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PhD-THESIS
ABSTRACT

RETENTION BEHAVIOUR OF COMPOUNDS WITH BIOCHEMICAL IMPORTANCE USING HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

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Table of Contents

Introduction 4

1.1. INTRODUCTION TO RETENTION MECHANISMS USED IN LIQUID CHROMATOGRAPHY

1.1.1. Classification of methods used in liquid chromatography according to the working mechanism 8

1.1.2. Modeling retention using liquid-liquid partition equilibrium 13

1.1.3. Modeling retention using solid-liquid adsorption equilibrium 15

1.1.4. Other types of equilibria involved in liquid chromatography 17

1.2. STATIONARY PHASES USED IN HILIC AND ZIC-HILIC SEPARATION MECHANISMS

1.2.1. Non-modified stationary phases 19

1.2.2. Chemically modified stationary phases 19

1.3. PREVIEW ON HILIC AND ZIC-HILIC RETENTION MECHANISMS

1.3.1. Role of the water layer immobilized to the surface of the stationary phases 30

1.3.2. Role of the electrostatic interactions 34

1.3.3. Role of the hydrogen bonds 35

1.3.4. Chromatographic retention mechanisms in HILIC and ZIC-HILIC 37

1.4. MODELING RETENTION IN HILIC AND ZIC HILIC

1.4.1. Influence of the organic solvent in the mobile phase composition 41

1.4.2. Influence of the ionic strength of the mobile phase 42

1.4.3. Influence of the pH of the mobile phase 43

1.4.4. Influence of temperature of the chromatographic column 43

1.5. CURRENT APPLICATIONS OF THE HYDROPHILIC INTERACTIONS MECHANISM FOR THE SEPARATION OF POLAR COMPOUNDS

2.1. RETENTION BEHAVIOUR OF SOME AMINOACIDS USING SULFOBETAINIC STATIONARY PHASES IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

2.1.1. Compound Characterization 49

2.1.2. Experimental Design 50

2.1.3. Influence of the organic modifier of the mobile phase on the chromatographic retention
Introduction 51
Chromatographic Conditions 51
Results and Discussions 52
Conclusions 60
2.1.4. Influence of the ionic strength of the mobile phase on the chromatographic retention
Introduction 61
Chromatographic Conditions 61
Results and Discussions 62
Conclusions 66
2.1.5. General Conclusions 66
2.2. RETENTION BEHAVIOUR OF SOME DIPEPTIDES USING SULFOBETAINIC STATIONARY PHASES IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY
2.2.1. Compound Characterization 67
2.2.2. Experimental Design 69
2.2.3 Influence of the organic modifier of the mobile phase on the chromatographic retention
Introduction 69
Chromatographic Conditions 69
Results and Discussions 70
Conclusions 78
2.2.4. Influence of the ionic strength of the mobile phase on the chromatographic retention
Introduction 79
Chromatographic Conditions 79
Results and Discussions 79
Conclusions 83
2.2.5. General Conclusions 83
2.3. INFLUENCE OF TEMPERATURE ON THE RETENTION BEHAVIOUR IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY
2.3.1. Compound Characterization 84
2.3.2. Experimental Design 85

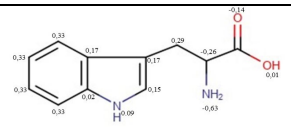
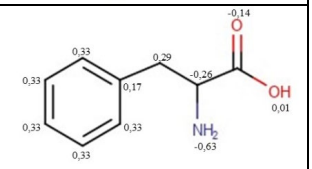
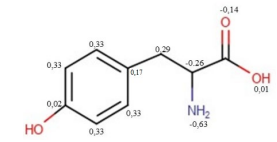
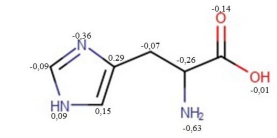
2.3.3. Temperature Influence On The Retention Behavior Of Some Aminoacids In Hydrophilic Interaction Liquid Chromatography
Introduction 86
Chromatographic Conditions 86
Results and Discussions 87
Conclusions 100
2.3.4. Temperature Influence On The Retention Behavior Of Some Dipeptides In Hydrophilic Interaction Liquid Chromatography
Introduction 101
Chromatographic Conditions 101
Results and Discussions 102
Conclusions 114
2.3.5. Temperature Influence On The Retention Behavior Of Some Polar Compounds In Hydrophilic Interaction Liquid Chromatography
Introduction 115
Chromatographic Conditions 115
Results and Discussions 116
Conclusions 128
2.3.6. General Conclusions 129
2.4. INFLUENCE OF THE SAMPLE SOLVENT AND INJECTION VOLUME ON THE CHROMATOGRAPHIC PARAMETERS IN HYDROPHOBIC INTERACTION LIQUID CHROMATOGRAPHY USING A SULFOBETAINIC STATIONARY PHASE
Introduction 131
Experimental Design and Chromatographic Conditions 131
Results and Discussions 132
Conclusions 140
2.5. FINAL CONCLUSIONS 142
PUBLISHED PAPERS 144
PRESENTATIONS 145
BIBLIOGRAPHY 146

Experimental Part

2.1. RETENTION BEHAVIOUR OF SOME AMINOACIDS USING SULFOBETAINIC STATIONARY PHASES IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

2.1.3 Influence of the organic modifier of the mobile phase on the chromatographic retention

Table 1. pK_a, log K_{ow}, log D (pH=6), μ, Sw values and charge /atom distribution for the studied aminoacids

Aminoacid	pk_a		log K_{ow}	log D (pH=6)	μ (D)	Sw (mg/L)	Charge/Atom
	$-NH_2$	-COOH					
Triptophan	9.396	-1.05	-1.05	-0.19	2.77	4193	
Phenylalanine	9.477	-1.28	-1.28	-1.18	2.22	3026.8	
Tyrosine	9.125	-1.76	-1.76	-1.49	3.56	94372	
Hystidine	9.253	-3.22	-3.22	-3.59	2.64	4193	

As seen in Fig.1. retention of all compounds is stronger for the case when using acetonitrile as organic modifier of the mobile phase than in the case of using methanol for the same purpose. This behavior is attributed to the disintegration of the water layer existing at the surface of the stationary phase. Briefly, water molecules are substituted by methanol molecules, a protic solvent with a higher capacity of forming hydrogen bonds, resulting in a more hydrophobic stationary phase [1]. On the other hand, the elutropic strenght of acetonitrile is lower compared to the one of methanol having as a concequence longer retention times [2].

