Advances in Sample Preparation 6 (2023) 100063

Contents lists available at ScienceDirect

Advances in Sample Preparation

journal homepage: www.elsevier.com/locate/sampre

Effect of the solvent on the chromatographic selectivity in reversed-phase and HILIC

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ARTICLE INFO

Keywords:
HILIC
Linear free energy relationships
Column characterization
Chromatographic retention
Chromatographic selectivity

ABSTRACT

In this work, the characterization of several reversed-phase and HILIC chromatographic systems is presented by means of the Abraham's solvation parameter model, focusing on the impact of solute polarizability, dipolarity, hydrogen bonding, and molecular volume on chromatographic retention. Although retention times in octadecylsilane columns are clearly dependent on the nature and content of the organic modifier in the mobile phase, similar chromatographic selectivities are reported for eluents containing acetonitrile or methanol in the range between 40 and 80%. The most relevant analyte properties affecting retention are the hydrogen bond acceptor capacity and the molecular volume, the former favoring partition into the mobile phase and the latter into the stationary phase. The behavior of HILIC systems greatly depends on the nature of the support (silica or polymeric), the bonded phase (zwitterionic, aminopropyl, dihydroxypropyl) and the organic solvent used in the eluent (acetonitrile or methanol), but they have in common that larger solute volumes allow more favorable partition into the organic solvent-rich mobile phase. The evaluation of the chromatographic retention of ionized analytes in HILIC should be performed with care, since they may interact with ionized buffering species, leading to unexpected lower retentions.

1. Introduction

Analytical problems involving complex samples with multiple analytes with a similar behavior are often difficult to solve. In these cases, an approximation based on chromatographic methods is usually the most common election, but finding the most convenient experimental conditions may not be easy and straightforward. Here the experience of the chromatographer plays a paramount role because a method development based only on blind trial and error might be excessively time consuming, and therefore expensive in terms of the instrumental, material, and human resources involved. When facing a new challenge, falling out of our previous experience, it is interesting to have some tools helping us to choose a few promising chromatographic systems to solve a particular analytical problem. Please notice that a chromatographic system is not just the column, meaning the bonded phase and support responsible for the stationary phase behavior, but also the eluent being pumped through, the mobile phase.

In this work, we aim to evaluate the effect of analyte properties on its interactions with the chromatographic stationary and mobile phases and consequently their effect on retention. For this purpose, we selected a variety of chromatographic systems consisting of different supports (silica or polymer), bonded phases (octadecyl, cyanopropyl, zwitterionic sulfobetaine, dihydroxypropyl, and aminopropyl), and mobile phase compositions (acetonitrile or methanol as organic solvents). A very convenient tool for this study is the Abraham's solvation parameter model, which relates chromatographic retention with solute properties such as polarizability, dipolarity, hydrogen bonding and molecular volume [1–4].

In 1993, Michael H. Abraham [5] proposed a model based on linear free energy relationships based on solution properties allowing to correlate and to interpret a wide variety of physicochemical and biochemical processes. According to this approach, the free energy change involved in a solvation process, such as the partition of a solute between liquid stationary and mobile phases, can be modeled as the sum of energy changes related to solute-solvent interactions. In the case of a chromatographic process the solvation property directly related to the free energy change is expressed as the decimal logarithm of the retention factor (k), and the model is mathematically expressed as follows:

$$\log_{10} k = c + e \cdot E + s \cdot S + a \cdot A + b \cdot B + v \cdot V \quad (1)$$

The different terms in Eq. (1) are described in Table 1. On the one hand, *E, S, A, B*, and *V* represent molecular descriptors that only depend on the solute and are independent of the chromatographic system

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https://doi.org/10.1016/j.sampre.2023.100063
Received 3 March 2023; Received in revised form 29 March 2023; Accepted 30 March 2023

Table 1
Description of the different terms in the Abraham's solvation parameter model (Eq. (1)). *E, S, A, B*, and *V* are solute descriptors, either experimentally determined or calculated. *e, s, a, b*, and *v* are the system coefficients, reflecting the difference in solute interaction between the solvated stationary phase and the mobile phase.

Term	Description
$\log_{10} k$	Decimal logarithm of the retention factor
c	System constant (accounting for the chromatographic phase ratio, normalization of descriptors and other factors that are not solute dependent)
e·E	Excess polarizability contributions from solute *n*- and π-electron pairs
s·S	Dipole-type interactions (orientation and induction)
a·A	Hydrogen bond donation from the solute to the solvent
b·B	Hydrogen bond donation from solvent to solute
v·V	Cavity formation in the solvent together with residual solute-solvent dispersion interactions (with *V* being the McGowan volume of the solute in $cm^3\ mol^{-1}/100$)

under study. These descriptors, which have been scaled in order to show values of similar magnitude (typically between 0 and 3), are available from either open access [6] or subscription [7] databases. On the other hand, *e, s, a, b*, and *v* are the coefficients allowing the characterization of the chromatographic system. They reflect differences between mobile and stationary phases in the complementary property of the molecular descriptor. A positive value of a system coefficient informs about more favorable interactions of the solute with the solvent molecules of the stationary phase, responsible for an increase in retention. If it is negative, interactions with mobile phase are more relevant, reducing retention. Finally, a system coefficient close to zero shows no significant differences between mobile and stationary phase regarding the feature under consideration.

