

HILIC characterization: estimation of phase volumes and composition for a zwitterionic column

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Abstract

A methodology for the estimation of the different phase volumes in HILIC is presented. For a ZIC-HILIC column the mobile phase volume (hold-up volume) is determined in several acetonitrile- and methanol-water compositions by a Linear Free Energy Relationships (LFER) homologous series approach involving *n*-alkyl-benzenes, -phenones, and -ketones. We demonstrate that the column works as a HILIC column when the mobile phase contains high and medium proportions of methanol or acetonitrile. However, for acetonitrile contents below 20%, or 40% for methanol, same column works in RPLC. In between, a mixed HILIC-RPLC behavior is observed, and solutes of low molecular volume are retained as in HILIC mode, but the largest ones show RPLC retention. From the homologous series retention data and pycnometric measurements involving the pure organic solvents and their mixtures with water, the mean solvent composition of the water-rich transition layers between column functionalization and the bulk mobile phase, which act as stationary phase, is estimated. Finally, the phase ratio between stationary and mobile phases is also estimated for each eluent composition, allowing the calculation of the corresponding stationary phase volumes. All volumes are strongly dependent on the water content in the eluent, especially when acetonitrile is selected as mobile phase constituent. In HILIC mode, when the water content in the hydroorganic mobile phase increases, the volumes of mobile phase decrease, but the volumes of stationary phase (mainly the water layer adsorbed onto the bonded-phase and the water-enriched interface) increase. However, at high water concentrations, where the column works in RPLC mode, the mobile phase volume increases and the stationary phase (which is now the bonded zwitterion) decrease when increasing the water percentage in the mobile phase.

Keywords

HILIC; LFER; Mobile phase; Phase ratio; Stationary phase; Phase volume

1. Introduction

1.1 HILIC and water uptake by the stationary phase

Hydrophilic interaction liquid chromatography (HILIC) is an especially suitable tool for the determination of polar compounds, such as organic contaminants in environmental samples [1], components in foods [2], pharmaceuticals [3] and biopharmaceuticals [4]. In reversed-phase liquid chromatography (RPLC) these kinds of substances present insufficient retention. Normal-phase liquid chromatography (NPLC) and ion-exchange chromatography (IEX) allow the separation of polar analytes, but polar compounds are often poorly soluble in organic NPLC mobile phases and IEX is only applicable to ionic compounds. Similarly to NPLC, HILIC employs traditional polar stationary phases, but in contrast mobile phases (MP) are similar to those used in RPLC (mixtures of an aqueous buffer with a miscible organic solvent). However, as pointed out in several reviews published in the recent years [5–13], retention mechanisms in HILIC are complex and are currently under investigation. Even the nature of electrolytes play a role on retention and selectivity [14–16].

Polar materials employed as stationary phases (SP) in HILIC present a high affinity for polar solvents, particularly water from the hydroorganic MP. Thus, in organic solvent/water MPs, water is preferentially adsorbed in the surface of the polar stationary phase. These interactions have been widely studied in silica columns with acetonitrile/water MPs [5,17–21]. Surface residual silanols interact much more strongly with water than with acetonitrile and thus water is preferentially adsorbed in the surface, creating water-rich layers. The water adsorption increases when the proportion of acetonitrile increases, with a maximum excess of water around 80/20 (v/v) of acetonitrile/water in the eluent [21]. For eluents with 75-90% of acetonitrile about 4-13% of the pore volume of the silica phase is occupied by the water-rich layer [20]. In fact, molecular dynamics studies with silica and acetonitrile-water mixtures [22,23] revealed the existence of three solvent regions of different composition inside a nanopore: I) a rigid quasi-immobilized water layer at the silica surface; II) a diffuse hydroorganic interface region, enriched in water, of reduced translational mobility between the water layer and the bulk mobile phase; and III) the nominal acetonitrile-water mixed MP. The diffuse hydroorganic layer was found to be dependent on the bulk acetonitrile-water composition. However, since adsorbed water is in dynamic equilibrium there is not a clear separation between these three regions and most likely a gradient of water rich solvent concentration and mobility from the adsorbent surface into the bulk mobile phase is formed [5,22–26]. The layer in the support surface is mostly strongly adsorbed water with very reduced mobility, but water adsorption decreases and mobility increases in the consecutive layers approximating composition and mobility of the flowing mobile phase. All these layers are labile and can be interchanged with the flowing mobile phase, but they have a variable reduced mobility in reference to the one of mobile phase. Thus, they act as stationary phase because solute in these layers is delayed in reference to the flowing mobile phase.

The behavior is comparable to the one of the charged micelles or microemulsions used as pseudo-stationary phases in micellar or microemulsion electrokinetic chromatographies.

As postulated by Alpert in his seminal HILIC study [27], it is usually assumed that the main retention mechanism is the partition of solutes between the bulk MP and all these water-enriched labile layers. However, other interactions (e.g. electrostatic) can take place between the solute and the fully immobilized stationary phase. In fact, the silica surface or the functionalized silica or the polymeric support can also interact with the solute and a dual hydrophilic interaction normal-phase and reversed-phase liquid chromatography mechanism has been proposed [11,13,28]. For several compounds and HILIC columns, a decrease of retention with the increase of the water content in organic-rich MP compositions have been observed (HILIC retention), in contrast with the retention increase in the low organic solvent compositions (RPLC retention). This dual mode produces a U-shaped curve in the plot of retention ($\log k$) vs. MP composition.

The aim of the present study is the proposal of a methodology in order to characterize the behavior of columns in HILIC, particularly the composition and volumes of the eluent and the water-enriched labile stationary phase layers inside the column, and their relations with the volume of stationary phase. This methodology is tested in a common HILIC zwitterionic column (ZIC-HILIC), based on a sulfobetaine phase covalently attached to porous silica, in the full range of acetonitrile/water and methanol/water mobile phases compositions.

1.2 Measurement of solvent volumes inside the column

In 2008 McCalley and Neue [20] used a pycnometric method for the measurement of the volume of solvent in HILIC silica columns using pure solvents of significantly different densities, i.e. water and methanol or acetonitrile, which can be applied to our zwitterionic column as follows.

The measured weight of the column filled with a solvent (w_{column}) is the sum of a constant contribution due to the weight of the column tube, endfittings and SP packing (w_{constant}), and a variable weight corresponding to the labile solvent filling the column (w_{solvent}), which is the volume of water being replaced by the organic solvent when the column is successively filled and weighed with the two solvents. If the column is not purged enough with the organic solvent, some of the adsorbed water may remain in the column and will be considered as part of the w_{constant} term. The labile solvent inside the column is replaced by the new flowing MP, and since the weight of this labile solvent depends on its density (ρ_{solvent}) and volume (V_{solvent}), the following expression can be derived:

$$w_{\text{column}} = w_{\text{constant}} + w_{\text{solvent}} = w_{\text{constant}} + V_{\text{solvent}} \cdot \rho_{\text{solvent}} \quad (1)$$

From this equation it follows that the labile solvent volume (V_{solvent}) can be measured from the weights of the column filled from the two different solvents (usually one of them water) according to:

$$V_{\text{solvent}} = \frac{w_{\text{column,water}} - w_{\text{column,organic}}}{\rho_{\text{water}} - \rho_{\text{organic}}} \quad (2)$$

where $w_{\text{column,water}}$ and $w_{\text{column,organic}}$ are the weights of the same column after being consecutively purged with water and a less dense organic solvent, and their corresponding densities (ρ_{water} and ρ_{organic} , respectively). McCalley and Neue found very similar solvent volumes for Waters Atlantis and AMT Halo columns when either acetonitrile or methanol were used as organic solvents [20].

In fact, as introduced before, the V_{solvent} value obtained by Eq. (2) is the overall volume of solvent that can be interchanged between water and the organic solvent. Thus, in HILIC organic solvent/water eluents, the labile solvent volume above mentioned (V_{solvent}) would comprise the hydroorganic MP flowing through the column and the labile mixed layers enriched in water between the MP and the surface of the bonded phase. These water-rich labile layers will act as the main stationary phase in HILIC conditions. In case of RPLC behavior, where the silica or bonded phase is expected to be the unique SP, the water-enriched eluent is expected to occupy the whole solvent volume inside the column. Figure 1 shows a schematic representation of these regions inside a chromatographic column in HILIC (Fig. 1A) and RPLC (Fig. 1B) modes.

V_{solvent} is often considered an estimation of the hold-up volume (or void volume), which corresponds to the volume of flowing eluent inside the column (V_M). Although the volume of solvent measured by pycnometry can be a good estimation of V_M for RPLC [29], where water and the organic solvent behave simply as the MPs and the bonded phase as the SP, this may be not especially right for HILIC. In HILIC conditions V_{solvent} will include the volume of the mobile phase (V_M) and the volume of the adsorbed water-rich layers acting as labile stationary phase.

The study of the volume and composition of these HILIC regions, their relationships with V_{solvent} and V_M and their dependence on the mobile phase composition is one of the further goals of this work. Pycnometric (V_{solvent}) and chromatographic data with homologous series (V_M) along all mobile phase compositions will be combined to determine these different volume parameters and estimate the mean composition of the adsorbed water rich layers HILIC stationary phases (labile stationary phase). Estimation of the phase ratio will also allow calculation of the overall volume of stationary phase which will comprise the labile stationary phase and the immobilized (bonded) stationary phase (which may act in different degrees depending on the preponderance of HILIC or RPLC retention mechanisms).