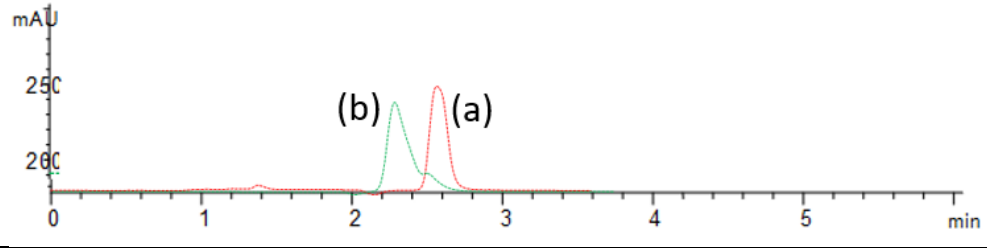


Fig.1. Overlayed Chromatograms of Tyrosine.
Stationary Phase:Sequant ZIC-HILIC, 5µm d.p.; Mobile Phase: (a)80%ACN and 20% 10 mM AcNH4 and (b) 80%MeOH and 10 mM 20% AcNH4

The complexity of the retention mechanism in HILIC is highlighted by the variation of the chromatographic behaviour of the studied analytes when the mobile phase contains diffrent solvents as organic modifiers. In the case when acetonitrile is used as organic modifier one can see a decrease of the retention times in relation with the decrease of the percentage of the organic modifier in the mobile phase for all studied compounds (behaviour considered to be a typical one in this kind of separations) whereas in the case of methanol the retention time decreases for the whole range of organic modifier studied in the case of tyrosine, but for the other compounds the retention time decreases in the range of 70-55% organic modifier, followed by an increase in the range of organic modifier between 40-30% or 40-50%(in the case of triptophan). In the range of 55-40% organic modifier the retention times for histidine, phenylalanine and triptophan the retention times follow no trend, behaviour that might be caused by increasing the salt concentration in the mobile phase which modifies the values of the pK_a of the mentioned compounds or might be a direct consequence of the dissociation of the residual silanol groups found at the surface of the stationary phase.

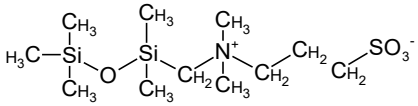


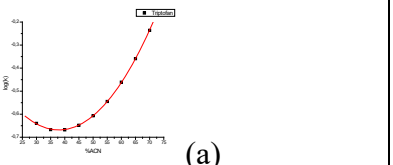
Fig.2. Chemical structure of the sulfobetainic stationary phase

Log k values for all studied compounds at diffrent mobile phase compositions were plotted against the organic modifier percentage in the mobile phase. These dependences can be modeled by a polynomial function of second degree, as follows:

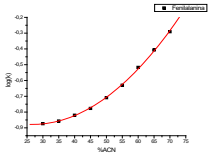
$$\log k = A + B1 \cdot C_m + B2 \cdot C_m^2 \qquad (1)$$

where A, B1 şi B2 are the regerssion paramaters calculated using Origin software and C_m represents the percentage of organic modifier in the mobile phase. The values of these

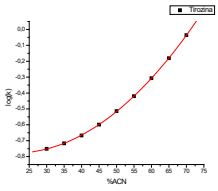
parameters are listed in Table 2 and 3. As one cand see, these regressions are characterized by corelation oefficients higher tahn 0.99.



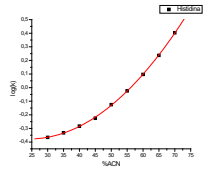
(a)



(b)



(c)



(d)

Fig.3. Plots of log k versus % acetonitrile content for (a)tryptophan, (b)phenylalanine, (c)tyrosine, (d)hystidine

Tabel.2. Regression parameters for studied compounds using mobile phase ACN/H2O (10 mM AcNH4)

ACN	**ACN**	**ACN**	**ACN**	**ACN**
A	-0.0621	-0.6526	-0.6458	-0.15944
B_1	-0.0319	-0.0169	-0.01273	-0.01801
B_2	$4.2177*10^{-4}$	$3.1718*10^{-4}$	$3.0619*10^{-4}$	3.7164*10-4
R^2	0.9999	0.9992	0.9999	0.9997

"U-shaped" polynomial dependences are reported in literature both for reversed phase liquid chromatography and hydrophilic interaction liquid chromatography. These dependences are characterized by a minimum situated in the studied range of organic modifier of the mobile phase or outside this interval. These elution curves are described by equation (1), mentioned earlier. In this equation, A represents the intercept, the extrapolated value of retention factor, k, which corresponds to a mobile phase containing 100% aqueous component, parameter usually denoted k_w. From a mathematic point of view this curve is characterized by a minimum, log k_{min} , reached when the condition $\frac{\partial \log(k)}{\partial C_m} = 0$ is fuffilled. This graphical point is characterized by certain ccomposition of the mobile phase, $(C_m)_{min}$ and the corresponding value of log *k*. These values are obtaioned when solving the following equations:

$$(C_m)_{min} = -\frac{B_1}{2B_2} \quad (2)$$

$$log\ k_{min} = A - \frac{B_1^2}{4B_2} \quad (3)$$

The calculated values of these parameters are shown in the table below.

Tabel.3. "U-shaped" elution curves parametersfor the studied compounds

Compound	Tyrosine	Histidine	Phenylalanine	Triptophan
Organic Modifier Acetonitrile				
log k_w	-0.062	-0.652	-0.645	-0.159
$(C_m)_{min}$	21.054	24.217	25.225	36.755
log k_{min}	-0.779	-0.376	-0.853	-0.510
Organic Modifier Methanol				
log k_w	-0.019	-0.427	-0.418	-0.053
$(C_m)_{min}$	45.690	39.062	30.090	40.350
log k_{min}	-0.293	-0.602	-0.508	-0.2547

According to literature, in the case when the retention follows a partition mechanism, the plots of retention vs the percentage of organic modifierin the mobile phase are given by the following equation :

$$\log k = \log k_w - S\phi \tag{4}$$

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(the organic modifier). 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:

$$\log k = \log k_B - \frac{A_S}{n_w} \log \phi \tag{5}$$

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 A_s/n_w 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 [24].

The retention mechanism of the four model compounds was investigated according to the two theoretical models. As resulting from Fiures 3 and 4, the dependences of log k as a function of mobile phase composition can be modeled by a polynomial mathematical function having the regression parameters presented in Table 2 and 3. Figure 5 presents the values of the decimal logarithm of the retention factor as a function of the decimal logarithm of the aqueous

composition in the mobile phase when the organic modifiers are both acetonitrile and methanol. These graphical representations can be considered polynomial for all the studied compounds.

Due to the fact that both log-lin and log-log dependences are described by polynomial functions, one can conclude that the studied analytes are retained both by direct interactions with the stationary phase (indicating an adsorption mechanism) and by other interactions which indicate their partition towards the water layer situated at the surface of the stationary phase [25].

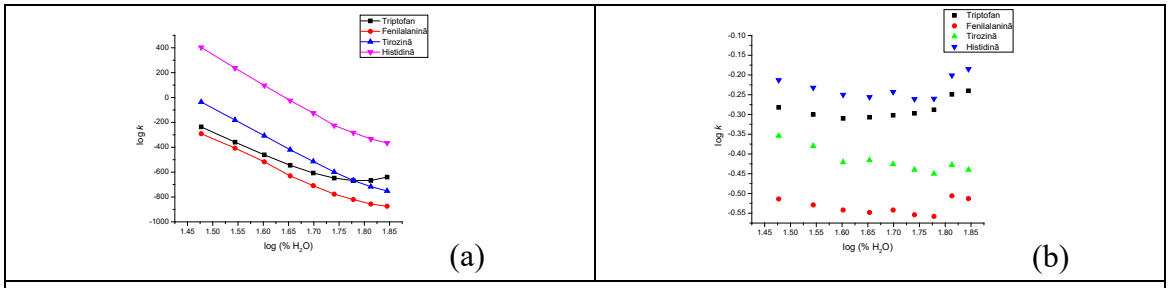


Fig.4. Plots of log k versus log (water content) for the studied aminoacids when the organic modifier of the mobile phase is (a) acetonitrile and (b) methanol

The parameters of the chromatographic peaks do not significantly change when varying the mobile phase composition, regardless of the organic modifier used. However, in most of the cases, symmetry values are found to be greater than 1, specific to the tailing phenomenon whereas the chromatographic efficiency tends to increase linear with the organic modifier percentage. Overall, these parameters tend to improve due to an increase in the salt composition of the mobile phase which has aa a direct consequence the suppression of electrostatic interactions between the functional groups of the analytes and those of the stationary phase [26]. Also, as reported in literature, the poor peak shapes might be attributed to the mismatch between the probe solvent and the mobile phase [27].