The application of Abraham's solvation parameter model assumes that the main retention mechanism in liquid chromatography is based on the partition of the solutes between the mobile and stationary phases. The presence of other retention mechanisms, such as adsorption, might introduce some deviations in the accuracy of the model, but even though it will provide information about the solute properties affecting retention in a chromatographic system. In the last part of this work, we study the chromatographic behavior of some acidic and basic compounds in hydrophilic interaction liquid chromatography (HILIC) over a wide range of pH values and using different buffering systems. The eluents used in HILIC have a very high content of organic solvent, which may result in a very low salt solubility and the consequent insufficient buffering capacities). Furthermore, under these mobile phase conditions electrostatic interactions can be established between charged species, leading to an unexpected retention behavior.

2. Material and methods

2.1. Instrumentation

The chromatographic system consisted of two LC-10ADvp pumps, an SIL-10ADvp autosampler, an SPD-M10Avp diode array detector, a CTO-10ASvp oven, and an SCL-10Avp controller, all from Shimadzu (Kyoto, Japan). The system was controlled by LC Solutions software from Shimadzu.

The columns studied were: Chrom-Clone C18 (150 × 46 mm 5 μm 100 Å), Luna NH2 (150 × 46 mm 5 μm 100 Å), and Luna CN (150 × 46 mm 5 μm 100 Å) from Phenomenex (Torrance, CA, USA); YMC-Triart Diol-HILIC (150 × 46 mm 5 μm 120 Å) from YMC Co. Ltd. (Kyoto, Japan); and ZIC-HILIC (150 × 46 mm 3.5 μm 100 Å) from Merck (Darmstadt, Germany).

2.2. Methods and chromatographic conditions

Several different acetonitrile-water and methanol-water mixtures were used as mobile phase. The mobile phase flow rate was 0.5 mL min^{-1} for the ZIC-HILIC column and 1 mL min^{-1} for the rest of the columns. The injection volume was 1 μL. All separations were performed at 25 °C, in duplicate. The extra-column volume of the HPLC instrument was subtracted from all the measured gross retention volumes. Hold-up volumes needed for the calculation of retention factors (*k*) were determined by the homologous series LFER approach described in reference [8].

A Crison 5014 glass electrode connected to a GLP22 pH-meter (Hach, Barcelona) was calibrated using standard aqueous buffers of pH 4.01 and 7.00 (25 °C), and pH was measured in the hydroorganic eluent ($_{w}^{s}$pH scale).

2.3. Chemicals and solvents

Water was obtained from a Milli-Q plus system from Millipore (Billerica, USA) with a resistivity of 18.2 MΩ cm. Acetonitrile and methanol, both HPLC gradient grade, were purchased from Panreac (Barcelona, Spain). Acids and bases used in the preparation of buffered mobile phases were for analysis grade obtained from different providers.

The compounds used in the present work for the characterization of chromatographic systems are presented in the supplementary material (Tables SP1) and were obtained from several different manufacturers, all high purity grade (≥ 98%).

2.4. Sample preparation

Stock solutions of the solutes were prepared in methanol at a concentration of 5 mg mL^{-1} and diluted to 0.5 mg mL^{-1} before injection. *n*-Alkyl ketones were injected at stock solution concentration due to their lower UV absorbance.

3. Results and discussion

3.1. Characterization of reversed-phase and HILIC chromatographic systems

Retention factors (*k*) of the compounds used for the characterization of the chromatographic systems (Table SP1) were measured for the five columns used in this work (Chrom-Clone C18, Luna CN, Luna NH2, YMC-Triart Diol-HILIC, and ZIC-HILIC). Acetonitrile and methanol are the most common organic solvents employed in the preparation of reversed-phase mobile phases. Although acetonitrile is more expensive than methanol, it is extensively used due to its higher elution strength (and thus a lower amount of organic solvent is needed), lower absorbance at short UV wavelengths, lower viscosity, and higher boiling points. The eluent composition selected in this study for reversed-phase columns was 60% acetonitrile/40% water. In HILIC the solvent strength is roughly the opposite of reversed-phase, being water the strongest solvent and acetonitrile is one of the weakest. Therefore, acetonitrile is the most common choice in HILIC, since it provides a much higher increase in retention compared to methanol. Provided that water plays a fundamental role in the constitution of HILIC stationary phases [9,10], the proportion of water in the mobile phase must be kept below certain limits to be different enough from the water-rich stationary phase and allow retention, and thus we chose in this work the eluent composition of 90% acetonitrile/10% water. Although Luna CN was thought to behave as a HILIC column, in fact this mode was only clearly

Table 2
Results of the characterization (Eq. (1)) of some reversed-phase (RPLC) and HILIC columns in acetonitrile-water mobile phases. Chrom-Clone C18, Luna CN, ZIC-HILIC, YMC-Triart Diol-HILIC, and Luna NH2 columns were characterized in the present work. The constants (*c*) and coefficients (*e, s, a, b*, and *v*) of the chromatographic systems (standard errors in parentheses), the number of solutes used in the characterization (*N*), and the determination coefficients (R^2) are reported.

