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Dedicated to Professor Dumitru Oancea on the occasion on his 75th anniversary

RETENTION MECHANISM IN ZWITTERIONIC HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY (ZIC-HILIC) STUDIED FOR HIGHLY POLAR COMPOUNDS UNDER DIFFERENT ELUTION CONDITIONS

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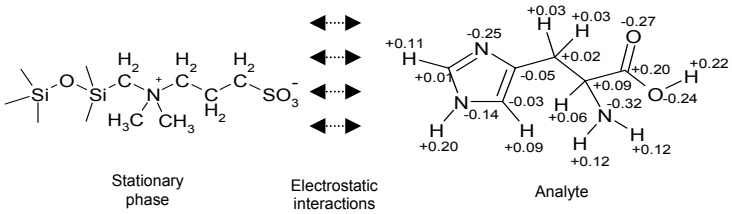
The retention behavior of six highly polar compounds on zwitterionic sulfobetaine stationary phase was studied, under different elution conditions. The influence of organic modifier type (acetonitrile, methanol), the organic solvent content, ammonium acetate concentration added to the mobile phase and column temperature on the retention of studied compounds were investigated. Influence of temperature on the retention of studied compounds was also investigated and van't Hoff plotts were used in estimating thermodynamic parameters of the chromatographic process. Two mathematical models usually used to differentiate between partition and adsorption processes were applied to the dependences of retention factor on mobile phase composition. These obtained results suggest that a mixed retention mechanism relying on both partition and adsorption processes may contribute to the chromatographic behavior of studied compounds on ZIC-HILIC stationary phase.



INTRODUCTION

The term hydrophilic interaction liquid chromatography (HILIC) was introduced for the first time by Alpert in his paper[1] from 1990, being a separation mode that combines stationary phases typically used in normal-phase chromatography (highly hydrophilic stationary phases) and mobile phases used in reversed phase chromatography (polar organic solvent that contains up to 30 %

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water with different salt). HILIC implies partitioning of polar analytes between a stationary phase rich in water (by the formed water layer over the surface) and the bulk mobile phase. In HILIC the more polar/hydrophilic is the analyte, the equilibrium is shifted towards the water layer immobilized in the stationary phase.[2-4]

Silica based stationary phases have been used for HILIC separations since 1990, although many of them were primary used in normal phase liquid chromatography.

HILIC stationary phases can be divided in three different groups: *a*) **neutral** (with no charge over the whole pH range typically used for HILIC, such as diol-silica, amide-silica, cyanopropyl-silica); *b*) **charged** (positively or negatively depending on pH used in HILIC separations, such as silica, aminopropyl-silica), and *c*) **zwitterionic** (positively and negatively charged groups in close proximity in the structure of functional ligands, usually designated by ZIC-HILIC). In the past decade more variations in the surface chemistry have emerged, such as stationary phase based on zwitterionic based silica (sulfobetanine and phosphorylcholine), and newly synthesized stationary phase with high hydrophilicity imidazolium and cysteine-silica,[5,6] carboxylate modified porous graphitic carbon,[7] methacrylate based monolithic capillary column,[8] and silica-bonded sufonated cyclofructan.[9] Currently, HILIC mechanism is used in the separation of very polar compounds that are usually difficult to be retained on reversed-phase stationary phase, in saccharide and glycoside separations,[10] nucleosides and nucleobases,[11,12] flavonoids,[13] or acidic agricultural compounds.[14] HILIC has been used for the quantitation of drugs and metabolites in complex biological matrices,[15] in pharmaceutical analysis,[16] in metabolomics and proteomic studies,[17,18] in food and environmental analysis.[19]

The mechanism of retention in HILIC is not completely understood, although the retention mechanism is primarily considered to be determined by the partitioning of the compounds on the water layer formed on the surface of stationary phase, but can also be influenced by the complex interaction between the polar compound, the type of organic solvent and of buffer salt ions with the polar stationary phase and residual silanols, causing secondary interactions (dipole-dipole, weak electrostatic interactions either attractive or repulsive depending on the charges of the atoms from molecules, increasing retention for charged basic compounds and less retention for charged acidic compounds) which implies that both partition and adsorption play important roles in retention in HILIC.[20]

The aim of this work was to investigate the behavior of several polar compounds on a zwitterionic HILIC (ZIC-HILIC) stationary phase in different chromatographic conditions and to study the effect of organic solvent content in the mobile phase, effect of ammonium acetate concentration, and of column temperature.

