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# A revisit to the retention mechanism of hydrophilic interaction liquid chromatography using model organic compounds

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ABSTRACT

In this work, a revisit to the retention mechanism of HILIC was attempted to point out critical factors that contribute to the chromatographic regime as well as to bring out subtle details of the relative contribution of partitioning and surface adsorption. In this vein, the retention behaviour of a set of water-soluble vitamins (WSVs) and toluene on three silica based columns was evaluated under varying chromatographic conditions. The data obtained were associated with the hydration degree of the stationary phases and the ability of the organic solvents to disrupt the formation of the water-enriched layer. Moreover, the elution behaviour of toluene at different buffer salt concentrations in the mobile phase, confirmed the preferential partition of salt ions into the stagnant layer, as ACN content was increased. The results from the fitting of partitioning and surface adsorption models indicated differences in the contribution of the two retention mechanisms to both neutral and charged compounds. The occurrence of surface adsorption and the retentivity differences for neutral WSVs depend on the hydration degree and the hydrogen bonding properties of the solutes and the column surface, respectively. For charged solutes experiencing electrostatic repulsion, the contribution of the adsorption mechanism at highly organic mobile phases, emanates from both the weak effect of buffer salt ions on the electrostatic interaction and the strong effect of hydrophilic interactions. On the other hand, the chromatographic retention of electrostatically attracted solutes indicates that the surface adsorption dominates, even at mobile phases rich in water.



## 1. Introduction

Reversed-phase liquid chromatography (RPLC) is the most widely used separation method in modern chromatographic science. However, it is not always the best choice for the separation of polar compounds, as they may show inadequate retention and thus poor resolution [1]. Hydrophilic interaction liquid chromatography (HILIC) has proven to be a useful analytical tool for the separation of polar analytes, offering a complementary selectivity compared to RPLC [2]. This separation mode utilizes a polar stationary phase in conjunction with a less polar mobile phase, which in most cases is a binary aqueous-organic mixture with water being the strongest eluting solvent [3].

It has been suggested that the driving force for the retention in HILIC is a mixed-mode mechanism. Partitioning of polar compounds between a water-enriched layer partially immobilized (or "slow moving") on the surface of the stationary phase and a highly organic mobile phase is postulated to be the primary retention mechanism [3,4]. Hydrogen bonding participates in this process, possibly as a driving force in the partitioning [5–8] while electrostatic interactions contribute to the retention mechanism, to a varying degree, depending upon the nature of the stationary phase, the ionization of the target compounds and the type and concentration of buffer salts in the mobile phase [4,5,7,9–17]. However, little work has been devoted to the transition between partitioning and/or adsorption mechanisms in HILIC mode and on the factors that influence this alteration.

Critical parameters for the retention mechanism in HILIC are the structure of the solute, the composition of the mobile phase and the polar functional moieties on the stationary phases. The surface of a HILIC stationary phase can be acidic, neutral or basic. Consequently, the retention mechanism may vary if compounds with assorted functional groups, from neutral to acidic and basic, are applied on different stationary phases. Polar stationary phases commonly used in HILIC separations consist of bare silica and silica phases modified with diol, amino, amido and zwitterionic functional groups. A great variety of stationary phases for HILIC applications has been extensively reviewed by Hemström and Irgum [1] followed up by the work of Ikegami and his colleagues [18]. Finally, the nature of the organic solvent influences significantly the retention and selectivity of polar compounds in this chromatographic mode [19].

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The objective of this study is to afford further insights into the retention mechanism of HILIC. A set of test compounds was opted for to provide a diversity of charge states as well as structural variations, position of charges etc. The objective was fulfilled by scrutinizing the chromatographic behaviour of polar water-soluble vitamins (WSVs) on three commercially available silica-based columns. Three models were evaluated in order to assess the contribution of partitioning and surface adsorption to the mechanism. The hydration degree of the stationary phases was estimated based on the elution behaviour of the apolar toluene, which was taken to correlate with the transition from partition to surface adsorption mechanism. Finally, the disturbance of the aqueous layer adsorbed on the polar phase was probed by monitoring the elution time of toluene in the presence of different organic solvents.

## 2. Experimental

### 2.1. Chemicals and reagents

Ammonium acetate, acetic acid, thiamine, nicotinic acid, nicotinamide, pyridoxine, DL-dithiothreitol, were obtained from Fluka Chemie (Buchs, Switzerland). Ammonium formate and riboflavin were obtained from Sigma–Aldrich Hellas (Athens, Greece) and formic acid from Scharlau Chemie (Barcelona, Spain). Ammonia (25%) was purchased from Merck (Darmstadt, Germany) and L-ascorbic acid from Reidel-de Haën (Seelze, Germany). HPLC grade acetonitrile (ACN), methanol (MeOH), isopropanol (IPA) and tetrahydrofuran (THF) were purchased from Fisher Scientific (Leicester, UK). Double-distilled water filtered through a 0.45 μm nitrocellulose membrane was used throughout. All chemicals and solvents were of analytical-reagent grade.

### 2.2. Standard solutions

Stock solutions of WSVs (500 mg/L) were made by dissolving appropriate amounts in ACN/water (1/1) in volumetric flasks of 10 mL. DL-Dithiothreitol was used as a stabilizer for L-ascorbic acid solutions. Complete dissolution of riboflavin was achieved with the addition of 40 μL of ammonia (25%) under magnetic stirring. All stock solutions were stored refrigerated in dark vials. Stock solution of toluene (1000 mg/L) was prepared by diluting the appropriate volume in 100% ACN. Working standard solutions were prepared daily from concentrated stock solutions by appropriate dilution with mobile phase with respect to the initial mobile phase conditions.

### 2.3. Instrumentation—chromatography

Liquid chromatographic analysis of the WSVs was conducted on a Shimadzu (Duisburg, Germany) HPLC system consisting of a LC20AD pump, a CTO 10AS column oven and a SPD-M20A diode array detector. Injections were made through a manual 7725i Rheodyne (Cotati, CA, USA) injector using a 20 μL sample loop. The columns tested were: Inertsil silica, HILIC-diol with endcapping and amino without endcapping (GL Sciences, Tokyo, Japan). The columns were of the same dimensions *viz.* 150 mm × 4.6 mm, 5 μm particle size, 100 Å. The LC-solution software Version 1.21 SP1 was used for data analysis and processing. Detection wavelengths were 254 and 272 nm.

The mobile phase of the HPLC system consisted of aqueous ammonium acetate (or ammonium formate) at various concentrations and pH values (solvent A) and ACN/water 90/10 (v/v) containing ammonium acetate (or ammonium formate) (solvent B), at concentrations equal to those in solvent A. The pH of solvent A was adjusted with acetic acid (or formic acid). The same volume of acid was added to the solvent B, at every pH value. A 0.45 μm nitrocellulose membrane and a 0.45 μm Teflon membrane were used for filtering the aqueous and the organic mobile phase, respectively. Mixed aqueous-organic mobile phases were delivered isocratically. The retention time of the solvent peak (baseline disturbance) was used as the void time for calculating retention factor.

## 3. Results and discussion

### 3.1. Retention of WSVs and selectivity of polar stationary phases

#### 3.1.1. Effect of acetonitrile content

The effect of ACN content on the retention was studied in the range of 50–90% (v/v), at a constant buffer salt concentration of 10 mM. The aqueous portion was adjusted to pH 5.0. Fig. 1 shows the variation of retention factor of the selected WSVs as a function of ACN content in the mobile phase.