1.3 Measurement of hold-up volumes

A common alternative way of measuring the hold-up volume is by injecting a suitable unretained marker in the chromatographic system. This is the case, for instance, of inorganic salts in RPLC. In HILIC these salts cannot be used because of its strong polarity and several studies propose

toluene as hold-up volume marker due to its non-polar hydrophobic nature [15,20,24,30–32], assuming that it is unable to penetrate into the water layer adsorbed on the surface of the bonded phase. However, this is a simplified approximation since toluene was found to be more retained in a ZIC-pHILIC column than many other compounds in acetonitrile-water and methanol water MPs [33]. Extrapolation from retention volumes of the compounds of homologous series is an alternative for better hold-up volume determination. Therefore, in the present work hold-up volumes are measured for each studied MP composition using a homologous series approach derived from Abraham’s solvation model [33,34]. Briefly: since all the homologues in a series share nearly the same properties (polarity, polarizability, hydrogen bonding...), except for the molecular volume, retention can be modelled as:

$$V_R = V_M + r \cdot 10^{\nu V} \quad (3)$$

where V_R is the retention volume of the homologue, V_M is the hold-up volume (the flowing MP volume), r and ν are constant values depending on the particular chromatographic system and homologous series, and V is the McGowan characteristic volume of the homologue (in units of mL mol⁻¹/100). r depends on the dispersion, dipole-dipole, dipole-induced dipole, polarizability and hydrogen bond interactions [34], which are similar for all members of the homologous series, but different for each series. The coefficient of the McGowan volume (ν) measures the endoergic work of separating solvent molecules to provide a cavity of suitable size for the solute molecule, and thus for a particular solvent this energy depends only on the size of the solute. Solutes of large volume favors the transfer from polar to less polar solvents. In RPLC, the creation of a cavity in the non-polar SP is less energetically demanding than in the polar MP. As a consequence, big solutes tend to partition into the SP, increasing thus their chromatographic retention, leading positive values of ν . The opposite trend is observed in HILIC, provided that the labile water-rich SP is more cohesive than the flowing MP, and therefore ν takes negative figures.

1.4 Estimation of the phase ratio

In 2015 Moldoveanu and David [35] proposed a procedure for the estimation of the ratio between the volume of the stationary phase (V_S) and the void volume (V_M) of the column (phase ratio, Φ) in reversed-phase liquid chromatography, based on the solvophobic theory of interactions in solution. In summary, the constant governing the partition equilibrium between two solvents mainly depends on the geometry of the solute [36]:

$$\log K_i = aA_i - b_i \quad (4)$$

where A_i is the van der Waals surface area of the solute and a is approximately constant for a particular partition system. b is a correction of the molecular surface in case the solute includes polar functional

groups, which are assumed to not contribute to solvophobic interactions. Its value is zero for hydrocarbons and presents specific values depending on the polar groups of the molecule (OH, C=O, COOH, NH₂, NH, Cl, NO₂...) and the partition system. Assuming that the main chromatographic retention mechanism is the partition of the analytes between the stationary and the mobile phases, the retention factor (k_i) can be expressed as:

$$k_i = K_i \Phi \quad (5)$$

where K_i is the partition constant of the analyte between both chromatographic phases, and Φ is the phase ratio ($\Phi = V_s/V_M$). Combining Eqs. (4) and (5):

$$\log k_i = (aA_i - b_i) + \log \Phi \quad (6)$$

In the case of aromatic hydrocarbons, assuming that the b value in Eqs. (4) and (6) is very close to zero for aromatic hydrocarbons [35,37], a system of two equations can be set relating the octanol/water partition system ($\log P_{o/w}$) with the chromatographic retention ($\log k_{\text{chrom},i}$) measured with a particular column and mobile phase composition:

$$\begin{cases} \log P_{o/w,i} = a_{o/w} A_i \\ \log k_i = a_{\text{chrom}} A_i + \log \Phi \end{cases} \quad (7)$$

Provided that the same compound is tested in both partition systems, the van der Waals surface area (A_i) can be eliminated and Eq. (7) takes the form of:

$$\log k_i = (a_{\text{chrom}}/a_{o/w}) \log P_{o/w} + \log \Phi \quad (8)$$

This equation should allow the estimation of the phase ratio of a particular chromatographic system from the measured retention factors of alkyl benzene homologues of well-known octanol/water partition ratios, since $\log \Phi$ would be the intercept in the linear regression. This methodology was used for the determination of $\log \Phi$ values for three C18 columns in mobile phases containing acetonitrile and methanol as organic modifiers, using benzene, toluene, ethyl-, propyl-, and butylbenzene as test compounds [37].

2. Materials and methods

2.1 Instrumentation

The HPLC instrument, manufactured by Shimadzu (Kyoto, Japan), consisted of two LC-10ADvp pumps, an SIL-10ADvp auto-injector, an SPD-M10AVvp diode array detector, a CTO-10ASvp oven set at 25 °C, and an SCL-10Avp controller. The system was controlled by LCSolutions software from Shimadzu. Extra-column volume (i.e. the volume between the injection and the detection points, excluding the column) was 0.118(±0.004) mL; this was subtracted from gross retention volumes in order to eventually allow the accurate comparison of data obtained from different instruments.

The studied column was a ZIC-HILIC from Merck (Darmstadt, Germany), 3.5 μm , 150 x 4.6 mm.

The analytical balance used in pycnometric measurements was a AT 261 DR from Mettler-Toledo (Columbus, Ohio, US) with an uncertainty at the sample amount (column weight of about 38 g) of 1 mg. The balance is located in a climatized room (22 ± 2 °C, 50 ± 5 % humidity) and yearly calibrated by an accredited calibration laboratory (Mettler-Toledo, Spain).

2.2 Methods and chromatographic conditions

Extra-column volume was determined by injecting 0.5 mg mL^{-1} aqueous solution of potassium bromide (Baker, >99%) in absence of column and using not only water as eluent, but also several acetonitrile/water and methanol/water mixtures. The injection volume was set 1 μL for all analytes the mobile phase flow rate was 0.5 mL min^{-1} in all cases. The column was equilibrated for at least 20 min before the first injection, then the compounds of the three homologous series were injected sequentially, one after the other, and finally replicates were obtained following the same procedure. No significant differences between replicates were observed.

Pycnometric measurements were carried out after two hours of purging the column with water, acetonitrile, and methanol (either pure or mixed) at a flow rate of 0.5 mL min^{-1} and 25 °C. After purging, the column was immediately capped with its corresponding endfittings and weighed in a calibrated analytical balance.

2.3 Chemicals and solvents

The injected *n*-alkyl benzenes, phenones, and ketones were purchased from Acros Organics, Alfa Aesar, Fluka, Merck, and Sigma-Aldrich, all of high purity grade ($\geq 97\%$). Stock solutions of the injected analytes were prepared in methanol at a concentration of 5 mg mL^{-1} . *n*-Alkyl ketones were directly injected, but *n*-alkyl benzenes and -phenones were diluted with methanol to 0.5 mg mL^{-1} before injection.

Water was obtained from a Milli-Q plus system from Millipore (Billerica, USA) with a resistivity of 18.2 M Ω cm. The organic modifiers used as mobile phase, acetonitrile and methanol, were HPLC gradient grade and from Fisher and Panreac.

2.4 Calculation

Fitted coefficients were optimized by using the MS ExcelTM macro "Ref_GN_LM", which is based on the Levenberg-Marquardt modification of the Gauss-Newton non-linear least-squares iterative algorithm [38].

3. Results and discussion

3.1 Measurement of solvent volume inside the column

First, the overall labile solvent volume inside the column was pycnometrically determined by means of Eq. (1) using water, acetonitrile, and methanol as pure solvents. Their respective densities at 25 °C are 0.9971 g mL⁻¹, 0.7766 g mL⁻¹ [39], and 0.7866 g mL⁻¹ [40]. The solvent volume measured from differences in water and acetonitrile was 1.693 mL, and 1.689 mL when methanol was used as the low density solvent. Virtually the same volume was found for both organic solvents in our zwitterionic functionalized silica column, which is consistent with McCalley and Neue findings for underivatized silica SPs [20]. Since the total volume inside the column (labile solvent + SP and support) can be easily calculated from the dimensions of the empty tube ($\pi \cdot (0.46/2)^2 \cdot 15 = 2.49$ mL), a mean solvent volume of 1.69 mL suggests that nearly the 68% of the total column volume is occupied by the labile solvent.

Secondly, in addition to the pycnometric measurements with pure solvents above mentioned, hydroorganic mixtures of different compositions in the range between 50 and 90% of acetonitrile or methanol (in volume), commonly used as mobile phases, were considered. The densities corresponding to each mixed solvent were calculated by interpolation from experimental literature data measured at 25 °C [39,40]. According to Eq. (1), a straight line should be obtained when the weight of the column is plotted against the density of the mobile phases if the solvent volume (1.69 mL) remains constant. As shown in Fig. 2, the column weight after purging the column with methanol-water mixtures was consistent with the solvent volume measured pycnometrically with pure solvents, since all experimental points (filled squares in the figure) lay very close to the dashed straight line with a slope of 1.69 mL (mean calculated V_{solvent} value according to Eq. (2)). This suggests that the mean solvent composition inside the column is very similar to that of the flowing MP. In contrast, the column is heavier than expected (according to the MP composition) when acetonitrile is part of the eluent. This indicates an enrichment of the mean solvent composition in the more dense solvent, i.e. water, in relation to the flowing eluent. This enrichment must be in the adsorbed water-rich layers of the labile stationary phase with a reduced mobility in reference to the free flowing eluent of the mobile phase.