EXPERIMENTAL

All solvents were HPLC (gradient) grade. Acetonitrile and methanol were purchased from Sigma-Aldrich. Reagent (ammonium acetate) was pro-analysis grade purchased from Merck. Water (resistivity minimum 18.2 MΩ cm and the total organic content, TOC, maximum 30 ppb) was produced within the laboratory with a TKA Lab HP 6UV/UF instrument.

The six analytes used: four amino acids (histidine, phenylalanine, tryptophan, and tyrosine), adenine, and metformin hydrochloride are commercially available chemical compounds with purities higher than 99 % and they were purchased from Sigma Aldrich.

Working solutions were prepared in water with concentration of 400 µg/mL histidine, phenylalanine, tryptophan, tyrosine, and 200 µg/mL metformin; solution of 400 µg/mL adenine was prepared in acidified water.

All experiments were performed on an Agilent 1100 Series liquid chromatograph (Agilent Technologies) consisting in the following modules: degasser (G1379A), binary pump (G1312A), auto sampler (G1313A), column thermostat (G1316A), and diode array detector (G1315A). The chromatographic system was operationally qualified before the study. Chromatographic data were acquired by means of Agilent Chemstation software rev. B.01.03.

A SeQuant ZIC-HILIC (Merck) stationary phase containing a zwitterionic sulfobetaine covalently attached to porous silica (5 µm particle size), according to producer guide, packed in a column with 150 mm length and 4.6 mm i.d., was used under isocratic elution conditions. All experiments were performed at 25°C (unless otherwise specified) using a flow-rate of 1 mL/min. UV detection was used by monitoring the absorbance at 220 nm wavelength. Injection volume was set up to 1 µL (unless specified).

The aqueous component of the mobile phase contained ammonium acetate ($AcNH_4$) at different concentrations. Methanol (MeOH) or acetonitrile (ACN) were used as organic modifier of the mobile phase. Column re-equilibration with different mobile phase compositions was always done (approx. 20 min).

Retention factor (k) was calculated according to the relationship $k = (t_R - t_0)/t_0$. Absolute retention time values (t_R) are considered as mean resulting from triplicate analysis. Toluene peak was used as dead time (t_0) indicator for each composition or temperature set-up value.

RESULTS AND DISCUSSION

Molecular modeling

Amino acids, the primary building blocks of proteins, are polar zwitterionic compounds, with different properties from acidic to basic, or from

hydrophilic to hydrophobic depending on the side chain. The charge of amino acids is strongly influenced by the pH of medium, at pH lower than their dissociation constant (pK_a) are protonated and the carboxylate moieties are deprotonated at pH above their pK_a.[21,22]

Phenylalanine, tryptophan, and tyrosine are neutral amino acids and in the pH range used in this study exist as neutral zwitterions, furthermore histidine is a basic amino acid with a positively side chain (imidazole group) under the conditions mentioned. Metformin (N,N-dimethylbiguanide) is an oral antihyperglycemic drug used in the treatment of non-insulin dependent diabetes mellitus, and adenine is a purine base component of the DNA with important roles in physiological and pathological activities. The octanol-water partition coefficient (log K_{ow}) and pK_a values of the studied compounds are presented in Table 1.[23-26] Out of the six compounds studied herein two of them (metformin and histidine) have positive charges in the conditions of the experiments herein. The structures in Fig. 1 show the degree of polarity of different atoms from the molecules investigated (obtained with Marvin Sketch)[27].

The SeQUANT ZIC-HILIC sulfobetaine stationary phase used in this study has a positively charged quaternary ammonium group, $(CH_3)_3N^+$, and a negatively charged sulphonic group $(-SO_3^-)$ in 1:1 which make its surface overall neutral; with high selectivity towards polar compounds. Sulfobetaine stationary phases were found to possess a slight negative surface charge because of the spatial arrangement with the sulphonic group at the distal end of the zwitterionic moiety.[28]

Table 1

Some physico-chemical parameters log K_{ow} and pK_a of the six studied compounds

Compound	CAS Number	Molecular mass	log K_{ow}	pK_{a1}	pK_{a2}	pK_R	Reference
Histidine	71-00-1	155.155	-3.22	1.82	9.17	6.0	[24]
Phenylalanine	150-30-1	165.189	-1.28	1.83	9.13	-	[24]
Tyrosine	60-18-4	181.189	-1.76	2.20	9.11	-	[24]
Tryptophan	73-22-3	204.225	-1.22	2.38	9.39	-	[24]
Adenine	657-24-9	135.127	-0.73	4.15	9.8	-	[25]
Metformin	73-24-5	129.164	-2.64	2.8	11.5	-	[26]

