



Volume and composition of semi-adsorbed stationary phases in hydrophilic interaction liquid chromatography. Comparison of water adsorption in common stationary phases and eluents

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ABSTRACT

Pycnometric and homologous series retention methods are used to determine the volume and mean composition of the water-rich layers partially adsorbed on the surface of several hydrophilic interaction liquid chromatography (HILIC) column fillings with acetonitrile-water and methanol-water as eluents. The findings obtained in this work confirm earlier studies using direct methods for measuring the stationary phase water content performed by Jandera's and Irgum's research groups. Water is preferentially adsorbed on the surface of the HILIC bonded phase in hydroorganic eluents containing more than 40% acetonitrile or 70% methanol, and a gradient of several water-rich transition layers between the polar bonded phase and the poorly polar bulk mobile phase is formed. These layers of reduced mobility act as HILIC stationary phases, retaining polar solutes. The volume of these layers and concentration of adsorbed water is much larger for acetonitrile-water than for methanol-water mobile phases.

In hydroorganic eluents with less than 20-30% acetonitrile or 40% methanol the amount of preferentially adsorbed water is very small, and the observed retention behavior is close to the one in reversed-phase liquid chromatography (RPLC). In eluents with intermediate acetonitrile-water or methanol-water compositions a mixed HILIC-RPLC behavior is presented.

Comparison of several HILIC columns shows that the highest water enrichment in the HILIC retention region for acetonitrile-water mobile phases is observed for zwitterionic and aminopropyl bonded phases, followed in minor grade for diol and polyvinyl alcohol functionalizations. Pentafluorophenyl bonded phase, usually considered a HILIC column, does not show significant water adsorption, nor HILIC retention.

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1. Introduction

1.1. HILIC stationary phase and dual HILIC/RPLC behavior

Hydrophilic interaction liquid chromatography (HILIC) allows the separation of polar compounds showing weak retention in reversed-phase liquid chromatography (RPLC), employing polar stationary phases similar to normal-phase liquid chromatography (NPLC) but in combination with hydroorganic mobile phases containing more than 50% of organic solvent. Polar compounds are often only slightly soluble in the relatively non-polar organic mobile

phases used in NPLC, but the solubility of this kind of compounds is normally enhanced in hydroorganic mixtures, such as the mobile phases used in RPLC. The relatively high polarity of the stationary phase in HILIC enables the formation of water-rich layers of reduced mobility on its surface, that can act as stationary phase.

Although the HILIC technique is being widely applied, the retention mechanisms are complex and still not fully understood. Alpert was the first to introduce the term HILIC and suggested that the main retention mechanism is derived from different solute-solvent interactions that contribute to the solutes partitioning between the bulk hydroorganic mobile phase and the water-rich layer partially immobilized on the stationary phase [1]. However, other interactions like adsorption, hydrogen-bonding, dipole-dipole interactions, electrostatic interactions, molecular shape selectivity, and hydrophobic interactions could also be involved in the retention

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depending on the solutes characteristics, the functional groups of the bonded phase and support, and the solvent composition of the mobile phase, especially the water content [2–5].

In RPLC, Monte Carlo simulations of octadecylsilane and stationary phases with amide and ether embedded groups in methanol-water show that, due to hydrogen bond interactions, polar-embedded phases are more ordered and take up more solvent than their alkyl counterparts. Consequently, retention of polar analytes is increased due to hydrogen bonding with the polar-embedded groups and the increased volume of sorbed solvent [6]. In fact, solvent penetration and retentive properties are depending on the chain length, nature of the embedded polar groups and the pore shape, but not significantly on column pressure [7]. For octadecylsilane stationary phases and acetonitrile-water or methanol-water as solvent mixtures, the C18 chains show increased extension into the mobile phase with the content of the organic component in the solvent, and acetonitrile or methanol molecules start to penetrate into the bonded chain region. The presence of water in the bonded phase is very low, with the exception of the water molecules bond to residual silanols. Interestingly, for methanol-water mixtures about 80% of residual silanols are involved in hydrogen bonds with at least one solvent molecule (mainly water but also methanol), but this fraction is reduced to 50% for acetonitrile-water (15% for neat acetonitrile) due to the aprotic nature of this organic solvent [8].

From a HILIC point of view, Melnikov and coworkers [8–10] carried out molecular dynamics simulations for uncoated silica pores in contact with acetonitrile-water mixtures, in order to study the water-silica coordination shells. A first tight coordination shell of water molecules at the silanol surface through hydrogen bonding (region I), followed by middle coordination shells populated by mobile water molecules that still interact with the nearest-surface immobilized water (region II), and finally a region where water assumes bulk-like dynamics (region III). Transferring these findings in a chromatographic context, these simulations point out that the solvent inside the column can be divided into three different regions: I) a water layer immobilized on the bonded phase and support, II) an adjacent diffuse interface layer containing a gradually decrease amount of water with a translational mobility that increases until the mobility of the bulk mobile phase is reached, and III) the bulk hydroorganic mobile phase flowing through the column. The diffuse transition layers between immobilized water stationary phase and flowing mobile phase have an intermediate composition and mobility between those of water stationary phase and organic solvent-water mobile phase. Mobility of solutes in the layers close to the bulk mobile phase is slightly lower than that of the mobile phase, but solutes in the layers close to the water immobilized one have very low mobilities. On average, solutes in these layers will be delayed in reference to the flowing mobile phase, i.e., will be somewhat retained. Statistically, part of the transition layers can be considered as an effective stationary phase and part as an effective mobile phase. The main purpose of this study is to characterize the volume and composition of these layers for several typical HILIC columns and eluents and compare the water enrichment in them.

Several studies [11–18] have shown that HILIC columns, using the same mobile phase, present large differences in retention and selectivity and conclude that the bonded phase not only acts as an inert support for the water layer into which solutes can partition but it can also interact with the solutes. In general, HILIC columns are available as underivatized or functionalized silicas. The latter can be divided into polar bonded phases prepared by reactions of the silica with trialkoxysilanes containing polar and alkyl groups (cyano-, diol-, amino-, pentafluorophenylpropyl-, ...), and active layers of polar polymers grafted on the silica gel (e.g. zwitterionic sulfoalkylbetaine or phosphorylcholine). Diol phases do not con-

tain ionizable groups but show high polarity and hydrogen bonding properties, which made them an interesting option for the separation of peptides, proteins and polar drug molecules. Polar compounds are expected to be less retained in cyanopropyl bonded phases due to the lack of hydrogen bond donor capabilities. Amino functional groups show increased affinities for acidic compounds, such as amino acids, due to ion exchange effects. Regarding the pentafluorophenyl bonded phase, analyte π - π electrons are expected to interact with the carbon ring (in non-acetonitrile mobile phases), besides the electrostatic and hydrogen bonding effects of fluorine groups. Zwitterionic functionalizations were originally intended for ion exchange separations, allowing the simultaneous determination of anionic and cationic compounds, but these kinds of columns have been successfully employed in the separation of broad variety of compounds such as acids and bases, carbohydrates, metabolites, amino acids, peptides, protein digests... Depending on the chemistry of the bonded phase and support, the water uptake capacity of the column strongly differs. Direct measurements of excess adsorption of water in HILIC columns revealed that polymeric grafted zwitterionic columns show the greatest levels of water uptake, closely followed by aminopropyl functionalized silicas. Less polar moieties have a lower affinity to water reducing the water uptake of the bonded phase.