2.1.4. Influence of the ionic strength of the mobile phase on the chromatographic retention

Ionic strenght is a parameter that affects HILIC retention, the salts in the mobile phase influencing both attarction and repulsion electrostatic interactions. When increasing the salt concentration in the mobile phase's aqueous component the chromatographic retention decreases if electrostatic attractions are present and increases in the case of electrostatic repulsions [7].

In the present study, when the mobile phase is rich in organic modifier, the retention mechanism is not significantly affected by the ammonium acetate concentration added to the aqueous part of the mobile phase. However, the retention time decreses for histidine and

triptophan. For phenylalanine and tyrosine the trend is of increasing in the concentration range between 2.5-10 mM but it decreases for concentrations between 10 and 20 mM. This behaviour is attributed to the fact that the dissociated ions of the salt act as titrating agents for the counterions of the stationary phase [28].

When the mobile phase contains less organic modifier retention times are found to be lower(especially in the case of histidine) and no trend can be seen regarding the variation of the retention time as a function of the concentration of the salt added to the aqueous component of the mobile phase. If HILIC retention is governed mainly by ion exchange mechanism the plots of the retention factor represented against the reciprocal value of the salt concentration added to the mobile phase should be described by a linear function.

For the case when the mobile phase contains 80% organic modifier in the mobile phase the asmentioned plots can be considered as being described by a linear function, indicating that the retention mechanism is of adsorptive type, dominated by ionic interactions as can be observed in Fig.6(a)). In the case when the mobile phase contains 40% organic modifier, these dependences are not linar (Fig.6.(b)), indicating a mixt retention mechanism, influenced both by adsorption and partition [5].

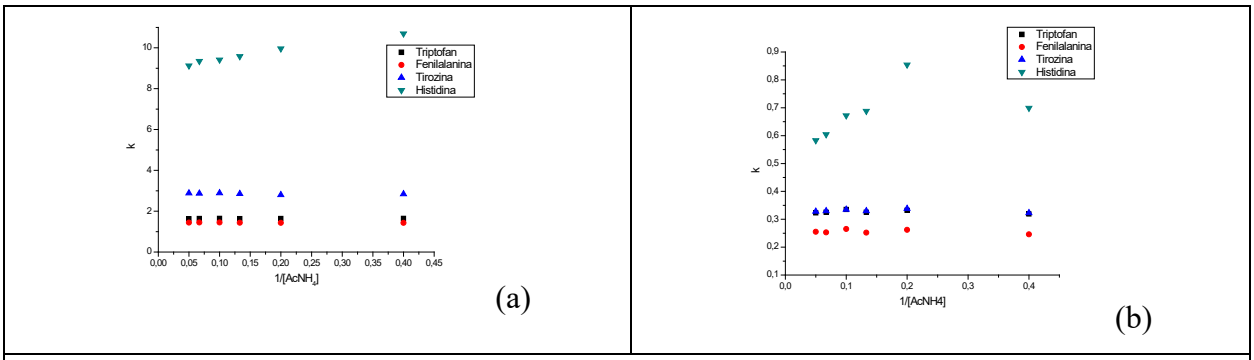


Fig.5. Plots of k versus 1/[AcNH4] for the studied compounds for (a) 80% acetonitrile and (b) 40 % acetonitrile in mobile phase.

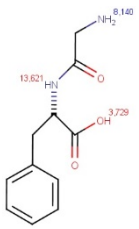
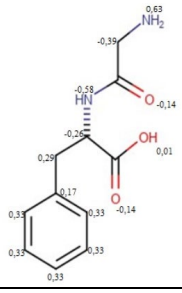
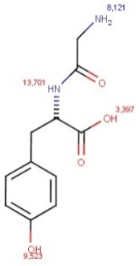
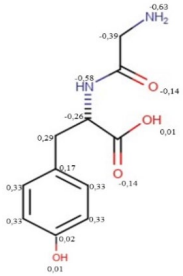
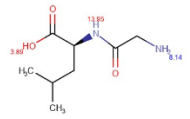
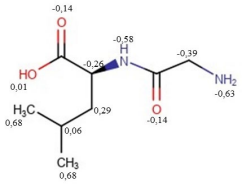
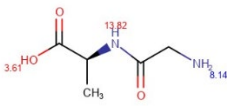
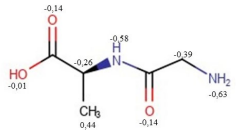
As seen in the study of the organic modifier of the mobile phase on chromatographic retention of the four aminoacids studied no significant variation in the values of the parameters of the chromatographic peaks is observed. Even so, in the case when the mobile phase is rich in organic modifier, but for histidine, all the other compounds are found to have symmetry values higher than 1, specific for the fronting phenomenon. This type of behavior is described in literature as being caused by the alteration of the aqueous layer situated at the surface of the stationary phase. For the other compounds, the obtained symmetry values of less than 1 indicate

multiple retention mechanisms [29]. The pronounced increase in chromatographic efficiency is attributed to the suppression of the electrostatic interactions.

2.2. RETENTION BEHAVIOUR OF SOME DIPEPTIDES USING SULFOBETAINIC STATIONARY PHASES IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

2.2.3. Influence of the organic modifier of the mobile phase on the chromatographic retention

Table 4. pK_a, log K_{ow}, log D (pH=6), μ, Sw values and charge /atom distribution for the studied dipeptides

	pK_a	**log K_{ow}**	**log D (pH=6)**	**μ(D)**	**Sw (mg/L)**	**Charge/atom**
Glycyl-L-phenylalanine		0.03	-2.29	5.42	1541	
Glycyl-L-tyrosine		-0.45	-2.6	5.08	12350	
Glycyl-L-leucine		-0.43	-2.69	4.64	4229	
Glycyl-L-alanine		-1.61	-3.95	4.74	104300	

HILIC retention mechanism is based on the differential distribution of the analyte molecules between the mobile phase and the water layer at the surface of the stationary phase. As the hyrophilicity of the analyte increases, the partition equilibrium is shifted towards right

and the analytes are stronger retained. [9]. Also, as the values of the dipole moments are lower, retention times should decrease [19]. In the case of the four dipeptides studied retention times are found to increade in order of the log k_{ow} and log D values, and in reversed order towards the dipole moment: glycyl-L-phenylalanine, glycyl-L-tyrosine, glycyl-L-leucine, glycyl-L-alanine for mobile phase compositions consisting in 55-70% acetonitrile as organic modifier. Between 70-80% organic modifier, the elution oreder is changed between glycyl-L-tyrosine and glycyl-L-leucine, possible due to changes in the values of the analyes pk_a-ului compared to values reported for aqueous solution [30].

In the case of using acetonitrile as organic modifier, rete retention of the analytes decreases when increasing the percentage of organic modifier in the mobile phase, behavior that is considered typical for HILIC separations. As the organic modifier concentration increases the water is strongly adsorbed at the stationary phase surface supporting liquid-liquid partition [31]. For mobile phase compositions with less than 55% organic modifier, retention times of glycyl-L-leucine and Glycyl-L-alanine, fall below the dead time of the column, indicating stronger interactions between the analyte and the mobile phase compared to the interactions between the analyte and the stationary phase .

