. [6] recently

reviewed zwitterionic stationary phases used in ion separations (ion chromatography and HILIC). True zwitterionic stationary phases contain equal amounts of groups bearing opposite permanent charges in their ligands. Indeed, these functional groups are not sensitive to pH, as is the case with the quaternary ammonium and sulfonate groups present in the sulfobetaine stationary phases. In the latter stationary phase, the positive charge is closest to the silica surface, while the sulfonate groups are the terminal end of the bonded ligand and are thus more accessible for interaction with the analytes. As a result, cations are generally more retained than anions on this stationary phase. However, oppositely charged functional groups may also self-associate, yielding a weak ion-exchanger. Sulfobetaine phases have proven to be useful for the HILIC separation of a variety of polar compounds [4,7–17].

A major objective of this study was to investigate the retention characteristics of sulfobetaine stationary phases and hopefully gain some insights into chromatographic retention mechanisms in the HILIC mode. The zwitterionic columns were chosen for their popularity, as a starting point to develop a stationary phase characterization method.

First of all, the retention of 12 model analytes was systematically investigated on the above two commercial sulfobetaine phases, by varying the chromatographic conditions. Plots of k as a function

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of chosen operating parameters were expected to provide some starting information on retention mechanisms.

In addition, a better understanding of the mechanisms that govern the retention of solutes in a particular chromatographic system can be achieved through the use of quantitative structure–retention relationships (QSRRs). A QSRR is a mathematical model relating the retention of a given analyte to physicochemical and structural parameters. Thus QSRRs can help us to gain some insights into the separation mechanisms that occur at the molecular level.

HILIC has only rarely been the topic of QSRR studies. The development of retention prediction models on unmodified silica, diol-, polyvinyl alcohol- and polyamine-bonded stationary phases under the HILIC mode has been described [18–22]. Models were derived using multiple linear regression analyses and artificial neural networks. Jandera et al. [23] used linear solvation energy relationships (LSERs) to describe retention of phenolic acids and flavone compounds on a variety of columns, including the ZIC-HILIC stationary phase. They compared the models obtained for each column used either in the RPLC mode, or in the HILIC mode. Michel et al. used QSRRs to compare different stationary phases, comprising the ZIC-HILIC phase, for the separation of peptides [24]. However, as the latter study was carried out in the RPLC mode, it is not very informative regarding the HILIC retention mechanism. In another study, Michel [25] performed QSRR studies to describe the retention of pesticides, this time using the ZIC-HILIC phase in the HILIC mode and comparing it to other polar stationary phases used in RPLC mode. But in this work, eight-descriptor models were used to describe the retention of only five solutes, which does not seem a reasonable basis for drawing generally applicable conclusions.

The second purpose of this work was thus to develop a reasonable and chemically sound QSRR for the description of retention in HILIC and to ascertain the possibility of using this model for the conjoint modelling of the retention of neutral compounds and ionized compounds with different charge state (anions, cations and zwitterions).

2. Experimental

2.1. Chemicals and reagents

HPLC-grade acetonitrile (ACN) and methanol (MeOH) were purchased from J.T. Baker (Noisy-le-Sec, France) and perchloric acid from VWR Prolabo (Darmstadt, Germany). Ammonium acetate and acetic acid were purchased from Fluka (St.-Quentin-Fallavier, France).

The 76 solutes used in this study are presented in Table 1, along with their structures and molecular descriptors. The chemicals were obtained from several different manufacturers.

Deionized (18 MΩ) water, purified using an Elgastat UHQ II system (Elga, Antony, France) was used for preparation of analyte and mobile phase solution.

2.2. Standards

Stock standard solutions of each analyte prepared at a concentration of $1000 \, \mu g \, mL^{-1}$ were obtained by dissolving a weighed amount of each compound with the appropriate solvent. For compounds 8, 9, 27–32, 37, 39, 50–52 and 57–59 MeOH was used; for catecholamine, indolamine, and metabolite (compounds 65–76) 0.2 M perchloric acid was used; for all the other compounds in Table 1 the standard solutions were prepared in deionized water.

The use of perchloric acid, for catecholamine dissolution, is dictated by the fact that the neurotransmitter analysis was inscribed in a larger study aiming at analyzing these molecules in brain extracts, which are prepared in perchloric acid. We wished to maintain

identical conditions throughout the whole method development process. All catecholamine stock solutions were stored at $-80 \, ^\circ C$.

The solutions used were obtained by diluting the corresponding stock standard solutions in buffer/organic modifier mixture in order to have an injection solvent as close as possible to the mobile phase and a final analyte concentration of $10 \, \mu g \, mL^{-1}$ for the catecholamines and $50 \, \mu g \, mL^{-1}$ for all the other compounds. In the HILIC mode the injection solvent composition is very important. Retention time shift can be caused by the slightest difference in the organic phase/aqueous phase ratio between the injection solvent and the mobile phase, particularly for solutes experiencing little retention. Moreover, it is imperative to have similar salt concentration and nature in the injection solvent and the mobile phase in order to obtain good peak symmetry [26].

The mobile phase was a mixture of acetonitrile and ammonium acetate buffer $_w^w pH$ 4. PhoEBus, an application program aid for buffer studies (Analis, Namur, Belgium) was used for the preparation of aqueous salt solutions. It was prepared by specifying the salt concentration and $_w^w pH$. The $_w^w pH$ value of the ammonium acetate solution was adjusted with 1 M acetic acid aqueous solution. The buffer $_w^w pH$ was then adjusted before the addition of organic solvent, then the buffer–acetonitrile $_w^S pH$ was measured, with the pH-meter calibrated in aqueous buffers. When mixing the $_w^w pH$ 4 buffer to 80% acetonitrile, the resulting $_w^S pH$ was 6.2.

2.3. Instrumentation

The chromatographic systems consisted of a Merck-Hitachi quaternary pump model Lachrom L-7100 (Darmstadt, Germany), a Rheodyne (Cotati, CA, USA) model 7725 injection valve fitted with a 20 μL loop, column oven Jet Stream 2 Plus, a 785A UV–visible HPLC Detector (Applied Biosystems, Courtaboeuf, France) and an evaporative light scattering detector ELSD model Sedex 55 (SEDERE, Alfortville, France). The UV detection was carried out at 280 nm for catecholamine analysis and 254 nm for the rest of the analysed compounds. The usual ELSD settings were as follows: photomultiplier, 7; evaporative temperature, $50 \, ^\circ C$; air pressure, 2.3 bar. The chromatographic data handling was accomplished using EZChrom Server software (Merck, Darmstadt, Germany).

The two columns studied were: ZIC-HILIC (SeQuant/Merck) 150×4.6 mm, 5 μm and Nucleodur HILIC (Macherey-Nagel) 125×3 mm, 3 μm. The mobile phase flow rate was $0.5 \, mL \, min^{-1}$ for the ZIC-HILIC column (in accordance with recommendations from the manufacturer) and $0.3 \, mL \, min^{-1}$ for the Nucleodur HILIC column.

2.4. Methods

The neurotransmitters (solutes 65–76 in Table 1) were injected one-at-a-time, and no experimental design was considered. The effect of acetonitrile percentage was studied with a $_w^w pH$ 4 40 mM ammonium acetate buffer; temperature was set at $20 \, ^\circ C$. Temperature effect was investigated with a mobile phase comprised of ACN and 25 mM aqueous solution of ammonium acetate $_w^w pH$ 4 80:20 (v/v). Salt concentration effect was investigated with a mobile phase comprised of ACN and aqueous solution of ammonium acetate $_w^w pH$ 4 80:20 (v/v) at $20 \, ^\circ C$. Retention factors (k) were recorded under all conditions tested.

For the linear solvation energy relationship (LSER) characterization, all solutes in Table 1 were analyzed under the following mobile phase conditions: ACN/100 mM ammonium acetate aqueous solution $_w^w pH$ 4, 80:20 (v/v) at $20 \, ^\circ C$. As our aim was to investigate the differences in stationary phase properties, it was important to choose some operating conditions that would be suitable to both columns under investigation. The operating conditions also needed to be consistent with common practice of HILIC today,

Table 1
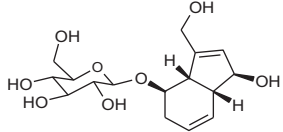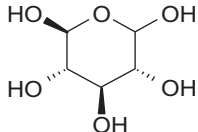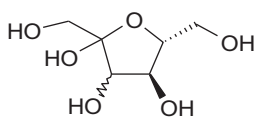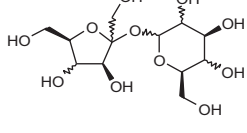Chromatographic solutes, their structures and molecular descriptors.
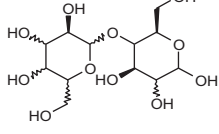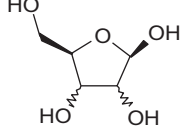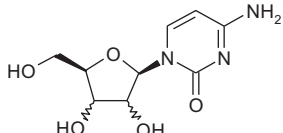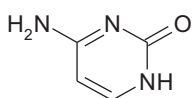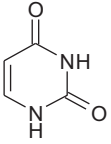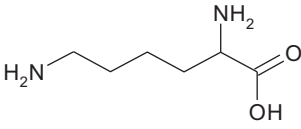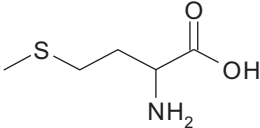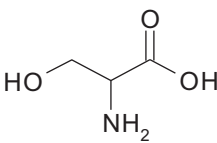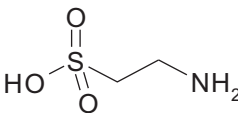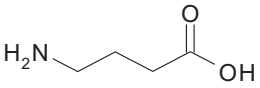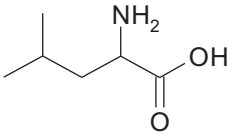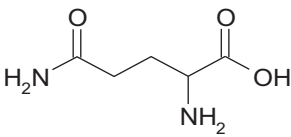
	Compound	Structure	E	S	A	B	V	D^-	D^+	log $D_{o/w}$ (pH 6.2)	Acid pK	Basic pK	Charge state at pH 6.2
1	Aucubin		2.33	2.34	1.62	2.62	2.4208	0.00	0.00	−1.94			0
2	Glucose		1.31	1.68	1.33	1.77	1.0567	0.00	0.00	−2.42			0
3	Fructose		1.30	1.61	1.31	1.83	1.1976	0.00	0.00	−2.51			0
4	Saccharose		1.97	2.50	2.10	3.00	2.2279	0.00	0.00	−3.81			0
5	Lactose		2.12	2.44	1.76	3.10	2.3101	0.00	0.00	−2.92			0
6	Ribose		1.13	1.39	1.04	1.54	0.9980	0.00	0.00	−2.33			0
7	Cytidine		2.09	2.21	0.87	2.62	1.6234	0.00	0.02	−2.52		4.4	0
8	Cytosine		1.43	1.90	0.60	1.02	0.7927	0.00	0.02	−1.02		4.4	0

Table 1 (*Continued*)

	Compound	Structure	E	S	A	B	V	D⁻	D⁺	$\log D_{o/w}$ (pH 6.2)	Acid pK	Basic pK	Charge state at pH 6.2
9	Uracil		0.81	1.00	0.44	1.00	0.7516	0.00	0.00	−4.54	9.7		0
10	Lysine (Lys)		0.58	1.26	0.99	1.48	1.2280	1.00	1.00	−3.24	2.3	10.6	Zwi
11	Methionine (Met)		0.72	1.08	0.78	1.06	1.1508	1.00	1.00	−2.48	2.3	9.1	Zwi
12	Serine (Ser)		0.60	1.15	1.03	1.30	0.7642	1.00	1.00	−3.64	2.1	9.1	Zwi
13	Taurine		0.49	1.64	0.52	1.34	0.8298	1.00	1.00	−3.25	1.7	8.8	Zwi
14	γ-Aminobutyric acid (GABA)		0.37	0.94	0.78	0.91	0.8464	1.00	1.00	−2.88	3.8	10.6	Zwi
15	Leucine (Leu)		0.39	0.92	0.78	0.97	1.1282	1.00	1.00	−1.78	2.3	9.9	Zwi
16	Glutamine (Gln)		0.79	1.84	1.27	1.48	1.1028	1.00	1.00	−4.03	2.3	9.1	Zwi
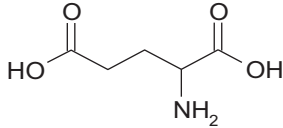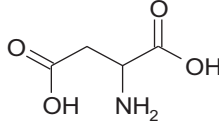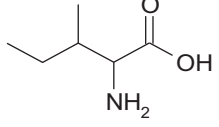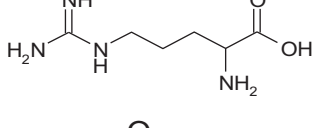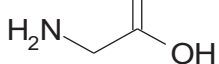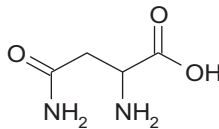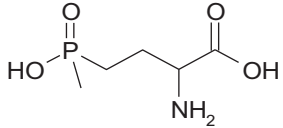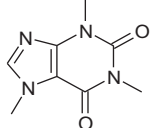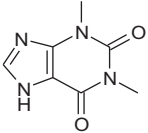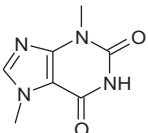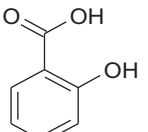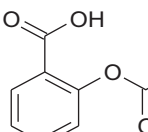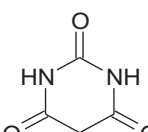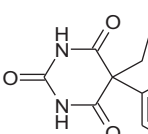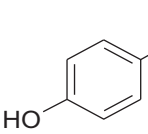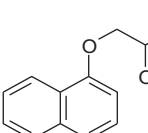
#	Name	Structure											
17	Glutamic acid (Glu)		0.55	1.37	1.35	1.26	1.0617	2.00	1.00	−4.75	2.3/3.8	9.9	Zwi
18	Aspartic acid (Asp)		0.55	1.37	1.18	1.26	0.9208	2.00	1.00	−5.25	2.3/2.5	9.9	Zwi
19	Isoleucine (Ile)		0.39	0.92	0.78	0.97	1.1282	1.00	1.00	−1.78	2.3	9.9	Zwi
20	Arginine (Arg)		1.06	1.24	1.26	1.95	1.3846	1.00	1.00	−3.84	1.9	12.1	Zwi
21	Glycine (Gly)		0.37	0.93	0.78	0.90	0.5646	1.00	1.00	−3.26	2.3	9.9	Zwi
22	Asparagine (Asn)		0.80	1.84	1.27	1.48	0.9619	1.00	1.00	−4.05	2.3	9.9	Zwi
23	Glufosinate		0.73	1.25	1.09	1.76	1.3093	2.00	1.00	−4.23	2.3/3.5	9.9	Zwi
24	Caffeine		0.50	1.72	0.05	1.28	1.3632	0.00	0.00	−0.45			0