Typical HILIC behaviour was observed for almost all six vitamins on the diol, silica and amino columns, as the retention of the compounds increased with increasing ACN content. The elution of thiamine from the silica column was feasible only when the water content in the mobile phase was 50% (v/v) due to the strong electrostatic attraction to the negatively charged surface. When ACN was held at 90% (v/v), nicotinamide and pyridoxine were more strongly retained on the silica column as compared to the amino and diol phases. On the other hand, riboflavin was retained more strongly on the amino column. The elution order of these compounds hinges on the presence of different (in kind and number) polar functionalities exhibiting weak hydrogen bonds ("π hydrogen bonds") to medium strength hydrogen bonds (e.g. via –OH, $-NH_2$) [21] if one allows for the possibility of a direct interaction between the polar solutes and the stationary phase [6–9,20].

For thiamine, electrostatic repulsion was evident on the amino column, which had the same charge. This solute was retained more weakly than nicotinic and L-ascorbic acids, which experienced electrostatic attraction [9], at 50–80% ACN (v/v). However, thiamine is evidently more hydrophilic than the acids. Therefore, at 90% ACN, where hydrophilic interaction dominates the chromatography [4], it fails to elute within 50 min despite the electrostatic repulsion. The acids were similarly well-retained on the silica column at 90% ACN despite the electrostatic repulsion in that case. This behavior is compared to that obtained with a neutral column such as the diol phase [22,23]. However, at lower levels of ACN (50–70%), where hydrophilic interaction was weak, the electrostatic repulsion of the acidic solutes on the silica column resulted in their earlier elution than the "neutral" ones: nicotinamide, pyridoxine, and riboflavin. Apparently, the electrostatic interactions between the solutes and a stationary phase of the same charge become less important at highly organic mobile phases and hydrophilic interactions dominate [4].

The retention factor of nicotinic acid (being negatively charged at pH 5.0) on the amino column decreases as the ACN content is increased from 50 to 70% and increases with further increment of ACN. The same retention behaviour, yet to a lower degree, holds true for L-ascorbic acid on the amino column when ACN content is increased from 50 to 60% (v/v). This chromatographic behaviour deviates from the typical HILIC retention trend, reflecting the complexity of the overall retention mechanism, and could be related to the competition between the negatively charged compounds and the acetate anions for the anion-exchange sites of the amino column. At highly organic mobile phases, these solutes partition more efficiently into the aqueous layer and hence they may be rendered more competitive for the positively charged sites of the stationary phase.

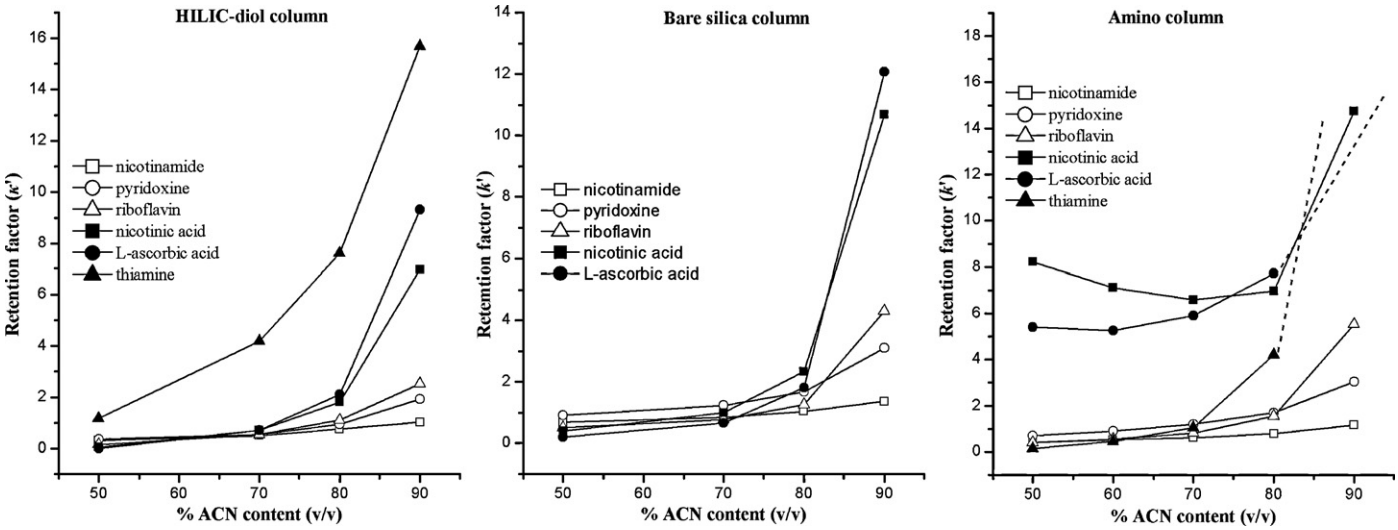


**Fig. 1.** Plots of *k*' vs. ACN content on different polar stationary phases. Mobile phase: ACN/$H_2O$ at various percentages; ammonium acetate, 10 mM; pH 5.0; column temperature, 25 °C; flow rate, 0.6 mL/min.

### 3.1.2. Effect of mobile phase pH

In this part of the study, ammonium acetate at a concentration of 10 mM was utilized in the pH range of 4.0–6.0. At pH 3.0, ammonium formate of the same concentration was the choice due to better buffering capacity at low pH values. The diol column has a neutral bonded phase, the aminopropyl phase possesses a significant positive charge at acidic and neutral pH values and the unbonded silica (p*K*a ~ 4) carries a fully negative charge at pH > 6.0.

Fig. 2 mirrors the effect of mobile phase pH upon retention and selectivity of WSVs. No significant difference was observed on the chromatographic behaviour of nicotinamide, which was practically uncharged (differences in *k*', less than 0.1) at the working pH conditions (p*K*a = 3.3). The same elution order was recorded for pyridoxine and riboflavin on the diol and amino columns. As for the silica column, pyridoxine was retained more strongly (*k*' = 3.55) than riboflavin (*k*' = 3.18) at pH 3.0, resulting in a reversed elution order. A reasonable explanation of this behaviour is the existence of weak electrostatic interactions between the positively charged pyridoxine (p*K*a of pyridinyl-group = 5.0) and the stationary phase, considering that silica is not fully charged at pH 3.0. Electrostatic repulsion could also explain the moderate decrease in the retention of pyridoxine on the amino column (reduction in *k*', from 3.15 to 2.81), when the mobile phase pH was lowered from 6.0 to 4.0.

The elution order of nicotinic and L-ascorbic acids was quite similar on the diol and silica columns. However, on the silica column at pH 3.0, L-ascorbic acid was the first-eluting compound, in contrast to nicotinic acid, which was the last eluting one in the chromatogram (Fig. 2B). Nicotinic acid exists as a zwitterion (p$Ka_1$ = 2.2 for carboxyl- and p$Ka_2$ = 4.7 for pyridinyl-group) and thus, it is more hydrophilic than L-ascorbic acid, which is mainly protonated at pH 3.0 (p*K*a = 4.0). The electrostatic attraction between the positively charged pyridinyl group of the solute and the partially charged surface of the silica column could be an additional rationale. In addition, stronger retention was recorded for these two acids on the silica column as compared to the diol phase. Nicotinic and L-ascorbic acids, both being negatively charged at pH 5.0, were more strongly retained on the amino column as compared to the diol and silica columns by virtue of the electrostatic attraction [13,17,24] even if the mobile phase is of high eluting strength with regard to hydrophilic interaction.