Figure 6 deplicts the plots for the values of the decimal logarithm of the retention factor represented as a function of the concentration of the organic modifier in the mobile phase for glycyl-L-phenylalanine and glycyl-L-tyrosine as model analytes. These dependencies can be considered as a polynomial function of second degree , with correlation coefficients higher than 0.9, indicating the presence of secondary retention mechanisms associated with adsorption, especially hydrogen bonds and ionic interactions. [24,32].

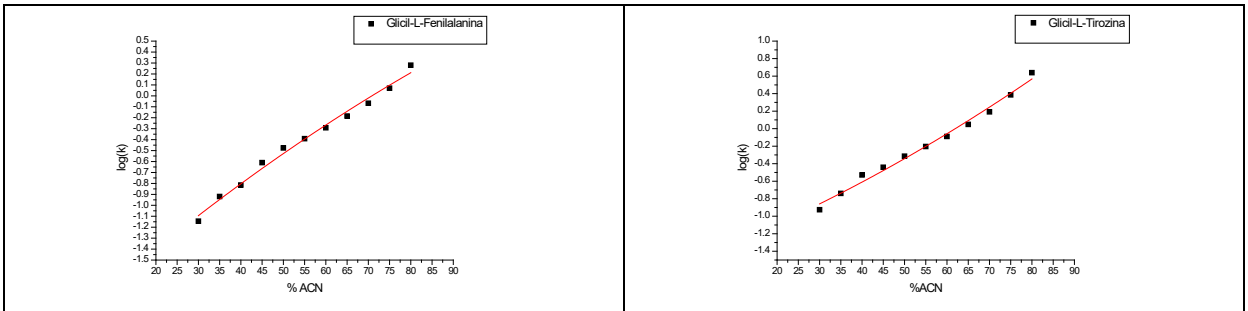


Fig.6. Plots of log k versus % acetonitrile content for glycyl-L-phenylanaline and glycyl-L-tyrosine when using a sulfobetainic stationary phase

Tabel.6. Regression parameters for studied compounds using mobile phase ACN/H2O (10 mM AcNH4)and a sulfobetainic stationary phase		
	Glycyl-L-phenylalanine	**Glycyl-L-tyrosine**
A	-2.05561	-1.4985
B_1	0.034	-0.0186
B_2	$-7.420*10^{-5}$	$9.0282*10^{-5}$
R^2	0.987	0.986

When replacing acetonitrile with methanol as organic modifier of the mobile phase, for glycyl-L-phenylalanine and glycyl-L-tyrosine, the retention is weaker, even though the retention behavior follows the same trend, meaning that the retention decreases when the organic modifier concentration increases. The calculated values for the parameters described by equation (2) and (3), presented in the previous chapter, for the "U-shaped" elution curves, in the case of glycyl-L-phenylalanine and glycyl-L-tyrosine, for mobile phase using acetonitrile and methanol as organic modifiers are presented in Tabel 7.

Tabel.7. "U-shaped" elution curves parametersfor the studied compounds		
Compound	**Glycyl-L-phenylalanine**	**Glycyl-L-tyrosine**
Organic Modifier Acetonitrile		
log k_w	-2.055	-1.498
$(C_m)_{min}$	22.91	9.960
log k_{min}	-1.665	1.587
Organic Modifier Methanol		
log k_w	-2.017	-1.771
$(C_m)_{min}$	23.220	23.610
log k_{min}	-1.797	-2.015

Similar to the study of aminoacids chromatographic behavior as a function of the mobile phase composition data were studied according to equation (4) and (5).

The log-lin and log-log plots can be considered as being polynomial for these model compounds confirming tthe presence of multiple retention mechanisms based both on partition and adsorption.

Retention times are found to be higher when using a bare silica stationary phase than in the case of using a sulfobetainic one. This observation is based on the presence of stronger interactions between the dipeptides and this type of stationary phase.

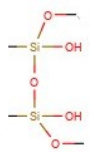


Fig.7. Chemical structure of the bare silica stationary phase

Tabel.8. Regression parameters for studied compounds using mobile phase ACN/H_2O (10 mM $AcNH_4$)and a bare silica stationary phase				
	Glycyl-L-phenylalanine	**Glycyl-L-tyrosine**	**Glycyl-L-leucine**	**Glycyl-L-alanine**
A	-0.006	-0.0119	0.1646	-0.0223
B_1	-0.00558	-0.0031	-0.0193	-0.0113
B_2	$1.9916*10^{-4}$	$2.1086*10^{-4}$	$3.5613*10^{-4}$	$3.4186*10^{-4}$
R^2	0.985	0.992	0.990	0.996

For all studied compounds the regresiion parameters are higher when using a bare silica stationary phase owing to a higher aqueous layer deposited at the surface of this type of stationary phase caused by a higher concentration of polar residual silanol groups. This characteristic allows greater partition processes.

2.2.4. Influence of the ionic strength of the mobile phase on the chromatographic retention

HILIC retention mechanism is strongly influenced by the ionic strength of the mobile phase. Salts present in the mobile phase composition undergo electrostatic interactions between the analyte and the stationary phase. Ammonium acetate and ammonium formate are the most utilized salts in this type of chromatographic separations due to their high solubility in the mobile phase.

From the experimental data obtained in this part of the study a first observation is that the retention time is higher when using ammonium formite than in the case of using ammonium acetate, owing to the fact that the elution strength of the formate ion is greater than the one of the acetate ion [9].

In general, increasing the salt concentration in the mobile phase decreases the electrostatic interactions wen using a sulfobetainic stationary phase, especially for ionizable compounds. Retention time for all analytes increases when increasing the salt concentration indicating the presence of electrostatic repulsions between the SO_3^- moiety of the stationary phase and the carboxyl functional group of the analytes.

Due to the fact that the plots k vs $1/[AcNH_4]$ (Fig. 10) are not described by a linear function, one may conclude that ion exchange interactions are mot the one dominating the retention mechanism under HILIC separations for the studied compounds.