3.2 Measurement of flowing mobile phase (hold-up volume)

Hold-up volumes were measured using an approach derived from Eq. (3) and involving three homologous series in a single model:

$$V_R = V_M + \sum_{i=1}^n (r_i \cdot f_i) \cdot 10^{v \cdot V} \quad (9)$$

where n is the number of homologous series included in the model, and f_i are binary flag descriptors (1 or 0) used as independent variables in the fitting (i.e. for homologues of i -th series, $f_i = 1$ and $f_{i \neq 1} = 0$). Analysis of all homologous series data in one single equation gives more precise and reliable hold-up volumes than doing them from the separated series. In this work n -alkyl benzenes, n -alkyl phenones, and n -alkyl ketones have been considered as complementary homologous series for the measurement of hold-up volumes, since they show different degrees of interactions with solvent molecules regarding polarizability contributions from n - and p -electron pairs (E Abraham descriptor), dipole-type interactions (S), and hydrogen bond donation from solvent to solute (B) (Fig. 3 and Table S1 in supplementary material). As already described in section 1.3, the different degree of these interactions will produce different values of the r parameter, one for each homologous series, which results in different degrees in the convex curvature of the V_R vs. V plot. The sign of the ν parameter determines the type of retention [33]. If $\nu < 0$, the curve decreases because an increase in volume results in a decrease in retention (HILIC retention). As an example of HILIC behavior, Fig. 4 shows for two different MPs (80% acetonitrile and 80% methanol) the retention vs. molecular volume for the three series of homologues. Retention volumes decrease with the molecular size of the homologues, since the energy required for the creation of a cavity in the water-rich SP increases with the volume of the solute, reducing thus the partition into the SP and consequently the chromatographic retention. The reverse trend is observed in RPLC conditions, where $\nu > 0$ and retention increases with the molecular size of the homologues [33]. Hence, the V_R vs. V plot increases. In the studied column, this RPLC behavior is clearly observed for the organic mobile phases with low content of organic solvent ($< 20\%$ of acetonitrile or $< 40\%$ of methanol)

For certain intermediate mobile phase compositions a U shape is observed when plotting chromatographic retention against the molecular volume of the homologues. This clearly suggests a mixed retention mechanism: mostly HILIC behavior for the smallest solutes (reduction of retention with analyte size) and a RPLC trend for the largest ones (the higher molecular volume, the higher retention). These trends can be observed for the studied ZIC-HILIC column in mobile phases containing around 20% acetonitrile or 40% methanol (Fig. 5). In this case, assuming that the mobile phase volume should be the same for both retention modes, Eq. (9) needs to take into account both HILIC and RPLC contributions to the retention volume:

$$V_{R(\text{HILIC}+\text{RPLC})} = V_M + \sum_{i=1}^n (r_{\text{HILIC},i} \cdot f_i) \cdot 10^{\nu_{\text{HILIC}} \cdot V} + \sum_{i=1}^n (r_{\text{RPLC},i} \cdot f_i) \cdot 10^{\nu_{\text{RPLC}} \cdot V} \quad (10)$$

where the subscripts HILIC and RPLC refer to the solutes of the series following HILIC or RPLC behavior, respectively. Figure 5 shows examples of the U-shaped curves due to the additive contribution of both the HILIC and RPLC retention mechanisms (Eq. (10)), in this case for the phenone homologues at 20% acetonitrile and 40% methanol. Plots for other mobile phase

compositions are also presented to show the evolution from pure HILIC (for instance, 80%) to mixed HILIC-RPLC and to RPLC behaviors (10-20%) when the water content in the mobile phase increases.

When the content of organic solvent is larger than 50% acetonitrile or 70% methanol only the HILIC behavior is apparent, but when the organic solvent content decreases the solutes with larger volumes become to show RPLC behavior. For instance, at 50% acetonitrile and 70% methanol dodecylbenzene was excluded from HILIC correlations because it is more retained than the smaller homologue octylbenzene and deviates markedly from the fitting. For lower organic solvents concentrations (40-30% acetonitrile, 60-50% methanol), more large solutes (octylbenzene, decanophenone, pentadecanone, ...) were deviating and thus excluded from HILIC correlations. This can be interpreted as the emergence of a RPLC mechanism on the basis of the predominant HILIC mode, triggered by the relatively high content of water in the eluent [13].

The V_M values and the rest of the coefficients fitted in Eqs. (9) and (10) for the range of organic solvent/water (v/v) MP compositions between 10 and 100% of acetonitrile and between 20 and 100% of methanol are presented in Table S2 of the supplementary material. The Table also indicates the observed HILIC, RPLC or mixed behavior in the homologous series. The ZIC-HILIC column follows a mainly HILIC behavior for mobile phase organic modifier content higher than 30% for acetonitrile but only higher than 60% for methanol. For mobile phases with high water content (< 20% of acetonitrile, or < 40% of methanol) the column behaves as a reversed-phase column.

Figure 6 summarizes the variation of hold-up volumes with the compositions of the studied mobile phases. V_M value obtained for pure acetonitrile as mobile phase (1.55 mL) is slightly lower than the V_{solvent} value of 1.69 mL, suggesting that there may be a tiny layer of water or acetonitrile-water of reduced mobility between the fully immobilized water layer and the acetonitrile bulk mobile phase, which was not fully removed when purging the column. Notice the hold-up volume decreases in the HILIC and mixed regions when the water content of the acetonitrile/water mobile phase increases (from 100% to 30% of acetonitrile), likely because the increase in water content increases the thickness and volume of the labile stationary phase and thus decreases the mobile phase volume. However, for methanol/water the hold-up volume in the corresponding regions (from 100% to 50% of methanol) is rather constant, probably because the higher similarity of methanol to water keeps the volume of the adsorbed labile stationary phase more constant. In both mobile phases a sharp increase of the hold-up volume is observed when the solvent composition reaches the RPLC region. The hold-up volume when the column acts in reversed-phase is even somewhat larger than the V_{solvent} value of 1.69 mL. In RPLC, the stationary phase is the zwitterionic bonded phase, without labile stationary phase, and thus all the solvent volume in the column is available as mobile phase.

3.3 Measurement of the mean solvent composition of the labile stationary phase transition layers between column functionalization and bulk mobile phase

The hold-up volumes of the eluents containing 50%, 80% and 90% of organic solvent are especially relevant in order to find the differences between the total labile volume inside the column (pycnometrically measured for these compositions in the previous section) and the volume of the flowing MP.

The volume of HILIC labile stationary phase (V_L) can be estimated from the difference between the overall labile solvent volume inside the column (V_{solvent} , pycnometrically determined – section 3.1) and the mobile phase (or hold-up) volume (V_M , estimated from homologous series – section 3.2):

$$V_L = V_{\text{solvent}} - V_M \quad (11)$$

On the other hand, the labile solvent weight inside the column (w_{solvent} , measured with an analytical balance after column equilibration) should consist of the weight of the bulk mobile phase (w_M) and the weight of the labile stationary phase (w_L):

$$w_{\text{solvent}} = w_M + w_L = \rho_M V_M + \rho_L V_L \quad (12)$$

where ρ_M and ρ_L are the densities of the flowing mobile phase and HILIC labile stationary phase, respectively. The former can be easily known because it is the density of the eluent. The latter, which will provide information about the composition of the labile stationary phase, can be estimated combining Eqs. (11) and (12):

$$\rho_L = \frac{w_{\text{solvent}} - \rho_M V_M}{V_{\text{solvent}} - V_M} \quad (13)$$

Finally, from this ρ_L solvent density it is possible to estimate the mean acetonitrile- or methanol-water composition of all these water-rich layers of labile stationary phase.

This procedure has been employed to calculate the mean solvent composition of the HILIC labile SP layers in MPs containing acetonitrile or methanol in the range between 50 and 100% (Table 1 and Fig. 7). Details in the calculation of mean solvent compositions are given in the supplementary material (Table S3).

In the presence of water in the mobile phase, the mean organic solvent content in the labile stationary phase layers is always lower than that of the bulk eluent. In the case of acetonitrile, for eluents with only a 10-20% of water a mean value of water above 50% is calculated for the labile stationary phase layers, which means a water excess in relation to the flowing mobile phase beyond 40%. Then the water content increases gradually with the eluent composition, but reducing the excess to a 24% when a 50% of water is used in the MP. These results are in good agreement with the ones obtained by Gritti et al. [21] from the adsorption isotherms for acetonitrile/water and a HILIC silica

column. When methanol is employed the water excess is found to be much smaller, below 13% in the studied range of eluent compositions.

3.4 Estimation of phase ratios

Phase ratios (Φ) were estimated for the ZIC-HILIC column in several acetonitrile- and methanol-water compositions using *n*-alkyl benzenes by means of two different procedures. Firstly, according to the methodology proposed by Moldoveanu in the characterization of RPLC columns [37] (Eq. (8)), and secondly by means of Eq. (6) but with the assumption of a zero b_i value for aromatic hydrocarbons. The experimental octanol-water partition ratios and calculated van der Waals surface areas for the *n*-alkyl benzenes used in the study are presented in Table 2. The detailed results of the fittings are presented in Table S4 (supplementary material) and Fig. 8 shows the estimated phase ratios at different eluent compositions. Very similar $\log \Phi$ values were obtained when using either the van der Waals surface or the octanol-water partition ratio as independent variables in the fittings. With the exception of the eluents with the smallest content of organic solvent (10% acetonitrile and 20% methanol) the fitted slopes for both equations were negative, indicating a main HILIC mode for the tested alkyl benzenes. From these calculated phase ratios and the mobile phase volumes (V_M , section 3.2) reported in Tables S2), the volume of stationary phase (V_S) for each chromatographic system can be estimated, since $V_S = V_M \cdot \Phi$. Results are presented in Table S5 of the supplementary material.