The so called HILIC columns, besides their main purpose of hydrophilic interaction liquid chromatography, can also show an RPLC or even a mixed HILIC-RPLC retention mechanism in the same column depending on the mobile phase composition. The water content in the hydroorganic mobile phase establishes the change from HILIC to RPLC mode: HILIC in mobile phases with a low concentration of water and RPLC in water-enriched mobile phases. This dual behavior depends on the polarity of the solutes and their tendency to partitioning into the water-rich layers [12,19]. Since the amount of adsorbed water appears to be dependent of the bonded phase nature, in addition to the mobile phase composition, the aim of the present work is the characterization of the water uptake capability of different HILIC columns using a combination of pycnometry and chromatographic retention of homologous series [20], through the estimation of the different solvent volumes inside the column, their mean composition, and how they take part in the retention of the solutes playing the role of stationary phase.

1.2. Measurement of the different solvent volumes inside the column

For a long time pycnometry have been used to measure the overall labile volume of solvent inside the column (V_{solvent}) using pure solvents of different density (for instance, water and acetonitrile or methanol) and to estimate hold-up volumes [21]. This represents all the volume inside the column cylinder that can be replaced by changing the eluent composition, and can be related to column weight according to Eq. (1) [22]:

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

where w_{column} is the measured weight of the column filled with a solvent. w_{constant} is the constant weight involving the column cylinder, endfittings, the bonded phase and support, and a possible fraction of water strongly adsorbed (Region I) on the polar surface of the bonded phase [22,23], that cannot be desorbed when the column is purged with the organic solvent. Thus, according to Eq. (1), V_{solvent} is the slope of the linear relationship between the total weight of the column (w_{column}) and the density of the solvent filling the column, being w_{constant} the intercept. Therefore, V_{solvent} can be calculated from the following equation [21]:

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

where $w_{\text{column,water}}$ and $w_{\text{column,organic}}$ are the weights of the same column after being consecutively purged first with water and then

with the organic solvent. ρ_{water} and ρ_{organic} are their corresponding densities, respectively.

In reversed-phase, particularly for C18-type columns, the organic eluent modifier is adsorbed on the bonded phase, with the methanol adsorption of mono-molecular nature and that of acetonitrile, on average, four times higher [24]. Another interesting feature of these two organic modifiers is their different interactions with residuals silanols in hidroorganic eluents: with acetonitrile silanols are covered by water molecules (acetonitrile does not compete for polar adsorption sites), whereas in the case of methanol there is a competition with water molecules because of its hydrogen bond formation capabilities. Nevertheless, this competition only takes place in methanol-water mobile phases if the coverage density is low enough for methanol molecules to penetrate the bonded phase and reach the silica surface [25]. Since the thickness of absorbed acetonitrile or methanol layers on RPLC bonded phases is expected to be in the molecular size (monolayer for methanol, three or more layers for acetonitrile [26,27]), the volume of solvent flowing inside the column (i.e. the hold-up volume, V_M) must be nearly the same as V_{solvent} . In contrast to RPLC, in HILIC V_{solvent} is the combination of two different solvent volumes which are strongly associated with the mobile phase composition: the volume of the mobile phase itself flowing through the column (V_M) (Region III) and the volume corresponding to the HILIC labile water-rich transition layers that act part as effective stationary phase (V_L) and part as effective mobile phase (Region II), $V_{\text{solvent}} = V_M + V_L$. Region I would be also included in V_L if the water in this layer is not fully immobilized in the bonded phase and support, and the column is purged enough during pycnometric determination of V_{solvent} .

Another classical approach to estimate hold-up volumes (V_M) in chromatography is through the variation of the retention of homologous series members [21]. Several models have been developed elsewhere to relate retention of homologous to member number and to estimate V_M from these relationships [28].

We have developed a similar model from the Linear Free Energy Relationships (LFER) of Abraham [29,30]. The LFER model of Abraham, also called Solvation Parameter Model when applied to chromatography, is a well-known equation that relates a free energy related property to solute-solvent interactions of cavity formation (vV), hydrogen bonding from solute to solvent (aA) and from solvent to solute (bB), dipolarity/polarizability (sS) and excess polarizability (eE). Solute descriptors are in upper case letters (V , A , B , S , and E) and solvent descriptors (coefficients of the equation) in lower case letters (v , a , b , s , and e). For partition properties between two solvents (such as $\log k$ in liquid chromatography), solvent coefficients measure the difference between the properties of the two partitioning phases.

In its application to liquid chromatography [31–37], the solvation parameter model takes the form:

$$\log k = c + e \cdot E + s \cdot S + a \cdot A + b \cdot B + v \cdot V \quad (3)$$

and since c , e , s , a , b , and v are system constants, and all homologous series members show common hydrogen bonding, dipolarity and polarizability descriptors (see Table S1 in Supplementary material) the term $c + eE + sS + aA + bB$ is constant. Thus, retention in a homologous series only depends on the volume of the series member, which is linearly related to the homologue number used in the classical approaches [21,28].

k is directly related to retention (V_R) and hold-up volumes (V_M) and we can relate retention volume to hold-up volume and Abraham descriptors through the equation:

$$V_R = V_M + r \cdot 10^{v \cdot V} \quad (4)$$

with

$$r = V_M \cdot 10^{c + e \cdot E + s \cdot S + a \cdot A + b \cdot B} \quad (5)$$

The hold-up volume can be calculated by fitting equation parameters (r , v , and V_M) to the retention data of the homologous series members.