The overall chromatographic behaviour of the two acids on the amino column warrants further investigation. As expected, going from pH 3.0 to 4.0, the retention time of the compounds increased, due to the significant degree of deprotonation. Further increment of the pH of mobile phase from 4.0 to 6.0 gradually decreased the retention of nicotinic acid (reduction in *k*', from 13.37 to 10.10). The same was true for L-ascorbic acid, when the mobile phase pH was raised from 5.0 to 6.0 (reduction in *k*', from 21.90 to 19.16). This retention behaviour is presumably the result of weaker electrostatic attraction between the acidic compounds and the amino column due to the decrease in the net positive charge on its surface [4]. In addition, at pH > 4.0, a significant amount of acetate anions are present in the negative form (p*K*a = 4.8), thus competing more effectively with the active sites of the amino column [14].

Thiamine, which carries a permanent positive charge (thiazolium ring) was retained more strongly on the diol column (up to 10 times longer retention times) as compared to the rest of the WSVs [25]. Thiamine was not eluted from the silica column within the pH range of 3.0–6.0, even at 30% water (v/v), due to the existence of strong cation-exchange interactions [5,20,26,27]. On the positively charged amino column, the existing electrostatic repulsion differentiates the elution pattern and peak shape of thiamine from that of the other charged solutes [15]. Lowering the pH of mobile phase from 6.0 to 3.0, a decrease in the retention of thiamine on the amino column was noticed. This can be accounted for by the increase in the positive charge density on the surface of the stationary phase and the existence of an additional positive charge of pyrimidinyl-group (p*K*a = 4.8) that contributes to the overall repulsive interactions.

### 3.1.3. Effect of buffer salt concentration

The effect of buffer salt concentration on the retention was investigated in the range of 5–20 mM. For the diol and silica columns, ammonium acetate was used at pH 5.0, with mobile phase consisting of ACN/water 90/10 (v/v). For the amino column, ammonium formate was used to attain a pH 3.0 with mobile phase consisting of ACN/$H_2O$ 85/15 (v/v). The results are portrayed in Fig. 3.

The retention of nicotinamide was practically unaffected (differences in *k*', less than 0.1) on all three columns employed as the buffer concentration was increased. A slight to moderate increase in the retention was observed for pyridoxine on the diol column and for riboflavin on all three columns with increasing buffer salt concentration. The retention of nicotinamide and riboflavin is mainly through partitioning, as they both remain at pH 5.0. The higher the salt concentration in the mobile phase the greater the

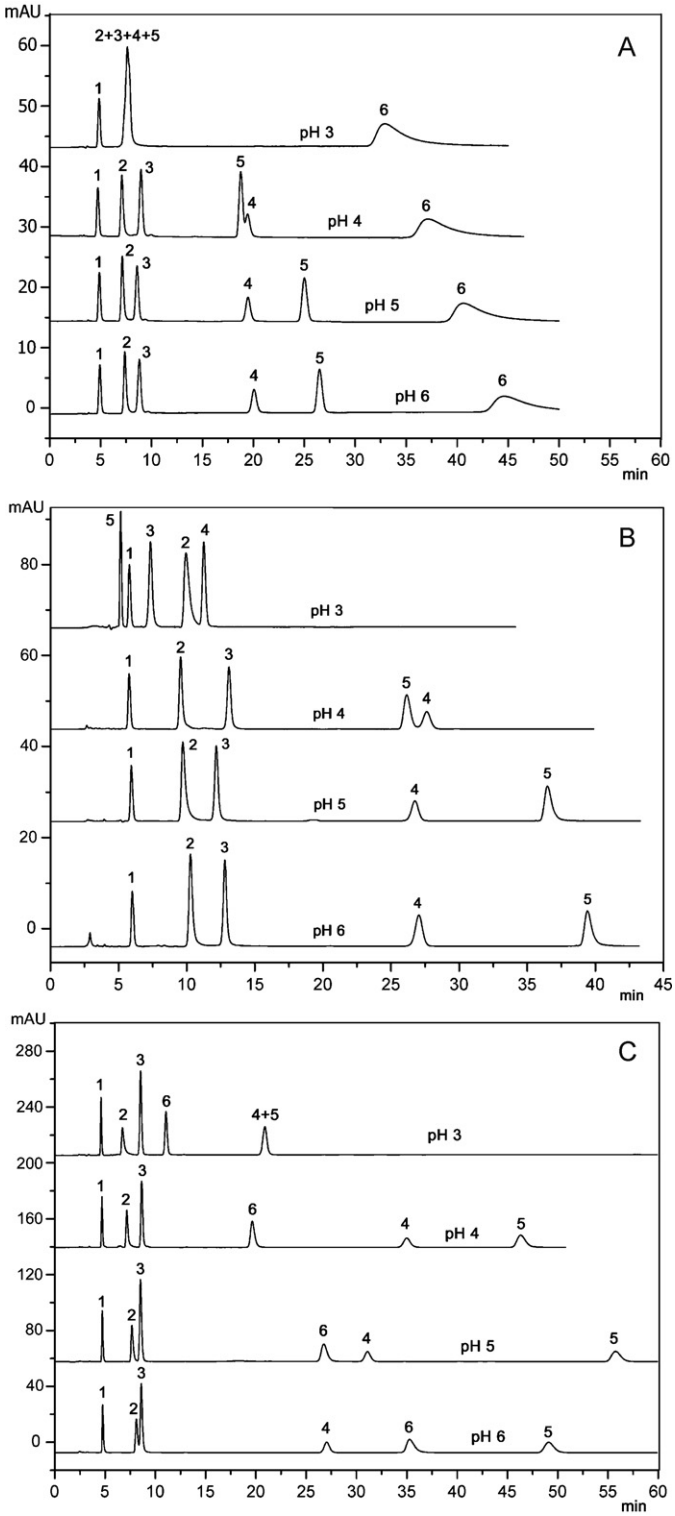


**Fig. 2.** Effect of pH on separation of WSVs. (A) HILIC diol, (B) bare silica, and (C) amino column. Conditions for A and B: ACN/$H_2O$, 90/10 (v/v); ammonium acetate/formate, 10 mM; flow rate, 0.6 mL/min; column temperature, 25 °C. (C) ACN/$H_2O$, 85/15 (v/v); other conditions as given for columns (A) and (B). Detection wavelength, 272 nm. Peak assignment: (1) nicotinamide, (2) pyridoxine, (3) riboflavin, (4) nicotinic acid, (5) L-ascorbic acid, and (6) thiamine.