*R – side chain of histidine (imidazole group)

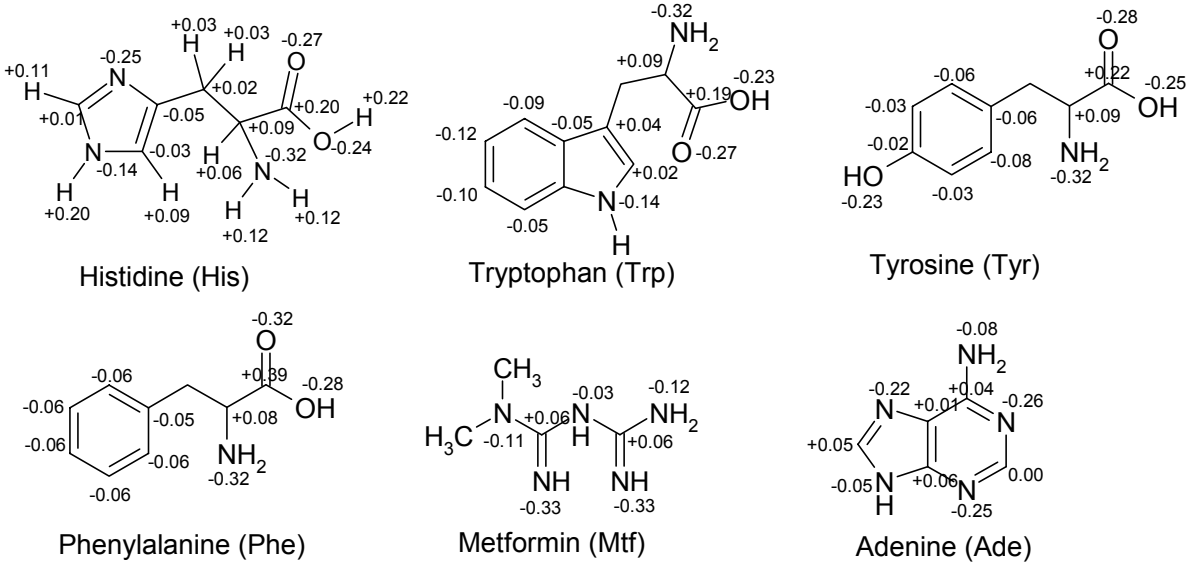

Fig. 1 – Chemical structures of studied compounds.

Effect of organic modifier on retention

The effects on retention of studied compounds for two solvents (acetonitrile and methanol) was investigated and compared on the ZIC-HILIC stationary phase (sulfobetaine silica). The influence of mobile phase composition was studied on a broad interval: acetonitrile content from 30 to 70%, with 5% increment, at 10 mM $AcNH_4$ kept constant in water; and for methanol between 30 to 80% with 5% increment, at the same concentration of ammonium acetate as in the case of acetonitrile. The log *k* value of each compound was plotted against organic solvent content, the weakest solvent in HILIC mechanism, as usually describing the dependence of retention on the mobile phase composition, at 25°C for column temperature (Fig. 2).

As observed elsewhere,[29] in HILIC mechanism methanol has an elution strength greater than acetonitrile, which leads to retention times much smaller for compounds studied with this solvent in mobile phase (see Fig. 2), close to the void volume of the column. This retention loss in methanol could be assigned to the disruption of water layer at the surface of stationary phase because of replacement of water with methanol molecules, a protic solvent with strong hydrogen bonding capability thus producing a more hydrophobic stationary phase.[29,30] In the case of acetonitrile, the hydrogen bond donor strength is much weaker than for methanol and the amino acids are retained more strongly.

Typical HILIC behavior was observed for the amino acids in the case of acetonitrile as organic modifier. Retention in HILIC increases with increasing polarity of the compounds and with increasing ACN content in the mobile phase, but there has to consider that as the percentage of the organic solvent increases, there is a change in the pH of the mobile phase and also the pK_a of the compounds studied because of dielectric constant decreases. As seen in Fig. 2, the retention decreased with the increasing water content, which is the strong eluent in HILIC. The retention time increased in the following order: phenylalanine, tryptophan, tyrosine, and histidine. This trend is somehow similar to the log K_{ow} values of the amino acids: histidine is the most polar/hydrophilic compound, and hence it was expected to have the strongest retention in HILIC. In general, analytes will prefer partition into mobile phase when the water concentration is increased, causing a smaller retention time.