When only a single behavior is observed, HILIC or RPLC, V_M can be obtained from fitting to Eq. (6):

$$V_R = V_M + \sum_{i=1}^n (r_i \cdot f_i) \cdot 10^{v \cdot V} \quad (6)$$

where V_R is the retention volume of the homologue, V is the McGowan characteristic volume of the homologue (in units of $\text{mL mol}^{-1}/100$), and r and v are constant values depending on the chromatographic system. r also depends on the homologous series used, because it is related to solute-solvent dispersion, dipole-dipole, dipole-induced dipole, polarizability, and hydrogen bond interactions [30]. In particular, the sign of v provides information about the prevailing retention mode of the column depending on the mobile phase composition. In HILIC v takes negative values because of the relatively high energy involved in the creation of a cavity in the water-rich layer, which acts as stationary phase, to accommodate the analyte in the partitioning process. Therefore, the higher the molecular size of the homologue the lower the retention volume. In contrast, the positive sign of v in RPLC indicates that chromatographic retention increases with the molecular size of the homologue because of their tendency to partition into the non-polar stationary phase, which in this case is the bonded phase. n is the number of homologous series included in the model of Eq. (6) and in this work $n=3$ (n -alkyl benzenes, n -alkyl phenones, and n -alkyl ketones series used). To the extent possible, it is recommended to select different series covering a wide range of different solute-solvent interactions, with the aim of providing a more accurate estimation of V_M . f_i in Eq. (6) are the binary flag descriptors (0 or 1) that allows the simultaneous adjustment of the n homologous series. The value of f is 1 for a particular series data and 0 for the rest of the series analyzed in the same dataset. For example, when fitting the retention data of alkyl benzenes ($V_{R, \text{alkyl benzenes}}$) as a function of their McGowan volume (V), the value of $f_{\text{alkyl benzenes}}$ is set to 1 whereas $f_{\text{alkyl phenones}} = f_{\text{alkyl ketones}} = 0$.

When a mixed HILIC-RPLC behavior is observed, two different trends in the variation of retention with v are noticed (HILIC and RPLC) showing the plots a characteristic U shape, where the minimum retention is the transition from HILIC to RPLC. This minimum does not necessarily correspond to the hold-up volume, which normally drops clearly below this transition minimum. The following equation allows V_M determination:

$$V_{R(\text{HILIC+RPLC})} = V_M + \sum_{i=1}^n (r_{\text{HILIC},i} \cdot f_i) \cdot 10^{v_{\text{HILIC}} \cdot V} + \sum_{i=1}^n (r_{\text{RPLC},i} \cdot f_i) \cdot 10^{v_{\text{RPLC}} \cdot V} \quad (7)$$

where r_{HILIC} and v_{HILIC} refer to the HILIC retention behavior and r_{RPLC} and v_{RPLC} to the RPLC behavior [20].

The determined V_M value is an estimation of the effective hold-up volume, i.e., the volume of the bulk mobile phase flowing freely inside the column (region III) and the statistic average of the transition water-rich layers that act as mobile phase (statistic part of region II).

From the difference between the total labile solvent volume inside the column (V_{solvent} , pycnometrically measured, Eq. (2)) and the effective hold-up volume (V_M , from homologous series approach Eqs. (6) or (7)), the volume of the HILIC labile water-rich transition layers (V_L) can be estimated as:

$$V_L = V_{\text{solvent}} - V_M \quad (8)$$

1.3. Measurement of mean composition of the water-rich transition layers of stationary phase

In HILIC conditions the total weight of labile solvent inside de column (w_{solvent}) is the sum of the weights of the effective mobile phase (w_{M}) and the effective water-rich transition layers of stationary phase between water fully immobilized in bonded phase and support surface (region I) and mobile phase (w_{L}), which in turn depends on their respective volumes (V_{M} and V_{L}) and densities (ρ_{M} and ρ_{L}):

$$w_{\text{solvent}} = w_{\text{L}} + w_{\text{M}} = V_{\text{L}} \cdot \rho_{\text{L}} + V_{\text{M}} \cdot \rho_{\text{M}} \quad (9)$$

Consequently, the density of the stationary phase transition layers (ρ_{L}) can be easily calculated from the density of the flowing eluent, the weight of the column and the volumes of mobile and HILIC transition layers stationary phase according to Eq. (10):

$$\rho_{\text{L}} = \frac{w_{\text{L}}}{V_{\text{L}}} = \frac{w_{\text{solvent}} - \rho_{\text{M}} \cdot V_{\text{M}}}{V_{\text{solvent}} - V_{\text{M}}} \quad (10)$$

w_{solvent} can be easily determined at any mobile phase composition after weighing the column (w_{column}) and subtracting the column constant weight (w_{constant} , origin ordinate of Eq. (1) when applied to pure solvents, water and methanol or acetonitrile). From these calculated densities, the fractions of organic modifier in the HILIC water-rich stationary phases can be easily determined through the published [38,39] relationships for acetonitrile- and methanol-water mixtures at 25 °C presented in Eqs. (11) and (12):

$$\%_{\text{acetonitrile}}(\text{v/v}) = -38.4 \cdot \rho^3 + 95.8 \cdot \rho^2 - 83.3 \cdot \rho + 25.9 \quad (11)$$

$$\%_{\text{methanol}}(\text{v/v}) = -41.1 \cdot \rho^3 + 96.0 \cdot \rho^2 - 77.6 \cdot \rho + 22.6 \quad (12)$$

Notice that this composition is an average of all the gradient compositions of layers of region II (between fully immobilized water on column surface and free flowing eluent) that acts as effective stationary phase.

2. Materials and methods

2.1. Instrumentation

A Shimadzu (Kyoto, Japan) HPLC system consisting of two LC-10ADvp pumps, an SIL-10ADvp auto-injector, an SPD-M10Avp diode array detector, a CTO-10ASvp oven set at 25 °C, and an SCL-10Avp controller were employed for chromatographic measurements. The system was controlled by LCSolutions software from Shimadzu.

The analytical balance used to weight the columns was an AT 261 DR from Mettler-Toledo (Columbus, OH, USA) with an uncertainty at the sample amount of 1 mg. The balance is located in a climatized room (22 ± 2 °C, $50 \pm 5\%$ humidity) and yearly calibrated by an accredited calibration laboratory (Mettler-Toledo, Spain).

The details of the six columns characterized are shown in Table 1.

2.2. Methods and chromatographic conditions

The extra-column volume was measured from the retention volume of several injections of 0.5 mg mL⁻¹ aqueous solution of potassium bromide (Baker, >99%), without column, and using as mobile phase water and a wide range of acetonitrile-water and methanol-water mixtures. A concordant value of 0.118(±0.004) mL with all eluents was found and subtracted from all the measured retention volumes.

The flow rate of the mobile phase was 0.5 mL min⁻¹ for ZIC-HILIC and ZIC-cHILIC and 1 mL min⁻¹ for Luna NH2, Kinetex F5,

YMC-Pack PVA-Sil, and YMC-Triart Diol-HILIC. The column was equilibrated during 20 min every time the mobile phase composition was modified. The injection volume was 1 µL. Retention times were determined at a detection wavelength of 210 nm for *n*-alkyl benzenes, 245 for *n*-alkyl phenones, and 275 nm for *n*-alkyl ketones.

2.3. Column equilibration study

In a first approach, in the two-pump high-pressure mixing chromatograph, each column was conditioned with water at a flow rate of 1 mL min⁻¹ for 1 h and weighed. Then, the second pump purged the column with the organic solvent, acetonitrile or methanol, at a flow rate of 1 mL min⁻¹, and the column was weighed every 15 min (15 mL of eluent) until reaching a time of 60 min (60 mL). With the aim of better characterizing the effect on equilibration of the very first mL of eluent, equilibration of column was repeated with a lower flow rate. After the first step of conditioning with water, the organic solvent was pumped at 0.2 mL min⁻¹ and the column weight was carefully measured every 5 min (1 mL of eluent) until reaching 50 min (10 mL). The column oven was always set to 25 °C and the column was capped with its endfittings before being weighed.