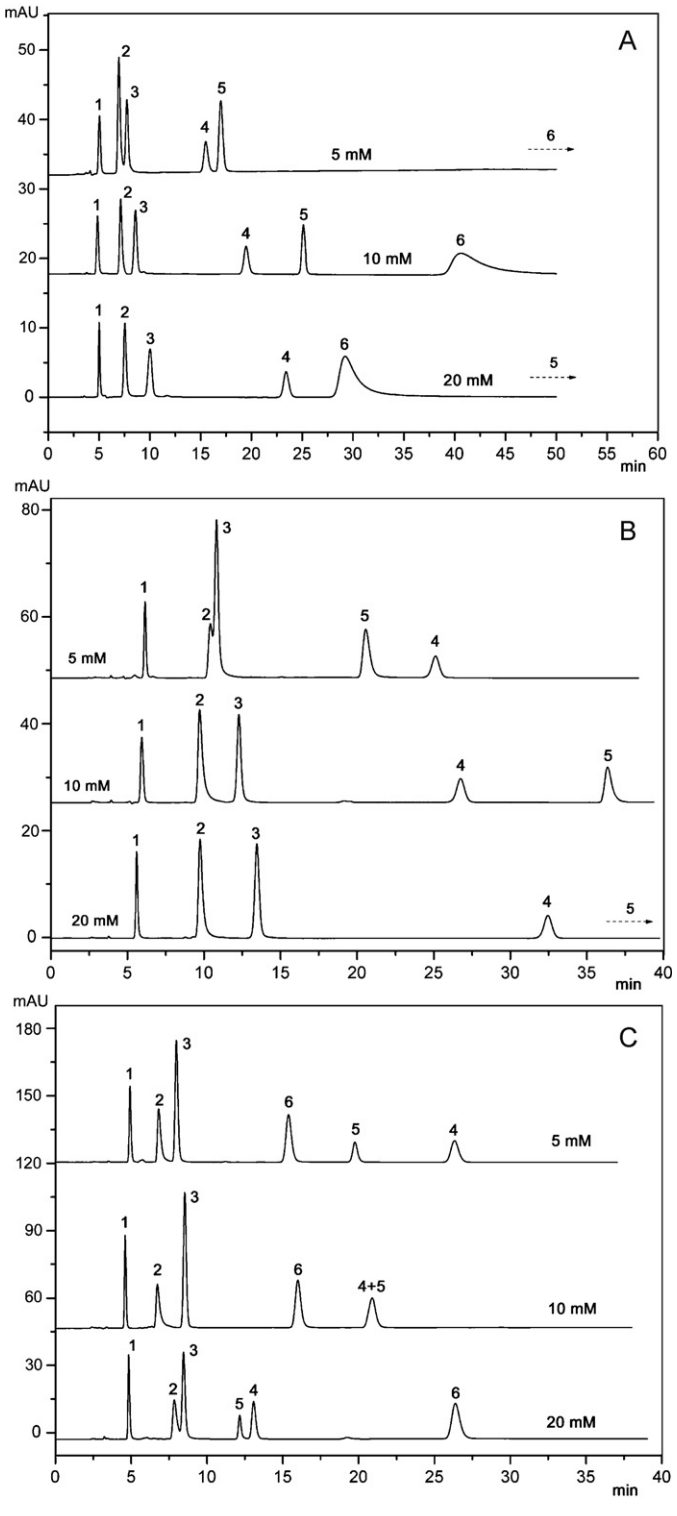


**Fig. 3.** Effect of buffer salt concentration on the separation of WSVs. (A) HILIC diol, (B) bare silica, and (C) amino column. Conditions for (A) and (B): ACN/$H_2O$, 90/10 (v/v); pH, 5.0; flow rate, 0.6 mL/min; column temperature, 25 °C. (C) ACN/$H_2O$, 85/15 (v/v); pH, 3.0; other conditions as given for columns (A) and (B). Detection wavelength, 272 nm. Peak assignment as in Fig. 2.

volume of the water-enriched layer or the higher its hydrophilicity giving rise to longer retention times through partitioning [13,17]. At pH 5.0, pyridoxine is ~50% positively charged while at pH 3.0 the nitrogen of the pyridinium ring is fully protonated (p*K*a = 5.0). As a result, increasing the buffer salt concentration leads to a moderate decrease in the retention on the silica column. The opposite behaviour was observed on the amino column at pH 3.0, as ammonium formate concentration is increased from 10 to 20 mM, evidence that the salt is shielding electrostatic repulsion.

Nicotinic and L-ascorbic acids tended towards longer retention times on both diol and silica columns when buffer concentra-

tion increased. This behaviour is attributed to the competition of ammonium cations for the active silanol sites, thus reducing the electrostatic repulsion [4,9,16,28]. It is worth mentioning that L-ascorbic acid was repelled more strongly than nicotinic acid from the silica at a buffer concentration of 5 mM, resulting in a reversal of the elution order. Not surprisingly, the opposite was true of the amino column, where the retention of the two acids was decreased markedly [5] when switching from 10 to 20 mM. Finally, at pH 3.0, where nicotinic acid carries both positive and negative charges, the electrostatic attraction dominates over repulsion. In partition- or adsorption-based (e.g. ion-exchange chromatography) chromatographic mode, solute molecules can be orientated in such a way that interactions with the stationary phase are thermodynamically favoured [29–31].

Thiamine exhibits a significantly decreased retention on the diol column when the buffer concentration is increased, presumably due to weakened ion-exchange interactions with residual silanol groups [7]. On the other hand, thiamine still showed strong retention on the silica column in the range of 5–20 mM of ammonium acetate to the point that it did not elute. In contrast, there was an increase in the retention of the solute on the amino column with increasing ammonium formate concentration. This effect was clearly evident when 20 mM of ammonium formate was used and the behaviour is in line with that of other basic compounds on amino-bonded phases [12]. The retention of the solute was less affected as the buffer salt concentration was increased from 5 to 10 mM. It should also be mentioned that at higher water contents (i.e. ≥30%), changing the amount of salt in the mobile phase had no significant effect on the retention of most of the solutes employed except those which are electrostatically attracted (Figs. S1–S3 of Supplementary Material). As proposed by Alpert, a salt concentration greater than 20 mM is needed for suppressing the electrostatic effects between solute/stationary phase [4]. However, the results herein indicate that the occurrence and the strength of electrostatic interactions hinge on both water content and buffer concentration in the mobile phase. The retention behaviour of the charged compounds on the amino column reveals that the electrostatic interactions (attractive or repulsive) exist at ACN/water 85/15 (v/v) and in the buffer concentration range of 5–20 mM. On the other hand, operating at highly organic mobile phases (i.e. above 85%), as is the case for the silica column, a salt concentration of 10 mM can effectively suppress electrostatic interactions (Fig. 3B). It should also be stated that the selectivity of the amino column at ACN 90% with 10 mM of ammonium formate (Fig. S3) resembles that of the diol and silica columns, as thiamine becomes again the most retained compound.

To stress the role of the buffer salt ions on the retention mechanism, relevant chromatograms were run injecting toluene in the presence and absence of a buffer salt in the mobile phase (Fig. 4). The results indicated that the elution time of toluene gradually decreased (reduction in the elution time, from 1.6 to 3.2%) in the presence of ammonium acetate as the ACN content was increased from 70 to 90%. This is evidence of the tendency of salt ions to partition into the aqueous layer, increasing the volume (or polarity) of the layer.