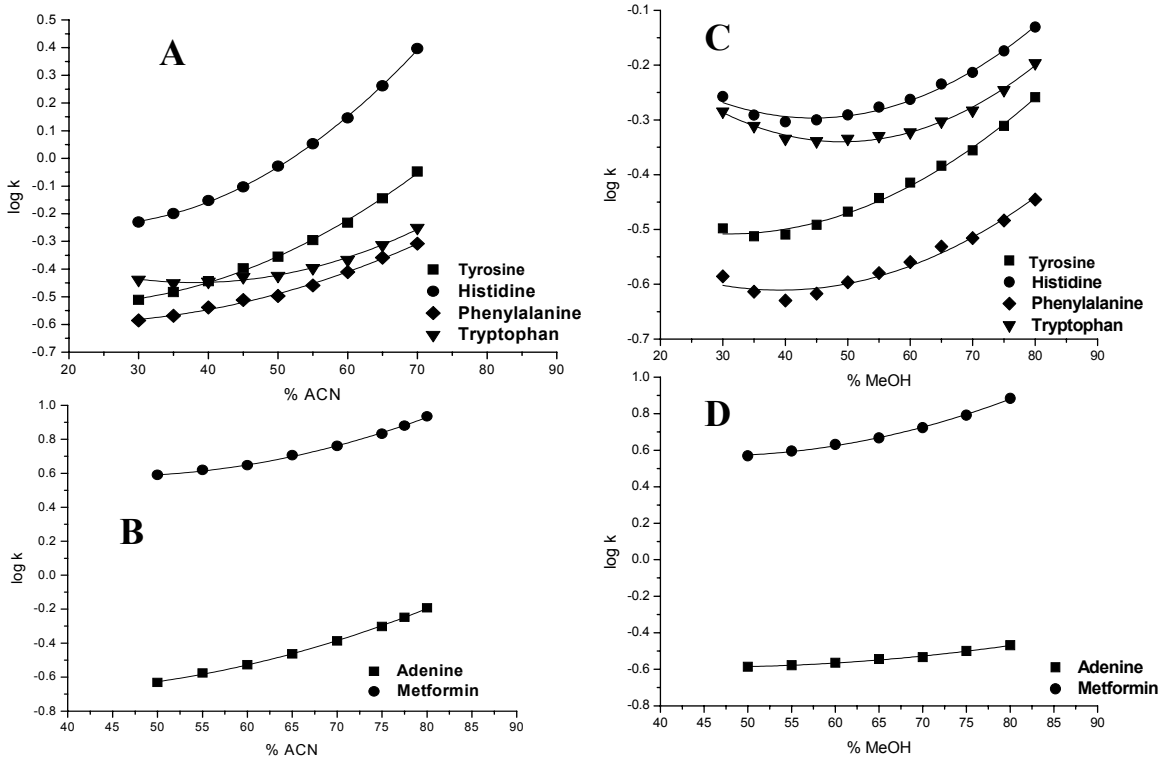

Fig. 2 – Plots of log *k versus* % acetonitrile content for A) amino acids and B) metformin and adenine; log *k versus* % methanol content for C) amino acids and D) metformin and adenine.

Replacing acetonitrile with methanol, the retention of all compounds decreased from 20 to 55 % water, which is in the accordance with HILIC mechanism, followed by a slight increase of retention in the interval 60 to 70% because at larger % aqueous component the salt concentration is higher. The compounds elute close to dead volume of the column. In this case the elution order was: phenylalanine, tyrosine, tryptophan, and histidine, which is different from ACN solvent. This might be explained by the fact that the organic modifier can also contribute to the degree of dissociation of buffer compound, the pK_a values of the analytes and the dissociation of residual silanol groups from the silica surface. The shift in elution order between tyrosine and tryptophan in ACN and MeOH mobile phase could be an indication of the complexity of retention process in HILIC.

According to a recent study[31] based on molecular dynamics it was highlighted the existence of water layer at the stationary phase because of the presence of a hydrogen bond network extending from the silanol moieties, which contribute to the accumulation of water molecules. This layer is involved in the retention mechanism, either in the partition mechanism or in adsorption mechanism. However, a real situation of the complexity of this process is given by both mechanisms. If the retention is governed by the partition mechanism then the following empirical equation describes the process:[32]

$$\log k = \log k_w - S\varphi \qquad (1)$$

where φ is the volume fraction of a stronger solvent (in this case water) in the mobile phase, S is the slope when fitted with a linear regression model, and $\log k_w$ is the retention for the weaker component (organic solvent) only. A plot of log k *vs* volume fraction water should yield a straight line for a partition mechanism.