2.4. Pycnometric measurements

Columns were purged at 25 °C with a flow rate of 0.5 mL min⁻¹ for ZIC-HILIC and ZIC-cHILIC and 1 mL min⁻¹ for Luna NH2, Kinetex F5, YMC-Pack PVA-Sil, and YMC-Triart Diol-HILIC. After 2 h for the first two columns and one hour for the rest, 60 eluent volumes in all cases, columns were capped with their respective endfittings and weighed. ZIC-HILIC and ZIC-cHILIC were pycnometrically measured for 100%, 90%, 80%, 50%, and 0% of organic solvent, while for Luna NH2, Kinetex F5, YMC-Pack PVA-Sil, and YMC-Triart Diol-HILIC all the range of organic solvent compositions was measured at 10% intervals.

2.5. Chemicals and solvents

Water was obtained from a Milli-Q plus system from Millipore (Billerica, CA, USA) with a resistivity of 18.2 MΩ cm. Acetonitrile and methanol, both HPLC gradient grade, were purchased from Chem-Lab.

The solutes of the homologous series (*n*-alkyl benzenes, *n*-alkyl phenones, and *n*-alkyl ketones) were obtained from Acros Organics, Alfa Aesar, Fluka, Merck, and Sigma-Aldrich, all of high purity grade (≥ 97%) and are reported in Table S1 in supplementary material along with their Abraham's molecular descriptors [40]. Stock solutions of the homologues were prepared in methanol at a concentration of 5 mg mL⁻¹. Ketones were directly injected but benzenes and phenones were diluted with methanol to 0.5 mg mL⁻¹. All the solutes were injected in duplicate.

2.6. Calculation

All calculations were done in MS ExcelTM. 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 [41].

3. Results and discussion

3.1. HILIC columns and organic modifier selection

For this study, six commercially available HILIC columns were selected based on their different polar stationary phases (Table 1).

Table 1
Specifications of the HILIC columns employed in the present work.

Column	Manufacturer	Support	Functionality	Particle size (mm)	Pore size (Å)	Surface area (m ² g ⁻¹)	Column size (mm)	Bonded phase structure
ZIC-HILIC	Merck	Porous silica	Polymeric zwitterionic sulfobetaine	3.5	100	180	150 × 4.6	
ZIC-CHILIC	Merck	Porous silica	Polymeric zwitterionic phosphorylcholine	3	100	-	150 × 4.6	
Luna NH2	Phenomenex	Fully porous silica	Aminopropyl with TMS encapping	5	100	400	150 × 4.6	
Kinetex F5	Phenomenex	Core-shell silica	Pentafluorophenyl with TMS endcapping	5	100	200	150 × 4.6	
YMC-Pack PVA-Sil	YMC	Polymerized silica	Polyvinyl alcohol	5	120	330	150 × 4.6	
YMC-Triart Diol-HILIC	YMC	Hybrid silica	1,2-Dihydroxypropyl	5	120	360	150 × 4.6	

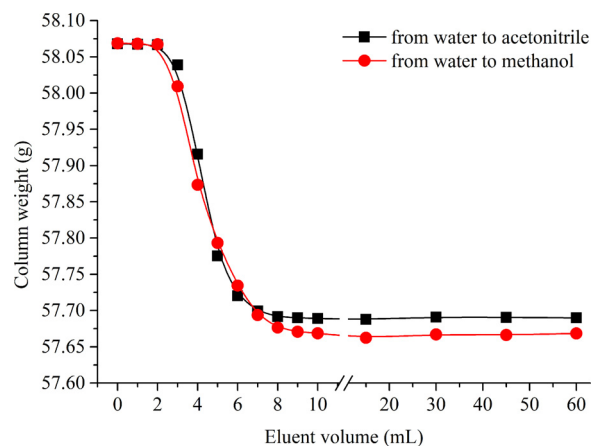
Merck (Darmstadt, Germany); Phenomenex (Torrance, CA, USA); YMC Co. Ltd. (Kyoto, Japan)

All columns have a silica-based support, share the same dimensions, and have similar characteristics in terms of particle size, pore size, and surface area. The significant difference resides on the bonded phase chemistry: polymeric zwitterionic sulfobetaine for ZIC-HILIC, polymeric zwitterionic phosphorylcholine for ZIC-CHILIC, aminopropyl with TMS endcapping for Luna NH2, pentafluorophenyl with TMS endcapping for Kinetex F5, polyvinyl alcohol for YMC-Pack PVA-Sil, and 1,2-dihydroxypropyl for YMC-Triart Diol-HILIC. The fillings of these columns are representative of some of the most common ones in HILIC applications.

Regarding the selection of organic modifiers included in the study, acetonitrile is by far the most common solvent used in HILIC mobile phases, followed by methanol. They significantly differ in their hydrogen bonding acidity, which leads to different chromatographic behavior in HILIC, as already observed for a polymeric zwitterionic column in a previous study [30]. In RPLC it is well known that the preferential adsorption of acetonitrile on alkyl bonded phases is much stronger than that for methanol, leading to concentrations of acetonitrile in the stationary phase higher than those in the acetonitrile-water mobile phase [42,43]. Interestingly, water is preferentially adsorbed in short alkylamide and amino-propyl groups under methanol-water eluents [44].

3.2. Column equilibration

After changing the mobile phase composition, it is very convenient to ensure a full equilibration of the column under the new chromatographic conditions, since partial equilibrations might affect retention behavior and selectivity [45]. In consequence, a py-

**Fig. 1.** Volume of acetonitrile or methanol needed to equilibrate the studied YMC-Triart Diol-HILIC column initially filled with water.

cnometric study was performed in order to figure out the volume of eluent required to achieve the full equilibration conditions for all the studied columns.

Fig. 1 shows, as an example, the weight reduction of the YMC-Triart Diol-HILIC column when water is replaced by acetonitrile or methanol. The column weight continued unchanged for the first 3 mL of the flowing mobile phase due to the dwell volume of the employed HPLC system, followed by a decrease in weight consistent with the lower density of the organic solvent in relation to

Table 2

Measured volume of the labile solvent inside each chromatographic column (V_{solvent} , Eq. (2)) at 25 °C and its relation to the total volume inside the column cylinder ($V_{\text{column}} = 2.49$ mL).