Chromatographic system	Bonded phase	*c*	*e*	*s*	*a*	*b*	*v*	*N*	R^2	Ref.
RPLC (60% acetonitrile)										
Spherisorb ODS-2	C18	−0.21(0.04)	0.18(0.03)	−0.40(0.02)	−0.46(0.02)	−1.09(0.03)	1.10(0.03)	127	0.980	[11]
ERC-1000 (ODS)	C18	−0.26(0.04)	−0.02(0.03)	−0.17(0.04)	−0.52(0.03)	−1.34(0.04)	1.37(0.03)	51	0.992	[11]
Unisil C18	C18	−0.37(0.09)	0.28(0.09)	−0.21(0.04)	−0.18(0.03)	−0.69(0.04)	1.00(0.05)	37	0.974	[11]
XTerra MSC18	C18	−0.30(0.03)	0.00(0.05)	−0.33(0.04)	−0.32(0.03)	−1.11(0.04)	1.19(0.03)	58	0.982	[12]
XTerra RP18	C18	−0.33(0.02)	0.12(0.03)	−0.34(0.02)	−0.20(0.02)	−1.00(0.03)	1.03(0.02)	55	0.992	[12]
Chrom-Clone C18	C18	−0.16(0.02)	0.07(0.03)	−0.26(0.02)	−0.51(0.03)	−1.31(0.04)	1.19(0.04)	80	0.962	–
Luna CN[a]	Cyanopropyl	−0.29(0.01)	0.12(0.01)	−0.18(0.01)	−0.24(0.02)	−1.02(0.02)	0.91(0.01)	80	0.991	–
HILIC (90% acetonitrile)										
Kinetex HILIC	Unbound silica	−0.84(0.05)	0.00(0.07)	0.03(0.07)	−0.04(0.07)	0.83(0.09)	−0.49(0.04)	74	0.780	[19]
ZIC-HILIC	Sulfobetaine	−0.64(0.05)	−0.22(0.06)	0.27(0.05)	0.29(0.05)	1.01(0.07)	−0.89(0.04)	78	0.938	–
ZIC-pHILIC	Sulfobetaine[c]	−0.51(0.05)	−0.04(0.09)	0.09(0.08)	0.83(0.08)	0.77(0.09)	−0.71(0.04)	56	0.930	[13]
YMC-Triart Diol-HILIC	1,2-Dihydroxypropyl	−0.72(0.03)	−0.03(0.04)	0.10(0.02)	0.13(0.03)	0.68(0.04)	−0.45(0.02)	75	0.941	–
Luna NH2	Aminopropyl	−0.53(0.03)	−0.04(0.04)	0.01(0.03)	0.36(0.03)	0.46(0.04)	−0.27(0.02)	84	0.908	–
Luna CN[b]	Cyanopropyl	−1.76(0.05)	0.04(0.06)	0.54(0.05)	0.05(0.07)	0.51(0.08)	−0.55(0.05)	74	0.892	–

[a] 40% acetonitrile.
[b] 100% acetonitrile.
[c] polymeric particle platform.

observed at 100% acetonitrile. For this reason, it was also assayed with a mobile phase containing 40% acetonitrile to ensure its reversed-phase behavior.

The set of solutes was carefully selected to be sufficiently large to ensure the statistical significance of the system coefficients and to have well-known and wide variated *E, S, A, B*, and *V* molecular descriptors. Table 2 shows the system constants (*c*) and coefficients (*e, s, a, b*, and *v*) calculated by multiple linear regression analysis of the retention factors (dependent variable) and the molecular descriptors (independent variable) of the solutes using Eq. (1). Compounds with residuals higher than 2.5 times the standard deviation of the linear regression were considered as outliers and removed from the set for the analysis. Additionally, the table shows several systems from the literature also characterized by the Abraham's solvation parameter model, with the aim of comparing chromatographic systems showing reversed-phase and HILIC behaviors. Whereas retention mechanisms in reversed-phase are relatively simple and well known, in HILIC it is much more complex. It is generally accepted that the main retention mechanism in HILIC is based on the partition of analytes between the organic solvent-rich mobile phase and water-enriched layers adsorbed on the bonded phase and chromatographic support (generally silica), acting as stationary phase. In addition to this hydrophilic partitioning, hydrogen bonding and electrostatic interactions between the solute and the bonded phase/support are also possible [9,10].