If the retention is due to adsorption then the best equation to describe this is the Snyder-Soczewinski equation:[32]

$$\log k = \log k_B - \frac{A_S}{n_w}\log\varphi \qquad (2)$$

where φ is the volume fraction of a stronger solvent (in this case water) in the mobile phase, $\log k_B$ is retention with pure B as solvent, A_S and n_w are the cross sectional areas occupied by the solute molecule in the surface and solvent molecules (the ratio *As*/*nw* can be considered as a constant, which can be estimated from the plot log k *vs* log φ). In this case, the plot log k *vs* log of water content in mobile phase (φ) should yield a straight line for an adsorption mechanism.[2-4,21]

The retention mechanism for the six compounds was investigated based on the two models presented above. As represented in Fig. 2, the dependences of log k on the water content (%ACN or MeOH) are fitted by polynomial regressions (regression parameters are given in Tables 2 and 3). On the other hand, the log-linear plots and log-log plots for the studied compounds on the both solvent (ACN and MeOH) depicted in Fig. 3 show clear linear dependences for some compounds (adenine metformin, and phenylalanine) and non-linear dependences for the other three studied compounds. This interpretation is in a fair agreement with the most accepted point of view describing the HILIC mechanism, emphasizing that this mechanism is dependent on both partitioning and adsorption processes at which the studied compounds are taking part.[32]

Table 2

Regression parameters for studied compounds using mobile phase ACN/H_2O (10 mM $AcNH_4$)

Compound	R^2	Intercept	B1	B2
Histidine	0.9996	-0.0946	-0.0129	$2.841\ 10^{-4}$
Tryptophan	0.9903	-0.1751	-0.0144	$1.887\ 10^{-4}$
Tyrosine	0.9982	-0.0458	-0.0072	$1.846\ 10^{-4}$
Phenylalanine	0.9949	-0.5569	-0.0041	$1.095\ 10^{-4}$
Metformin	0.9979	1.1191	-0.0242	$2.730\ 10^{-4}$
Adenine	0.9989	-0.4424	-0.0149	$2.251\ 10^{-4}$

Table 3

Regression parameters for for studied compounds using mobile phase MeOH/H_2O (10 mM $AcNH_4$)

Compound	R^2	Intercept	B1	B2
Histidine	0.9853	-0.0317	-0.0119	$1.334\ 10^{-4}$
Tryptophan	0.9891	0.0120	-0.0143	$1.458\ 10^{-4}$
Tyrosine	0.9936	-0.4120	-0.0063	$1.022\ 10^{-4}$
Phenylalanine	0.9635	-0.4542	-0.0079	$1.018\ 10^{-4}$
Metformin	0.9974	1.0914	-0.0232	$2.568\ 10^{-4}$
Adenine	0.9915	-0.3933	-0.0086	$9.603\ 10^{-4}$