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Table 1 (*Continued*)

	Compound	Structure	E	S	A	B	V	D⁻	D⁺	log $D_{o/w}$ (pH 6.2)	Acid pK	Basic pK	Charge state at pH 6.2
25	Theophylline		1.50	1.60	0.54	1.34	1.2223	0.00	0.00	0.12	8.7		0
26	Theobromine		1.50	1.60	0.50	1.38	1.2223	0.00	0.00	−0.80	9.9		0
27	Salicylic acid		0.89	0.84	0.71	0.38	0.9904	1.00	0.00	−1.16	3.0		−
28	Acetylsalicylic acid		0.78	0.80	0.49	1.00	1.2879	1.00	0.00	−1.48	3.5		−
29	Barbituric acid		1.09	1.19	0.46	1.16	0.8103	0.99	0.00	−8.25	4.0		−
30	Phenobarbital		1.63	1.80	0.73	1.15	1.6999	0.05	0.00	1.40	7.5		0
31	Paracetamol		1.06	1.63	1.04	0.86	1.1724	0.00	0.00	0.23	10.2		0
32	Propranolol		1.88	1.43	0.17	1.42	2.1480	0.00	1.00	−0.36		9.6	+
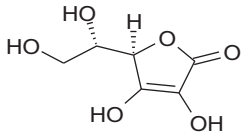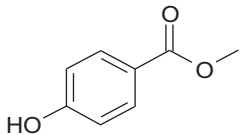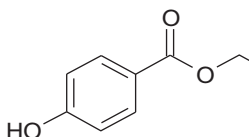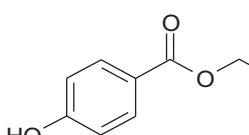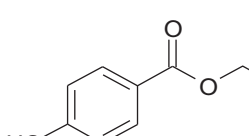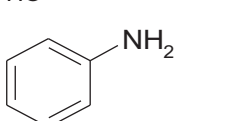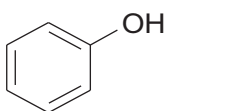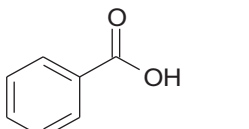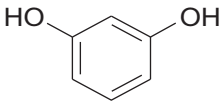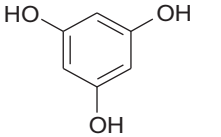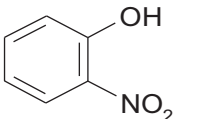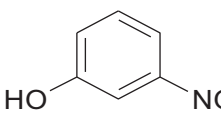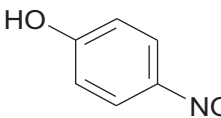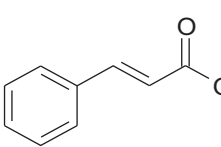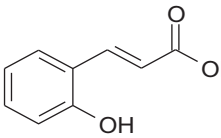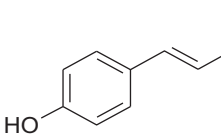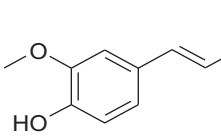
#	Compound	Structure											
33	Ascorbic acid		1.23	1.68	1.12	1.65	1.1116	0.99	0.00	−4.60	4.2		−
34	Methylparaben		0.90	1.37	0.69	0.45	1.1313	0.00	0.00	1.60	8.5		0
35	Ethylparaben		0.86	1.35	0.69	0.45	1.2722	0.00	0.00	2.08	8.5		0
36	Propylparaben		0.86	1.35	0.69	0.45	1.4131	0.00	0.00	2.57	8.5		0
37	Butylparaben		0.86	1.33	0.71	0.46	1.5540	0.00	0.00	3.05	8.5		0
38	Aniline		0.96	0.96	0.26	0.41	0.8162	0.00	0.02	1.14		4.6	0
39	Phenol		0.81	0.89	0.60	0.30	0.7751	0.00	0.00	1.59	10.3		0
40	Benzoic acid		0.73	0.90	0.59	0.40	0.9317	0.99	0.00	−0.06	4.1		−

Table 1 (*Continued*)

	Compound	Structure	E	S	A	B	V	D⁻	D⁺	log $D_{o/w}$ (pH 6.2)	Acid pK	Basic pK	Charge state at pH 6.2
41	Resorcinol		0.98	1.11	1.09	0.52	0.8338	0.00	0.00	0.84	9.6		0
42	Phloroglucinol		1.36	1.12	1.40	0.82	0.8925	0.00	0.00	−0.04	9.0		0
43	2-Nitrophenol		1.02	1.05	0.05	0.37	0.9493	0.05	0.00	1.99	7.5		0
44	3-Nitrophenol		1.05	1.57	0.79	0.23	0.9493	0.00	0.00	1.85	8.6		0
45	4-Nitrophenol		1.07	1.72	0.82	0.26	0.9493	0.09	0.00	1.65	7.2		0
46	Cinnamic acid		1.14	1.00	0.58	0.57	1.1705	0.99	0.00	0.16	4.2		−
47	o-Coumaric acid		1.13	1.39	1.07	0.79	1.2292	0.99	0.00	−0.46	4.2		−
48	p-Coumaric acid		1.13	1.39	1.07	0.79	1.2292	0.99	0.00	−0.46	4.2		−
49	Ferulic acid		1.11	1.46	0.85	0.87	1.4288	0.99	0.00	−0.61	4.2		−
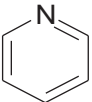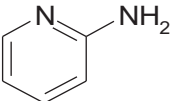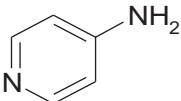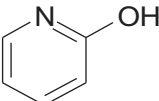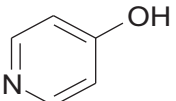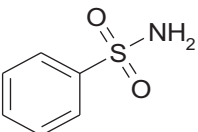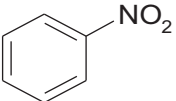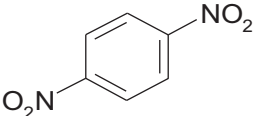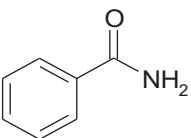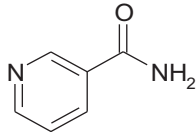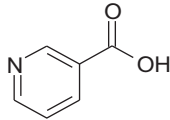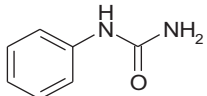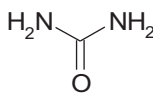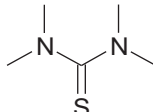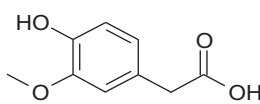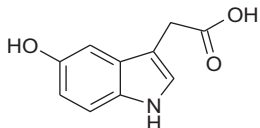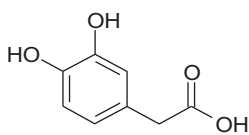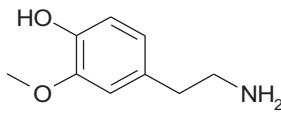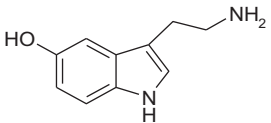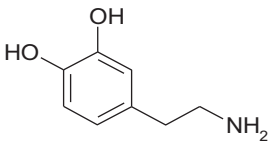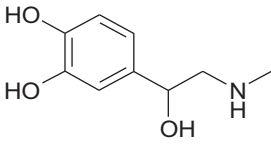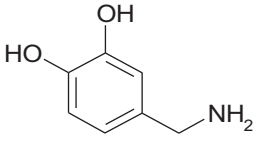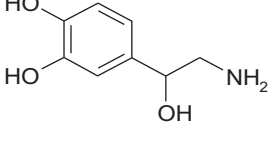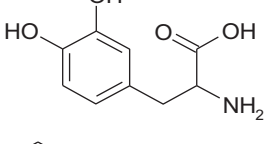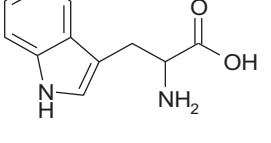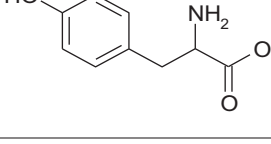
No.	Compound											
50	Pyridine	0.63	0.84	0.00	0.52	0.6753	0.00	0.07	0.83		5.1	0
51	2-Aminopyridine	0.98	1.10	0.32	0.63	0.7751	0.00	0.83	−0.38		6.9	+
52	4-Aminopyridine	0.90	1.21	0.23	0.71	0.7751	0.00	1.00	−2.96		9.3	+
53	2-Hydroxypyridine	0.83	1.03	0.50	0.67	0.7340	0.00	0.00	0.40		3.5	+
54	4-Hydroxypyridine	0.83	1.03	0.50	0.67	0.7340	0.00	0.00	0.08		3.5	+
55	Benzenesulfonamide	1.13	1.55	0.55	0.80	1.0971	0.00	0.00	0.47	9.7		0
56	1,4-Benzoquinone	0.75	0.55	0.00	0.81	0.7908	0.00	0.00	0.43			0
57	Nitrobenzene	0.87	1.11	0.00	0.28	0.8906	0.00	0.00	2.00			0
58	1,4-Dinitrobenzene	1.13	1.63	0.00	0.46	1.0648	0.00	0.00	1.73			0
59	Benzamide	0.99	1.50	0.49	0.67	0.9728	0.00	0.00	0.59			0

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Table 1 (*Continued*)

	Compound	Structure	E	S	A	B	V	D^-	D^+	log $D_{o/w}$ (pH 6.2)	Acid pK	Basic pK	Charge state at pH 6.2
60	Nicotinamide		1.01	1.09	0.63	1.00	0.9317	0.00	0.00	−0.27		3.4	+
61	Nicotinic acid		0.79	1.21	0.57	0.73	0.8906	1.00	0.03	−0.67	2.0	4.7	−
62	Phenylurea		1.11	1.33	0.79	0.79	1.0726	0.00	0.00	0.95			0
63	Urea		0.50	1.49	0.83	0.84	0.4648	0.00	0.00	−1.68			
64	1,1,3,3-Tetramethylthiouruea		0.81	0.80	0.00	1.41	1.1332	0.00	0.00	0.79			0
65	Hydroxyvanillic acid (HVA)		0.96	1.35	0.85	0.80	1.3309	0.99	0.00	−1.13	4.3		−
66	5-Hydroxyindole-3-acetic acid (5HIAA)		1.64	1.70	1.38	0.93	1.3613	0.98	0.00	−1.01	4.6		−
67	3,4-Dihydroxyphenylacetic acid (DOPAC)		1.12	1.47	1.35	0.86	1.1900	0.99	0.00	−1.44	4.3		−
68	3-Methoxytyramine (MT)		0.99	1.23	0.49	1.02	1.3563	0.00	1.00	−1.96	10.0	8.8	+

No.	Compound	Structure	E	S	A	B	V	D⁻	D⁺				
69	Serotonine (S)		1.67	1.58	1.02	1.15	1.3867	0.00	1.00	−2.14	10.5	8.8	+
70	Dopamine (DA)		1.35	1.46	1.20	1.04	1.2154	0.00	1.00	−2.28	10.5	8.8	+
71	Adrenaline (A)		1.35	1.34	1.15	1.51	1.4150	0.00	1.00	−2.81	10.0	8.6	+
72	3,4-Dihydroxybenzylamine (DHBA)		1.15	1.34	0.98	1.08	1.0745	0.00	1.00	−3.01	10.0	9.0	+
73	Noradrenaline (NA)		1.40	1.49	1.23	1.61	1.2741	0.00	1.00	−3.29	10.0	8.6	+
74	3,4-Dihydroxyphenylalanine (DOPA)		1.33	1.77	1.56	1.44	1.4307	1.00	1.00	−1.93	2.1	9.1	Zwi
75	Tryptophan (Trp)		1.62	1.80	1.09	1.23	1.5433	1.00	1.00	−1.31	2.1	9.1	Zwi
76	Tyrosine (Tyr)		1.18	1.60	1.28	1.29	1.3720	1.00	1.00	−2.01	2.1	9.1	Zwi

E, excess molar refraction; S, dipolarity/polarizability; A, hydrogen bond acidity; B, hydrogen bond basicity; V, McGowan's characteristic volume; D⁻, Negative charge; D⁺, positive charge.