The main solute properties affecting the retention in the reversed-phase and HILIC chromatographic systems (Table 3) are the molecular volume (*V*) and the hydrogen bond basicity (*B*). The difference between both retention modes is the sign of the system coefficients (*v* and *b*). In reversed-phase, positive values of *v* indicate that the stationary phase is less cohesive than the mobile phase, and thus the formation of a cavity in the octadecyl stationary phase requires less energy to overcome the intermolecular solvent-solvent interactions than in the acetonitrile-water mobile phase. Consequently, the higher the molecular volume of a solute, the higher the retention in reversed-phase ($v{\cdot}V > 0$ (positive values) → log *k* ↑, Eq. (1)). In HILIC, the water-rich layers acting as stationary phase are more cohesive than the mobile phase, favoring the partition into the eluent because of the lesser energy required for the solute to create a cavity, and therefore reducing retention ($v{\cdot}V < 0$ (negative values) → log *k* ↓). In fact, in the absence of bonded phase and just unbound silica (Kinetex HILIC), *b* and *v* are the only system coefficients significatively different from zero. Concerning hydrogen bond basicity, we need to observe again the differences between mobile and stationary phases to find the reasoning behind the negative value of *b* for reversed-phase and positive for HILIC. C18 and cyanopropyl lack acidic hydrogens, and therefore interactions with solutes with hydrogen bond acceptor capabilities are favored with the hydroorganic mobile phase, reducing retention ($b{\cdot}B < 0$ → log *k* ↓). In HILIC, the higher proportion of water reinforces the hydrogen bond donor features of the stationary phase, in relation to the acetonitrile-rich eluent, increasing retention ($b{\cdot}B > 0$ → log *k* ↑).

In reversed-phase, solute hydrogen bond acidity ($a{\cdot}A < 0$) and dipolarity/polarizability interactions ($s{\cdot}S < 0$) also favors partition into the mobile phase, although to a lesser extent than hydrogen bond basicity. Just the opposite behavior is generally observed for HILIC. The system coefficient *e* is generally small and close to zero for both chromatographic models, suggesting that the extent of solute-solvent dispersion interactions is similar for both the stationary and the mobile phases ($e{\cdot}E \approx 0$ → log *k* ∼).

Comparison of the two columns with the same bonded phase (sulfobetaine) but different support (ZIC-HILIC, silica; ZIC-pHILIC, polymer), shows for the polymeric a reinforced retention for solutes with hydrogen bond acidity at the expense of basicity. This shows that, in fact, support also plays an active role in the chromatographic behavior of HILIC systems.

For the characterized chromatographic systems, provided that solute descriptors can be easily estimated from the molecular structure of any compound [6], retention and selectivity factors can be roughly estimated, allowing to find *a priori* the most promising column and mobile phase for the separation of two analytes in a mixture.

3.2. Effect of mobile phase composition on chromatographic systems

3.2.1. Reversed-phase

Anyone who has ever worked in chromatography knows well that the retention time of a compound depends on the composition of the mobile phase. In an octadecylsilane column, for example, the retention factor of a poorly polar analyte decreases with increasing organic solvent content in the eluent. This phenomenon, translated to the Abraham model (Eq. (1)), must necessarily affect the system coefficients modeling the behavior of the chromatographic system. *A priori* we might not know the extent of the variation for each coefficient, but we can be sure that the sum of the different terms in the equation will lead to a smaller value of the dependent variable log *k*.

Fig. 1A shows the *e, s, a, b*, and *v* values of a reversed-phase Spherisorb ODS-2 column in different acetonitrile-water and methanol-water mixtures [11]. For both organic solvents, a decrease in the positive

Table 3
Preparation of the 90% acetonitrile buffered mobile phase used in this work and measured mean pH values in the aqueous buffer and the eluent throughout column conditioning and chromatographic runs (standard deviation in parentheses).

	Aqueous buffer				Mobile phase
Initial species	pK_a [20]	Conc. (mM)	pH adjusted with	pH	$^{s}_{w}$pH
Oxalic acid	1.25	50	Potassium hydroxide	1.51(0.01)	2.34(0.04)
Chloroacetic acid	2.87	50	–	2.07(0.01)	3.21(0.05)
Citric acid	3.13	50	Potassium hydroxide	3.14(0.01)	4.69(0.01)
Formic acid	3.75	100	Ammonia	2.96(0.01)	5.54(0.02)
				3.44(0.01)	6.16(0.06)
				4.70(0.02)	7.00(0.01)
HEPES[a]	7.56	50	Potassium hydroxide	7.51(0.01)	7.42(0.02)
Ammonium acetate	–	50	–	6.97(0.02)	7.90(0.02)
Ammonia	9.25	50	Acetic acid	10.00(0.10)	8.68(0.09)
Pyrrolidine	11.31	50	Acetic acid	11.20(0.10)	9.86(0.04)
CAPS[b]	10.50	100	Potassium hydroxide	10.38(0.04)	10.03(0.06)
Dimethylamine	10.73	100	Acetic acid	11.40(0.04)	10.13(0.04)

[a] HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid).
[b] CAPS (3-(cyclohexylamino)-1-propanesulfonic acid).