### 3.1.4. Effect of organic modifier

Fig. 5 shows the effect of different organic solvents, namely, ACN, MeOH, IPA and THF on the retention of the WSVs. Poor or absence of retention for compounds that are retained mainly by partitioning and hydrogen bonding arose from the use of protic solvents, such as MeOH and IPA [5,7,19]. The relatively weaker strength of IPA is attributed to its more hydrophobic character [5]. For compounds that experience electrostatic attraction, retention is adequate. Such is the case for thiamine on the diol and silica columns and nicotinic and L-ascorbic acids on the amino column. It is worth noticing that

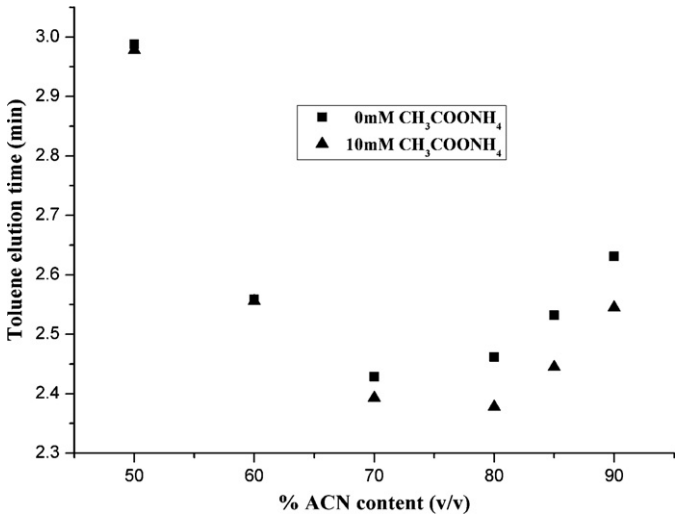


**Fig. 4.** Effect of buffer salt concentration on the elution time of toluene. Conditions: diol column; ACN/$H_2O$ at various percentages; flow rate, 0.6 mL/min; column temperature, 25 °C; detection wavelength, 254 nm.

the elution of thiamine was realized on the silica column only with the use of MeOH as the weak eluent. Finally, the substitution of THF for ACN caused a significant decrease in the retention of almost all compounds, presumably because of the higher hydrogen-bonding acceptor capability of THF [5]. In contrast, the use of THF caused a drastic increase in the retention of thiamine on the amino column. The same effect was evident on the diol column but to a lesser degree. This finding suggests that, the retention of thiamine is determined by the hydrogen bond-acceptor properties of ACN and THF.

In all three columns, the eluting strength follows the order: ACN < THF < IPA < MeOH, which is correlated with the different hydrogen bonding potentials of the organic solvents [5,7]. The retention loss of polar solutes in the presence of MeOH has also been attributed to the disruption of the formation of the water-enriched layer by the replacement of water with organic molecules [5,32]. To yield further insights into this phenomenon, for the three stationary phases, an experiment was carried out to monitor the elution behaviour of toluene in the presence of the four organic solvents, on the three stationary phases. Considering that toluene is an apolar molecule, the increase in the elution time would be evidence of the formation of a less hydrophilic layer immobilized on the surface of the stationary phase. The results are shown in Fig. 6. On the diol column the elution time of toluene in MeOH was approximately twice than in ACN, while the increase in the elution time in IPA was less than that in MeOH. It is noticeable, though, that toluene was eluted at longer times using ACN as compared to THF. On the amino column, except for the organic–aqueous mixture of 50/50 (v/v), the disruption of the aqueous layer is greater in MeOH, followed by IPA, THF and ACN. As for the silica column, this order is hardly applicable. However, it can be said that for most of the organic solvents the replacement of the water molecules is more efficient with MeOH than with IPA, THF and ACN. In addition, THF causes greater disruption than does ACN and less than IPA.

Based on the above observations it is clearly evident that MeOH replaces water molecules more efficiently than any other organic solvent assessed, thus toluene can penetrate a larger pore volume. Comparing MeOH and IPA, in all three columns, the disruption upon the aqueous layer is highly dependent of the hydrogen-bonding acidity of the organic solvent. As for ACN and THF on the diol column, the disruption is controlled by the same factor but on the silica and amino columns, the disruption seems to be influenced by the hydrogen-bonding basicity of the solvent molecules.

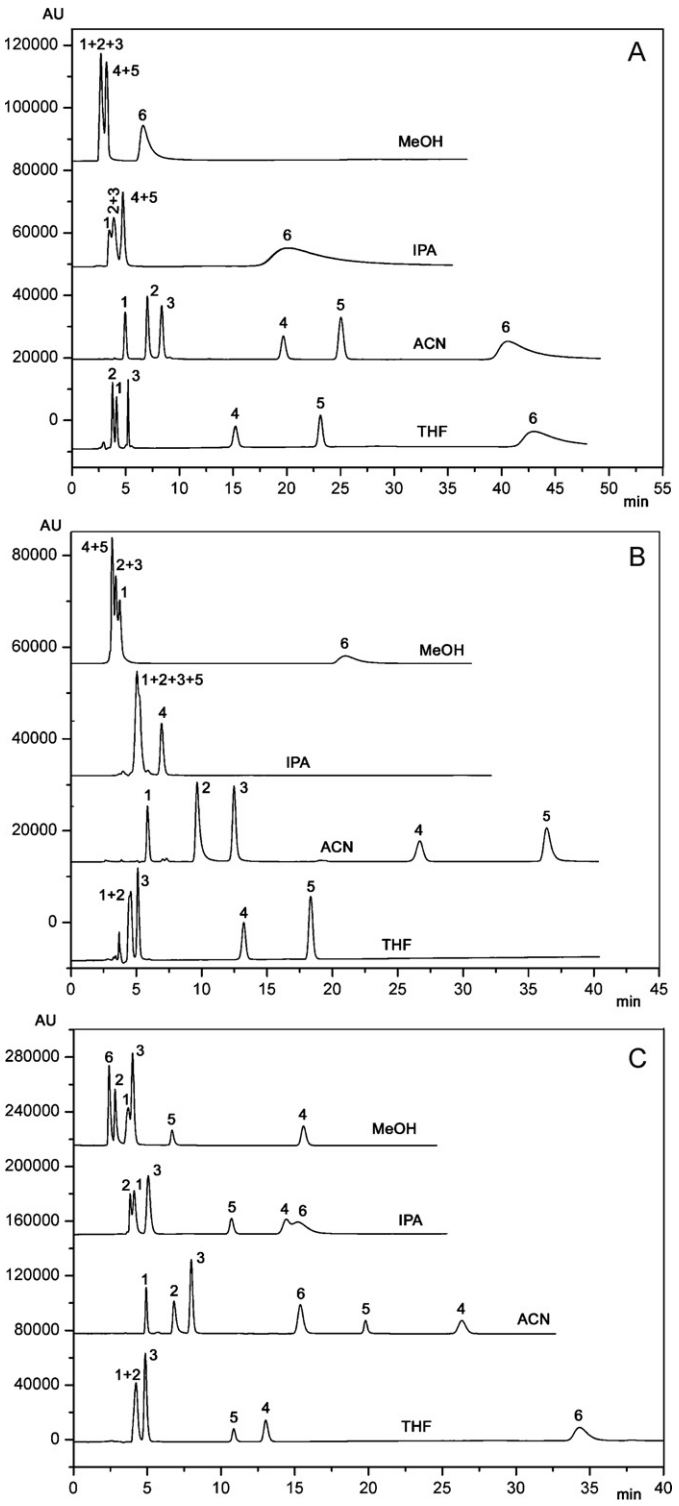


**Fig. 5.** Effect of different organic solvents on the separation of the WSVs. Conditions (A) diol and silica columns: organic/$H_2O$, 90/10; $CH_3COONH_4$, 10 mM; pH, 5.0. (B) Amino column: organic/$H_2O$, 85/15; $HCOONH_4$, 5 mM; pH, 3.0; flow rate, 0.6 mL/min; column temperature, 25 °C. Detection wavelength, 272 nm. Peak assignment as in Fig. 2.