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thus acetonitrile was selected as the preferred organic solvent, and ammonium acetate was selected for its compatibility with MS detection. Another important point is that the chosen mobile phase should allow measuring appropriate retention factors for all columns: the elution strength must be sufficient, so that the analysis time remains reasonable, but not too short otherwise the precision on the measurement of retention factors is poor.

2.5. Data analysis

Abraham descriptors, pK and $\log D_{o/w}$ values at ^S_wpH 6.2 were determined using Absolv Webboxes program, based on ADME Boxes version 3.5 (Pharma Algorithms, ACD Labs, Toronto, Canada). Whenever an exact match was found in the Absolv database, the experimental values were preferred. When no exact match could be found, the descriptors calculated by Absolv were used. Extra descriptors (negative and positive charge) were computed using pK values as indicated below. $\log D_{o/w}$ is the logarithm of the ratio of the equilibrium concentrations of the neutral species of a molecule in octanol to all species (unionized and ionized) in the water phase at 25 °C. It differs from $\log P_{o/w}$ in that ionized species are considered as well as the neutral form of the molecule and thus is more representative of hydrophobic character in buffered conditions.

Multiple linear regression analyses were performed with XLSTAT 2009.2.03 software (Addinsoft, New York, NY, USA). The logarithm of the retention factor ($\log k$) was used as the dependent variable.

3. Theory

The QSRR approach furnishes a detailed and reliable description of the role and extent of the different molecular interactions that can be established between the analytes and the chromatographic system. Among QSRRs, the solvation parameter model using Abraham descriptors has gained acceptance as a general tool to explore the factors affecting retention in chromatographic systems [27–29]. The retention of selected probes in a dense fluid can be related through this relationship, also known as linear solvation energy relationship (LSER), to specific interactions by the following equation:

$$\log k = c + eE + sS + aA + bB + vV \tag{1}$$

In this equation, capital letters represent the solute descriptors, related to particular interaction properties, while lower case letters represent the system constants, related to the complementary effect of the phases on these interactions. c is the model intercept term, which when the retention factor is used as the dependent variable is dominated by the phase ratio. E is the excess molar refraction (calculated from the refractive index of the molecule) and models polarizability contributions from n and π electrons. S is the solute dipolarity/polarizability. A and B are the solute overall hydrogen-bond acidity and basicity. V is the McGowan characteristic volume in units of cm^3 mol^{-1}/100; it is the actual volume of a mole when the molecules are not in motion. The system constants (e, s, a, b, and v), obtained through a multilinear regression of the retention data for a certain number of solutes with known descriptors, reflect the magnitude of difference for that particular property between the mobile and stationary phases. Thus, if a particular coefficient is numerically large, then any solute having the complementary property will interact very strongly with either the mobile phase (if the coefficient is negative) or the stationary phase (if the coefficient is positive). Eq. (2) can be deduced from Eq. (1):

$$\log \alpha = e\,\Delta E + s\,\Delta S + a\,\Delta A + b\,\Delta B + v\,\Delta V \tag{2}$$

where α is the separation factor between two solutes and ΔX represents the difference in the X coefficient between these two solutes.

Consequently, the coefficients also reflect the system's selectivity towards any particular molecular interaction.

Moreover, characterising different stationary phases while always using the same mobile phase and operating conditions ensures that the LSER coefficients can be compared to provide a comparison of the stationary phase properties.

Furthermore, based on the method suggested by Ishihama and Asakawa [30], the angle between two solvation vectors (ω) associated with two chromatographic systems can be calculated according to the following equation, based on the solvation parameter model coefficients of the two systems noted i and j:

$$\cos \theta_{ij} = \frac{\vec{\omega}_i \times \vec{\omega}_j}{\left|\vec{\omega}_i\right| \times \left|\vec{\omega}_j\right|}$$
$$= \frac{e_ie_j + s_is_j + a_ia_j + b_ib_j + v_iv_j}{\sqrt{e_i^2 + s_i^2 + a_i^2 + b_i^2 + v_i^2}\sqrt{e_j^2 + s_j^2 + a_j^2 + b_j^2 + v_j^2}} \tag{3}$$

The angle between two columns provides a mean to measure the informational equivalence of different chromatographic systems. However, this information is not sufficient to judge whether two stationary phases are similar, as it does not take into account the confidence limits associated with the system constants.

The similarity between two chromatographic systems is thus evaluated through the calculation of the J similarity factor, determined through Eqs. (4)–(6):

$$J = \cos \theta_{ij} - \cos(\theta_{di} + \theta_{dj}) \tag{4}$$

$$\cos(\theta_{di} + \theta_{dj}) = \sqrt{\left(1 - \frac{D_i^2}{\left|\vec{\omega}_i\right|^2}\right)\left(1 - \frac{D_j^2}{\left|\vec{\omega}_j\right|^2}\right)} - \frac{D_iD_j}{\left|\vec{\omega}_i\right|\left|\vec{\omega}_j\right|} \tag{5}$$

$$D = \text{TINV}(1 - 0.99, \ N)\text{SE} \tag{6}$$

where TINV is the inverse of the Student's t-distribution for the specified degrees of freedom N, and SE is the average of the standard errors of the solvation parameter model coefficients.

In Eq. (4), when J is positive, the systems compared are found to be similar; in the opposite case, they are considered to be different.

When two stationary phases are similar, it indicates that the elution order of analytes will be very similar in the two chromatographic systems. However, retention times might be different. The global intensity of the interactions can be compared through the values of the solvation vector length, calculated as follows:

$$u_i = \sqrt{e_i^2 + s_i^2 + a_i^2 + b_i^2 + v_i^2} \tag{7}$$

Thus when u_i and u_j are close, retention will be similar on both phases (provided phase ratio is close), while different values of vector length indicates that retention and separation factors will be larger in the chromatographic system providing larger values of u. Eq. (1) and the five Abraham descriptors were designed to describe neutral molecules. As such, Eq. (1) is not sufficient for a complete description of HILIC processes as most compounds of interest for the HILIC mode are ionic or ionizable species.

To account for extra interactions associated with the presence of charges present on totally or partially ionized species, two elements must be considered:

(i) The E, S, A, B and V descriptors of the neutral form of one species may vary when this species is in its ionized form.
(ii) Additional descriptors could be introduced to account for electrostatic interactions that are not properly taken into account in Eq. (1). For consistency with the rest of the equation the new solute descriptor(s) should be related to free energy.

Zhao et al. [31,32] have considered the first point for partitioning solvent systems. First of all, the molecular volume is different when a compound is in its ionized form. Anions are slightly larger than the neutral atoms or molecules they are derived from. This is essentially due to the repulsive forces resulting from the introduction of additional electrons. Cations, on the contrary, are much smaller than the corresponding atoms or molecules, due to the attraction of extra valence charge on the electrons. As a result, some recalculation of the McGowan volume used in Eq. (1) was required for ions. The authors determined it for a set of compounds [31].

The E descriptor can be obtained from the ionic molar refraction [33].
S, A, and B were determined for a series of anions and cations through an experimental method [34].

The S values thus obtained were generally much larger for anions and cations than for the corresponding neutral species. Indeed, it seems reasonable that the ionized form of an acid or base should have more capabilities for dipole–dipole interactions than the neutral form.

The A values should be small or zero for anions but large for cations, which is in accordance with the definition of the A descriptor. Indeed, although A is generally associated to the Bronsted definition of hydrogen bond acidity (proton donor), it is actually based on the more general definition of acidity according to Lewis (electron acceptor). Besides, it seems logical that the ionized form of an acid (carboxylic acid or phenol, for instance) should contain fewer hydrogen-bond acid donor sites than the neutral form. On the other hand, the protonated form of a base has more hydrogen-bond acid donor sites than the neutral form. Bolliet et al. [35] have suggested that the A descriptor be modified using the proportion of the species in its neutral form at the mobile phase $_w^S$pH However, this can only work for molecules having only one acidic function so it would not be a correct assumption for molecules possessing different functional groups contributing to the A character, such as phenolic acids.

In the same manner, the B values should be small or zero for cations but large for anions (electron donors).

In this respect, the experimental values obtained by Abraham and Zhao [32] were not entirely chemically reasonable and some other means to estimate them is still wanted. As a result, even using the modified E, S, A, B and V descriptors, no satisfying correlation coefficients could be obtained for ionic species, leading the authors to conclude that using only descriptors that are the same in kind as those for neutral compounds was not a satisfactory solution. Thus two additional terms were introduced. The following equation was adopted:

$$\log SP = c + eE + sS + aA + bB + \nu V + j^+ J^+ + j^- J^- \tag{8}$$

J^+ and J^- were not introduced simultaneously in the equation. J^+ was used for univalent cations (it is zero for anions and neutral molecules), and seemed to be related to structural effects of cations, such as hydrophobic hydration. It was reasonably correlated to cation radius. J^- was used for univalent anions (it is zero for cations and neutral molecules) and apparently accounted for specific interactions between anions and hydroxylic solvents.

To determine J^- and J^+ values the authors set $j^- = +3$ in the partition systems where anions should experience extra interactions, and $j^+ = -3$ in the partition systems where cations should experience extra interactions, then back-calculated J^- and J^+ based on the partition coefficient of the considered ionic species. This procedure produced much more reasonable descriptor values. However, the system constants $j^- = +3$ and $j^+ = -3$ were general constants used by Abraham and Zhao, and there is no reason why these should be the same for all partitioning systems. On the contrary, it is preferable to let these system constants vary, as the authors later showed [36].

Other authors have suggested additional descriptors and interaction terms for Eq. (1), without modifying the usual Abraham descriptors. Rosés et al. [35,37] also looked for an additional descriptor, which would be zero for neutral solutes and non-zero for ionic species, to study retention of ionizable species in RPLC. They suggested two different descriptors. The first one they introduced was the P descriptor or "scaled effective acid dissociation constant" [35]. It is equivalent to an effective dissociation constant and was defined for acidic species. For a weak acid, P is defined as follows:

$$P = \frac{14 - pK_*}{10} \tag{9}$$

where pK^* is the dissociation constant of the acid in the hydro-organic mobile phase prepared at pH*. The latter is the effective $_S^S$pH obtained after mixing buffer and organic solvent, and is thus different from the aqueous $_w^w$pH of the buffer, measured before mixing with the organic solvent. For P to be zero for neutral compounds, it was arbitrarily decided that pK^* would be equal to 14 for these solutes. The 10 value at the denominator is also arbitrary, but was retained because it produced a convenient P scale on a comparable range to other Abraham descriptors.

P is then introduced in the LSER equation to yield:

$$\log k = c + eE + sS + aA + bB + \nu V + pP \tag{10}$$

Eq. (10) proved useful to describe the retention of neutral and acidic ionizable compounds on a large pH scale. However, the P descriptor requires multiple definitions for different types of solutes: the above definition does not stand, neither for neutral species nor for fully ionized solutes such as strong acids, bases, or salts of strong acids and bases.

Rosés et al. [37] thus introduced another descriptor, defined as follows:

$$D = \frac{10^{(pH_* - pK_*)}}{1 + 10^{(pH_* - pK_*)}} \tag{11}$$

Then D was introduced in the LSER equation according to Eq. (12):

$$\log k = c + eE + sS + aA + bB + \nu V + dD \tag{12}$$

As the correlations obtained were still not satisfactory, and actually worse than those obtained with Eq. (10), the authors modified this equation in the following manner:

$$\log k = c + eE + sS + aA + bB + \nu V + d \log[1 - D(1 - f)] \tag{13}$$

where f is a parameter that is characteristic of the chromatographic system and equal to the ratio of retention factors for the ionic species and the neutral species. The f parameter is supposed to be constant for one pair of compounds in defined chromatographic conditions.

The authors have shown that Eq. (13) was much more satisfying than Eq. (10), and that the new descriptor could be used in a single equation to describe retention of acids, bases and amphiprotic compounds. It also pointed out that the degree of ionization of the analyte was a key parameter in the retention of ionizable compounds.