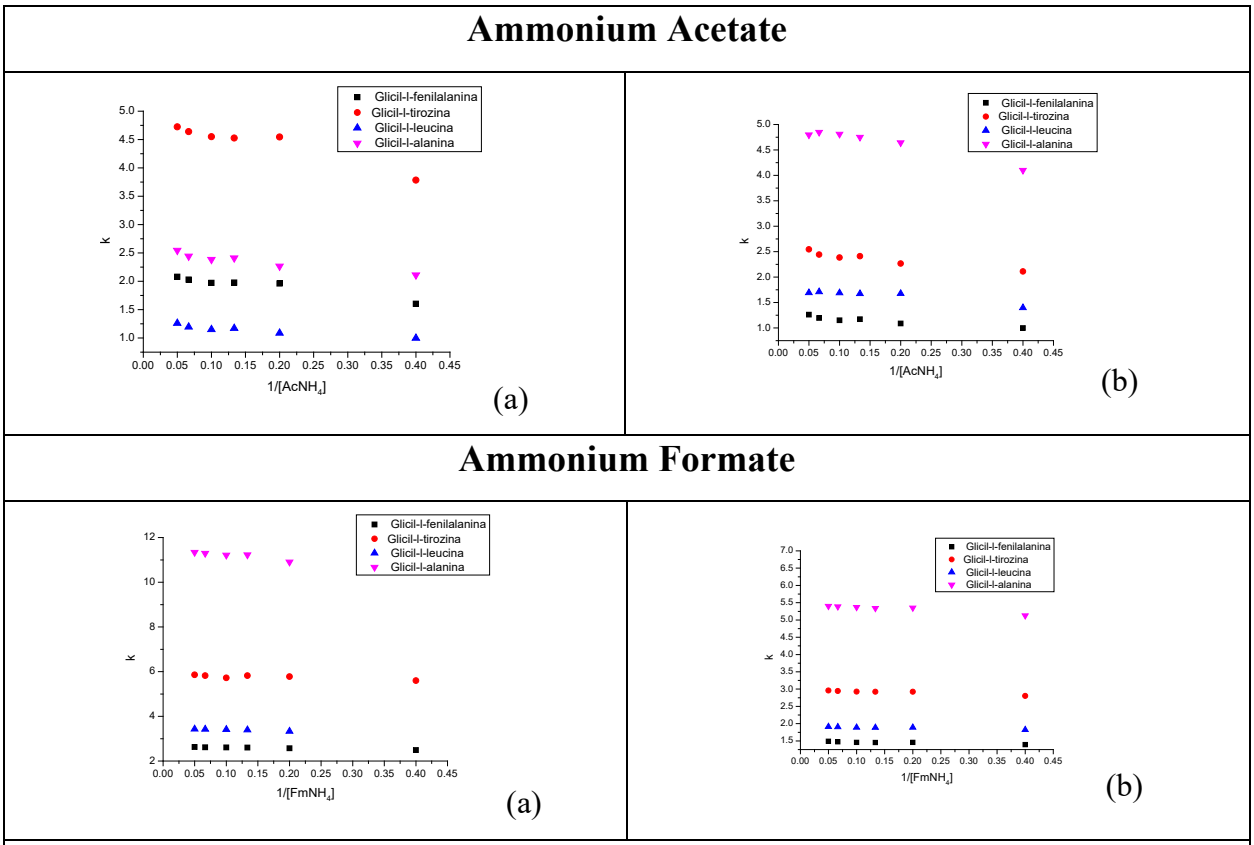


Fig.8. Plots of k versus 1/[AcNH4] for the studied compounds for (a) 80% acetonitrile and (b) 75 % acetonitrile in mobile phase.

2.3. INFLUENCE OF TEMPERATURE ON THE RETENTION BEHAVIOUR IN HYDROPHILIC INTERACTION LIQUID CHROMATOGRAPHY

2.3.3. Temperature Influence On The Retention Behavior Of Some Aminoacids In Hydrophilic Interaction Liquid Chromatography

In HILIC separation the temperature of the stationary phase plays an important role in the retention mechanism. The relationship that links the column temperature to the retention factor in the van't Hoff equation:

$$\ln k = -{\Delta H^0}/{RT} + {\Delta S^0}/{R} + \ln\phi \qquad (6)$$

Where ΔH^0 is the standard enthalpy for the transfer of the analyte from mobile phase to the stationary phase, ΔS^0 is standard entropy for the same partition process, R is the universal gas constant (8.314 J K^{-1} mol^{-1}), T is the absolute column temperature (namely t + 273.15, t being

column temperature in Celsius degree) and ϕ represents the phase volume ratio (the volume of stationary phase to that of the mobile phase).

Even though the retention time of the studied aminoacids does not vary significantly when increasing the temperature of the stationary phase, one can observe three different trends, regardless of the organic modifier (acetonitrile or methanol) used in the mobile phase composition. The retention time decreases for phenylalanine and histidine all over the temperature range, whereas in the case of tryptophan and increase is observed. In the case of tyrosine an increase is seen between 20-30 ^{o}C, followed by a decrease in the 30-50^{o}C temperature range, following a "U-shaped" curve similar to the study where the influence of the organic modifier of the mobile phase was observed. In all cases, retention times were lower when methanol was used as organic modifier compared to the same study using acetonitrile, according to the observations detailed in Chapter 2.1.

Ln k values, calculated for all the studied compounds were plotted against the reciprocal of the column temperature (1/T). Some of these dependences can be considered as being linear, according to the following equation:

$$ln\, k = A + B \cdot 1/T \quad (7)$$

whereas others might be considered polynomial:

$$ln\, k = A + B1 \cdot 1/T + B2 \cdot (1/T)^2 \quad (8)$$

The values of standard enthalpy change (ΔH^0) associated with the HILIC retention process can be estimated using the values of slope (B) calculated from the linear regreesions ($\Delta H^0 = -R \bullet B$). Taking this into account, in the case of tryptophan, when the mobile phase consist in 80% Acetonitrile, the calculated value of the standard enthalpy change is ΔH° =7.050kJ/mol. For the cases ΔH° has a positive value,the retention process is considered to be am endothermal one.

In Figure 9 and Tabel 9 are represented the plots and the regression parameters obtained when representing the natural logarithm of the retention factor as a function of the reciprocal of the absolute temperature of the chromatographic column using a second degree polynomial function.

The curved shape of these plots is attributed to a chromatographic retention based both on partition processes as well as some overlayed secondary interactions of adsorptive type, most

probably ionic ones [33]. Deviations from van't Hoff linearity are reported in literature for reverse phase liquid chromatography studies [15,16].

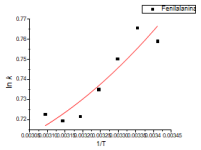
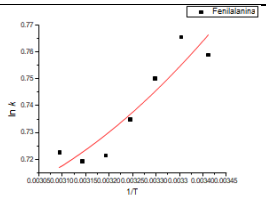
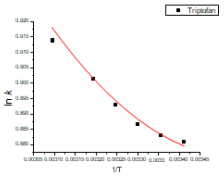
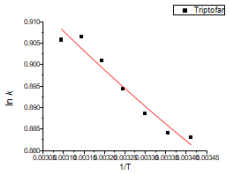
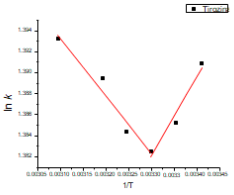
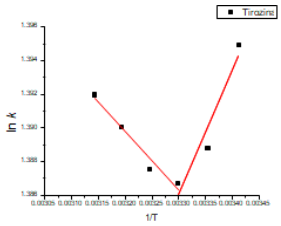
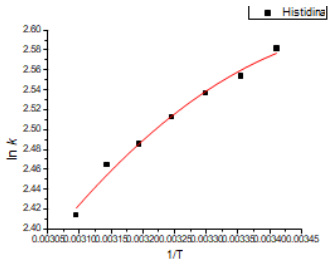
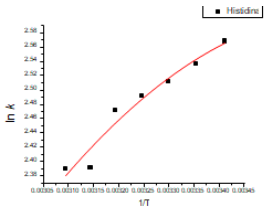
POLYNOMIAL REGRESSIONS	
Phenylalanine	
80%ACN	**75%ACN**
	
Triptophan	
80%ACN	**75%ACN**
	
Tyrosine	
80%ACN	**75%ACN**
	
Histidine	
80%ACN	**75%ACN**
	

Fig.9. Polynomial plots of ln k vs 1/T for the studied aminoacids when the mobile phase uses ACN as organic modifier

Tabel 9. Regression parameters obtained for the studied compounds when the stationary phase is of sulfobetainic type and the mobile phase consists of 80 or 75% acetonitrile as organic