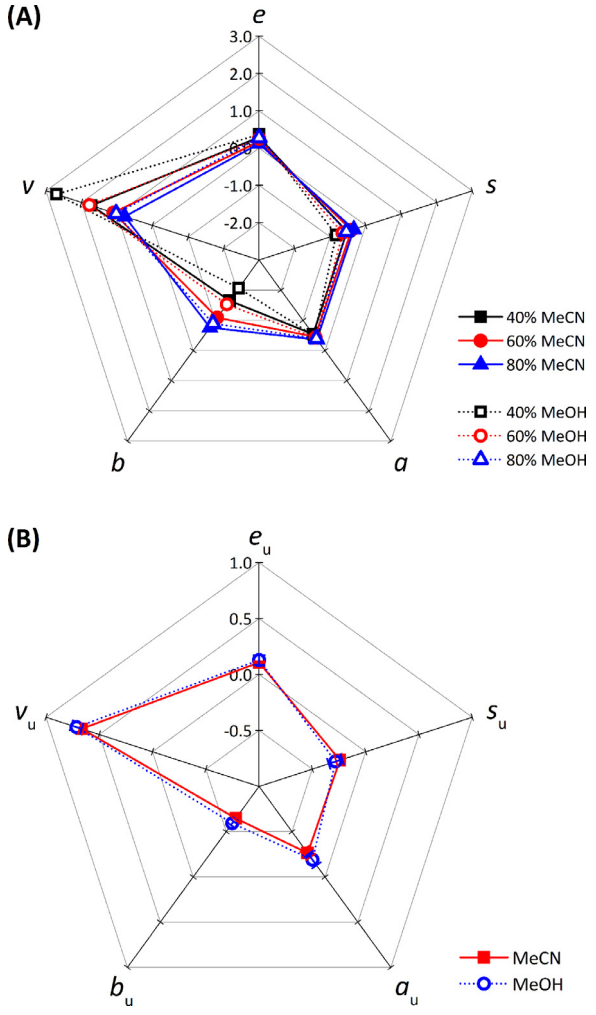

Fig. 1. (A) System coefficients (Eq. (1)) for a Spherisorb ODS-2 column in several acetonitrile- (MeCN) and methanol (MeOH)-water compositions. (B) Mean unitary coefficients for the considered acetonitrile- and methanol-water mobile phases.

coefficients (*e* and *v*) and an increase in the negative ones (*s*, *a*, and *b*) is observed when the proportion of acetonitrile or methanol in the eluent increases, but this variation is more pronounced for *b* and *v*. Notice that these latter descriptors are related to the solute hydrogen bond basicity and the molecular volume, which are the most relevant features affecting retention in C18 columns, as commented in the previous section for acetonitrile-water mobile phases.

At this point, if we want to analyze the contribution of each descriptor within a certain chromatographic system and compare it with another with a different range of log *k* values, we need to apply a normalization procedure. To do so, we divide each coefficient by the square root of the sum of squares of all coefficients (for example, $a_u = a/(e^2+s^2+a^2+b^2+v^2)^{\frac{1}{2}}$), and the resulting system coefficients (e_u, s_u, a_u, b_u, and v_u) are the components of a vector of unitary length [12].

Normalization of the system coefficients presented in Fig. 1A leads to very similar values not only for all compositions sharing the same organic modifier, but also between acetonitrile and methanol. The mean values obtained for acetonitrile- and methanol-water eluents, together with their corresponding standard deviations (error bars), are shown in Fig. 1B. As a practical implication of these results, we can conclude that similar selectivities are expected for this column in the studied range of mobile phase compositions, independently of the water content in the eluent and the election of organic modifier. This behavior has been also observed for several reversed-phase columns and mobile phases containing acetonitrile, methanol, and tetrahydrofuran as organic solvents [13], confirming the relevance of the solute molecular volume ($v_u >> 0$) and hydrogen bond acceptor capacity ($b_u << 0$) in reversed-phase retention.

3.2.2. HILIC

In the following part of the study, we compare the effect of the mobile phase composition on the chromatographic behavior of different HILIC columns using eluents with high contents of acetonitrile and methanol. Fig. 2 shows the obtained Abraham coefficients (*e, s, a, b,* and *v*) for a representative sample of the chromatographic systems considered (full and detailed results are in Table SP2 of the supplementary material). In the case of the underivatized silica column (Kinetex HILIC) we observe that changing acetonitrile to methanol only affects the hydrogen bond acceptor capacity of the chromatographic system (*a*), whereas for the zwitterionic column with polymeric support (ZIC-pHILIC) choosing one organic solvent or the other, or even the water content in the eluent, significantly affects the five system coefficients. The use of acetonitrile or methanol has an impact on the hydrogen bonding features (particularly *b*, but also *a*) and dispersion interactions (*e*) of the aminopropyl (Luna NH2) and the 1,2-dihydroxypropyl (YMC-Triart Diol-HILIC) columns, whereas dipolarity/polarizability coefficient (*s*) seems to remain unaltered.

With the aim of better comparing the behavior of the studied HILIC systems in terms of chromatographic selectivity, we normalized the system coefficients and calculated the average values for each column and type of organic modifier. From these results, presented in Fig. 3, we can