Still there are some constraints associated with the D descriptor:

(i) An accurate measurement of pH* and pK^* is required.
(ii) The retention of the neutral and ionic species in the chromatographic system must be known, requiring additional experiments.

(iii) No distinction is made between cationic and anionic species, as solute ionization was expected to cause a retention reduction in RPLC, whatever the solute charge.

Li proceeded differently [38]. The objective was to propose a molecular descriptor that would not require knowledge of the pK^*, the dissociation constant of ionizable compounds in the hydroorganic mobile phase, but would rather rely on aqueous pK. The equation proposed was the following:

$$\log k = c + eE + sS + aA + bB + vV + \frac{U}{1 + V10^{\mp(pH-pK)}} \tag{14}$$

where U and V are regression coefficients, calculated as follows:

$$\log\left(\frac{1 + f10^{\pm(pH-pK)}}{1 + 10^{\pm(pH-pK)}}\right) = \frac{U}{1 + V10^{\mp(pH-pK)}} \tag{15}$$

And where f is defined as previously described.

As the proposed model (Eq. (14)) is not linear, the data treatment is more complex than the usual multiple linear regression used for Eq. (1).

In this paper, we sought to modify the solvation parameter model to enable retention to be described for both neutral and ionic species, in which the latter could be anionic, cationic or zwitterionic. Indeed, it is expected that the presence of a positive or negative charge could have different effects on solute retention in the HILIC mode, especially with stationary phases possessing charged ligands such as the sulfobetaine phases investigated here. We thus suggest yet another equation, derived from Eqs. (11) and (12):

$$\log k = c + eE + sS + aA + bB + vV + d^-D^- + d^+D^+ \tag{16}$$

where D^- represents the negative charge carried by anionic and zwitterionic species, and D^+ represents the positive charge carried by cationic and zwitterionic species, according to the following equations:

$$D^- = \frac{10^{(pH*-pK*)}}{1 + 10^{(pH*-pK*)}} \tag{17}$$

$$D^+ = \frac{10^{(pK*-pH*)}}{1 + 10^{(pK*-pH*)}} \tag{18}$$

For neutral species, D^- and D^+ are zero so that Eq. (16) reverts to Eq. (1).

pH* is the effective pH in the mobile phase, also known as ${}^S_S$pH according to IUPAC notation. However, for purposes of simplicity, the mobile phase ${}^S_w$pH (pH measured in the hydro-organic mixture, with the electrode calibrated in aqueous buffers) and aqueous ionization constants were used in the following.

4. Results and discussion

4.1. Comparison of chromatographic behaviours in varied operating conditions

The columns are both silica-based and have the same bonded ligands, although they differ in column dimensions (150×4.6 mm vs. 125×3 mm), particle size (5 vs. 3 μm), pore size (200 vs. 110 Å) and specific surface area (135 vs. 340 m^2/g), for ZIC-HILIC and Nucleodur HILIC respectively. Bonding density may also be different: based on the information provided by the manufacturer, the Nucleodur HILIC phase has a 7% carbon content. The manufacturer of the ZIC-HILIC phase remains vague on this point, indicating "approximately 10% carbon content". Lämmerhofer et al. [39] indicate a 8.74% value of unknown origin, possibly measured in their laboratory by elemental analysis. Based on these data, the bonding density in ZIC-HILIC would be larger than that of Nucleodur

HILIC. But the exact surface chemistry might be different for the two columns: for instance, the length of spacer arm, which is a carbon chain thus participating in the measured carbon content, is unknown to us. Hemström and Irgum [40] indicate that, in the ZIC-HILIC phase, the sulfoalkylbetaine moieties reside on grafted organic polymer chains, thus providing a thick interactive layer. The exact surface chemistry and the concentration of residual – and accessible – silanol groups of both stationary phases is however unknown to us. The ZIC-HILIC column has been reported in varied HILIC applications [10,13–16,41–47], while the Nucleodur HILIC column, having appeared more recently on the marketplace, has not yet been reported in any publication (to the best of our knowledge).

A few experimental parameters were selected to study in more detail their influence on retention and selectivity of 12 selected compounds (compounds 65–76 in Table 1). The same test compounds were previously studied on eleven other stationary phases, in a variety of chromatographic conditions [3].

4.1.1. ACN percentage

The effect of the ACN fraction in the mobile phase was studied in the range 60–85%, with a 40 mM ammonium acetate buffer ${}^w_w$pH 4. Thus total salt concentration in the mobile phase varies between 6 and 16 mM, and the mobile phase ${}^S_w$pH varied between 5.4 and 6.6. Fig. 1 exemplifies the results obtained on an acid (DOPAC), a base (DA) and an amino-acid (DOPA). In all the following figures, the same acid, base and amino-acid were selected as an example, as their congeners all provided parallel curves. All 12 test compounds experienced a marked increase in retention when acetonitrile percentage is increased. This is naturally in line with a HILIC-type retention mechanism. However, it appears that the slope of the retention increase is not identical for all compounds, leading to reversals in the elution order of some solutes when the mobile phase composition is changed.

It also appears that the two columns exhibit very similar retention behaviour, and that peak shapes are marginally improved when the proportion of water in the mobile phase is increased.

According to the fundamental relationships between retention and elution strength established for partitioning and adsorption chromatography, a gross picture of the prevailing HILIC retention mechanism can be obtained by plotting different graphs:

(i) a plot of $\log k$ vs. volume fraction of ACN in the eluent. A linear plot indicates a predominantly partitioning process. Indeed, the retention in partition-like mechanisms like RP chromatography can be described by the empirical equation:

$$\log k = \log k_w - S\varphi \tag{19}$$

where φ is the volume fraction of solvent in the mobile phase and k_w is the hypothetical retention factor when the mobile phase is purely aqueous. Alternately, the second order empirical equation proposed by Schoenmakers et al. [48] can also be used to describe partitioning mechanisms:

$$\log k = A\varphi^2 + B\varphi + C \tag{20}$$

where φ is the volume proportion of water in the mobile phase.

(ii) a plot of $\log k$ vs. logarithm of the mole fraction of ACN in the eluent. A linear plot indicates a predominantly adsorption process, according to the Snyder-Soczewinski expression, when water is the stronger member in the eluent:

$$\log k = \log k_w - \frac{A_S}{n_w}\log N_w \tag{21}$$

where A_S and n_w are the cross-sectional areas occupied by the solute molecule on the surface and the water molecules, and N_w is the mole fraction of water in the eluent.
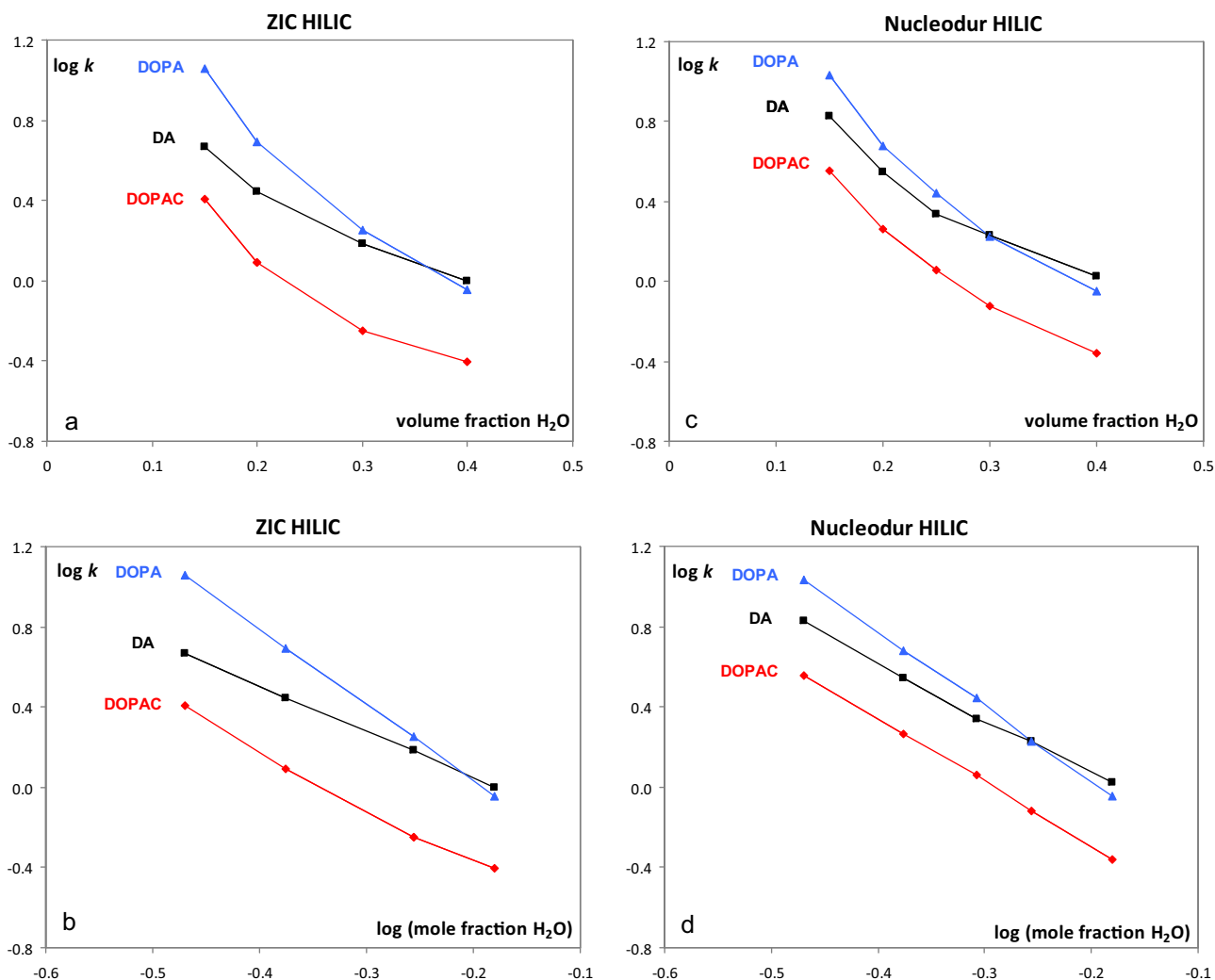
Fig. 1. Variation of $\log k$ with the proportion of water in the mobile phase for three standards. Buffer: ammonium acetate 25 mM, ^w_wpH 4; 20 °C. (a) ZIC-HILIC retention depending on volume fraction of water, (b) ZIC-HILIC retention depending on the logarithm of the mole fraction of water, (c) Nucleodur HILIC retention depending on volume fraction of water and (d) Nucleodur HILIC retention depending on the logarithm of the mole fraction of water.

Fig. 1a and c shows the retention of the selected three compounds ($\log k$) plotted against the volume fraction of water, while Fig. 1b and d shows the retention ($\log k$) plotted against the logarithm of the mole fraction of water. Trying to fit the log–log curves (Fig. 1a and c) to a linear regression curve (Eq. (19)) provides poor correlation coefficients, typically ranging between 0.90 and 0.98. However, the second order Eq. (20) provides much better correlation coefficients, all over 0.99.

On another hand, it is clear from Fig. 1b and d that the log–log curves also fit very well to a linear regression curve, with correlation coefficients over 0.999 in most cases. The only exception is the small deviation observed for the three acidic compounds (HVA, 5HIAA and DOPAC) when the fraction of water is the largest. Excluding the last point from the regression restores excellent correlation coefficients. This small deviation can result from two facts:

(i) Because of the way the solutions were prepared, the total salt concentration is significantly different when increasing the water content from 15% to 40%. Indeed, the total salt concentration varies from 6 to 16 mM. As will be discussed in the next section, the retention mechanism at low and high salt concentrations might be somewhat different.
(ii) The mobile phase ^S_wpH was reduced from 6.6 to 5.4 when the proportion of water increased from 15% to 40%. Judging from

the aqueous pK of the acidic compounds (4.3 and 4.6), it is possible that they would be partly neutral in the largest proportion of water (lowest ^S_wpH values) while they would be in their anionic form in the smallest proportion of water (largest ^S_wpH values). As a result, the possible interactions they would establish with the chromatographic system would be different.