Column	V_{solvent} (mL)		Mean value ($\pm$ SD)	$V_{\text{solvent}}/V_{\text{column}}$ (%)
	Acetonitrile and water	Methanol and water		
ZIC-HILIC	1.693	1.689	1.691 $\pm$ 0.003	68%
ZIC-CHILIC	1.810	1.799	1.804 $\pm$ 0.008	72%
Luna NH2	1.872	1.886	1.882 $\pm$ 0.010	75%
Kinetex F5	1.400	1.396	1.398 $\pm$ 0.003	56%
PVA-Sil	1.968	1.991	1.979 $\pm$ 0.016	79%
Diol-HILIC	1.777	1.805	1.791 $\pm$ 0.020	72%

water. After 15 mL the column weight remained constant, suggesting that the full equilibration was achieved. Similar patterns were obtained for the rest of the studied columns.

Equilibration studies are often referred to the number of column volumes necessary to achieve the steady state of the chromatographic systems. However, as discussed in Section 1.2, the definition of “column volume” in HILIC is not straightforward. Since the hold-up volume (V_M) strongly depends on the mobile phase composition, it might be more convenient to use the overall labile volume of solvent inside the column (V_{solvent}) instead. In this sense, the studied columns were equilibrated after purging with V_{solvent} volumes in the range between 7 and 11 times.

Consequently, 15 mL were considered to be the minimum required volume to achieve the steady state of the studied HILIC systems.

3.3. Labile solvent volume inside the column

The total labile solvent volume (V_{solvent}) inside the studied columns was pycnometrically measured using water and acetonitrile or methanol as organic solvents, and the results are presented in Table 2. It is worth noting that very similar volumes were obtained for each column regardless of the selected organic solvent for the assay. From the column dimensions, which in all cases were 150 mm length and 4.6 mm internal diameter, the total volume inside the column cylinder can be easily calculated ($V_{\text{column}} = (\pi(0.46/2)^2 15 = 2.49$ mL). The ratio between V_{solvent} and V_{column} (Table 2) is a relative measure of the volume inside the column filled by the labile solvent (i.e., the partially immobilized water-rich layers and the hydroorganic flowing eluent), being the rest of the space occupied by the bonded-phase, its support, and fully immobilized water. According to the obtained results for nearly all columns, 70–80% of the column is filled with labile solvent. In the case of the Kinetex F5 this ratio is reduced to 56%, due to the core-shell technology employed in this column. In contrast to the other studied columns packed with porous silica materials, Kinetex particles are made of a solid nonporous silica core surrounded by a porous shell layer, which results in a reduction of the overall porosity inside the column. As will be discussed later, unlike the rest of the columns characterized in this work, the Kinetex F5 column only shows RPLC behavior, even when acetonitrile- or methanol-rich mobile phases are employed.

3.4. Water enrichment of stationary phase transition layers

With the aim of providing evidence of the existence of water-rich hydroorganic stationary phase layers, the columns were additionally equilibrated with different solvent mixtures of acetonitrile or methanol with water and weighed. In case all the labile solvent inside the column (V_{solvent}) has the same composition than the flowing mobile phase, the plot of the weight column vs. the density of eluent is expected to result on a straight line of slope V_{solvent} (Eq. (1)). This behavior is expected in RPLC due to the small amount of organic solvent (methanol, acetonitrile) involved

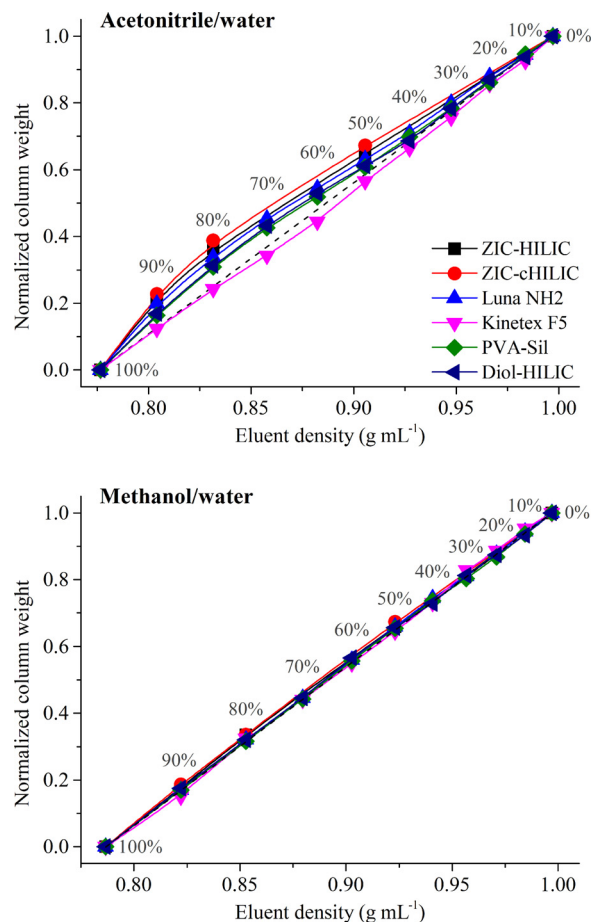


Fig. 2. Measured normalized weights of the studied columns at different solvent compositions (water and acetonitrile or methanol, and hydroorganic mixtures). The % (v/v) of organic solvent in the eluent is also provided. The dashed straight line corresponds to the expected weight when the solvent composition inside the column matches that of the flowing eluent.

in the preferential penetration and solvation of the bonded phases and the existence in the interfacial region of organic-rich layers of varying molecular thicknesses (about one layer for methanol, four for acetonitrile) [8,46]. The results obtained, Fig. 2, show a clear different behavior depending on whether acetonitrile or methanol is used as eluent. For the sake of better comparison, the column weights were normalized between that of the column equilibrated with organic solvent (0) and water (1). Interestingly, the linear relationship between the column weight and the eluent density is fulfilled in mobile phases containing methanol, suggesting a single solvent composition inside the column, with no significant water adsorption. However, when acetonitrile was used as organic modifier, positive deviations of this straight line were observed, with the only exception of the Kinetex F5 column. Higher column

weights indicate an average content of the hydroorganic mixture enriched in the denser solvent, i.e. water, in relation to the flowing eluent. ZIC-cHILIC, Luna NH2, and ZIC-HILIC, in this order, show the greatest water accumulation.

These results are in agreement with the water uptake isotherms of some HILIC materials measured by Irgum and coworkers [17]. In this study the authors clearly pointed out that water uptake greatly depends on the monomeric or polymeric nature of the functionalized silica. The former are silicas functionalized with polar ligands typically linked by short alkyl spacers, and the latter are polymer grafted silicas carrying one polar moiety for each polymeric unit. Monomeric functionalized silicas are prone to the formation of water monolayer, followed by multiple layer adsorption with an increase of water in the eluent, whereas water uptake on polymerically functionalized silica forms hydrogel layers which gradually expand with the water content in the mobile phase. As a result, the water uptake capacities of polymeric grafted phases are normally higher than that of monomeric ones. Therefore, it is not surprising that the ZIC-cHILIC (phosphorylcholine) and the ZIC-HILIC (sulfobetaine) columns employed in our study, both polymerically grafted zwitterionic columns, tend to show the greatest levels of water uptake. Soukup and Jandera [18] also pointed out that among the 16 stationary phases investigated using frontal analysis method and coulometric Karl–Fischer titration, ZIC-cHILIC showed the strongest affinity to water.