In the case of acetonitrile, the phase ratio is about 0.15 in the range comprised between 0 and 10% of water in the eluent, and then it progressively increases up to about 0.7 at 70% water, followed by a sharp decrease. A similar trend is observed for methanol, but less marked, with maximum phase ratio values between 50 and 60% of water in the eluent. This behavior is in good agreement with the results obtained for V_M . When in HILIC mode the water content of the eluent increases, the volume of water immobilized as SP increases as well and consequently the volume of mobile phase (V_M) inside the column decreases. In addition, in gaining importance the RPLC mode with the water content in the MP, interactions between solutes and the sulfobetaine bonded phase might also be expected. The overall effect is an increase of the phase ratio and the volume of the stationary phase. In the studied range of eluent compositions, V_S values range from about 0.2 to 0.8 mL for acetonitrile/water and from 0.3 to 0.8 mL for methanol/water. In RPLC mode, the SP is only the bonded zwitterionic sulfobetaine and all solvent volume becomes mobile phase. Maximum of stationary phase is obtained when combined HILIC and RPLC mechanisms actuate at the same time (around 20% acetonitrile and 40-50% methanol).

Fig. 9 shows the variation of V_S , as well as V_M , for acetonitrile and methanol. The overall volume of solvent and stationary phase reaches a maximum when the dual HILIC-RPLC retention

mechanism is clearly observed (20% acetonitrile and 40% methanol), and these maximum combined values of mobile and stationary phases volumes are 2.25 mL for acetonitrile and 2.28 mL for methanol mobile phases. Since the geometrical inner volume of the empty column is 2.50 mL, this means that about 0.25 mL corresponds to the support of the bonded zwitterionic phase. When the HILIC mechanism is clearly predominant in MPs containing higher proportions of organic solvent, the bonded zwitterionic phase is probably acting only as a support of the labile stationary phase.

Conclusions

The sulfobetaine based zwitterionic column works mostly in HILIC mode for high and medium contents of organic solvent for methanol/water (> 60% methanol) and acetonitrile/water (> 30% acetonitrile) mobile phases. However, for low organic solvent contents in the mobile phase (< 40% of methanol or < 20% acetonitrile), the same column works in RPLC mode. A mixed HILIC-RPLC behavior is clearly observed for intermediate mobile phase compositions.

It is widely assumed that in HILIC the main retention mechanism is based on the partition of analytes between the mobile phase and a water rich stationary phase formed by consecutive layers of of variable composition and mobilities between that of the mobile phase and the immobilized stationary phase support. Combination of our measurements by pycnometry and retention of homologous series confirms the presence and the stationary phase role of these hydroorganic layers of reduced mobility, and allows the calculation of its mean composition. When acetonitrile is used as eluent, these labile stationary phase layers are significantly enriched in water in relation to the mobile phase composition, up to a 50% of water for 90% acetonitrile mobile phase (where there is only a 10% of water). For methanol/water mobile phases, the excess of water in the labile stationary phase layers is much smaller, below 13% of excess water in reference to the water content in the mobile phase.

The volumes inside the column of the flowing hydroorganic mobile phase (hold-up volume) and the labile stationary phase water-rich layers depend on the eluent composition, affecting in turn the volume of overall stationary phase (bonded sulfobetaine + reduced mobility labile layers). When the column works in HILIC conditions (high and medium contents of organic solvent), the volume the stationary phase increases with the proportion of water in the eluent, and consequently the mobile phase volume decreases. This variation is much higher for acetonitrile/water than for methanol/water mobile phases. A sharp increase of mobile phase volume (and decrease of stationary phase volume) is observed when the proportion of organic solvent in the mobile phase decreases enough to reach the RPLC behavior. In this instance, the labile water-rich layers are replaced by the similarly water-rich flowing eluent and only the bonded phase (sulfobetaine) remains as stationary phase.

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.

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TABLES

Table 1

Mean composition of the transition layers between the bulk mobile phase and stationary phase.

Mobile phase composition (v/v)	ρ_M^a (g mL ⁻¹)	V_{solvent}^b (mL)	V_M^c (mL)	w_{solvent}^d (g)	ρ_L^e (g mL ⁻¹)	Transition layers composition (v/v) ^f
Acetonitrile	100%	0.7766	1.548	1.314	0.7766	100%
	90%	0.8040	1.693	1.383	1.395	46%
	80%	0.8316		1.278	1.450	37%
	50%	0.9056		1.219	1.556	26%
Methanol	100%	0.7866		1.454	1.329	0.7866
	90%	0.8221	1.689	1.474	1.392	86%
	80%	0.8528		1.480	1.448	67%
	50%	0.9231		1.453	1.563	42%

^a From refs. [39,40]; ^b Section 3.1; ^c From Table S2 (supplementary material) according to section 3.2; ^d Determined from Eq. (1) (w_{constant} measured for acetonitrile and methanol MPs were 36.737 and 36.741 g, respectively); ^e Eq. (13); ^f $\%(\text{acetonitrile}) = -38.4\rho_L^3 + 95.8\rho_L^2 - 83.3\rho_L + 25.9$, $\%(\text{methanol}) = -41.1\rho_L^3 + 96.0\rho_L^2 - 77.6\rho_L + 22.6$.

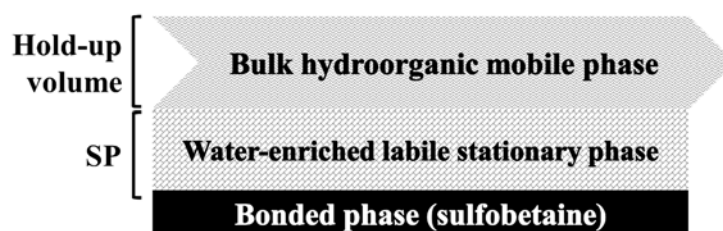
Table 2

Calculated van der Waals surface areas [41] and experimental octanol-water partition ratios [42] of the solutes employed for the estimation of phase ratios.

Compound	Van der Waals surface area (Å ²)	log $P_{o/w}$
Benzene	135.86	2.13
Toluene	168.11	2.73
Ethylbenzene	198.54	3.15
Propylbenzene	229.24	3.72
Butylbenzene	259.85	4.38

FIGURES

(A) HILIC MODE



(B) RPLC MODE

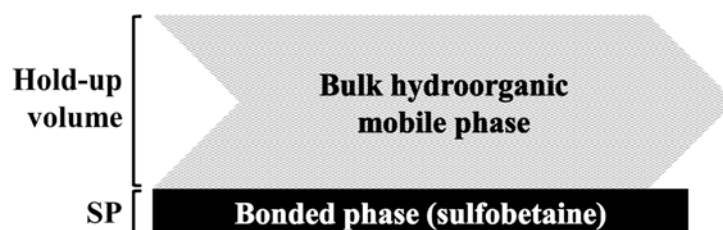


Fig. 1. Schematic representation of the different partitioning regions inside the ZIC-HILIC chromatographic column in: (A) HILIC mode and (B) RPLC mode.

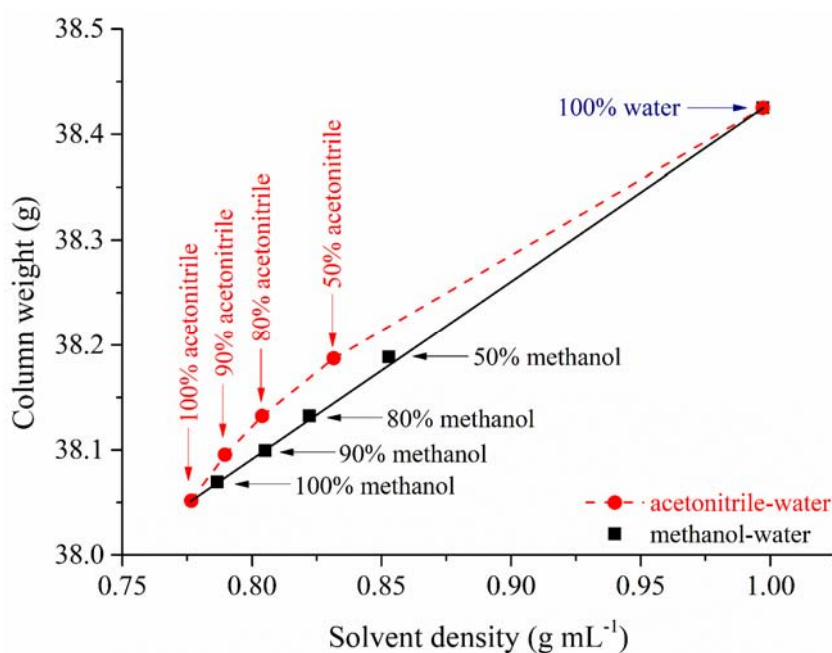


Fig. 2. Variation of total column weight (+ endfittings) with the eluent composition (water, acetonitrile and methanol, and hydroorganic mixtures at 90, 80, and 50% in volume of organic solvent). The continuous straight line was calculated from the pure solvents, water, acetonitrile, and methanol (slope of 1.69 mL).

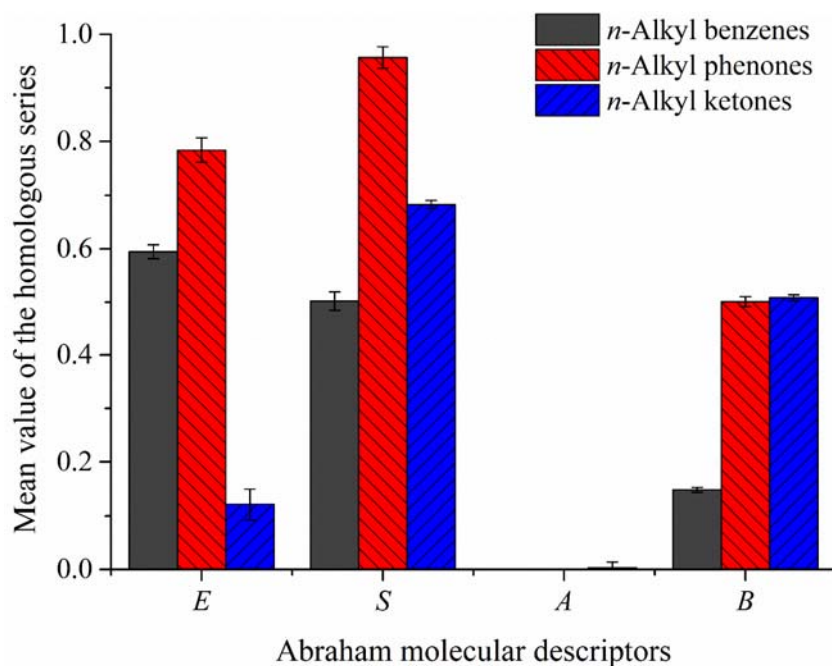


Fig. 3. Mean values of Abraham molecular descriptors [43] of the homologous series used for hold-up volume measurement: excess molar refraction (*E*), solute dipolarity-polarizability (*S*), solute hydrogen-bond acidity (*A*) and basicity (*B*). Standard deviation bars for the mean of the values of the homologous series members also included.