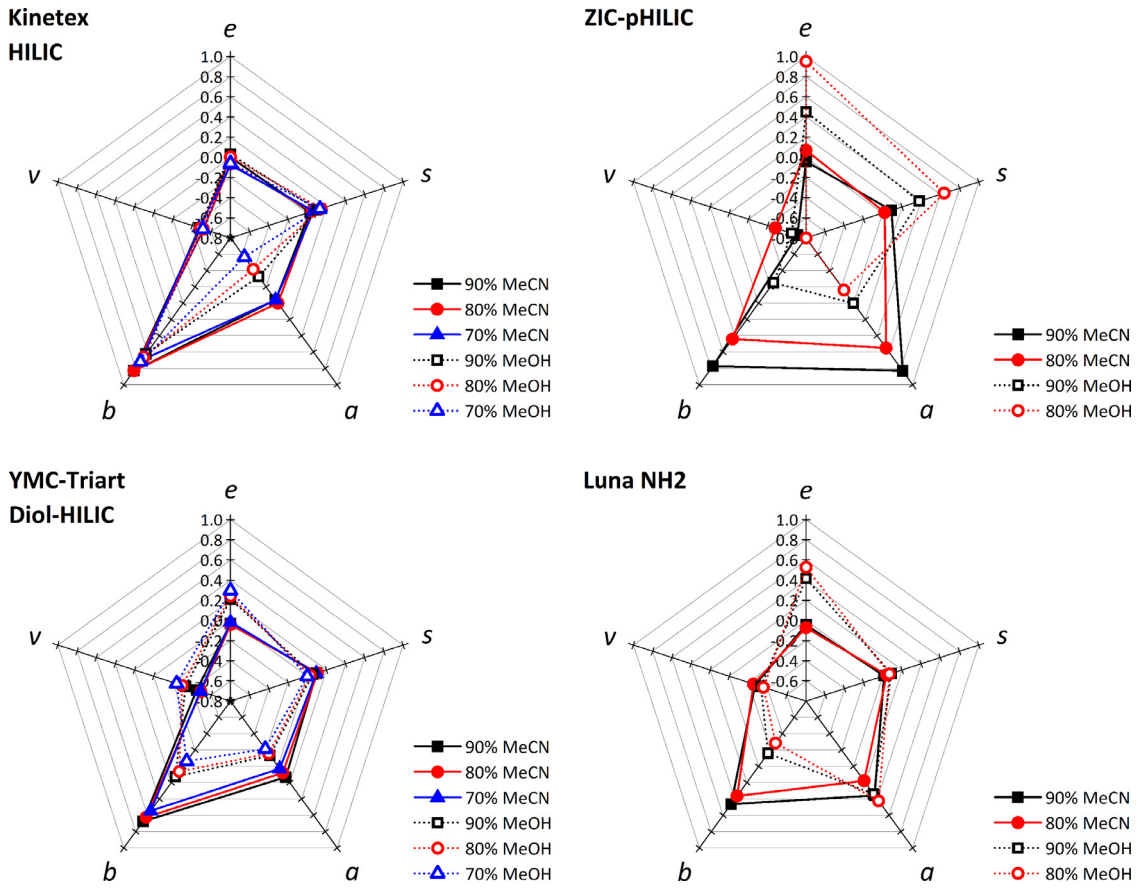

Fig. 2. System coefficients (Eq. (1)) for HILIC columns with different bonded phases in hydroorganic mobile phases containing variable proportions of acetonitrile (MeCN) or methanol (MeOH) as organic solvents.

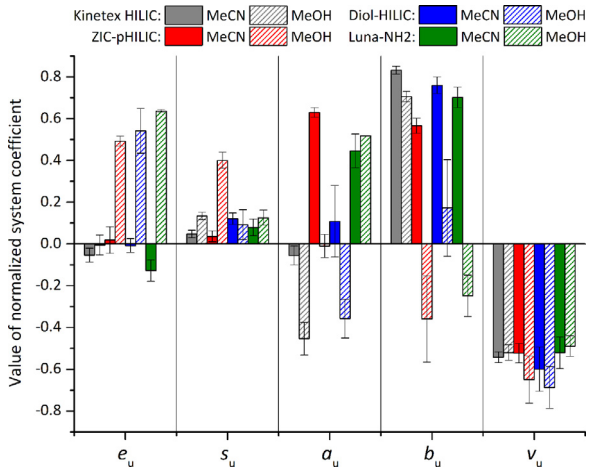

Fig. 3. Mean values of normalized system coefficients for Kinetex HILIC, ZIC-pHILIC, YMC-Triart Diol-HILIC, and Luna NH2 columns in acetonitrile- and methanol-water mobile phases (Table SP2).

draw three generalized observations. Firstly, the magnitude of the standard deviation (error bars) in relation to the mean value is generally small, indicating that little variations in selectivity are expected when tuning the water content in the mobile phase. Secondly, all chromatographic systems, regardless of the column and eluent, have in common a negative cavity coefficient (v) of similar magnitude. This is consistent with the assumed main retention mechanism in HILIC based on the hydrophilic partition of the solute between a water-rich stationary phase and a less cohesive hydroorganic mobile phase. Notice that this is a common feature for unbound silica (Kinetex HILIC), silica with bonded phases (Diol-HILIC and Luna NH2) and polymer with bonded phase (ZIC-pHILIC). Thirdly, dipolarity/polarizability interactions (s) contribute to increase retention, but to a lower extent (except for ZIC-pHILIC and methanol). The effect on retention of dispersion (e) interactions and hydrogen bonding (a and b) clearly depends on the bonded phase and the mobile phase solvent, which points out the importance of secondary interactions between the column functionalization and the solute, or even in the structure and composition of the adsorbed water-rich layers. Therefore, hydrophilic partition might be the main retention mechanism in HILIC, but the role of the bonded phase and support should not be underestimated.