Irgum's work [17] also stated the substantial affinity for water of amino phases, almost comparable to the polymeric grafted phases, which is again in good agreement with our results. Luna NH2 (aminopropyl) is a basic column, with protonated and positively charged amino groups, which favors the large increase of water content inside the column. YMC-Pack PVA-Sil (polyvinyl alcohol) and YMC-Triart Diol-HILIC (1,2-dihydroxypropyl) are both neutral columns showing a less affinity for water uptake compared to the columns with ionic or ionizable functional groups [11–13,17]. On the contrary, the slight negative deviation for Kinetex F5 suggests a small enrichment on the less dense solvent, i.e., acetonitrile.

3.5. Flowing mobile phase volume and column behavior

The volume occupied by the flowing mobile phase, the hold-up volume (V_M), was determined from the Abraham LFER approach (Eq. (3)) using *n*-alkyl benzenes, *n*-alkyl phenones, and *n*-alkyl ketones homologous series (descriptor data in Table S1 of supplementary material). For each column and mobile phase composition single V_M and v values were obtained, whereas specific r_i parameters were dependent on the particular homologous series (benzenes, phenones or ketones). These values and the fitting statistics can be consulted in Table S2 of the supplementary material.

Except Kinetex F5, the columns presented two different behaviors depending on the mobile phase composition. On the one hand, at high concentrations of organic solvent, either methanol or acetonitrile, retention decreases with the molecular volume of the homologues, showing a typical HILIC behavior. The larger the molecular volume of the homologue, the lower the retention because of the difficulty of the cavity formation in the water-enriched transition layers, due to relatively high cohesion between solvent molecules. The range of organic solvent compositions with a clear HILIC behavior was wider with acetonitrile than with methanol organic solvents: ZIC-HILIC, 100% to 40% vs 100% to 70%; ZIC-cHILIC, 100% to 60% vs 100% to 70%; Luna NH2, 90% to 60% vs 100% to 80%; YMC-Pack PVA-Sil, 100% to 60% vs 100% to 80%; YMC-Triart Diol-HILIC, 100% to 60% vs 100% to 70%. By increasing the water content, although the main behavior of the homologues was still HILIC, some of the largest ones were excluded from the correlation because their retention volumes were higher than the immediately preceding homologue member. This increase in the chro-

matographic retention of the largest homologues is due to the influence of RPLC retention mode, and it indicates the beginning of the general behavior change from HILIC to RPLC. In some cases, enough retention volumes of the homologous series were available showing clearly both behaviors in a single mobile phase composition and V_M could be well determined through Eq. (7). On the other hand, higher contents of water in the mobile phase led to increase retention with the molecular volume of the homologues, which constitutes the typical RPLC behavior because the cohesion of the water-rich mobile phases is higher than that of the less polar stationary phase (mainly the bonded phase). Therefore, due to the lower energy required for the solute to form a cavity in the stationary phase, largest homologues partition more favorably into the stationary phase increasing its chromatographic retention. The range of undoubted RPLC behavior for HILIC columns was also dependent on the water content in the acetonitrile- or methanol-water mobile phases: ZIC-HILIC and ZIC-cHILIC, from 90% vs 70%; Luna NH2, from 70% vs 50%. For most of the columns, when using mobile phases containing nearly 100% of water, homologues were strongly retained, and the measurement of their retention volumes (V_R) were excessively time consuming. In some mobile phases with higher water contents, although showing a clear RPLC behavior, some of the smallest homologues were excluded for the V_M adjustment because they showed more retention than expected because they still had a HILIC behavior.

In the Kinetex F5 column, only the RPLC chromatographic model was observed in all the studied range of both sets of organic solvents compositions, from 100% to 40%. Above 60% of water, the homologues were too much retained to be measured in a reasonable time window. This is consistent with the results presented in Section 3.4, showing the same composition of all the solvent inside the column than that of the flowing eluent, or even a slight enrichment in acetonitrile of the possible immobilized solvent in the column surface, for all the range of studied mobile phases. These observations point out the inability of the pentafluorophenyl bonded phase to generate the water-rich transition layers responsible for the HILIC partition mechanism.

Fig. 3 shows the V_M estimated for each column and mobile phase composition together with the V_{solvent} pycnometrically measured. Excluding Kinetex F5, the difference between V_{solvent} and V_M when using pure organic solvent as mobile phase proved the existence of the water-rich transition layers semi-absorbed on the bonded phase and support. The column was filled with the organic solvent after being purged previously with pure water. Using pure organic solvents, V_M was expected to be virtually V_{solvent} , as long as no water amount was introduced inside the column as mobile phase and thus, water-rich transition layers would be removed if the column is purged enough with the organic solvent. Instead, V_M was slightly below V_{solvent} for almost all columns and both organic solvents, suggesting the presence of a tiny transition layer acting as stationary phase according to the HILIC behavior observed from the injection of homologous series (with the exception of Luna NH2 in 100% acetonitrile). Gradient grade acetonitrile and methanol were used as received without further treatment, and according to the manufacturers their water contents were lower than 150 and 500 ppm, respectively. Flushing with these organic solvents can be not enough to displace some strongly adsorbed water in the column, and the small water contents in the organic solvent seems to be sufficient to create a tiny water-rich transition layer and to give rise to a HILIC behavior.

When acetonitrile was used as organic solvent, in the mobile phase range of HILIC behavior (solid lines in Fig. 3), it is clearly noticeable that V_M decreases when increasing the content of water in the eluent up to about 30%. This is consistent with an enlargement of the water-enriched layers, embedding water from the eluent, which reduces the available volume inside the column for

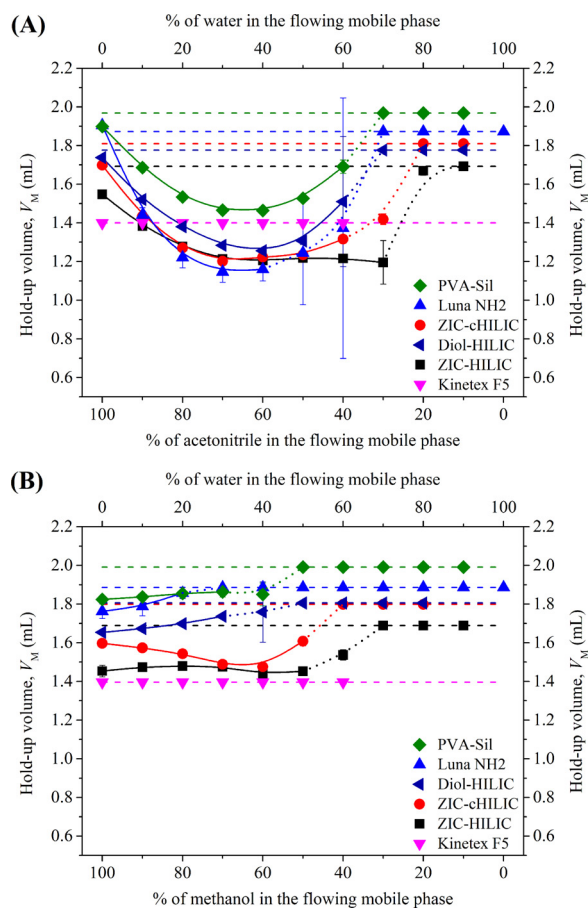


Fig. 3. Variation of the hold-up volumes (V_M) of the studied columns with the composition of the mobile phase: (A) acetonitrile-water and (B) methanol-water mixtures. Dashed straight lines correspond to the overall labile volume of solvent inside the column (V_{solvent}) pycnometrically measured. Solid and dotted lines represent V_M of HILIC and mixed HILIC-RPLC retention modes, respectively. Error bars for standard deviations are included.