The elution time of toluene using MeOH as the weak eluent, was longer on the diol column than on the silica and amino columns. This behaviour was less noticeable with IPA and THF, indicating that the retention loss of polar solutes is significantly influenced by the solute–solvent interactions, as well. However, the presence of MeOH seems to be beneficial for strongly retained compounds (i.e. thiamine on the diol and silica columns, nicotinic and L-ascorbic acids on the amino column). The retention is significantly decreased but the solutes still show adequate retention. The above observations are in line with a recent successful application of alcoholic mobile phases for the separation of polar and ionizable compounds [33].

### 3.1.5. Effect of column temperature

The effect of column temperature was investigated on all three columns by varying the column temperature over the range of 15–45 °C, in increments of 10 °C. For the diol and silica columns, a constant mobile phase composition of ACN/water 90/10 (v/v) was utilized containing 10 mM ammonium acetate at pH 5.0. For the amino column, a mobile phase composition of ACN/$H_2O$ 85/15 (v/v) was used containing 5 mM ammonium formate at pH 3.0. Squared correlation coefficients and retention enthalpies of WSVs as predicted by van't Hoff equation [19] are given in Table 1 (Van't Hoff plots are illustrated in Figs. S4–S6 of Supplementary Material). Acceptable linearity was found for nicotinamide and riboflavin on all three columns [34–36]. Linearity was also noticed for pyridoxine on the diol column, while the opposite was noticed on the silica and amino columns. Nonlinear relationships of the van't Hoff equation were also found for nicotinic and L-ascorbic acids on the silica and amino columns. Deviation from linearity was also observed for thiamine on both diol and amino columns while no data could be gained for the silica column due to the complete retention of the compound.

Enthalpy values ranged from $-2.5 \pm 0.2$ to $-20 \pm 1$ kJ/mol indicating that an exothermic process of transferring solutes from the mobile phase to the stationary phase occurs and the solutes become less retained as column temperature is increased [13]. Retention enthalpy values for the two acids on the amino column were close to zero. A moderate to large decrease in the retention was noticed for the neutral solutes as well as for the solutes carrying the same charge with the stationary phase. A reduction in hydrogen-bonding strength with increasing temperature could be a logical explanation. As the temperature increases, the thermal motion weakens the hydrogen bonds so that the polarity of water is reduced [37,38]. On the other hand, only a slight decrement was observed on the retention of compounds which are electrostatically attracted, such as thiamine on the diol column and nicotinic and L-ascorbic acids on the amino column.

When a solute is electrostatically attracted by the stationary phase, the motion of the molecules is restricted, as measured by a change in entropy [39]. It is expected that the amount of water adsorbed onto the surface of the stationary phase would change with temperature [16,40,41]. The elution time of toluene was monitored at several temperatures within the studied range and it was found that the variations were less than 0.8% as compared to its elution time at 25 °C. As an example, the variation of elution time of toluene on the diol column was as follows: $t_{15\,^\circ C} = 2.65 \pm 0.00$ min, $t_{25\,^\circ C} = 2.65 \pm 0.00$ min, $t_{35\,^\circ C} = 2.66 \pm 0.00$ min, $t_{45\,^\circ C} = 2.67 \pm 0.00$ min. The small variations on the elution time of toluene with temperature would be evidence of the inconsequential change in the thickness of the stagnant aqueous layer. Therefore, we may assume that, the differences in intercepts of the van't Hoff equation can be more related to entropy changes than changes of the phase ratio (term $\ln \phi$) itself [42]. On the diol column, thiamine has a positive entropy change ($\Delta S^0/R + \ln \phi = +0.22$), in contrast to the rest of the WSVs ($-1.69 < \Delta S^0/R + \ln \phi < -5.55$), which could be related to the electrostatic attraction of the solute from residual silanol groups. On the silica column, only nicotinic acid exhibits a positive entropy change ($\Delta S^0/R + \ln \phi = +0.39$). This finding is in accordance with the chromatographic behaviour of the solute (Fig. 3B), which is less susceptible than L-ascorbic acid to electrostatic repulsion as the buffer salt concen-

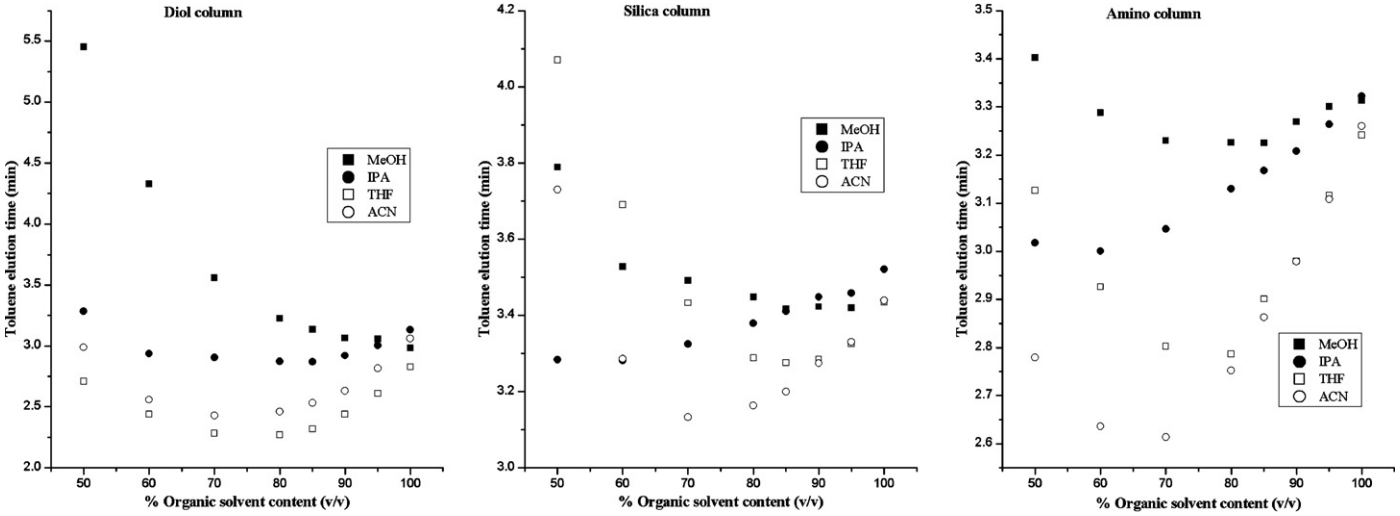


**Fig. 6.** Effect of organic modifier on the elution time of toluene. Conditions: organic/aqueous solvent at various percentages; flow rate, 0.6 mL/min; column temperature, 25 °C. Detection wavelength, 254 nm.

tration is decreased. Finally, on the amino column, nicotinic and L-ascorbic acids show a higher positive entropy change ($\Delta S^0/R + \ln \phi$, +2.00 and +1.85, respectively), which is in agreement with the abovementioned ion-exchange retention mechanism.

### 3.2. Mechanism of retention

#### 3.2.1. Hydration of the polar stationary phases

In HILIC, the existence of a layer on the surface of a polar stationary phase is postulated, which is rich in water even at highly organic mobile phases [1,43–45]. However, the water molecules are dynamically immobilized on the surface of the column; thus, the hydration of polar stationary phases would vary according to the nature of the column surface [19]. The estimation of the hydration of the three silica-based columns was attempted by comparing the chromatographic behaviour of toluene, as shown in Fig. 7. In all columns, the elution time of toluene has its lowest value at approximately 70% ACN (v/v) while at lower or higher percentages this time rises. The above results are in agreement with a previous work [43] and suggest a close relation of toluene elution behaviour to the thickness (or volume) of the aqueous layer formed on each column. In the work of McCalley and Neue, it has been mentioned that below 70% (v/v) of ACN the difference in the polarity between the aqueous layer and the bulk eluent is significantly decreased, whereby the increase in elution time of toluene through partitioning is accounted for [43]. However, the increased elution time above 70% of ACN could be explained by the decreased volume (or thickness) of the layer. Moreover, at a highly organic mobile phase, the stagnant layer is not purely aqueous, a fact which would permit partitioning of toluene to some degree.