3.3. Chromatographic retention of acid/base compounds in HILIC

In 1977 Horváth, Melander and Molnár described the effect of solute ionization on the retention of weak acids and bases in reversed-phase liquid chromatography [14]. Assuming a partition process of the solute between the mobile and the stationary phase, the retention behavior of a compound with acid/base properties can be modeled according to Eq. (2):

$$k = \frac{k_{HX} + k_X \cdot 10^{pH - pK_a}}{1 + 10^{pH - pK_a}} \quad (2)$$

where k_{HX} is the retention factor of the protonated species of the solute (for instance, HA for a neutral acid or BH^+ for a neutral base) and k_X for the deprotonated one (A^- or B). The degree of ionization is given by the relationship between the mobile phase pH and the pK_a value (acidity constant) of the solute in the particular mobile phase composition.

Therefore, reproducible retention factors of partially ionized compounds can only be achieved if the mobile phase has a constant pH value, and this is obtained by means of buffered mobile phases. This model should also be applicable to HILIC, as long as the retention mechanism is also based on the solute partition between the two chromatographic phases.

Notice that in the preparation of the organic solvent-rich mobile phases used in HILIC we are facing two major challenges. Firstly, ionized species are generally poorly soluble, and low concentrations might lead to insufficient buffering capacities and undesired pH variations throughout chromatographic runs. Therefore, when working under HILIC conditions in isocratic mode, we recommend periodic measurements of the pH of the eluent. Secondly, the pH of an aqueous buffer changes after mixing with the organic modifier, and the final pH of the eluent might be very different from that of the initial aqueous buffer. Due to its accuracy and simplicity of measurement, we propose to use the $^{s}_{w}$pH escale [15–17], where the glass electrode is calibrated with conventional aqueous standards (typically at pH 4 and 7) and the pH is measured in the hydroorganic mobile phase.

In this work we assayed different buffering systems in 90% acetonitrile mobile phases, using the ZIC-HILIC column as a case study, with the aim of covering the widest possible operational pH range [18]. We prepared 50 mM aqueous solutions of several acids and bases and adjusted the pH with concentrated potassium hydroxide, ammonia or glacial acetic acid. The pH was adjusted in the range of pK_a ± 1 unit, in order to have a sufficient buffer capacity even at the low 5 mM buffer concentration of the mobile phase, and it was periodically measured at the detector exit with a glass electrode. If pH readings were not stable (standard deviations higher than 0.1 pH units) and the buffer was sufficiently soluble in the mobile phase, the concentration of the aqueous buffers was raised up to 100 mM. The preparation and measured pH values of the finally selected buffering systems are shown in Table 3. Notice that for neutral acids (oxalic, chloroacetic, citric, and formic) the mobile phase $^{s}_{w}$pH increases in relation to the aqueous one of the same buffer, whereas for the cationic acids (conjugate acids of the neutral bases ammonia, pyrrolidine, and dimethylamine) it decreases. Ammonium acetate solution was prepared just dissolving in water the appropriate weight of salt, without further pH adjustment. HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) and CAPS (3-(cyclohexylamino)-1-propanesulfonic acid) are zwitterionic compounds, and the pK_a value reported in the table is the one corresponding to the basic piperazine and amino groups, respectively. For these buffers the pH shifts to lower values with the addition of acetonitrile, such as for the basic buffer above mentioned, but with smaller variations.

The dependence of retention with mobile phase pH was studied for several acids and bases with pK_a values in the range between 3 and 8. Fig. 4 shows the results of acetylsalicylic acid and lidocaine, which are representative of the retention profiles obtained for all the studied protolytes (Table SP3, Figure SP1 and Figure SP2 in the supplementary material). In contrast to reversed-phase, it can be observed that in HILIC retention increases with the ionization degree of the analytes, this is towards basic pH for acids and acidic pH for bases. However, it is clear at a first glance that an unexpectedly lower retention is observed for some basic buffers in the analysis of acidic compounds, and some acidic buffers in runs involving basic analytes. This might be related with ion pair formation between charged buffering species and ionized analytes, such as chloroacetate and hydrogencitrate with protonated lidocaine, or ammonium, pyrrolidonium and dimethylammonium with acetylsalicylate. The ion pair would present a reduced polarity and a larger volume, leading to a more favorable partition into the mobile phase in relation to the water-rich stationary phase.

In this work, in the neutral to basic pH range, two different buffering systems involving ammonium/ammonia and acetic acid/acetate were assayed. The first buffer ($^{s}_{w}$pH 7.90) was directly prepared from the ammonium acetate salt, whereas the second one ($^{s}_{w}$pH 8.68) was made by addition of small volumes of glacial acetic acid to an ammonia solution. The molar fraction of ammonium is higher in the second buffer, and this seems to support the hypothesis that it is the cationic buffer the responsible species of the unexpected chromatographic behavior of negatively charged analytes. Notice that this lower retention is not observed when uncharged species of bases or acids are chromatographed in these buffering systems.