Hemström and Irgum [40] indicate that, when adsorption is the prevailing mechanism and Eq. (21) is adequate to fit retention data, the slope of the regression lines should be related to the polarity of the solute. Indeed, solutes having a higher number of polar sites should interact with polar interaction sites on the stationary phase to a greater extent. To further investigate the possibility of an adsorption mechanism, we thus plotted the slope of the log–log curves (Eq. (21)) vs. the $\log D_{o/w}$ values at pH 6.2 (Fig. 2). Although the fits are far from perfect linearity, it appears that, within the different compound families (acids, bases and amino-acids), a linear tendency exists with a negative slope. This indicates that increased polarity of the solute (decreased $\log D_{o/w}$ values) is related to larger slopes in Eq. (21). This seems to indicate a rather good compliance of our retention data to an adsorption mechanism. Besides, acids and amino-acids seem to fit to an identical regression line, possibly indicating that they interact with the same adsorption sites of the stationary phase, while bases fit to a different regression line
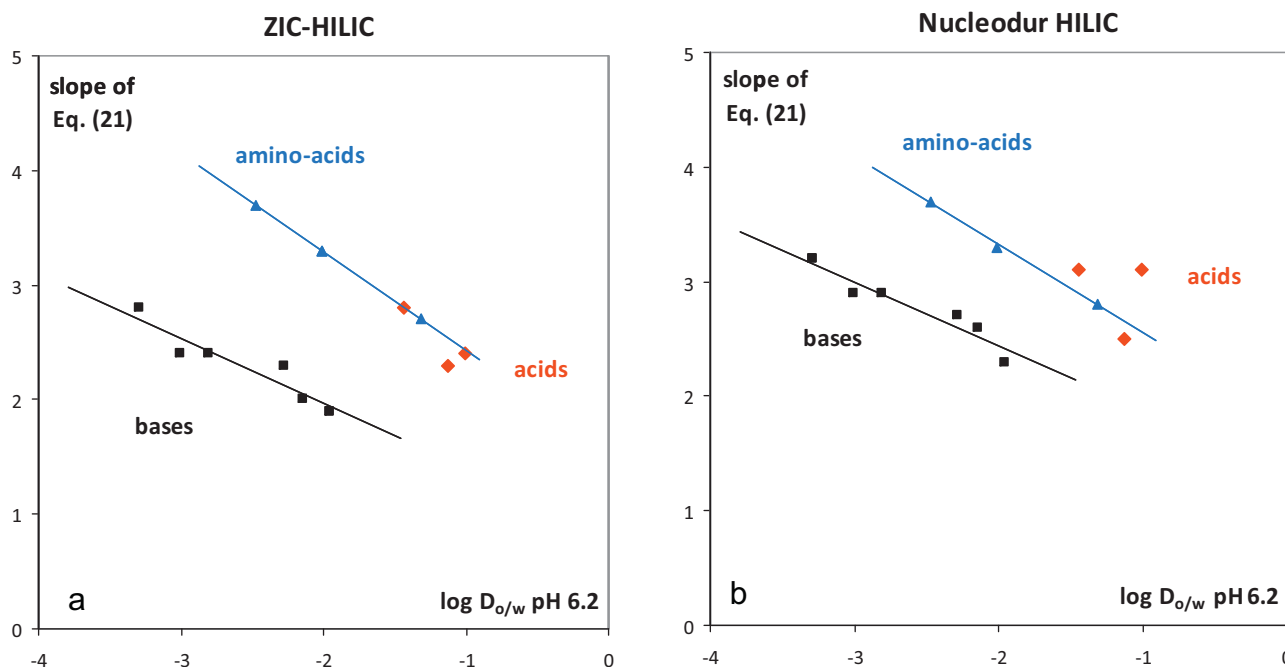
Fig. 2. Variation of the slope of Eq. (21) (see Fig. 1b and d) with solute polarity expressed by log $D_{o/w}$ at ^w_wpH 6.2 for solutes 65–76 in Table 1. (a) ZIC-HILIC and (b) Nucleodur HILIC

and thus would interact with other adsorption sites, or that the interaction may not involve adsorption at all.

Based on all these observations, it seems reasonable to assume a kind of "mixed-mode" retention mechanism, composed of both partitioning and adsorptive interactions. This is in accordance with McCalley's observations on the ZIC-HILIC column [49], that some compounds fitted better to Eq. (19) while other compounds fitted better to Eq. (21). Further discussions on this point will be developed in the following sections.

4.1.2. Salt concentration

Upon addition of salt in the buffered mobile phase, the general trend observed is an improvement in peak shape (asymmetry is reduced and column efficiency is slightly improved) passing from water to a "critical" salt concentration, which is different from the situation when the ACN percentage changes [8]. Also the nature of salt was critical, even at large salt concentrations, as replacing ammonium formate by ammonium acetate caused a marked deterioration of peak shapes and changes in the elution order (data not shown). As for retention changes with salt concentration, they were different for different compound types (acids, bases and amino-acids) but again quite consistent between the two stationary phases (Fig. 3). Although salt concentration impacts retention to a minor extent, compared to ACN concentration, it was significant because it could also induce elution order changes. Again the protonated bases appear to behave differently from the acids and amino-acids: retention of the latter increases with salt concentration, while retention of the former decreases. This supports the above hypothesis that acids and amino-acids interact with the same adsorption sites of the stationary phase, or may establish the same type of interactions with the stationary phase.

For all solutes, the retention variation is most significant up to a certain point then levels off. The curves are a little different between the two columns because the plateau seems to be reached at lower salt concentrations on the Nucleodur HILIC (10 mM) than on the ZIC-HILIC (15 mM). The concentration of charges at the surface of the two stationary phases may be different, possibly due to a higher

bonding density of the sulfobetaine ligands on the ZIC-HILIC phase. Whatever the origin of the difference, it takes less salt to titrate the ionic groups and eliminate electrostatic effects with the Nucleodur HILIC column than with the ZIC-HILIC column.

The proposed mechanism is the following:

At low salt concentrations, there exist electrostatic interactions between the sulfobetaine ligands and the solutes. These interactions may be attractive (particularly between protonated bases and the sulfonate groups) or repulsive (particularly between deprotonated acids and the sulfonate groups). However, hydrophilic interactions must also be present, or else anionic acids would not be retained, as mentioned by McCalley [49]. This combination has been described by Alpert [26] as ERLIC (electrostatic repulsion–hydrophilic interaction chromatography). This is also consistent with others observations [4]. Increasing salt concentration suppresses both electrostatic attraction and repulsion, causing decreasing retention of basic compounds and increasing retention of acidic ones [4,7,49,50]. Moreover, an increase in retention with salt concentration increases can also be related to hydrophilic partitioning. Presumably high concentrations of organic solvent in the mobile phase cause salt to partition preferentially into the water-rich pseudo-stationary phase. The presence of more solvated ions in this phase would increase its volume, potentially leading to stronger retention of polar solutes [7,49].

Besides, similarly to the observations of McCalley on the ZIC-HILIC column [49], plotting the retention factors (k) against the inverse of the counter-ion concentration in the mobile phase did not produce straight lines as should be the case for an ion-exchange mechanism, but produced curves (data not shown). This supports the hypothesis that a hydrophilic-partition mechanism is superimposed on the adsorption mechanism related to electrostatic interaction.

4.1.3. Column temperature

Column temperature was investigated in the range of 10–50 °C (Fig. 4) with a mobile phase comprised of 80% acetonitrile and 20% 25 mM ammonium acetate buffer, ^w_wpH 4 (^S_wpH 6.2). This corre-
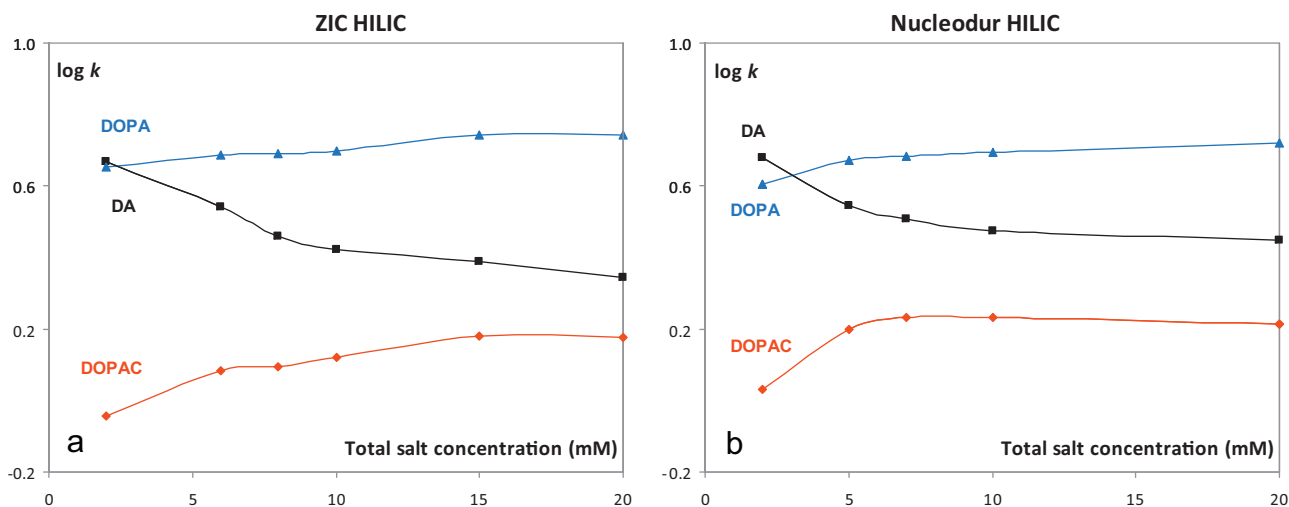
Fig. 3. Variation of log k with the total concentration of salt in the mobile phase for three example compounds. ACN percentage: 80%; ammonium acetate buffer $_w^w$pH 4 ($_w^S$pH 6.2); 20 °C. (a) ZIC–HILIC and (b) Nucleodur HILIC

sponds to an overall salt concentration of 5 mM. As a result, in these conditions, electrostatic interactions should be a significant part of the retention mechanism.

The relationship between retention factor and column temperature in RPLC is often described by the van't Hoff equation:

$$\ln(k) = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln(\Phi) \tag{22}$$

where ΔH° and ΔS° are retention enthalpy and entropy changes for the retention interaction, R is the gas constant and Φ the phase ratio.

If the retention in HILIC is through partitioning, the van't Hoff equation should apply.

Linearity of the van't Hoff plots was reasonable for all the analytes concerned on both columns under the conditions applied. Only the amino-acids on Nucleodur HILIC displayed a marked curvature. Deviations from linearity mostly appear in the low temperature range. This is however consistent with the behaviour of ionizable species, as reported previously [8].

Among our 12 test-solutes, different compound families exhibited different retention behaviour, while column efficiency generally improved for all solutes with increasing temperature.

Even more surprising, although the two columns had shown comparable properties up to this point, they display here a marked difference. Indeed, the six bases behave identically on both columns (retention increases when temperature increases, although the slopes are greater on ZIC–HILIC than on Nucleodur HILIC) while the acids and amino-acids have opposite behaviour between the two columns: on ZIC–HILIC, their retention increases with increasing temperature while on Nucleodur HILIC, their retention decreases with increased temperature. Exothermic transitions of solutes from the mobile to the stationary phase are generally observed in the literature on ZIC–HILIC column used in HILIC mode [4,7]. Endothermic transitions could result from adsorptive interactions superimposed on the HILIC partitioning mechanism [51–53]. Based on the discussion on the salt concentration effect, it is reasonable to assume that the retention mechanism in the current mobile phase is of the ERLIC type, thus comprising a combination of hydrophilic partitioning and electrostatic interaction. The extent of electrostatic interaction relative to the hydrophilic partitioning may be different between the two columns, resulting in different slopes of van't Hoff plots for the two columns. Indeed, different elements discussed in the preceding paragraphs suggest that the bonding density is larger on the ZIC–HILIC column than on the Nucleodur HILIC column. At 5 mM
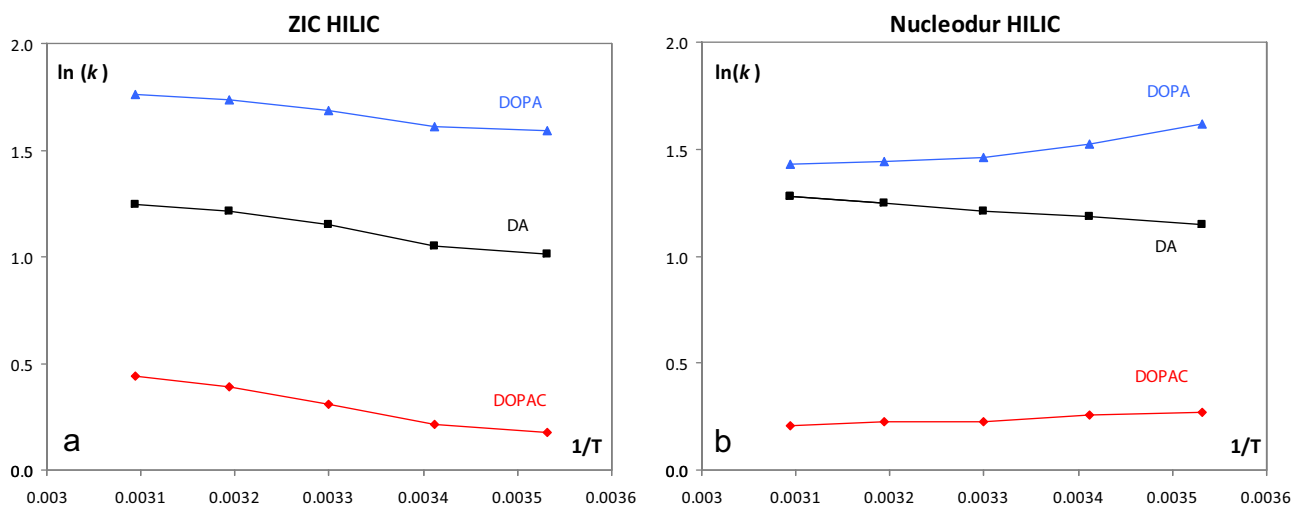
Fig. 4. Variation of log k with column temperature (10–50 °C) for three standards. ACN-buffer 80:20 (v/v); buffer was ammonium acetate 40 mM, $_w^w$pH 4 ($_w^w$pH 6.2). (a) ZIC–HILIC and (b) Nucleodur HILIC.

salt concentration, a greater concentration of free sulfonate groups might thus remain on the ZIC-HILIC phase than on the Nucleodur HILIC phase. As a result, electrostatic interaction might represent a greater part of the retention mechanism of the former than the latter, resulting in different transferring enthalpy.