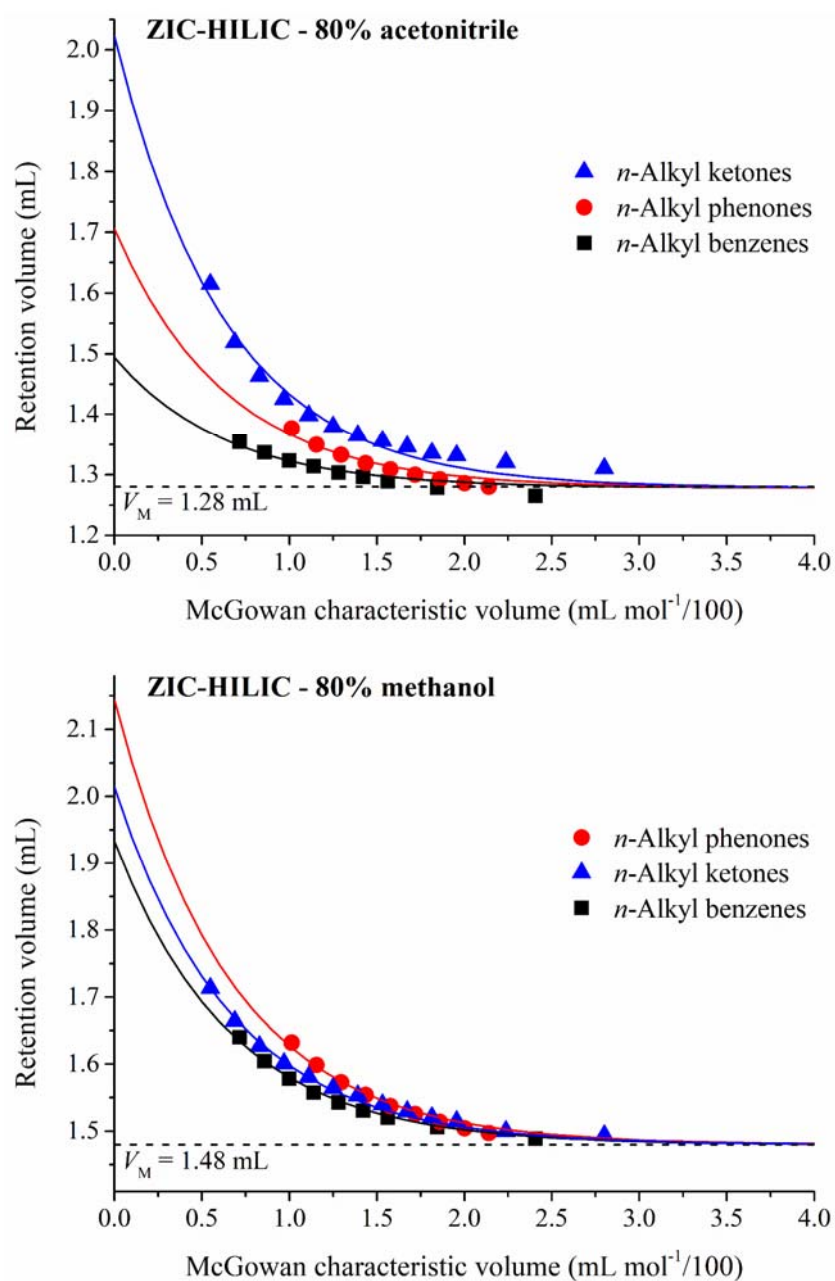


Fig. 4. Examples of hold-up volume measurements from retention data of homologous series (Eq. (9)) showing a typical HILIC behavior.

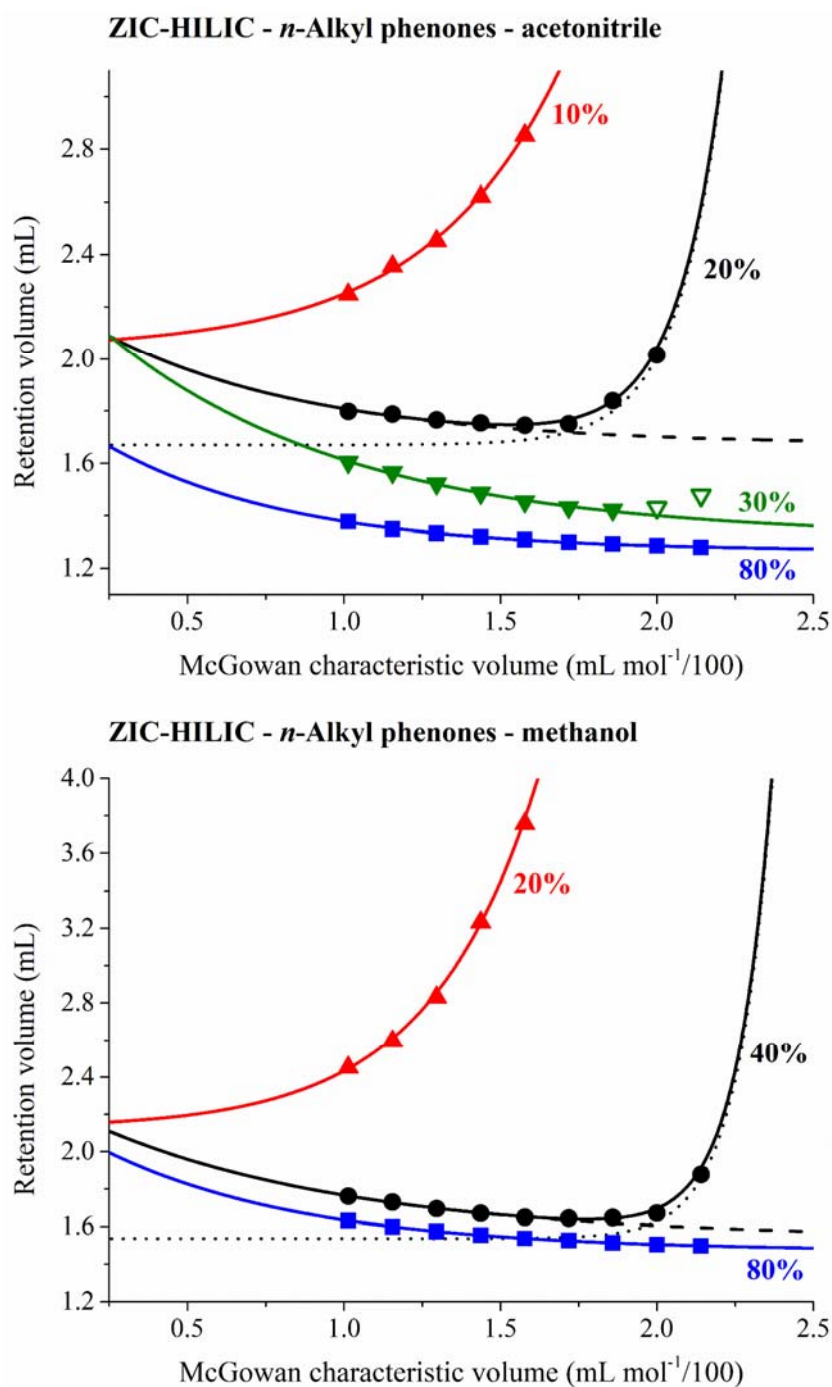


Fig. 5. Examples of pure HILIC (■), pure RPLC (▲) and mixed HILIC + RPLC (●) behavior in a ZIC-HILIC column. Solid lines represent fittings to Eq. (10); dashed and dotted lines show the contributions to the mixed mode of HILIC and RPLC, respectively. In some cases a main HILIC behavior is observed (▼), but the largest solutes show evidences of RPLC (empty ▼).

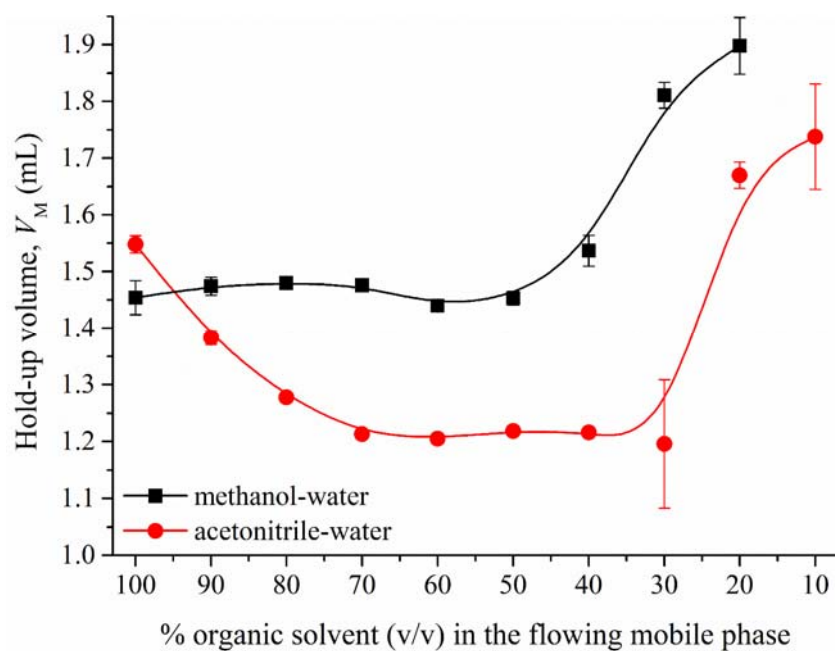


Fig. 6. Variation of the hold-up volumes (V_M) of a ZIC-HILIC column with the composition of acetonitrile/water or methanol/water mobile phases. Error bars included.