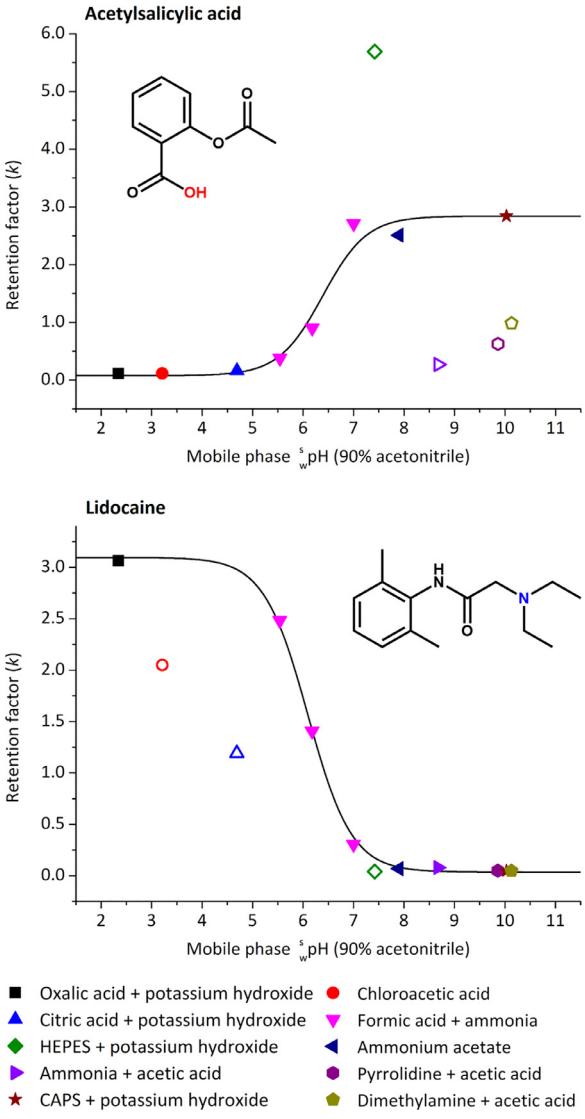

Fig. 4. Dependence of chromatographic retention with the mobile phase $^{s}_{w}$pH and nature of the buffering system for acetylsalicylic acid and lidocaine in a ZIC-HILIC column at 90% acetonitrile. Empty symbols represent data points excluded in the fitting to Eq. (2).

The results obtained with HEPES buffer should be treated with caution, not only because of the abnormal retention it gives rise to for acidic substances, but also because of the different behavior it produces in the chromatographic system. Measured hold-up volumes were about 0.3 mL lower than those determined for the rest of buffers, suggesting that HEPES is affecting somehow the formation of the water-rich layer acting as stationary phase in HILIC or is leading to some kind of exclusion mechanism.

According to the fittings of experimental data to Eq. (2), the pK_a values (referred to the $^{s}_{w}$pH scale) at 90% acetonitrile of acetylsalicylic acid and lidocaine are quite similar, 6.4 and 6.1, respectively. However, notice the pK_a shifts in relation to the aqueous values (3.48 and 7.95), +2.9 for the acid and −1.8 for the base. Although to a different extension, these positive and negative pK_a shifts for acids and bases,

respectively, are observed for the compounds included in the study (with the exception of benzyl nicotinalte, Table SP3).

4. Concluding remarks

Although retention factors in C18 columns clearly depend on the percentage and nature of organic modifier in the mobile phase, similar selectivities can be observed in a wide range of eluent compositions prepared with acetonitrile and methanol as organic solvents. The analyte properties that most affect reversed-phase retention are the hydrogen bond basicity, favoring interactions with the hydroorganic mobile phase and thus reducing retention, and the molecular volume, increasing retention due to a more favorable partition into the less cohesive non-polar bonded phase.

Even though HILIC columns share the same main retention mechanism, the partitioning of analytes into water-enriched layers, their chromatographic behavior, and selectivity largely depend on the nature of the support/bonded phase and the organic solvent used in the eluent. A common feature for the columns studied in this work in acetonitrile/water eluents is the importance of analyte hydrogen bond basicity and molecular volume in retention. These are also the most relevant solute properties in reversed-phase, but in HILIC its impact on retention is just the opposite.

The analysis of acids and bases requires the use of buffered mobile phases, which is not straightforward in HILIC eluents containing a high proportion of organic solvent. Since a pH shift takes place when an organic solvent is added to an aqueous buffer, it is strongly recommended to measure the pH in the hydroorganic mobile phase. The ionized species of compounds with acid/base properties, due to the increase in polarity, are expected to be more retained than the neutral ones. However, some charged buffering species can interact with these ionized compounds, leading to unexpectedly lower retention factors.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Xavier Subirats: Conceptualization, Methodology, Validation, Supervision, Writing – original draft. **Laura Casanovas:** Investigation. **Lídia Redón:** Investigation, Visualization, Writing – review & editing. **Martí Rosés:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition.

Data availability

Data will be made available on request.

Acknowledgements

This work was supported by Grant PID2020-115374GB-I00 funded by MCIN/AEI/10.13039/501100011033. The authors thank YMC Europe GmbH (Dinslaken, Germany) and Dr. Daniel Eßer for providing the YMC-Triart Diol-HILIC column.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sampre.2023.100063.

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