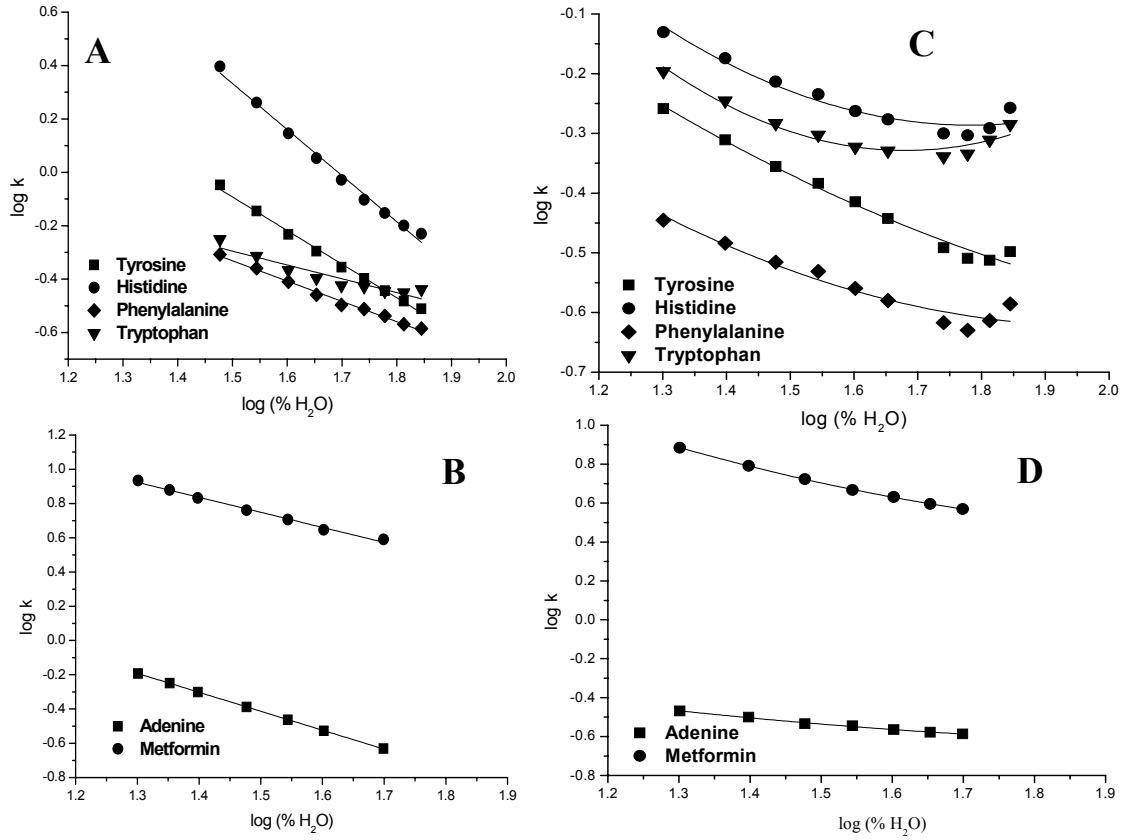

Fig. 3 – Dependence of log *k versus* log (water content) for A) amino acids and B) adenine and metformin, for ACN; log *k versus* log (water content) for C) amino acids and D) adenine and metformin for MeOH.

Effect of salt concentration on retention

The effect of salt concentration on the retention of four amino acids was investigated in the range 2.5 - 20 mM ammonium acetate (*i.e.*, 2.5; 5; 7.5; 10; 15; and 20 mM), at two different compositions of the mobile phase: one rich in acetonitrile (80/20 ACN/water) and the other with a higher content in water (40/60 ACN/water). The ammonium concentration represents the concentration in the aqueous component only, not the overall concentration in the mobile phase. Acetonitrile was chosen in this study because as mentioned before the retention of the compounds in methanol mobile phase lead to small retention times even at increasing MeOH content, and because ACN is miscible in all proportions with water and ammonium acetate.

As can be seen in Fig. 4 the retention has a slow decrease for histidine, a slight increase for phenyalanine and minor variations for tryptophan and tyrosine, at 80 % ACN in the mobile phase. But although slight modifications are observed, overall the retention was unaffected as the buffer concentration increased because the difference in *k* values was less than 0.1. The only exception is histidine for which a difference of 1.8 in retention was noticed. A possible explanation could be that at high level of acetonitrile content in the mobile phase the salt prefers to be in the rich water layer,[33] and in the case of histidine could be explained by the fact that the imidazole positive side chain would be attracted electrostatically to the negative sulphonic group of the stationary phase.