Moreover, control of phase temperature can affect the separation selectivity on a zwitterionic stationary phase, as the flexibility of the intercharge spacer arm increases with increased temperature (if sufficiently long), permitting the formation of internal salts and leading to reduced interactions with cationic analytes [6]. This would be in accordance with our observations because higher temperatures would favour a partitioning mechanism, thus linear van't Hoff plots. On the contrary, lower temperatures would favour possible electrostatic interactions, that is to say a more adsorption-like mechanism, resulting in non-linear van't Hoff plots. This too implies that the length of the ligands is shorter in the Nucleodur HILIC coating than in the ZIC-HILIC coating. This can however not be confirmed as, again, the exact surface chemistry of the two stationary phases remains unrevealed by the manufacturers.

In the whole study presented in Section 4.1, different arguments thus suggest the existence of a mixed-mode retention mechanism. To further evaluate this hypothesis and possibly quantify the part taken by each process in the retention mechanism on sulfobetaine stationary phases used in the HILIC mode, quantitative structure–retention relationships were established.

4.2. Comparison based on quantitative structure–retention relationships

4.2.1. Choice of analytes

The compounds selected are small molecules having, for the most part, properties relevant for biomedical and pharmaceutical studies (Table 1). The set of analytes investigated here was never used before and was especially designed for this study. Thus some validation must be performed, to ensure that it is appropriate to establish QSRRs. Indeed, there are some essential rules to follow in order to obtain meaningful results from multiple linear regression analyses. One is that the set of probe solutes must be sufficiently large to ensure the statistical significance of the calculated system constants. A rule of thumb indicates that a minimum of four solutes per variable should be used, although it is clearly better to overdetermine the system by using more input retention factors. In our case, intending to use as many as seven independent variables, we have chosen to use a much larger solute set with more than 10 probe solutes per variable. Besides, the system constants, particularly in small data sets, are strongly influenced by statistical outliers. This is another reason for increasing the initial data set so experimental errors have less weight on the final equation.

Size of the solute set is not the only requirement: an equilibrated set of solutes should have a wide variety of chemical functions, so that the introduction of additional solutes would not significantly modify the results. Additionally, the applicability of the calculated model is much larger when the data set that served to establish the coefficients is more diverse. This means that the chosen solutes must differ in physico-chemical properties and have different three-dimensional structures in order for the LSER to be considered general. As a counter-example, the equations calculated by Jandera et al. on the ZIC-HILIC stationary phase [23] were only based on two compound families with little diversity, thus the obtained models cannot be applicable to any other compound family than the ones that served to establish the equations. While building our solute set, we have been careful to introduce a great variety of functional groups, sizes and shapes. Besides, positional isomers and slightly different functional groups were intended to provide subtle details of factors influencing retention and selectivity. Fig. 5, showing the repartition of the solutes of Table 1 in

each descriptor space, demonstrates this point. It should be clear from this figure that the solutes are distributed in such a manner that each descriptor covers a wide range. Also, clustering should be avoided as much as possible. The only exceptions to this rule are the D^- and D^+ descriptors, because, due to the very definition of these parameters, a large proportion of solutes have at least one D value equal to zero. Also, as a result of the working pH ($_w^S$pH 6.2), most species are either completely neutral or completely ionized. Actually, the final set comprises compounds which are:

 (i) Neutral in the operating $_w^S$pH conditions (47.4%)
 (ii) Partly or totally anionic (17.1%)
(iii) Partly or totally cationic (13.2%)
(iv) Partly or totally zwitterionic (22.4%).

Minima, maxima, average and standard deviation values for each descriptor can be found in Table 2. It was found reasonable to compare the D^- and D^+ average and standard deviation values only for charged species. Each descriptor covers a wide range that defines the applicability domain of the models to be established, which in turn will ensure the predictability from the models.

The property being studied ($\log k$) should also span a wide range in values. In the present study, $\log k$ values range from -1.5 to 1.5 on both columns, and no cluster of values is observed.

Another essential rule of QSRRs is that the variables employed in the regression be independent, that is to say the descriptors used in one equation should be as orthogonal as possible. Cross-correlation must be avoided because it results in difficulties in the interpretation of the coefficients, as the multiple linear regression analysis is unable to distinguish between correlated descriptors. Thus it is necessary that the probe solutes be chosen so as to minimize correlation between the variables. Generally, a solute set with large functional diversity guarantees little cross-correlation among the descriptor values. This point is demonstrated in Table 3, representing the correlation matrix for the solutes in Table 1. Each descriptor was also plotted against another, and non-correlation was reflected by the random scatter of the data, without any particular compounds acting as levers. Only the E and V descriptors appear to present some correlation. However, it must be pointed out that covariance measured through the correlation coefficient is somewhat overestimated because this coefficient can be strongly influenced by a few points acting as levers, while the rest of the points are scattered. We have thus checked that the points on each plot of descriptors taken two by two were indeed widely scattered.

As a conclusion, we believe we have compiled a set of test solutes that is sufficiently wide and diverse for the characterization of the selected HILIC systems. The present solute set is thus suitable for retention description of the sulfobetaine stationary phases. However, whether it is appropriate for describing retention in any HILIC chromatographic system remains to be determined. Additional solutes may need to be introduced to this data set for stationary phases or mobile phase conditions which would be more or less retentive than the ones used here, in order to retain a reasonable range of retention factors.

4.2.2. Comparison to $\log D_{o/w}$ ($_w^w$pH 6.2)

Since hydrophilic interaction is one of the mechanisms that govern the retention in the HILIC mode, the hydrophilic properties of a compound should at least partly determine its behaviour in this chromatographic system. $\log D_{o/w}$, like $\log P_{o/w}$, is a measure of the hydrophilic character of a compound: high $\log D_{o/w}$ values are measured for compounds with low hydrophilic character. In HILIC, the solutes are postulated to partition between the organic-rich mobile phase and the water-rich pseudo-stationary phase that is immobilized on the stationary phase. Thus, the more hydrophilic the compound, the lower the $\log D_{o/w}$ value and the longer the
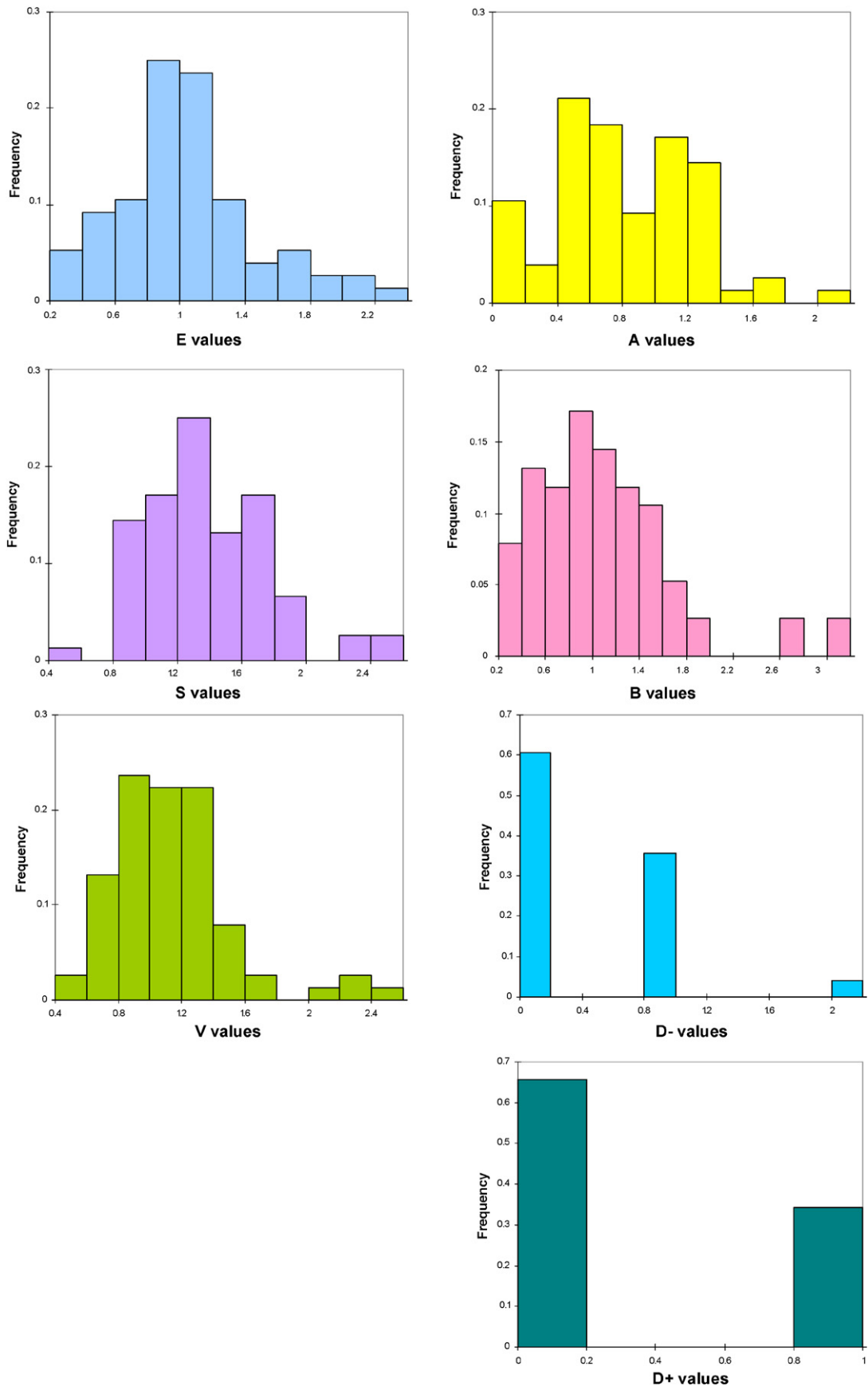
Fig. 5. Distribution of descriptor values among the solute set in Table 1.

Table 2
Figures of merit for the solute set in Table 1. (The average and standard deviation values for D^- and D^+ were calculated using only charged species.).

	E	S	A	B	V	D^-	D^+
Minimum	0.37	0.55	0.00	0.23	0.46	0.00	0.00
Maximum	2.33	2.50	2.10	3.10	2.42	2.00	1.00
Average	1.05	1.38	0.81	1.07	1.15	1.10	0.99
Standard deviation	0.42	0.38	0.45	0.59	0.37	0.31	0.03

chromatographic retention. If the HILIC retention mechanism is analogous to a partition mechanism, then the analytes partition between the aqueous pseudo-stationary phase of unknown pH and the buffer–acetonitrile mobile phase at $_w^S$pH 6.2. In addition, the retention data were acquired with a mobile phase composed of 80% acetonitrile and 20% 100 mM ammonium acetate buffer. This corresponds to an overall salt concentration of 20 mM. Judging from the above observations, the retention mechanism on both stationary phases in these conditions should be essentially of the HILIC type at such high salt concentration, and not of the ERLIC type.

Fig. 6 is well in accordance with theory, as $\log k$ is inversely correlated to $\log D_{o/w}$. The relationship with retention on both columns is shown. The plots also make it clear that no particular group of solutes is responsible for the poor fit as neutral species, anions, cations and zwitterions all scatter in a homogeneous fashion about the regression line. The worst correlation coefficient obtained for the ZIC-HILIC phase seems to result essentially from the least retained neutral species, for which accuracy of the retention measurement might be an issue. However, the correlation coefficient ($R^2 = 0.70$ and 0.87 for ZIC-HILIC and Nucleodur HILIC respectively) suggests that hydrophilic partitioning is only one of the mechanisms involved in retention in the HILIC mode, thus $\log D_{o/w}$ cannot furnish more than a rough estimate of solute retention. This seems to indicate that, even at the highest salt concentration, where the ionic groups of the stationary phase should be titrated, some adsorption interactions might still participate in the retention mechanism.

Indeed, the water layer is not built on an inert stationary phase; thus all functional groups of the stationary phase, the sulfobetaine ligands and the residual silanol groups, cannot be prevented by the immobilized water layer from interacting with analytes.

Similarly to the results presented in Fig. 6, in a study of Kadar et al. [54] correlation coefficients of about 0.7 were obtained when trying to correlate $\log k$ to $\log D_{o/w}$. Lämmerhofer et al. obtained comparable correlation coefficients [39]. Besides, when Quiming et al. [19] elaborated models to describe retention in the HILIC mode, $\log D_{o/w}$ did not prove sufficient to achieve a complete description of the retention mechanism. Other interaction terms had to be taken into account, namely hydrogen bonding, dipole–dipole interactions and other electrostatic interactions. In the following, we have devised a retention model to try and quantify these interactions.