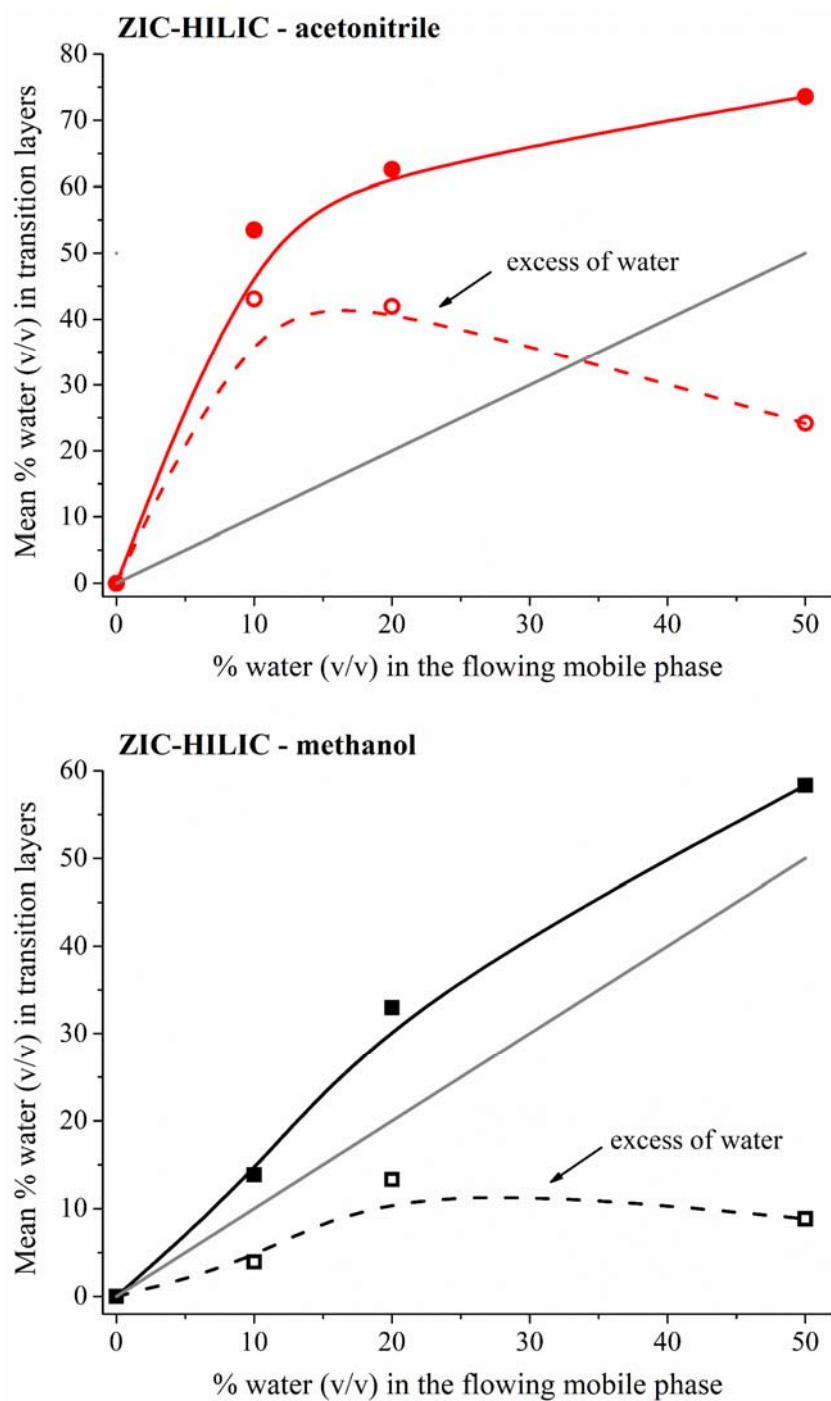


Fig. 7. Mean water content in transition layers between flowing mobile phase and the stationary phase. Empty symbols and dashed lines represent the excess of water in transition layers in relation to flowing mobile phase. A solid grey line of unitary slope and null intercept is also presented for comparative purposes.

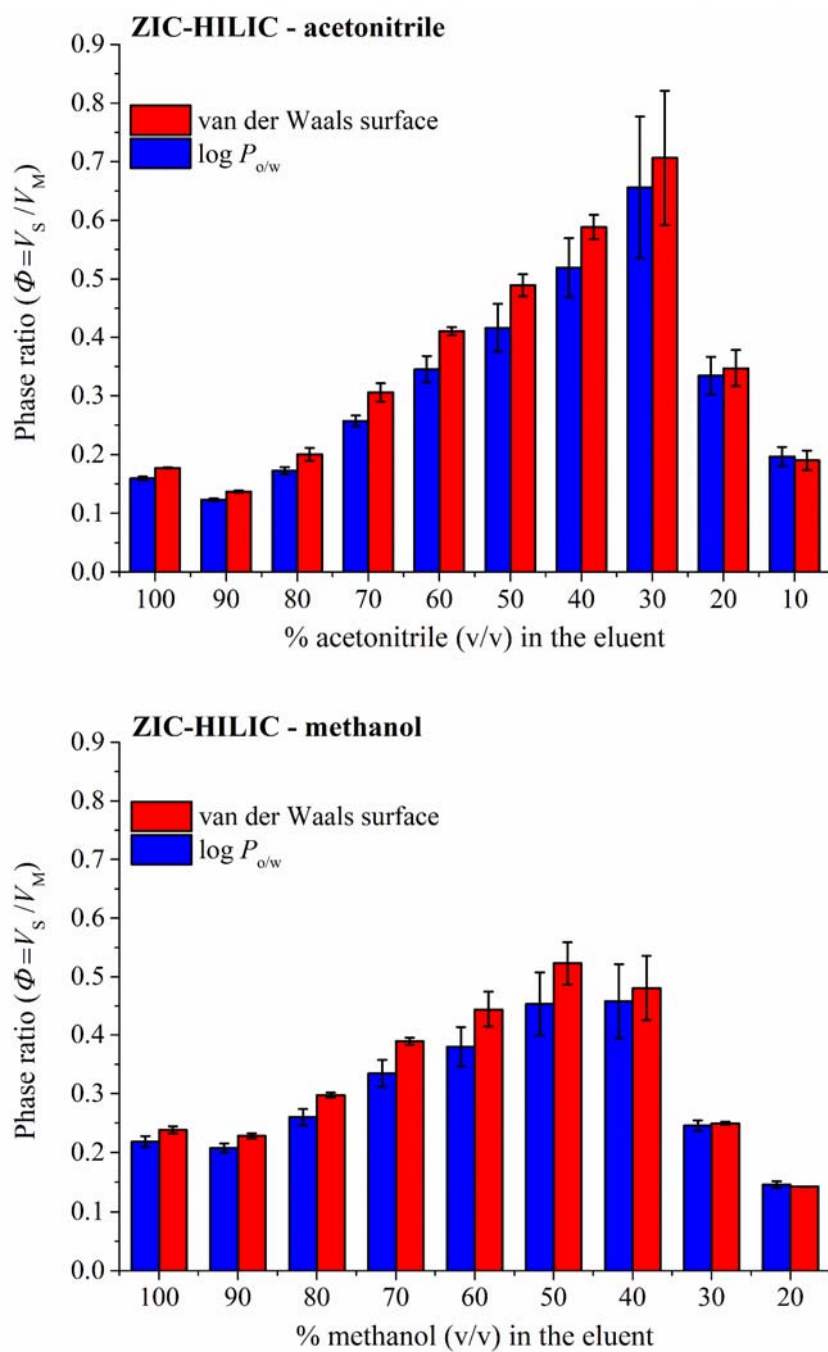


Fig. 8. Variation of the phase ratios with the mobile phase composition, estimated from retention factors of *n*-alkyl benzenes and their molecular van der Waals surface (Eq. (6)) or their octanol-water partition ratio (Eq. (8)).