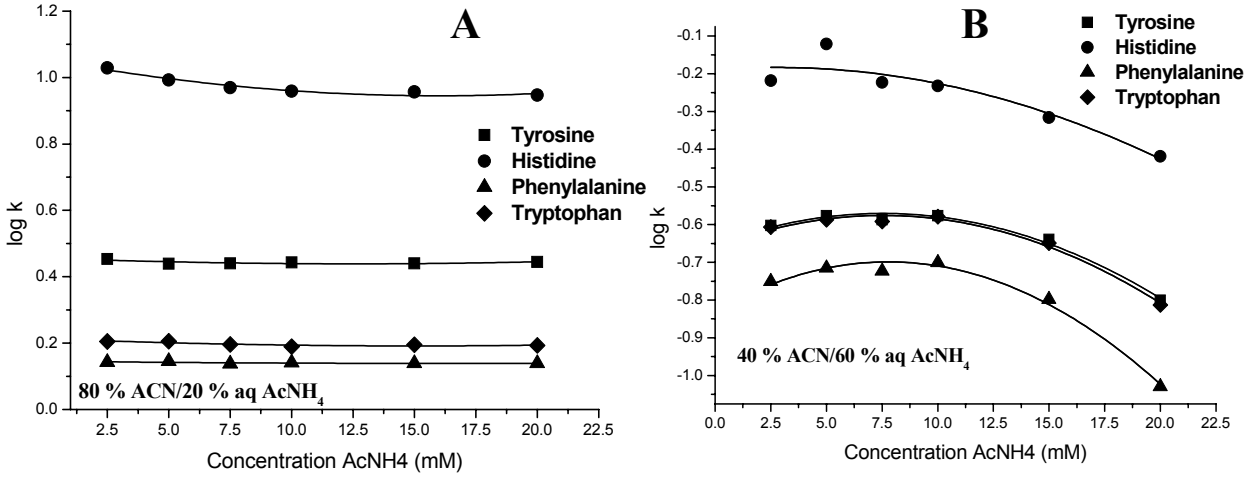

Fig. 4 – Effect of salt concentration on the retention of amino acids for A) 80% ACN; B) 40% ACN.

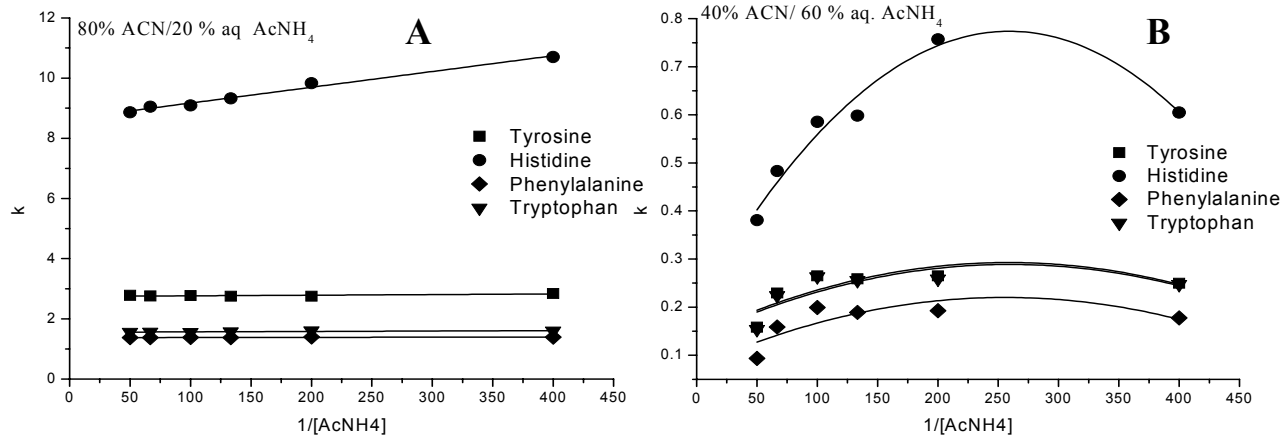

Fig. 5 – Plots of *k versus* 1/[$AcNH_4$] for the studied compounds for A) 80% acetonitrile and B) 40 % acetonitrile in mobile phase.

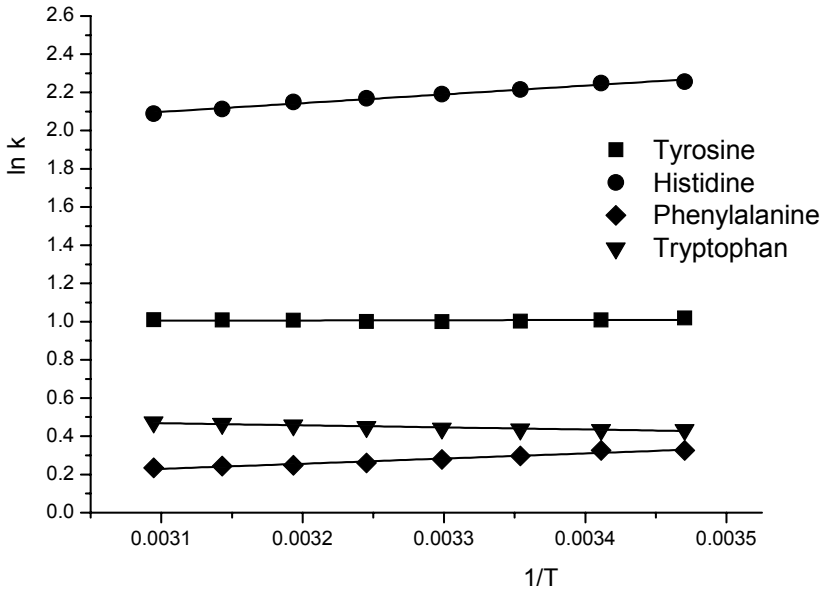

Fig. 6 – Van't Hoff plots of amino acids obtained for a mobile phase containing 80% ACN and 20% aqueous solution of 10 mM ammonium acetate.