the flowing mobile phase (i.e., the hold-up volume). As the water content in the eluent increases, differences in polarity between the mobile phase and the water layer adsorbed on the bonded phase become less pronounced, reducing the thickness and the HILIC relevance of the water-enriched transition layers. At this point the RPLC behavior starts to be noticed (dotted lines in Fig. 3), the progressive reduction of the transition layers allows the expansion of V_M with the water content in the mobile phase, until it reaches the maximum possible value of V_{solvent} when the RPLC mode takes chromatographic control of retention. In RPLC, due to the absence of differentiated water-enriched layers, all the available solvent volume inside the column is expected to be of the same composition than the flowing eluent.

In contrast to acetonitrile, in the HILIC range of methanol-water mobile phases the hold-up volume remains quite constant (Fig. 3), probably because the higher similarity of water to methanol than to acetonitrile.

3.6. Water-rich stationary phase transition layers volume

The effective volume acting as stationary phase of the water-rich layers (V_L) between the water adsorbed onto the bonded phase and the flowing mobile phase can be estimated by subtracting the hold-up volume (V_M) from the overall solvent volume inside the column (V_{solvent}) (Eq. (8)). V_L values were determined for the studied columns for a wide range of mobile phase composi-

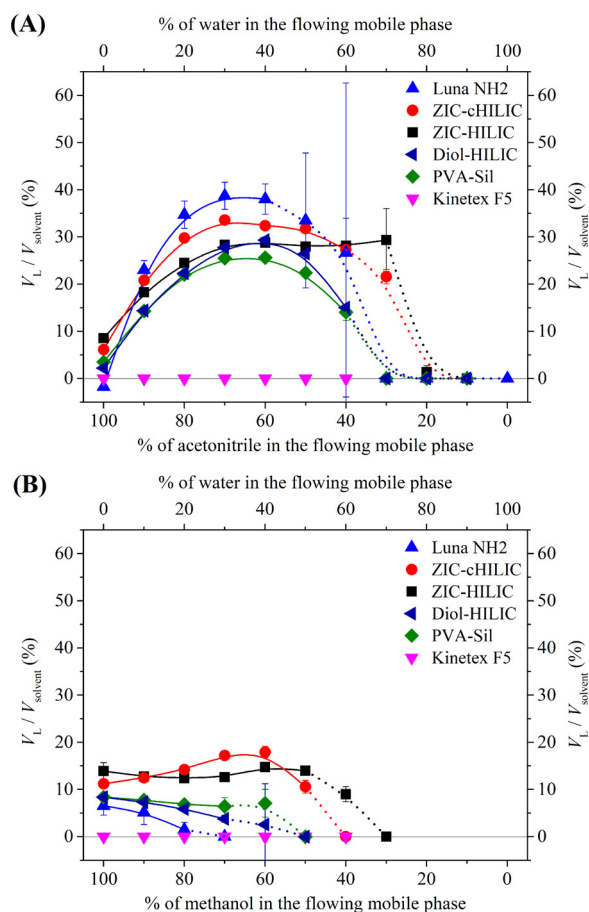


Fig. 4. Percentage in volume of the water-rich transition layers (V_L) over the overall labile volume of solvent inside the column (V_{solvent}): (A) acetonitrile-water and (B) methanol-water mixtures. Solid and dotted lines represent HILIC and mixed HILIC-RPLC retention modes, respectively. Error bars for standard deviation are included.

tions (Table S3) and, for ease of comparison, the ratios V_L/V_{solvent} were calculated and presented in Fig. 4. These ratios can be interpreted as the fraction of the total labile solvent volume inside the column occupied by the water-rich transition layers acting as effective stationary phase in HILIC mode. Using acetonitrile as organic modifier, the Luna NH2 column shows the thickest transition layers (up to almost 40% of solvent volume), followed by the zwitterionic ZIC-cHILIC and ZIC-HILIC, and finally the YMC-Triart Diol-HILIC and the YMC-Pack PVA-Sil. These results are in agreement with previous studies showing that charged bonded phases, including zwitterionic, are prone to higher levels of water uptake [17,18]. The aminopropyl functionalization of Luna NH2 is expected to be positively charged (the pK_a of 3-aminopropyltriethoxysilane in aqueous solution is around 10.5), in contrast to the neutral dihydroxypropyl (Diol-HILIC) or the polyvinyl alcohol (PVA-SIL) bonded phases.

Similar results are obtained for methanol-water eluents, although the volume of the water-rich adsorbed layers (less than 20%) is much lower than for acetonitrile-water. Volume of water-rich layers adsorbed in zwitterionic ZIC-cHILIC and ZIC-HILIC bonded phases is larger than that in YMC-Triart Diol-HILIC and YMC-Pack PVA-Sil columns. However, and contrary to acetonitrile-water, Luna NH2 column adsorbs lower water volumes than the other HILIC columns.

3.7. Water-rich transition layers composition

For all mobile phase compositions and columns showing a clear HILIC behavior, the mean compositions of the water-rich transi-