4.2.3. Retention models

There are different approaches to QSRRs. One of them is to start from numerous molecular descriptors and selecting the combination of descriptors providing the best correlation to reten-

tion. This is, for instance, the approach taken by Quiming et al. [18–22]. This leads to certain difficulties as different chromatographic systems would be best described by different molecular descriptors, thus comparison between different systems is quite complicated.

Another method is to start from a reduced number of selected solute descriptors, based on a priori knowledge of the interactions contributing to retention. We generally favour the latter approach, as comparison between different chromatographic systems is much easier this way.

Besides, we chose to focus principally on an existing model, the relevance of which has already been established, rather than trying to conceive a completely new model for the chromatographic system we wished to characterize. The solvation parameter model, fully described in the theory section above, has the advantage of having been widely used for the characterization of chromatographic systems in the liquid phase, thereby providing numerous references and comparison points for any new system.

The system constants for both sulfobetaine columns are presented in Table 4. Three equations are provided for each column. The first one was established based on Eq. (1), the usual solvation parameter model limited to Abraham descriptors, retaining only those compounds that are neutral at the mobile phase $_w^S$pH. The second one was calculated with the same Eq. (1), but for all compounds in Table 1, whatever their ionization state. The third one was calculated again for all compounds in Table 1, but based on our suggested equation comprising two additional descriptors to account for ionic interactions with anions and cations, Eq. (16).

The goodness of fit can be estimated with the adjusted determination coefficient (R^2_{adj}), standard error in the estimate (SE) and Fischer F statistic. If the fits are rather good when using only neutral compounds with Eq. (1), they seriously deteriorate upon introduction of the ionic species in the model calculation. This is natural because the solvation parameter model was designed for neutral species and is not appropriate to describe retention of ionic species. It should however be clear from these parameters that, when ionizable compounds are present in the data set, the goodness of fit is greatly improved on moving from Eqs. (1) to (16). On the Nucleodur HILIC phase, equally good correlation is obtained between the first and third equations. This is also visible on Fig. 7, where the fits associated to the six calculated models can be compared. It appears on these figures that the experimental retention of all ionic species is larger than the retention calculated when no term is present to account for ionic interactions (Fig. 7b and e). When the ionic interactions are taken into account, the scattering of points about the first bisector is more homogeneous (Fig. 7c and f). This suggests that the two additional terms d^-D^- and d^+D^+ adequately describe

Table 3
Covariance matrix (determination coefficients R^2) for the solutes in Table 1.

	E	S	A	B	V	D^-	D^+
E		0.69	0.40	0.54	0.72	−0.37	−0.21
S	0.69		0.57	0.64	0.63	−0.13	−0.05
A	0.40	0.57		0.59	0.45	0.30	0.22
B	0.54	0.64	0.59		0.64	0.07	0.21
V	0.72	0.63	0.45	0.64		−0.06	0.02
D^-	−0.37	−0.13	0.30	0.07	−0.06		0.43
D^+	−0.21	−0.05	0.22	0.21	0.02	0.43	

Fig. 6. Relationship between retention and the octanol–water partition coefficient of ionized species at $_w^S$pH 6.2 (log $D_{o/w}$) for the solutes in Table 1. The red line is the first bisector; the black line is the regression line. Open diamonds are neutral species at the mobile phase $_w^w$pH red diamonds are anionic species, black squares are cationic species, blue triangles are zwitterionic species. (a) ZIC-HILIC and (b) Nucleodur HILIC. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

extra ionic interactions in the studied HILIC systems. This also indicates that the change in retention caused by the ionization of an acidic or basic compound is too great for the predicted retention from the standard solvation parameter model (Eq. (1)) for the neutral form of the compound to be a reasonable estimate of retention when appreciably ionized.

Possible outliers were detected by inspecting the plot of the standardized residuals against the fitted values. Standardized residuals with absolute values higher than 2.0 (at 95% confidence level) can be considered outliers and removed from the data set. The regression must then be repeated using the remaining cases.

It was also ascertained that the normalized residuals in no way correlated with any of the descriptors used as independent variables.

Judging from the correlation coefficient values (R^2_{adj} = 0.940 and 0.934 respectively for ZIC-HILIC and Nucleodur HILIC), more than 90% of the variance observed in these chromatographic systems is explained with Eq. (16). The missing 6–7% can have different origins:

(i) For the purpose of simplicity, the E, S, A, B and V descriptor of the neutral species were used, although it was mentioned above that they should be somewhat different for ionic species.

Indeed, the Abraham descriptors were calculated with a software program that is unable to cope with ionized forms of an acid or base. For future works, they should thus be determined experimentally to possibly improve the retention description. For this purpose, a number of partitioning or chromatographic systems should be characterized with Eq. (16) so as to back-calculate the solute descriptors.

(ii) Again for the purpose of simplicity, the mobile phase $_w^S$pH and aqueous pK values were used, rather than $_S^S$pH and pK* values. This is questionable, because the $_S^S$pH experienced by the solutes in the hydro-organic mobile phase is somewhat different from the $_w^S$pH measured with the electrode calibrated in aqueous buffers, and the pK* values are different from aqueous pK. Indeed, as a general rule, pK* of acids increase and pK* of bases decrease when the percentage of organic solvent increases [55]. However, pH* may be different in the organic-rich mobile phase from what it would be in the water-rich pseudo-stationary phase, which is probably close to 4 (the prepared buffer $_w^w$pH). Moreover, ionic species could be surrounded by "shells" of solvent of a different composition from the bulk mobile phase [56]. Thus using the $_S^S$pH value in the mobile phase may well be as wrong as sticking to the mobile phase $_w^S$pH because the solutes would experience different

Table 4
System constants and statistics for both columns n is the number of solutes considered in the regression, R^2_{adj} is the adjusted correlation coefficient SE in the standard error in the estimate, F is Fischer's statistic and the numbers in italics represent 99.9% confidence limits.

Stationary phase	c	s	a	b	v	d⁻	d⁺	n	R^2_{adj}	SE	F
ZIC-HILIC	−0.673		0.355	0.950	−0.915			30	0.966	0.124	279
	0.076		*0.062*	*0.050*	*0.085*						
	0.253	−0.578	0.629	1.185	−1.103			68	0.743	0.365	49
	0.191	*0.183*	*0.137*	*0.118*	*0.180*						
	−0.385	−0.323	0.277	1.024	−0.727	0.335	0.497	67	0.940	0.176	173
	0.104	*0.098*	*0.069*	*0.059*	*0.082*	*0.046*	*0.052*				
Nucleodur HILIC	−0.765		0.468	0.889	−0.861			33	0.934	0.173	151
	0.092		*0.076*	*0.063*	*0.106*						
	0.243	−0.702	0.788	1.136	−0.973			74	0.715	0.400	47
	0.193	*0.184*	*0.147*	*0.122*	*0.194*						
	−0.388	−0.395	0.375	0.951	−0.611	0.283	0.550	70	0.934	0.189	164
	0.109	*0.102*	*0.073*	*0.062*	*0.087*	*0.047*	*0.055*				
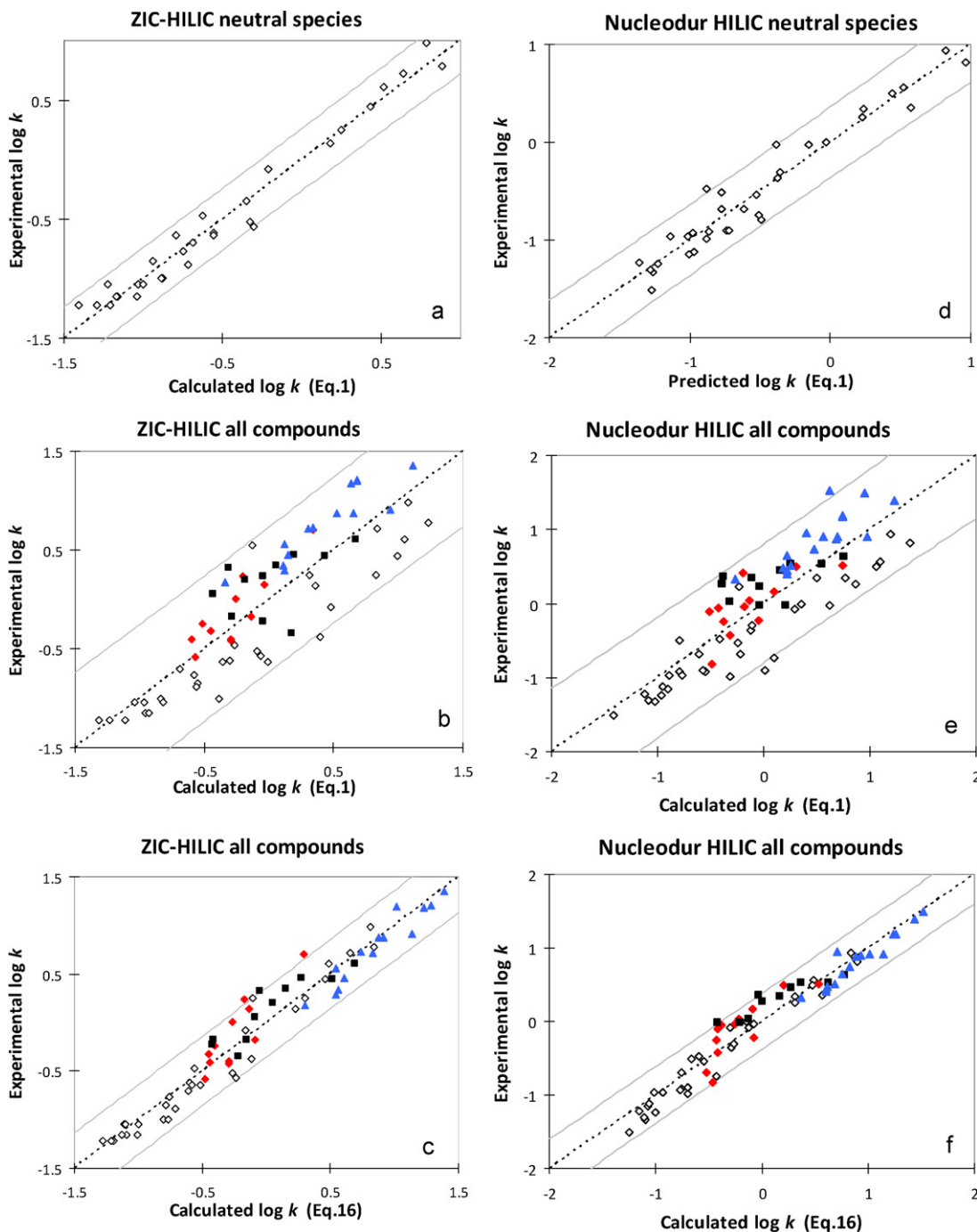
Fig. 7. Model fits (experimental *vs.* calculated log *k*) for the equations in Table 4. (a) ZIC–HILIC retention of neutral species calculated with Eq. (1), (b) ZIC–HILIC retention of all species in Table 1 calculated with Eq. (1), (c) ZIC–HILIC retention of neutral species calculated with Eq. (16), (d) Nucleodur HILIC retention of neutral species calculated with Eq. (1), (e) Nucleodur HILIC retention of all species in Table 1 calculated with Eq. (1) and (f) Nucleodur HILIC retention of neutral species calculated with Eq. (16). Open diamonds are neutral species at the mobile phase $_w^w$pH red diamonds are anionic species, black squares are cationic species, blue triangles are zwitterionic species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pH environments when moving from the mobile phase to the pseudo-stationary phase.

(iii) Simply using the ionization degree of a species as a measure of its ability to participate in ionic interactions is also a gross simplification because it does not take into account the possible shielding of the charge by other functional groups in the molecule, preventing it from direct interaction with the mobile and stationary phase. For most compounds in Table 1, however, this should be of little significance because most ionic entities should be easily accessible.

Still, we consider these results as reasonably good, and it is worth noting that the sign and magnitude of each regression coefficient obtained are in accordance with the chemical nature of the stationary–mobile phase systems under investigation, as will be further detailed below. As each coefficient is significantly larger than its standard deviation, the results are amenable to interpretation. It is also significant that the coefficients do not vary strongly between the first and third equations (neutral species with Eq. (1) compared to all species with Eq. (16)). The differences observed are most certainly due to the fact that some of the descriptor values in

the neutral species set are more strongly correlated than in the complete solute set. In particular, the S descriptor is strongly correlated to the V descriptor. As a result, it is impossible to keep them both in one linear regression. The suppression of the s coefficient thus mechanically results in a decreased value of the v coefficient, to compensate for this absence.

Since the descriptors represent the solute effect on various solute-phase interactions, the coefficients obtained from the multiple linear regression analyses correspond to the complementary effect of the stationary and mobile phases on these interactions. The regression coefficients thus encode chromatographic system properties. As the chromatographic conditions and mobile phase were kept constant for the two columns, the comparison of coefficients provides a comparison of the stationary phases.