	Fenilalanină		**Triptofan**		**Tirozină**		**Histidină**	
	80%ACN	75%ACN	80%ACN	75%ACN	80%ACN	75%ACN	80%ACN	75%ACN
A	5.561	1.179	3.496	1.4342	-	-	-6.8486	-7.038
B_1	$-3.181 \cdot 10^{3}$	$-6.033 \cdot 10^{2}$	$-1.478 \cdot 10^{3}$	$-2.472 \cdot 10^{2}$	-	-	$5.264 \cdot 10^{3}$	$5.277 \cdot 10^{3}$
B_2	$-5.211 \cdot 10^{5}$	$1.474 \cdot 10^{5}$	$2.087 \cdot 10^{5}$	$0.249 \cdot 10^{5}$	-	-	$-7.333 \cdot 10^{5}$	$-7.2186 \cdot 10^{5}$
R^2	0.975	0.801	0.914	0.935	-	-	0.983	0.934

Deviation from van't Hoff linearity are frequently seen in studies involving HILIC separation mechanism [34]. One possible cause might be the existence of different forms of the analyte in the mobile phase, causing different types of interactions with the polar surface of the stationary phase. For example, as seen in the case of histidine, depending on the pH of the mobile phase, multiple molecular forms might be present (Fig. 10). At a mobile phase pH ~ 7 given by the 10mM CH_3COONH_4 solution used in this study, histidine can be present in two dissociated molecular forms which will interact different with the polar groups associated with the stationary phase.

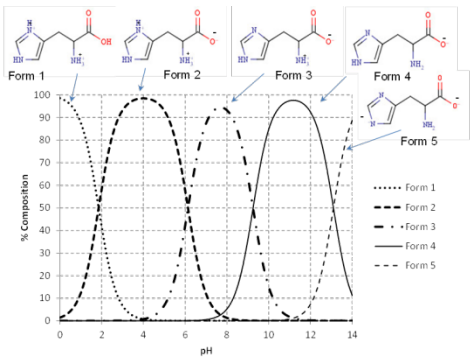


Fig.10. Different forms of histidine as a function of pH.

The second part of this study followed the influence of the temperature on retention behavior of hidtidine under HILIC mechanism when using a chromatographic column filled with a bare silica stationary phase when the mobile phase contained 75% acetonitrile as organic modifier and a 10mM ammonium acetate solution as aqueous component. Even though the retention behavior follows the same trend, experimental data underline the fact that the

interactions between the analyte and this kind of stationary phase are stronger than in the case when using a sulfobetainic stationary phase.

2.3.4. Temperature Influence On The Retention Behavior Of Some Dipeptides In Hydrophilic Interaction Liquid Chromatography

For all the studied compounds a decrease in the chromatographic retention is observed when increasing the temperature of the chromatographic column. This behavior is considered to be a clasic one for HILIC mechanism, indicating that the transfer process of the analytes between the mobile and the stationary phase is an exotermal one, favored by low temperatures of the chromatographic column [9].

When the plots ln k vs 1/T can be assigned to a liniar function, the retention mechanism is owed to the partition of the analytes between the mobile phase rich in organic modifier and the aqueous layer found at the surafec of the stationary phase [6]. This situation was found in the case of glycyl-L-phenylalanine when the mobile phase contained 80% organic modifier(acetonitrile) and also in the case of glycyl-L-leucine for 75% organic modifier in the mobile phase. The calculated values of ΔH^0, were -9.926 kJ/mol in the case of glycyl-L-phenylalanine and -8.837 kJ/mol in the case og glycyl-L-leucine.

The plots ln k vs 1/T described by a liniar mathematical function were characterized by values of R^2 higher than 0.99 for glycyl-L-phenylalanine and glycyl-L-leucine when the mobile phase used 80% organic modifier and for glycyl-L-leucine and glycyl-L-tyrosine when using 75% organic modifier in the mobile phase composition.

For the other studied cases, the correlation coefficients were better when data was fitted based on a second degree polynomial function, indicatind that the retention mechanism is influenced by electrostatic attractions between the functional groups of the analyte and those of the stationary phase.

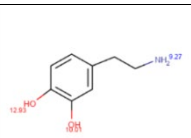
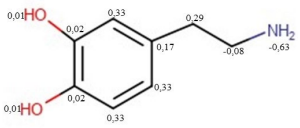
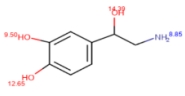
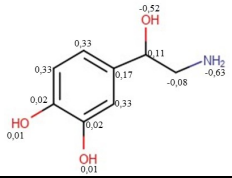
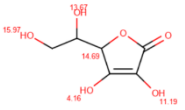
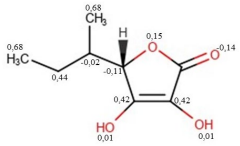
Another part of this study followed the retention mechanism of the proposed dipeptides when using a stationary phase characterized by particles of 3,5 μm in diameter. For all studied compounds the retention times were lower but the chromatographic behavior was the same.

For comparison, the influence of the temperature of the chromatographic column was studied for a bare silica stationary phase. The retention times were higher for all studied

compounds, due to the fact that the morphology of the water layer at the surface of this stationary phase facilitates the interactions with the analytes [35].

2.3.5. Temperature Influence On The Retention Behavior Of Some Polar Compounds In Hydrophilic Interaction Liquid Chromatography

Table 10. pK_a, log K_{ow}, log D (pH=6), μ, Sw values and charge /atom distribution for the studied aminoacids

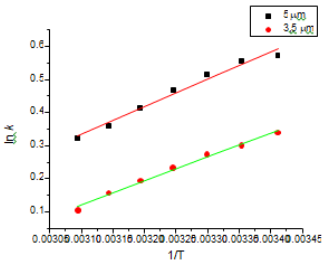
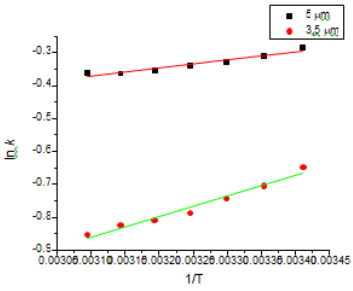
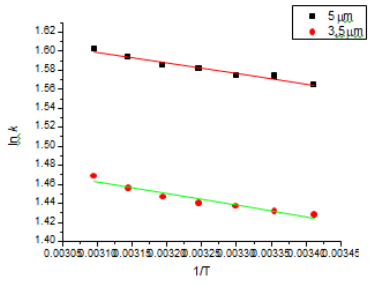
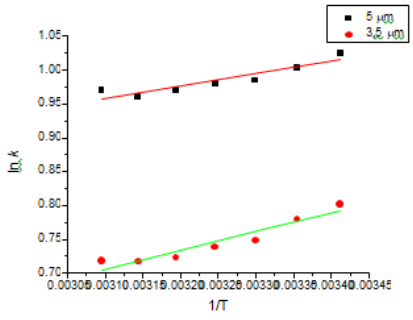
Compound	pK_a	log K_{ow}	log D (pH=6)	μ (D)	Sw (mg/L)	Charge/atom
Dopamine		0.78	-2.17	2.91	1000000	
Noradrenaline		-1.1	-2.90	2.06	1000000	
Ascorbic Acid		-1.91	-3.75	5.49	1000000	

When the mobile phase is rich in organic modifier, the retention mechanism of the studied compounds is mostly one of hydrophilic partition of the analytes between the mobile phase and the aqueous layer at the surface stationary phase. But for the case of dopamine and noradrenaline when the mobile phase uses 80% organic modifier and noradrenaline when the mobile phase uses 75% organic modifier the retention times are found to follow a descending tendency when the temperature of the chromatographic column increases, indicating a exothermal process, usually associated with partition [34]. Deviations from this behavior are also observed as a result of possible electrostatic attractions between the animo functional group of the analytes and the sulfonate group of the sulfobetainic stationary phase [36]. The same chromatographic behaviour was seen both when the particles of the stationary phase were 5μm or 3.5 μm in diameter.