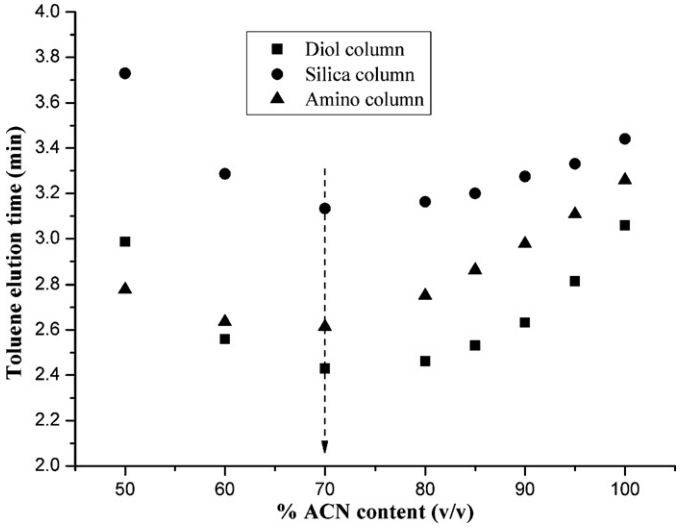


**Fig. 7.** Elution time of toluene on diol, silica and amino columns. Conditions: ACN/$H_2O$ at various percentages; flow rate, 0.6 mL/min; column temperature, 25 °C. Detection wavelength, 254 nm.

At an ACN content ≥60% (v/v), the elution time of toluene follows the order: $t_R^{silica} > t_R^{amino} > t_R^{diol}$. It seems that the aqueous layer is thinner on the silica column followed by the amino and diol columns. The thinner the aqueous layer formed on the surface of the stationary phase the greater the opportunity of toluene to reach and interact with the hydrophobic part (i.e. siloxanes, propyl group spacers) of the chromatographic stationary phases [27,43]. Taking

**Table 1**
Squared correlation coefficients and retention enthalpies of WSVs on different polar stationary phases, from Van't Hoff equation[a].

| Compound | Diol column[b] | | Silica column[b] | | Amino column[c] | |
|---|---|---|---|---|---|---|
| | $R^2$ | $\Delta H^0$ (kJ/mol) | $R^2$ | $\Delta H^0$ (kJ/mol) | $R^2$ | $\Delta H^0$ (kJ/mol) |
| Nicotinamide | 0.9966 | −5.6 ± 0.3 | 0.9915 | −2.5 ± 0.2 | 0.9951 | −3.3 ± 0.2 |
| Pyridoxine | 0.9900 | −8.5 ± 0.3 | 0.9725 | −6.6 ± 0.2 | 0.9851 | −3.5 ± 0.2 |
| Riboflavin | 0.9951 | −10.4 ± 0.4 | 0.9944 | −7.1 ± 0.4 | 0.9899 | −2.6 ± 0.3 |
| Nicotinic acid | 0.9847 | −9.5 ± 0.4 | 0.9460 | −4.8 ± 0.3 | 0.9122 | −0.7 ± 0.4 |
| L-Ascorbic acid | 0.9870 | −20 ± 1 | 0.9120 | −13.8 ± 0.6 | 0.9048 | −0.3 ± 0.2 |
| Thiamine | 0.9524 | −6.1 ± 0.8 | – | – | 0.9222 | −7.3 ± 0.6 |

[a] Van't Hoff equation: $\ln k' = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + \ln \phi$.
[b] ACN/$H_2O$, 90/10; $CH_3COONH_4$, 10 mM; pH 5.0.
[c] ACN/$H_2O$, 85/15; $HCOONH_4$, 5 mM; pH 3.0.

**Table 2**
Retention factors, $k'$, of neutral WSVs on the different stationary phases. Conditions: $CH_3COONH_4$, 10 mM; pH, 6.0; flow rate, 0.6 mL/min; column temperature, 25 °C.

| Compound | Diol column | | | Silica column | | | Amino column | | |
|---|---|---|---|---|---|---|---|---|---|
| | ACN (v/v) | | | ACN (v/v) | | | ACN (v/v) | | |
| | 70% | 80% | 90% | 70% | 80% | 90% | 70% | 80% | 90% |
| Nicotinamide | 0.49 | 0.70 | 1.10 | 0.82 | 1.03 | 1.46 | 0.62 | 0.83 | 1.16 |
| Pyridoxine | 0.66 | 1.03 | 2.08 | 1.24 | 1.78 | 3.20 | 1.37 | 1.89 | 3.15 |
| Riboflavin | 0.46 | 0.92 | 2.70 | 0.70 | 1.31 | 4.13 | 0.86 | 1.57 | 5.44 |

into account the different hydration of the phases and assuming that the retention mechanism for neutral compounds in HILIC takes place through pure partitioning, it would be expected that nicotinamide, pyridoxine and riboflavin are retained more strongly on the diol column because of the higher volume of the aqueous layer formed. Nonetheless, at pH 6.0, nicotinamide and pyridoxine are retained more strongly on the silica column followed by the amino and diol columns. The amino column is more retentive of riboflavin followed by the silica and diol columns (Table 2).

The above results suggest that neutral compounds, in addition to partitioning, can interact directly with the stationary phase through hydrogen bonding. The nonlinear retention times of the solutes at ACN content above 70% (v/v) support this conclusion as well. The above observations are further corroborated by the retention data of simple compounds (Supplementary Material: retention behaviour of simple organic molecules), where the elution order is well correlated with the hydrogen-bond acidity ($a_2{}^H$) and basicity ($\beta_2{}^H$) of the solutes. The stronger retention of neutral compounds on the silica column, followed by the amino and diol columns, is also evidence of the stronger propensity of the silica surface to form hydrogen bonds [46]. As for the diol and amino columns, the bonding density of the surface (i.e. the presence of residual silanol groups) seems to play an important role [47].

### 3.2.2. Retention models

To further probe the nature of the HILIC retention of WSVs on the different columns, some models of partitioning and surface adsorption were applied. For partitioning, the relationship is given by Eq. (1) [1,48,49].

$$\log k' = \log k'_{org} - S\varphi \quad (1)$$

where $k'_{org}$ is the retention factor for the weaker component (i.e. organic) only as mobile phase, $\varphi$ is the volume fraction of the stronger solvent and $S$ is the slope of $\log k'$ versus $\varphi$ when fitted to a linear regression model. For surface adsorption the relationship can be expressed by Eq. (2) [1,50,51].

$$\log k' = \log k'_w - \frac{A_S}{n_w}\log\varphi \quad (2)$$

where $k'_w$ is the solute retention factor with pure water as eluent, $A_S$ and $n_w$ are the cross-sectional areas occupied by the solute molecule on the surface and the water molecules, respectively, and $\varphi$ is the mole fraction of the stronger solvent (i.e. water) in the eluent.