In the case of 40 % ACN in the mobile phase, a similar pattern is observed for all four amino acids: a slight increase in retention when the salt concentration was increased from 2.5 to 5 mM $AcNH_4$ followed by a much greater decrease as the salt concentration increased in the interval 7.5-20 mM $AcNH_4$ (see Fig. 4). This can be explained by the fact that increasing the salt concentration in mobile phase may have the effect of suppressing both electrostatic repulsion and attraction.[34-38]

The possibility of ion exchange mechanism proposed for the retention in HILIC could be proved from the plots giving the dependence of the retention factor (k) on the reciprocal value of buffer cation concentration (1/[salt]).[36] In this case, the plot (k vs 1/[salt]) should be a straight line, assuming that no other retention mechanism exists. However, these plots given in Fig. 5 are not straight lines, which indicates that the buffer has an additional effect on the separation mechanism in HILIC other than the competition with the compounds for the stationary phase sites.

The data represented in Fig. 5 show rather different retention behaviour of studied compounds depending on the water content of the mobile phase, at the same concentration level of CH_3COONH_4. At high content of acetonitrile in mobile phase (Fig. 5.A) the dependences k vs 1/[salt] are linear, which means that contribution of adsorption based on ion exchange to the retention mechanism is dominant.[36] For low content of acetonitrile in mobile phase (Fig. 5.B) these dependences are not linear, and thus the ion exchange effects are no longer dominant in the retention mechanism because the salt concentration is higher.

Effect of column temperature

The effect of column temperature on retention was investigated for studied solutes under isocratic conditions by varying the column temperature within the range 15 - 50°C, at 5°C intervals, for acetonitrile and methanol used as organic solvents of the mobile phase and for 10 mM ammonium acetate added in aqueous component of the mobile phase. Higher temperature values were not investigated because of the instability of the column according to the manufacturer's data.

The retention data were graphically represented in order to find out the linearity of the natural logarithm of the retention factor (ln *k*) towards reciprocal absolute temperature (1/T), according to the van't Hoff dependence:

$$\ln k = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \phi \qquad (3)$$

In the above equation, ΔH^0 and ΔS^0 represent the variation of standard enthalpy and entropy change, respectively, for the transfer of solute from mobile phase to stationary phase, R is the gas constant value (8.314 J K^{-1} mol^{-1}) and ϕ is the phase ratio of the chromatographic column, i.e. the ratio between the volume of stationary phase to volume of mobile phase.

Good linearity was noticed for histidine, phenylalanine, tryptophan and tyrosine for acetonitrile/water mobile phase, but less for methanol/water mobile phase. Positive slopes of the van't Hoff plots for acetonitrile solvent were obtained (Fig. 6), which means negative and favorable enthalphy ΔH^0 of the transfer of solutes from mobile phase to stationary phase. However, the slopes of these plots were lower compared to slopes usually obtained for other important retention mechanisms in LC, such as reversed-phase mechanism, for instance. Consequently, the standard enthalpy change calculated for the studied compounds for a mobile phase containing 80% methanol and 20% aqueous 10 mM CH_3COONH_4, for example, were the following: -5.28 kJ/mol (tyrosine); -6.27 kJ/mol (histidine); -6.33 kJ/mol (tryptophan) and -8.40 kJ/mol (phenylalanine). These values of standard enthalpy change calculated for these compounds were below -10 kJ/mol, typically calculated in reversed-phase LC mechanism (*e.g.*[39,40]).

CONCLUSIONS

This study investigated the behavior of six different very polar compounds on ZIC-HILIC stationary phase under various chromatographic conditions. The experimental results obtained for different mobile phase compositions suggest that the retention mechanism in ZIC-HILIC is not governed by a unique process. This complex retention process can be explained by a combination of two possible mechanisms that are taking place simultaneously: 1) partition mechanism of polar solutes between the mobile phase and stationary phase; 2) adsorption mechanism of these solutes at the ionic sites from stationary phase due to the electrostatic interactions (ion-ion, dipole-ion).

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