The intercept, c, is not assigned any chemical significance. It represents a part of the retention factors that is not accounted for by the solvation parameters. Therefore, the c coefficients are not easily compared or interpreted, and they will be omitted in this discussion.

First of all, the general repartition of the coefficient values between positive and negative values is characteristic of a normal-phase type system. Indeed, most polar-type interactions are positive indicating that increased polarity of the solute causes increased retention, while non-polar interactions (dispersive interactions represented by the v coefficient) are negative, indicating that increased non-polar molecular volume causes decreased retention. This is an intuitive outcome because polar compounds are expected to have longer retention on the polar stationary phase than less polar ones in HILIC.

Besides, the dominant and opposite parts taken by the b and v coefficients are characteristic of partitioning systems where water is one of the two phases. Such large and opposite b and v coefficients are always observed, for instance, in RPLC systems. However, in the latter, b is negative and v is positive. This confirms that HILIC behaviour is a sort of "reversed RPLC" system.

The statistical significance of individual coefficients was evaluated using the t-ratio, which is defined as the ratio of the regression coefficient to its standard error. Based on this factor, the e coefficient appeared not to be a significant factor in explaining retention on both columns. This can have two possible meanings: either e-type interactions (interactions through π and non-binding electrons) do not participate to the retention mechanism in this chromatographic system, or e-type interactions have the same magnitude between the solute and stationary phase as between solute and mobile phase.

The s coefficient gives the tendency of the phase to interact with dipolar and/or polarizable solutes. In the present case, it is small and negative, indicating that dipole–dipole interactions are slightly stronger between solute and mobile phase than between solute and stationary phase.

The a coefficient denotes the hydrogen-bond basicity of the stationary phase (or pseudo-stationary phase), because acidic solutes (having a positive A coefficient) will interact with a basic phase. The positive contribution to such interactions by the stationary phase can stem from: (i) the immobilized aqueous layer or (ii) the sulfonate terminal functions of the sulfobetaine ligands. It must be pointed out that, in the definition of the A term, all chemical functions related to the overall acidity are taken into account: not only carboxylate but also hydroxyl and primary and secondary amine groups, for instance, contribute to the A descriptor. Moreover, as the A descriptor qualifies neutral species, the "acidic" species defined by a positive A value are protonated acids, not anionic species. This is obviously a defect in the present work because, as mentioned above, some descriptor values should be recalculated for the ionized species. In particular, the A values should be recalculated for the anionic acidic functions.

The b coefficient is a measure of the hydrogen-bond acidity of the phase, because basic solutes (having large B values) will interact with an acidic phase. Similarly to the above situation, large B values qualify electron-donor species in their neutral form and no correction of the descriptor was applied for the protonated bases. The large b coefficient observed here can be largely attributed to the aqueous pseudo-stationary phase, because the strong (Lewis) acidic character of water is generally responsible for large b coefficients. Besides, it is suspected that the electron-acceptor quaternary amine function at the base of the sulfobetaine ligand may not be fully accessible to interact with basic solutes.

In the QSRR analyses conducted by Quiming et al. [19], the hydrogen-bond acceptor character (equivalent to the B descriptor here) was found to have a significant influence on retention on a silica stationary phase used in the HILIC mode, while the hydrogen-bond donor character (equivalent to the A descriptor here) was not significant. This could be consistent with our observation that the a coefficient, although positive, is smaller than the b coefficient.

However, on a diol stationary phase [22], both the hydrogen-bond donor and acceptor characteristics had an influence on retention. On a polyvinylalcohol stationary phase [21], the hydrogen-bond donor character was significant, while the hydrogen-bond acceptor character was not. These comparisons do actually not make much sense, because $\log D_{o/w}$, which was also used as a solute descriptor in the above QSRRs, also comprises some hydrogen bonding component, which is not clearly quantified.

Jandera et al. [23] found much larger a coefficients than b coefficients when describing the HILIC retention of phenolic acids on the ZIC-HILIC phase. However, the significance of LSER equations based on only 12 solutes with little structural diversity is questionable.

The v coefficient is a combination of exoergic dispersion forces that make a positive contribution and an endoergic cavity term making a negative contribution. Clearly, dispersive interactions with the stationary and mobile phases here should be negligible, and the large negative value of the v coefficient is most probably associated with the difficulty in inserting the solute in the highly cohesive aqueous pseudo-stationary phase.

Both d coefficients are positive, indicating that the presence of a permanent charge in the solute structure induces increased retention. This can be attributed to enhanced solubility in the aqueous layer and reduced solubility in the acetonitrile-rich mobile phase, leading to hydrophilic partitioning. The d^+ coefficient is larger than the d^- coefficient, possibly due to remaining attractive electrostatic interactions occurring between cations and the sulfonate function of the stationary phase, while the latter would cause repulsive interactions with anions. Indeed, the sulfonate groups on the outside of the sulfobetaine ligands give the column cation-exchange properties despite the overall zwitterionic nature of the bonded ligand. Nevertheless the negatively charged acids do not elute in the dead volume, suggesting that the retention conferred by the hydrophilic interaction is stronger than the electrostatic repulsion with the concentration of ACN used here. Moreover, the possibility of an ion-pairing mechanism involving the buffer ions (acetate and ammonium) cannot be excluded to explain the significant d values.

For the purpose of clarity, the coefficients obtained with Eq. (16) on both columns were also represented in Fig. 8. Very little difference is observed between the two columns. Indeed, upon calculation of the θ angle between the two chromatographic systems, based on Eq. (3) adapted to take account of the two extra system constants, a 8° angle is found between the two columns. Moreover, the calculation of J based on Eqs. (4)–(6) indicates that the two columns are similar and cannot be distinguished based on their interaction properties measured with Eq. (16). Thus replacing one column by the other should provide essentially identical elution orders when working with mobile phase conditions comparable
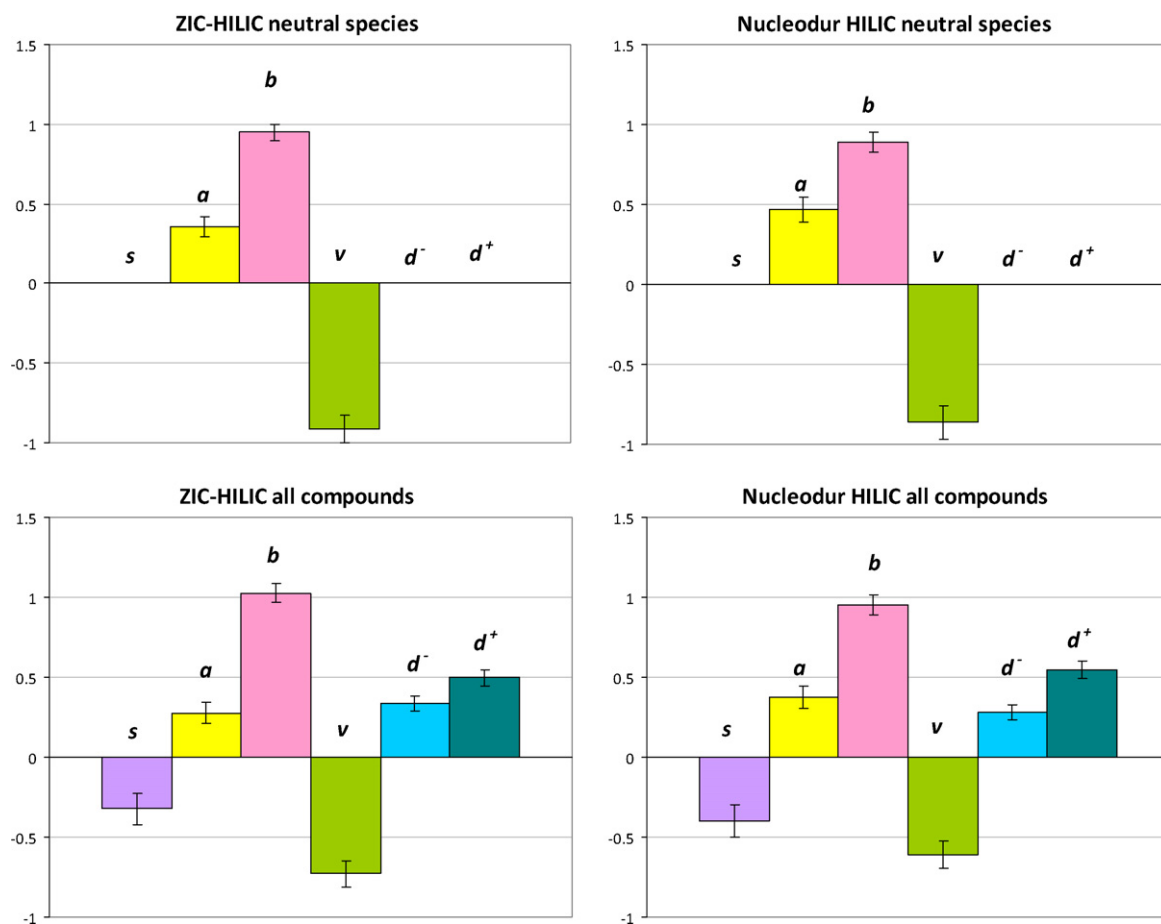
Fig. 8. System constants issued from the multiple linear regression analysis of retention factors of solutes in Table 1 on (a) ZIC-HILIC retention of neutral species calculated with Eq. (1), (b) ZIC-HILIC retention of neutral species calculated with Eq. (16), (c) Nucleodur HILIC retention of neutral species calculated with Eq. (1) and (d) Nucleodur HILIC retention of neutral species calculated with Eq. (16).

to the ones used here. This is also confirmed by Fig. 9, where the retention factors on both columns appear to be highly correlated, indicating highly similar retention behaviour of the two columns when used in identical operating conditions.
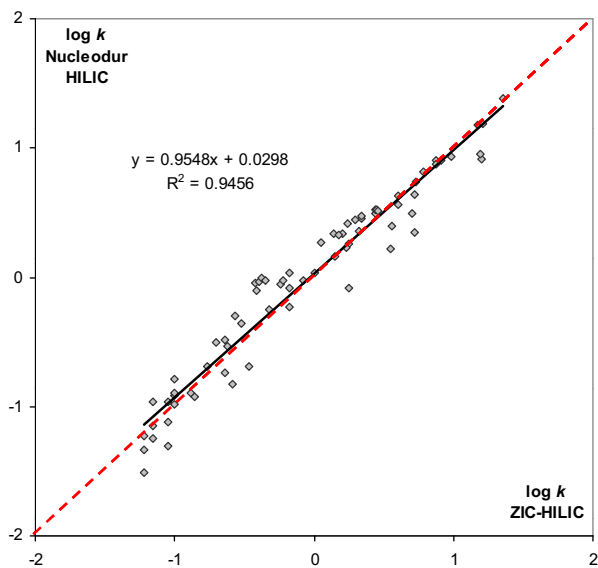
Fig. 9. κ–κ plot comparing the retention on the ZIC-HILIC phase to the retention on the Nucleodur HILIC phase for the solutes in Table 1. Mobile phase: acetonitrile–100 mM ammonium acetate buffer ^w_wpH 4 80:20 (v/v), 20 °C.

In addition, the calculation of the u vector length according to Eq. (7), again adapted to take account of the d coefficients, provides the following values: u (ZIC-HILIC) = 1.46 and u (Nucleodur HILIC) = 1.40. This indicates that retention and separation factors should be essentially of the same order between the two columns, which is in accordance with Fig. 9 where most data points appear to be close to the first bisector, indicating comparable retention on both columns, despite different specific surface area and possibly different bonding density.

5. Conclusion

Although the two sulfobetaine-bonded stationary phases appeared essentially identical, displaying identical retention behaviour under different operating conditions, some differences appear as regards temperature effect on retention, possibly resulting from different bonding density.

Generally better peak efficiency is noticed on the Nucleodur HILIC column, which was based on smaller silica particles, although significant salt concentrations were necessary to obtain symmetric peaks for some compounds (for instance A, DOPA, DHBA or NA).

A modified version of the solvation parameter model appeared to provide significant improvement to correlate the retention data measured in the HILIC mode.

The general repartition of all LSER coefficients is in accordance with good chemical sense. The large and opposite values of the b and v coefficients suggest that partition of the solutes between the organic-rich mobile phase and the water-rich pseudo-stationary

phase does occur, as initially suggested by the correlation between retention factors and octanol–water partition coefficients. Other system constants evince the multi-modal retention mechanism of the sulfobetaine stationary phases used in the HILIC mode. As a result, all polar compounds, whether they are neutral, anionic, cationic or zwitterionic, could prospectively experience sufficient retention to achieve a separation on these columns, rendering them suitable stationary phases for varied applications.

The degree of ionization as indicated by the pK values of the compounds seems an appropriate descriptor for describing the ionic interactions of the analytes with the stationary and mobile phase components. The significant contribution of d^- and d^+ to retention indicate that coulombic interaction is one mechanism of retention in the studied system that was not correctly assessed with the standard solvation parameter model.

There is currently no standardized HILIC characterization test. Whether the above suggested method is to become a standardized method remains to be demonstrated with further experiments on different stationary phases and with different operating conditions. This will be the object of future work.

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