In addition, a third model, recently introduced, was evaluated, which includes solute–solvent-stationary phase interactions [52,53]:

$$\ln k' = a + b\ln\varphi + c\varphi \quad (3)$$

where $\alpha$ is a constant related to the interaction energy between solutes with the stationary phase and the mobile phase, $b$ is a coefficient that relates to the direct solute-stationary phase interaction and $c$ relates to the interaction energy between solutes and solvents. In all cases, the mobile phase consisted of 10 mM ammonium acetate buffer at pH 5.0. The ACN content was varied over the range of 50–90% (v/v) acquiring thirteen data points in each case.

Neither log–linear plots nor log–log plots were satisfactory for the whole set of vitamins studied, hinting at the absence of pure partitioning or surface adsorption mechanism on all columns employed. (Plots of $\log k' = f(\phi)$, $\log k' = \log f(\phi)$ and squared correlation coefficients for the three columns are illustrated in Figs. S7–S9 and Tables S2–S4, respectively, of Supplementary Material.) Replotting the log-linear data for nicotinamide, pyridoxine and riboflavin (neutral molecules at the working pH), for water content ranging from 25 to 50% (v/v), a significantly improved linear relationship ($0.9907 < R^2 < 0.9915$, $p < 0.01$, $F > 428$, standard error of estimate < 0.020) was noticed. Likewise, the linear relationship for nicotinic acid was significantly improved ($R^2 = 0.9919$, $p < 0.01$, $F = 755$, standard error of estimate: 0.012) in the water content range of 25–50% (v/v) while for L-ascorbic acid ($R^2 = 0.9912$, $p < 0.01$, $F = 640$, standard error of estimate: 0.014) this was noticed for a water content over the range 20–50% (v/v).

The respective results for the diol column indicate that the linearity of the log-linear plot for nicotinamide, pyridoxine and riboflavin is significantly improved ($0.9900 < R^2 < 0.9929$, $p < 0.01$, $F > 381$, standard error of estimate < 0.021) at a water content of 20–50% (v/v) while for nicotinic and L-ascorbic acids this occurs ($0.9905 < R^2 < 0.9991$, $p < 0.01$, $F > 400$, standard error of estimate < 0.021) at a water content of 16–50% (v/v) [7]. The difference in the contribution of surface adsorption mechanism between the diol and silica column for the neutral WSVs can be attributed to the hydration of the surface of stationary phase. The thinner aqueous layer formed on the silica column allows the solutes to reach and interact with the surface at relatively higher water content. The contribution of the adsorption mechanism to the retention of L-ascorbic acid on the silica, at a water content $\leq$20% (v/v) can be rationalized by the strength of hydrophilic interactions in combination with the stronger hydrogen-bonding properties of the silica surface, as compared to the diol column. For nicotinic acid, its ability to directly interact with the surface of the silica column at water content $\leq$25% (v/v) seems to be correlated with the existence of both repulsive and attractive forces, depending on the orientation of the molecule.

As regards the amino column, replotting the log-linear data for nicotinamide, pyridoxine and riboflavin, a significantly improved linear relationship ($0.9924 < R^2 < 0.9943$, $p < 0.01$, $F > 528$, standard error of estimate < 0.016) was noticed over the same range of water content with the silica column (i.e. 25–50%, v/v). As to thiamine, the partition model fits the data more satisfactorily, for water content in the range of 50–16% (v/v). Given the hydration of the stationary phases, it would be expected that the partition model fits the retention data within a broader range of water content. An explanation could lie in the permanently positive charge of the molecule, which is less affected by the presence of organic solvent in the mobile phase. Consequently, thiamine is susceptible to electrostatic repulsion even at 85% of ACN. This is in accordance with the chromatographic behaviour of the compound with respect to buffer salt concentration (Fig. 3C). Nicotinic and L-ascorbic acids show a distinct behaviour of high relevance. The squared regression coefficients of these acids deviate significantly from both partitioning (0.6731 and 0.7219, respectively) and surface adsorption (0.8258 and 0.8544, respectively) models. Moreover, there is no range of water content in which the partitioning model fits the retention data satisfactorily. The above results are consistent with the retention behaviour of these two acids with increasing ACN content in the mobile phase (see Section 3.1.1). However, the chromatographic behaviour of these compounds implies that surface adsorption is the dominant retention mechanism.

Finally, as regards Eq. (3) for the diol and silica columns, the model satisfactorily predicts the data ($0.9951 < R^2 < 0.9997$), reinforcing the idea that retention in HILIC is governed by a mixed mode mechanism. For the amino column, the equation fits well the retention data for nicotinamide, pyridoxine, riboflavin and thiamine ($0.9930 < R^2 < 0.9995$) in the entire range of water content in the mobile phase. In contrast, deviation from linearity was observed again for nicotinic acid ($R^2 = 0.9636$) and L-ascorbic acid ($R^2 = 0.9796$). (Tables S5–S7 of Supplementary Material, provide the numerical values of constants $\alpha$, $b$ and $c$ for the WSVs on the three columns, along with the goodness-of-fit test data.)

## 4. Conclusions

In the present study, the retention mechanism in HILIC was investigated by evaluating the chromatographic behaviour of selected WSVs. Through careful observations of the effect of chromatographic conditions on the retention, some mechanistic details of HILIC can be better inferred. The relative contributions of partitioning and surface adsorption mechanism are highly connected with the nature of the stationary phase (i.e. the hydration and charge), the properties of the solutes (i.e. the kind and number of polar functional groups) and the mobile phase conditions. The aqueous–organic composition of the mobile phase in HILIC mode determines the kind and strength of specific interactions (e.g. hydrogen bonding, ion-exchange) between the solutes and the surface of the stationary phase, thus corroborating its great importance; it determines the relative contribution of partitioning and surface adsorption mechanisms.

Specific conclusions can be drawn, as follows:

- The transition from a partitioning to a surface adsorption mechanism for neutral WSVs occurs at ACN content ≥80% on the diol column and at ACN ≥75% on the silica and amino columns. The above difference on the contribution of surface adsorption is related to the different degree of hydration of the stationary phases.
- The transition from a partitioning to a surface adsorption for WSVs having the same charge as the stationary phase occurs at ACN contents >85%, where electrostatic repulsion becomes less significant and hydrophilic interaction dominates. On the other hand, for WSVs which are electrostatically attracted, surface adsorption remains the dominant retention mechanism.
- The change from ACN to other organic solvents of greater hydrogen bonding potential confirms the significant contribution of these interactions to the overall retention mechanism in HILIC. The disruption of the aqueous layer, which is confirmed by the elution behaviour of the apolar toluene, becomes more pronounced in the presence of MeOH than with IPA or THF. Consequently, the poor or absent retention of polar compounds follows from both the more hydrophobic character of the stagnant layer and the solvent–solute interactions.
- In highly organic mobile phases, an increase in buffer salt concentration renders the aqueous layer more hydrophilic as the mobility of the salt ions is significantly restricted towards this layer. The partition of salt ions begins at ACN ≥70% (v/v), where the difference in the polarity between the established aqueous layer and the bulk eluent increases significantly.
- As ACN content increases, electrostatic effects represent a lesser proportion of the total interactions while hydrophilic interaction becomes strong and dominates the retention mechanism in HILIC mode. The presence of buffer salt ions plays an important role towards the suppression of electrostatic interactions; however, this effect depends on both buffer concentration and water content in the mobile phase.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.02.069.

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