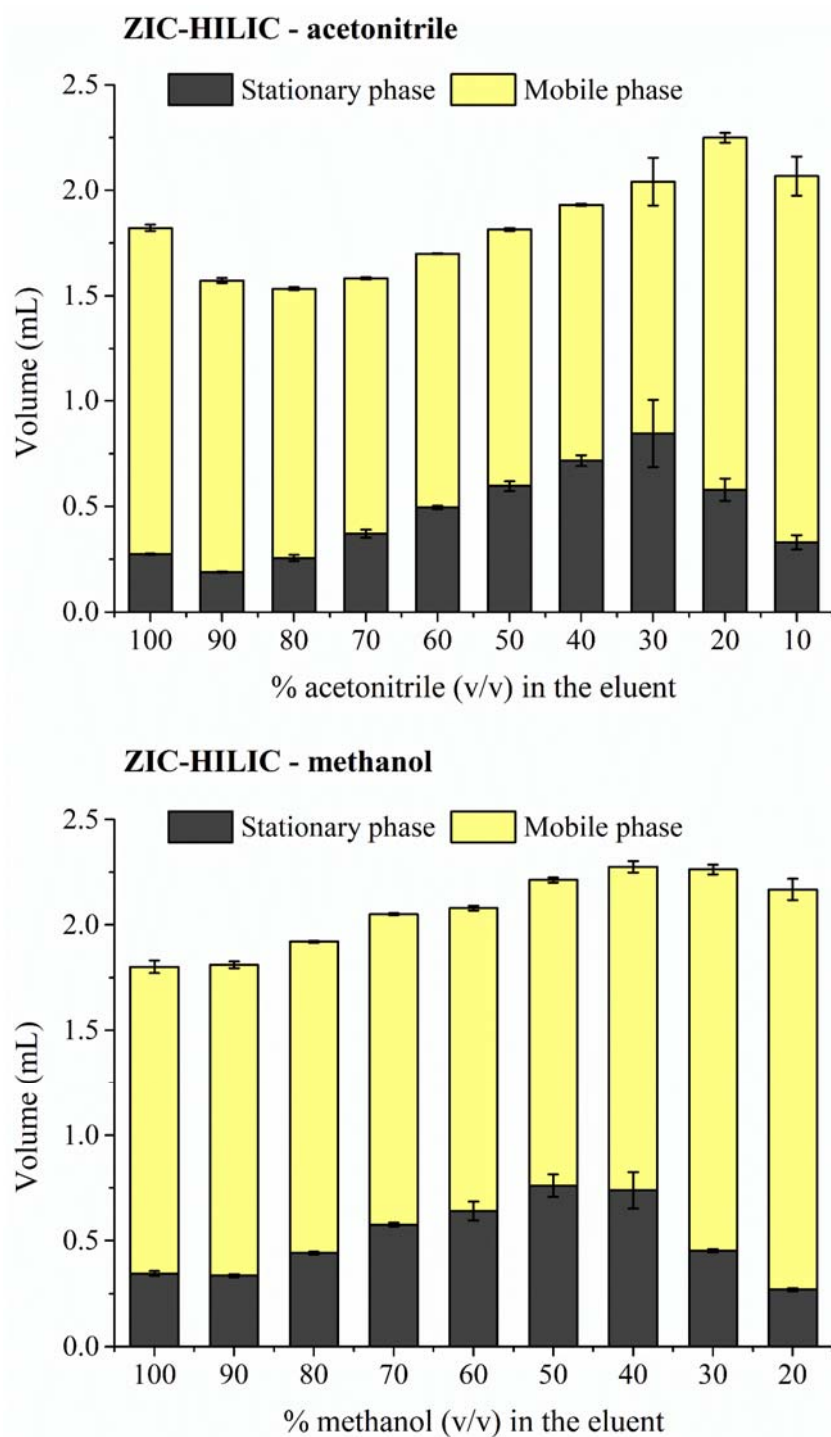


Fig. 9. Estimated volumes of stationary and mobile phases in the studied ZIC-HILIC column with acetonitrile- and methanol-water eluents. MP volumes were measured from homologous series retention data (Eq. (9)) and SP volumes from estimated phase ratios (Eq. (6) and Table S2 – supplementary material). Data in Table S5.

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SUPPLEMENTARY MATERIAL

HILIC characterization: estimation of phase volumes and composition for a zwitterionic column

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TABLES

Table S1

Homologues used for the measurement of hold-up volumes and their corresponding molecular descriptors (Abraham's solvation parameter model).

Homologous series	n_C	E	S	A	B	V
<i>n</i> -Alkyl benzenes						
Benzene	0	0.61	0.52	0.00	0.14	0.716
Toluene	1	0.60	0.52	0.00	0.14	0.857
Ethylbenzene	2	0.61	0.51	0.00	0.15	0.998
Propylbenzene	3	0.60	0.50	0.00	0.15	1.139
Butylbenzene	4	0.60	0.51	0.00	0.15	1.280
Pentylbenzene	5	0.59	0.51	0.00	0.15	1.421
Hexylbenzene	6	0.59	0.50	0.00	0.15	1.562
Octylbenzene	8	0.58	0.48	0.00	0.15	1.844
Dodecylbenzene	12	0.57	0.47	0.00	0.15	2.407
<i>n</i> -Alkyl phenones						
Acetophenone	2	0.82	1.01	0.00	0.48	1.014
Propiophenone	3	0.80	0.95	0.00	0.51	1.155
Butyrophenone	4	0.80	0.95	0.00	0.51	1.296
Valerophenone	5	0.80	0.95	0.00	0.50	1.437
Hexanophenone	6	0.78	0.95	0.00	0.51	1.578
Heptanophenone	7	0.77	0.95	0.00	0.50	1.718
Octanophenone	8	0.77	0.95	0.00	0.50	1.859
Nonanophenone	9	0.76	0.95	0.00	0.50	2.000
Decanophenone	10	0.75	0.95	0.00	0.50	2.141
<i>n</i> -Alkyl ketones						
Propanone	3	0.18	0.70	0.04	0.49	0.547
Butanone	4	0.17	0.70	0.00	0.51	0.688
Pentan-2-one	5	0.14	0.68	0.00	0.51	0.829
Hexan-2-one	6	0.14	0.68	0.00	0.51	0.970
Heptan-2-one	7	0.12	0.68	0.00	0.51	1.111
Octan-2-one	8	0.11	0.68	0.00	0.51	1.252
Nonan-2-one	9	0.11	0.68	0.00	0.51	1.392
Decan-2-one	10	0.11	0.68	0.00	0.51	1.533
Undecan-2-one	11	0.10	0.68	0.00	0.51	1.674
Dodecan-2-one	12	0.10	0.68	0.00	0.51	1.815
Tridecan-2-one	13	0.10	0.68	0.00	0.51	1.956
Pentadecan-2-one	15	0.10	0.68	0.00	0.51	2.238
Nonadecan-2-one	19	0.09	0.68	0.00	0.51	2.801

Table S2

Fitted V_M , r_i , and ν parameters (Eq. (9)) from n -alkyl benzenes, n -alkyl phenones, and n -alkyl ketones homologous series (Table S1) for the ZIC-HILIC column and each studied mobile phase composition (standard deviations of the fitted parameters in grey). Number of homologues (N) used in the fittings, the adjusted determination coefficients (R_{adj}^2), and the root-mean-square error ($RMSE$) and the main chromatographic behavior are also reported.

Organic modifier	(v/v)	V_M (mL)	r_{benz}	r_{phen}	r_{ket}	ν	N	R_{adj}^2	$RMSE$	Behavior					
Acetonitrile	100%	1.548	0.015	0.231	0.024	0.406	0.047	0.424	0.034	-0.451	0.076	31	0.937	0.01	HILIC
	90%	1.383	0.012	0.158	0.027	0.263	0.033	0.480	0.036	-0.446	0.058	31	0.954	0.01	HILIC
	80%	1.278	0.007	0.216	0.032	0.428	0.059	0.745	0.064	-0.682	0.060	31	0.969	0.01	HILIC
	70%	1.213	0.005	0.314	0.030	0.648	0.066	0.995	0.063	-0.779	0.044	31	0.986	0.01	HILIC
	60%	1.205	0.003	0.589	0.029	1.263	0.082	1.184	0.049	-0.921	0.029	31	0.996	0.01	HILIC
	50%	1.219	0.007	0.827	0.086	1.582	0.231	1.139	0.104	-0.917	0.068	30	0.980	0.01	HILIC
	40%	1.216	0.005	0.789	0.054	1.511	0.148	1.119	0.069	-0.912	0.049	25	0.992	0.01	HILIC
	30%	1.196	0.113	0.915	0.052	0.984	0.086	0.712	0.065	-0.363	0.135	23	0.946	0.02	HILIC-(RPLC) ^a
	20%	1.670	0.023	0.942	0.133	0.603	0.126	-	-	-0.640	0.095	14	0.995	0.01	HILIC part RPLC part
	10%	1.738	0.093	0.166	0.065	0.082	0.040	0.050	0.029	0.728	0.118	18	0.964	0.07	RPLC
Methanol	100%	1.454	0.030	0.274	0.032	0.403	0.029	0.464	0.023	-0.311	0.063	31	0.941	0.02	HILIC
	90%	1.474	0.016	0.298	0.019	0.394	0.031	0.444	0.025	-0.407	0.061	31	0.958	0.01	HILIC
	80%	1.480	0.004	0.454	0.023	0.665	0.047	0.534	0.024	-0.655	0.037	31	0.990	0.01	HILIC
	70%	1.476	0.005	0.573	0.037	0.793	0.070	0.581	0.032	-0.719	0.045	30	0.989	0.01	HILIC
	60%	1.440	0.011	0.530	0.051	0.661	0.085	0.643	0.058	-0.656	0.079	29	0.969	0.01	HILIC-(RPLC) ^a
	50%	1.453	0.012	0.745	0.067	0.805	0.089	0.660	0.047	-0.650	0.071	27	0.980	0.01	HILIC-(RPLC) ^a
	40%	1.537	0.027	0.839	0.059	0.773	0.066	-	-	-0.527	0.071	16	0.993	0.01	HILIC part RPLC part
	30%	1.811	0.023	0.025	0.008	0.009	0.004	0.000	0.001	1.000	0.100	15	0.976	0.04	RPLC
	20%	1.898	0.050	0.079	0.020	0.045	0.014	0.011	0.005	1.027	0.082	18	0.985	0.07	RPLC

^aData for the HILIC part. RPLC behavior observed only for a few solutes with the largest volumes.

Table S3

Mean composition of the transition layers between the bulk mobile phase and stationary phase.