Only in the case of noradrenaline when the mobile phase used 75% acetonitrile as organic modifier the calculated values of R^2 for the ln k vs 1/T plots were higher that 0.99. for these

situations the calculated values of ΔH^0 were -3.326 kJ/mol(for the stationary phase with particle diameter of 5µm) and -2.507 kJ/mol (for the stationary phase with particle diameter of 3.5µm), indicating an exothermal process.

When the same date are fitted using a second degree polynomial function, R^2 values increase, indicating once again the existence of supplementary interactions between the analytes and the stationary phase.

<table>
<tr><th colspan="2">LINEAR REGRESSIONS</th></tr>
<tr><th colspan="2">Ascorbic Acid</th></tr>
<tr><th>80%ACN</th><th>75%ACN</th></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><th colspan="2">Dopamine</th></tr>
<tr><th>80%ACN</th><th>75%ACN</th></tr>
<tr><td> 
</td><td> 
</td></tr>
</table>

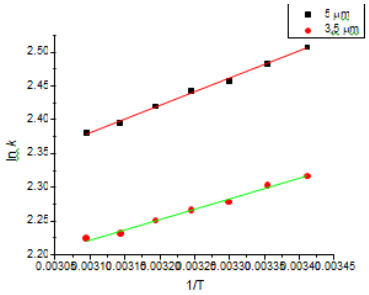
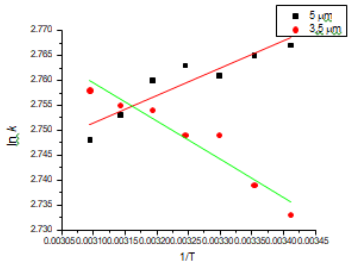
Noradrenaline	
80%ACN	**75%ACN**
	

Fig.11. Linear plots of ln k vs 1/T for the studied aminoacids when the mobile phase uses ACN as organic modifier

For comparison, acetonitrile was replaced by methanol in the mobile phase composition. As expected, the retention times were lower for all studied compounds [37]. The retention time of ascorbic acid decreased below the dead time of the chromatographic column, due tomits ability to form hydrogen bonds with the mobile phase, causing very low interactions with the stationary phase [23]. For dopamine and noradrenaline, retention times decrease, stregthening the fact that these analytes are more likely subjected to partition mechanisms between the mobile phase and the aqueous layer at the surface of the stationary phase. The presence of secondary interactions is pointed out by values of the R^2 coefficients when plotting ln k vs 1/T using a second degree polynomial function.

2.4. INFLUENCE OF THE SAMPLE SOLVENT AND INJECTION VOLUME ON THE CHROMATOGRAPHIC PARAMETERS IN HYDROPHOBIC INTERACTION LIQUID CHROMATOGRAPHY USING A SULFOBETAINIC STATIONARY PHASE

For the study of the injection volume influence on the retention behaviour under the ZIC-HILIC mechanism, glycyl-L-leucine and glycyl-L-alanine were studied as model compounds.

In the first part of the study the sample solvent is identical to the mobile phase, being comprised of 80% acetonitrile as organic modifier and a solution of ammonium acetate in diffrent concentrations (2.5, 5, 10, 20, 25 mM) as aqueous phase. As seen in Figure 12, the retention time, peak efficiency an chromatographic peak symmetry of the studied compounds do not vary in a significant manner when increasing the injection volume between 1 and 20 µl, even though the chromatographic parameters are better in the case of the last eluted parameter, glycyl-L-alanine.

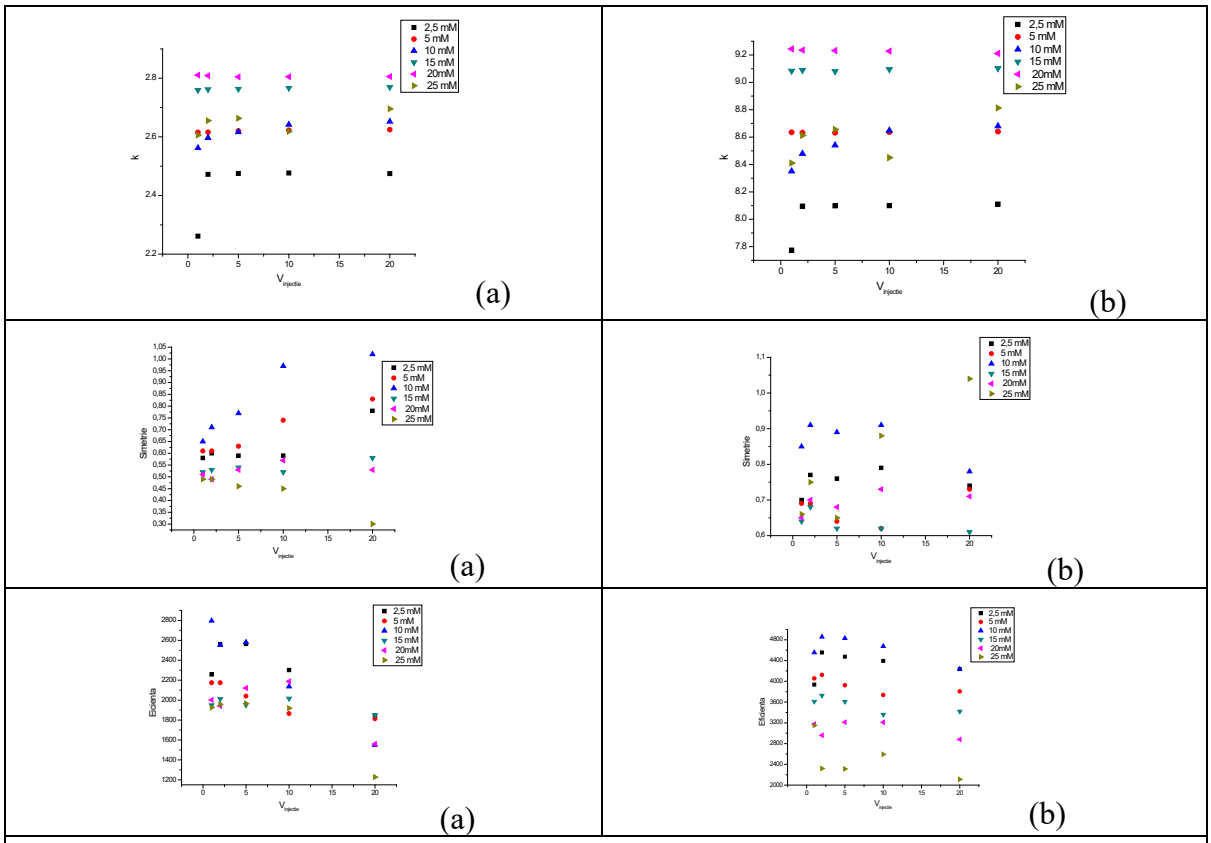


Fig.12. Influence of the injection volume on chromatographic parameters (k, symmetry, efficiency) when the sample solvent is identical to the mobile phase for (a) glycyl-L-leucine and (b) glycyl-L-alanine.