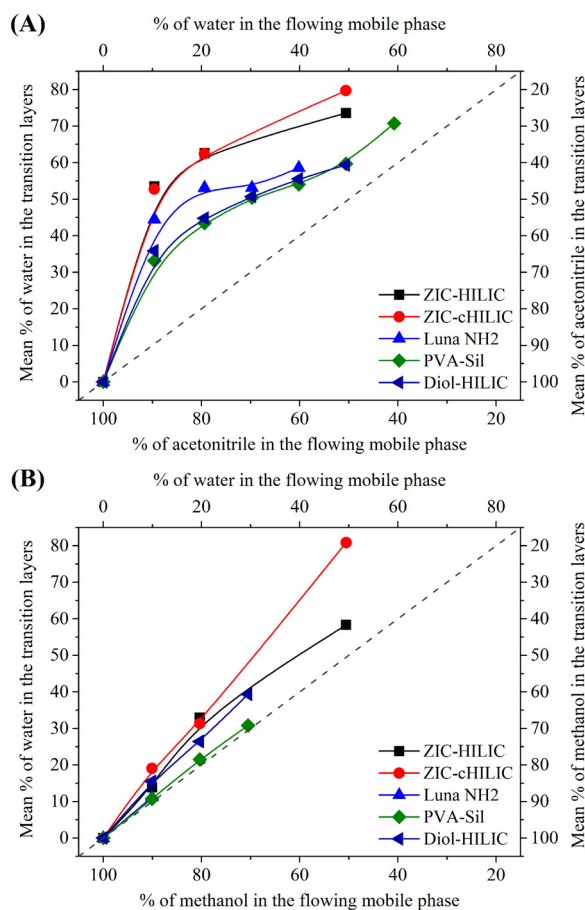


Fig. 5. Mean water content in the HILIC transition layers between the flowing mobile phase and the bonded phase and support: (A) acetonitrile-water and (B) methanol-water mixtures. A dashed grey line of unitary slope and null intercept would represent an exact match between transition layers and mobile phase composition.

tion layers were estimated according to the procedure described in Section 1.2, and the detailed results are presented in Table S3 of supplementary material. A summary is presented in Fig. 5. Only compositions with a clear water enrichment (relative errors less than 30%) are presented in this Figure. The error in the calculation of these compositions is a combination of the errors in the pycnometric (V_{solvent}) and chromatographic (V_M) measurements. Since V_{solvent} was determined from column weights measured in a calibrated analytical balance and the density of organic solvents at 25 °C, the error associated to its measurement was below 0.01 mL (<0.05%). The error in the measurement of hold-up volumes (V_M) for each studied chromatographic system was calculated from the fitting error of this parameter in Eqs. (6) and (7). It depends on the column and organic modifier employed, but the average error was 0.02 mL (1.2%). Since the uncertainties of V_{solvent} and V_M are comparable, the error related to the volume of the water-rich transition layers (V_L , Eq. (8)) obtained from the subtraction of one to the other is in the same range (0.02 mL). However, since V_L is smaller than V_{solvent} or V_M , the relative error is in fact much higher, with average values of about 4% and 14% for acetonitrile and methanol, respectively. Consequently, the uncertainty related to the determination of the density of the transition layers Eq. (10)) and its composition (Eqs. (11)-(12)) presented in Figure 5 are on average about 6% for acetonitrile and 20% for methanol.

For mobile phases containing acetonitrile as organic modifier a big excess amount of water on the transition layers in relation to the water provided by the flowing eluent is observed. Just with a

10% of water in the mobile phase, the transition layers had above 30% of water for the neutral columns PVA-Sil and Diol-HILIC, 40% for positively charged Luna NH2 and 50% for the zwitterionic ZIC-HILIC and ZIC-cHILIC columns, i.e., 3-5 times the amount of water in the mobile phase. When the percentage of water in the mobile phase increases, the percentage of water in the transition layers increases too as expected, but the proportion of excess water in transition layers in reference to the one in the mobile phase decreases. In mobile phases with 20% of water, the amount of water in transition layers is between 40% and 60% approximately (2-3 times the one in the mobile phase) and in 50% water between 60% and 80% (1.2-1.6 times). In any case, the excess of water follows the trend: ZIC-cHILIC $\approx$ ZIC-HILIC > Luna NH2 > Diol-HILIC $\approx$ PVA-Sil. Differences between aminopropyl and zwitterionic columns might be related to monomeric or polymeric grafted nature of the bonded phase on the silica support. The monomeric grafted Luna NH2 is expected to accumulate water in layers, whereas the grafted hydrophilic polymeric chains of both the ZIC-HILIC and ZIC-cHILIC columns are reported to form a hydrogel, and these grafted chains progressively extend when swelling [17].

For mobile phases containing methanol, the water enrichment of the transition layers is smaller than for acetonitrile-water eluents. The volume of these layers is very small too, as indicated in previous section (see also Fig. 4 and Table S3 of supplementary material). In consequence, the precision in the calculated mean water percentage in the transition layers is worse than for acetonitrile-water. Despite this problem, the results indicate that water adsorption is larger for the zwitterionic columns than for the polyvinyl and diol columns, as in acetonitrile-water. It is not so clear for aminopropyl Luna column because V_L is very small (less than 0.1 mL, Table S3) and the relative errors in the calculation of compositions are very large (more than 50%), but the calculated values seem to indicate that water enrichment with methanol-water eluents is similar to the ones of zwitterionic columns. The poor water enrichment and small volumes of the adsorbed water-rich layers produce a very small increase in the expected weight of the columns, which cannot be clearly seen in the plots of Fig. 2 for methanol-water. Hence, these plots are very close to the linearity expected for no significant water enrichment.

4. Conclusions

Combination of pycnometry and chromatographic volume retention measurements of homologous series provides information about the volume and composition of the water-rich transition layers semi-adsorbed in HILIC. Pycnometric measurements with pure solvents (water, acetonitrile, methanol) give the overall volume of labile solvent inside the HILIC column, whereas retention of homologous series allows calculation of the volume of solvent acting as mobile phase. The difference between both volumes is the effective volume of labile eluent in the transition layers of gradual variable composition between the water layer fully immobilized in column filling surface and the flowing mobile phase. Polar solutes are retained in these solvent layers, which can be considered HILIC stationary phases. Additional pycnometric measurements with the mixed eluents used as mobile phases (acetonitrile-water and methanol-water in this study) provide the weight of these water-rich stationary phases and combination with their volumes, the mean density and composition of the transition stationary phase layers.

Application of the method to several HILIC columns shows that the maximum water enrichment is produced for mobile phases of approximately 40% or more of acetonitrile, and more than 60% in the case of methanol. When the acetonitrile or methanol contents in the eluent decrease, water preferential adsorption decreases. In consequence, pure or almost pure HILIC retention is observed

for the rich acetonitrile and very rich methanol mobile phase compositions, a mixed HILIC-RPLC retention for the intermediate acetonitrile-water and methanol-water compositions, and a close to pure RPLC retention for the most water-rich mobile phases. Zwitterionic and aminopropyl HILIC columns show the largest water enrichment, followed by the polyvinyl alcohol and diol bonded columns. The HILIC pentafluorophenyl column studied shows no preferential water adsorption, nor HILIC behavior at all regardless of the mobile phase composition. Water adsorption is much larger for acetonitrile-water than for methanol-water eluents.

Declaration of Competing Interest

The authors declare that they have no known conflict of interest that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Lidia Redón: Investigation, Data curation, Writing – original draft. **Xavier Subirats:** Methodology, Validation, Formal analysis, Supervision, Writing – original draft, Visualization. **Martí Rosés:** Conceptualization, Methodology, Supervision, Writing – review & editing, Visualization, Project administration, Funding acquisition.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.chroma.2021.462543](https://doi.org/10.1016/j.chroma.2021.462543).

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