Mobile phase composition (v/v)	ρ_M (g mL ⁻¹)	w_{column} (g)	w_{constant} (g)	V_{solvent} (mL)	V_M (mL)	w_{solvent} (g)	w_M (g)	V_L (mL)	w_L (g)	ρ_L (g mL ⁻¹)	Transition layers composition (v/v)	
Acetonitrile	100%	0.7766	38.052		1.548	1.314	1.202	0.145	0.112	0.7766	100%	
	90%	0.8040	38.132	36.737	1.693	1.383	1.395	1.112	0.310	0.283	0.9148	46%
	80%	0.8316	38.187			1.278	1.450	1.063	0.415	0.387	0.9342	37%
	50%	0.9056	38.294			1.219	1.556	1.104	0.474	0.452	0.9554	26%
Methanol	100%	0.7866	38.070				1.454	1.329	1.144	0.235	0.185	0.7866
	90%	0.8221	38.132	36.741	1.689	1.474	1.392	1.212	0.215	0.180	0.8350	86%
	80%	0.8528	38.189			1.480	1.448	1.262	0.209	0.186	0.8881	67%
	50%	0.9231	38.304			1.453	1.563	1.341	0.236	0.222	0.9390	42%

Calculation of the mean solvent composition of the labile stationary phase transition layers

From experimental data found in the literature [1,2], relations between the composition (% of organic solvent in volume) and density (g mL⁻¹) of mobile phases at 25 °C were established:

$$\text{Acetonitrile/water: } \%_{\text{MeCN}} = -38.4\rho_{\text{MeCN/water}}^3 + 95.8\rho_{\text{MeCN/water}}^2 - 83.3\rho_{\text{MeCN/water}} + 25.9 \quad (SD=0.005; R^2=0.9998)$$

$$\text{Methanol/water: } \%_{\text{MeOH}} = -41.1\rho_{\text{MeOH/water}}^3 + 96.0\rho_{\text{MeOH/water}}^2 - 77.6\rho_{\text{MeOH/water}} + 22.6 \quad (SD=0.002; R^2=1.0000)$$

- 1) The mobile phase density (ρ_M) was calculated from its composition (% of organic solvent in volume).
- 2) The column filled with the mobile phase was weighted (w_{column}) in an analytical balance.
- 3) The constant column weight (w_{constant}) corresponded to the joint weight of the column tube, endfittings and stationary phase packing, and it was calculated as the intercept of Eq. (1) when pure solvents were used (water and methanol or acetonitrile).
- 4) The solvent volume inside the column (V_{solvent}) was estimated as the slope of Eq. (1) when pure solvents were used (water and methanol or acetonitrile).
- 5) The mobile phase volume inside the column (hold-up volume, V_M) was determined from the homologous series approach (Section 3.2).
- 6) The weight of the solvent inside the column (w_{solvent}) was calculated after subtracting w_{constant} from w_{column} .
- 7) The weight of the mobile phase inside the column (w_M) was estimated from its volume (V_M) and density (ρ_M).

- 8) The volume of the labile stationary phase (V_L) was calculated after subtracting the volume of the flowing mobile phase (V_M) from the total solvent volume inside the column (V_{solvent}).
- 9) The subtraction of the weight of the flowing mobile phase (w_M) from the total solvent weight inside the column (w_{solvent}) allowed the calculation of the weight of the labile stationary phase (w_L).
- 10) The density of the labile stationary phase (ρ_L) was calculated from its weight (w_L) and volume (V_L).
- 11) Finally, the composition of the labile stationary phase transition layers between column functionalization and bulk mobile phase (% of organic solvent in volume) was calculated from its density (ρ_L).

References

- [1] M. Nakamura, K. Tamura, S. Murakami, Isotope effects on thermodynamic properties: mixtures of $x(\text{D}_2\text{O or H}_2\text{O}) + (1 - x)\text{CH}_3\text{CN}$ at 298.15 K, *Thermochim. Acta.* 253 (1995) 127–136. doi:10.1016/0040-6031(94)02086-4.
- [2] J. V. Herráez, R. Belda, Refractive Indices, Densities and Excess Molar Volumes of Monoalcohols + Water, *J. Solution Chem.* 35 (2006) 1315–1328. doi:10.1007/s10953-006-9059-4.

Table S4

Fitted parameters to Eqs. (6) and (8) from retention factors of benzene, toluene, ethyl-, propyl-, and butylbenzene for the ZIC-HILIC column and each studied mobile phase composition (standard deviations of the fitted parameters in grey). Number of homologues (N) used in the fittings, the determination coefficients (R^2) and standard deviation of the fitting (SD) are also reported.

Organic modifier	(v/v)	$\log k_i = a_{\text{chrom}} A_1 + \log \Phi$							$\log k_i = (a_{\text{chrom}}/a_{\text{o/w}}) \log P_{\text{o/w}} + \log \Phi$						
		a_{chrom}		$\log \Phi$	N	R^2	SD	$a_{\text{chrom}}/a_{\text{o/w}}$		$\log \Phi$	N	R^2	SD		
Acetonitrile	100%	-2.6E-03	1.8E-05	-0.751	0.004	5	1.000	0.002	-0.147	0.005	-0.798	0.017	5	0.996	0.009
	90%	-2.6E-03	4.2E-05	-0.863	0.009	5	0.999	0.004	-0.145	0.005	-0.909	0.018	5	0.996	0.009
	80%	-3.8E-03	1.8E-04	-0.698	0.037	5	0.993	0.018	-0.215	0.008	-0.763	0.026	5	0.996	0.014
	70%	-4.3E-03	1.3E-04	-0.515	0.026	5	0.997	0.013	-0.243	0.006	-0.589	0.021	5	0.998	0.011
	60%	-4.3E-03	3.1E-05	-0.386	0.006	5	1.000	0.003	-0.240	0.009	-0.462	0.031	5	0.996	0.016
	50%	-3.9E-03	5.9E-05	-0.311	0.012	5	0.999	0.006	-0.217	0.011	-0.380	0.038	5	0.992	0.020
	40%	-3.0E-03	4.0E-05	-0.230	0.008	5	0.999	0.004	-0.169	0.008	-0.285	0.028	5	0.993	0.014
	30%	-1.7E-03	1.2E-04	-0.151	0.024	5	0.985	0.012	-0.094	0.010	-0.183	0.034	5	0.966	0.018
	20%	-1.9E-03	2.0E-03	-0.460	0.041	4 ^a	0.973	0.015	-0.111	0.021	-0.476	0.064	4 ^a	0.964	0.018
	10%	2.2E-04	3.4E-04	-0.721	0.063	4 ^a	0.946	0.024	0.121	0.019	-0.706	0.058	4 ^a	0.952	0.022
Methanol	100%	-2.1E-03	7.7E-05	-0.622	0.016	5	0.996	0.007	-0.116	0.009	-0.660	0.028	5	0.984	0.015
	90%	-2.3E-03	6.0E-05	-0.641	0.012	5	0.998	0.006	-0.129	0.007	-0.683	0.024	5	0.990	0.013
	80%	-3.3E-03	3.6E-05	-0.526	0.007	5	1.000	0.004	-0.182	0.009	-0.585	0.031	5	0.992	0.016
	70%	-3.7E-03	3.1E-05	-0.409	0.006	5	1.000	0.003	-0.206	0.010	-0.475	0.032	5	0.993	0.017
	60%	-3.8E-03	1.2E-04	-0.352	0.024	5	0.997	0.012	-0.214	0.011	-0.420	0.037	5	0.992	0.019
	50%	-3.4E-03	9.6E-05	-0.281	0.019	5	0.998	0.009	-0.190	0.012	-0.343	0.041	5	0.988	0.021
	40%	-2.4E-03	2.0E-04	-0.318	0.036	4 ^a	0.987	0.014	-0.141	0.016	-0.339	0.047	4 ^a	0.976	0.018
	30%	-1.1E-03	3.8E-05	-0.603	0.006	3 ^b	0.999	0.002	-0.068	0.008	-0.609	0.022	3 ^b	0.986	0.006
	20%	2.2E-03	1.3E-05	-0.847	0.002	3 ^b	1.000	0.001	0.131	0.012	-0.837	0.032	3 ^b	0.992	0.009

^aButylbenzene was excluded; ^bButyl- and propylbenzene were excluded.

Table S5

Phase ratios (Φ), mobile phase (V_M) and stationary phase volumes (V_S) of a ZIC-HILIC column using acetonitrile/water and methanol/water as eluents.

Organic modifier	(v/v)	$\Phi = V_S/V_M^a$		V_M (mL) ^b		V_S (mL)	
Acetonitrile	100%	0.177	0.001	1.548	0.015	0.274	0.003
	90%	0.137	0.001	1.383	0.012	0.190	0.003
	80%	0.201	0.011	1.278	0.007	0.256	0.014
	70%	0.306	0.016	1.213	0.005	0.371	0.019
	60%	0.411	0.007	1.205	0.003	0.495	0.008
	50%	0.489	0.019	1.219	0.007	0.596	0.023
	40%	0.589	0.021	1.216	0.005	0.716	0.026
	30%	0.707	0.115	1.196	0.113	0.845	0.159
	20%	0.347	0.031	1.670	0.023	0.580	0.052
	10%	0.190	0.017	1.738	0.093	0.331	0.034
Methanol	100%	0.239	0.006	1.454	0.030	0.347	0.011
	90%	0.229	0.004	1.474	0.016	0.337	0.007
	80%	0.298	0.004	1.480	0.004	0.441	0.006
	70%	0.390	0.006	1.476	0.005	0.575	0.009
	60%	0.444	0.031	1.440	0.011	0.640	0.044
	50%	0.523	0.036	1.453	0.012	0.760	0.053
	40%	0.481	0.055	1.537	0.027	0.739	0.086
	30%	0.249	0.003	1.811	0.023	0.452	0.008
	20%	0.142	0.000	1.898	0.050	0.270	0.007

^aFrom Table S4 (van der Waals surface area); ^bFrom Table S2.