The role of the added salt to the mobile phase is to supress the strong interactions of injected analyte with the stationary phase functional groups. Equations (9) and (10) present the competitive equilibriums that take place between the salts in the mobile phase, the stationary phase and the analytes. Salts are following a dissociation process, supressing the interactions between the functional groups of the analyte and the functional groups of the stationary phase, having as a result the improvement of the chromatographic shapes.

$$(X^+Y^-) + Silica - A^+B^- \leftrightarrow Silica - A^+Y^- - B^-X^+ \quad (9)$$

$$Analyte^{\pm} + Silica - A^+B^- \leftrightarrow Silica - A^+ - B^-(Analyte^{\pm}) \quad (10)$$

If the salt concentration is very low, it is possible that the electrostatic interactions between the molecule of the analytes and the functional groups of the stationary phase not to be sufficiently suppressed, whereas in the opprosite case (of higher salt concentration) it is possible for miscibility mismatches between the organic and the aqueous components of the mobile phase to occure [38]. Following this reason, the optimal value of ammonium acetate to be added to the

aqueous compoment of the mabile phase was chosen to be 10 mM, in accordance to the values reported in other literature studies.

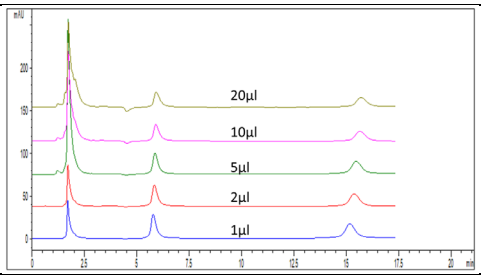


Fig.13. Overlaid chromatograms resulted from the injection of of different sample volumes containing Glycyl-L-leucine and Glycyl-L-alanine
Stationary Phase: Sequant ZIC HILIC, p.d. 5 µm
Sample solvent identical to the mobile phase consisting of 80%ACN/20%AcNH4 10 mM.

In the second part of this chapter, the sample solvent was different form the mobile phase composition (80% acetonitrile and 20% aqueous solution of ammonium acetate 20mM). Sample solvent consisted in 80% ACN and 20% ammonium acetate aqueous solution of different concentrations (2.5, 5, 10, 20 mM).

Chromatographic parameters (retention factor, peak efficiency and peak symmetry) for the second eluted analyte, glycyl-L-alanine are not affected by the variation of salts concentration in the composition of the sample solvent. As seen in Figure 13, in the case of glycy-L-leucine a negative peak is observed when the concentration of salts in the sample solvent increases, having as a consequence poor values for peak efficiency and symmetry when increasing the injected volume of the analyte in the chromatographic column. This observation leads to the conclusion that the equilibriums at which the analyte participates within the chromatographic system under HILIC mechanism are more sensible to the increase in the injection volume rather than to the sample solvent composition.

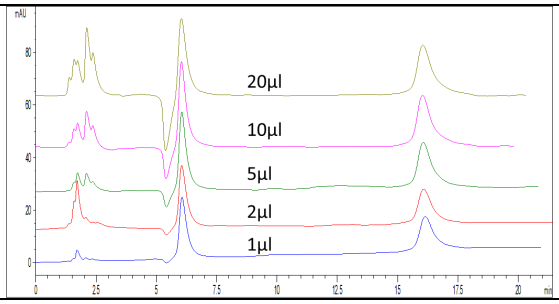


Fig.14. Overlaid chromatograms resulted from the injection of of different sample volumes containing glycyl-L-leucine and glycyl-L-alanine
Stationary Phase: Sequant ZIC HILIC, p.d. 5 µm
Mobile Phase: 80%ACN şi 20% $AcNH_4$ 20mM ;Sample Solvent: 80%ACN/20%AcNH4 10 mM.

2.5. GENERAL REMARKS

In the experimental part of the thesis "Retention Behaviour Of Compounds With Biochemical Importance Using Hydrophilic Interaction Liquid Chromatography", the influence of chromatographic parameters on the retention of polar analytes using liquid chromatography based of hydrophilic interactions mechanism was studied.

For two classes of compounds: amino acids (tyrosine, histidine, phenylalanine and tryptophan) and dipeptides (glycyl-L-phenylalanine, glycyl-L-leucine, glycyl-L-tyrosine, and glycyl-L-alanine) the effects of the mobile phase composition (organic modifier concentration and ionic strength) and chromatographic column temperature were studied. For the ascorbic acid, dopamine and noradrenaline the influence the temperature of the chromatographic column was observed. The influence of the sample solvent and the injected volume was studied for glycyl-L-leucine and g lycyl-L-alanine. The mobile phase contained acetonitrile or methanol as an organic modifier, and ammonium acetate and ammonium formate as salts added to the aqueous component of the mobile phase. The stationary phase was bare silica, or silica modified with a sulfobetainic ligand (which contains) a positive charged quaternary ammonium group and a sulfonate group with a negative charge at the distal end of the ligand attached to the silicagel surface.

Regarding the mobile phase organic modifier`s influence, favorable results were obtained for amino acids and dipeptides when using acetonitrile, rather than methanol. The graphical representation of the retention factor`s decimal logarithm against the organic modifier`s concentration led to "U-shaped" curves which points to a mixed retention mechanism, dominated by the hydrophilic partition of the analyzed compounds between the aqueous layer at the surface of the stationary phase and the mobile phase volume, especially when the ladder one contains a high percentage of organic modifier. Attraction or repulsion interactions of electrostatic type between the analyzed compound and the stationary phase overlap this process, as reported in the literature.

Supression of the electrostatic interaction was proved to occur when the salt concentration added to the mobile phase is increased, both for high and low concentrations of organic modifiers in the mobile phase composition. The comparative study of acetate and formate ions added to the mobile phase proves that chromatographic retention is influenced more by the concentration of salts added, rather then their kind.

Also, an extensive study of the temperature influence in hydrophobic interaction mechanism was conducted. For most compounds, the retention time decreased with the increase of the temperature, regardless of the kind of stationary phase or composition of the mobile phase. This points to the fact that the transfer process of the analytes from the mobile phase to the aqueous layer of the stationary phase is governed by partition. Deviations from the van't Hoff liniarity were caused by possible interactions between the analytes and the stationary phase, overlapped to the partition mechanism.

The attempt to correlate chromatographic retention in ZIC-HILIC mechanism with certain molecular descriptors (log Kow, log D sau μ) of the analyzed compounds did not yield acceptable results due to their high diversity.

Because of the unfavorable shapes of the chromatographic peak symmetry and efficiency values found, a study of the influence of sample solvent and volume injected in the chromatographic column was proposed. When the samples solvent was identical to the mobile phase in terms of composition, favorable result were obtained for injection volumes of up to 20μL. If the samples solvent was different to the mobile phase composition, influences on the first eluded compound retention can occur, and more so for medium injection volumes (up to 20μL). For this reason, it is recommended that if large injection volumes are needed, the samples solvent be identical or as close as possible to the mobile phase. If not possible, the injection volume used for chromatographic separation should be as low as possible.

To conclude, for the analyzed compounds, when using hydrophilic interaction liquid chromatography separations, two kinds of retention mechanisms overlap: the analytes partition between the aqueous layer at the surface of the stationary phase and the mobile phase volume, and electrostatic interactions or hydrogen bonds between the compounds of interest and the mobile or stationary phases.

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