University of Bucharest
Faculty of chemistry
Doctoral School in Chemistry

Doctoral Thesis

Retention studies regarding the influence of organic modifiers in reversed phased liquid chromatography

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2020

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2. Original contributions – Theoretical and experimental studies

Mobile phase is just as important as stationary phase while discussing HPLC separations since chromatographic partition phenomenon takes place between the two phases. Among the retention mechanisms this paper focuses on most important one, reversed phase (RP-HPLC). Although the current scientific literature has a lot of studies and research regarding this separation mechanism there is still ample interest in this field, with particular areas of interest as : i) the mechanism of chromatographic retention, ii) determination of molecular parameter (hydrophobicity/lipophilicity values, dissociation constants, solubility), iii) measuring certain thermodynamic parameters (enthalpy and entropy from retention data), iv) measurements of stationary phase volume active in retention. Chromatographic retention is a complex phenomenon that allows the determination of otherwise difficult to measure parameters [1]. In order to describe the chromatographic retention molecular descriptor of the studied compounds are used.

There are a multitude of approaches regarding retention modelling in RP-HPLC [2-4]. A first class of models are the empirical ones, which describe the variation of some chromatographic parameters measured from experiments depending on the experimental parameters, among which the composition of the mobile phase is the best known. These models also allow the estimation of molecular sizes of the studied compounds, by interpolation or especially by extrapolating the established functions, to certain values of the composition of the mobile phase. Molecular modeling of the partition process, being a complex one, is less studied and is practically limited to applying the solvophobic theory to the RP-HPLC partition equilibrium. Models based on the correlation of molecular sizes with chromatographic retention data (known as QSRR studies) are more intensively studied and in many cases have the ability to predict the chromatographic behavior of new compounds. A more useful model for studying the RP-HPLC process is the thermodynamic one, which knows many applications in the study of inter-phase distribution processes, allowing, for example, the measurement of the fundamental thermodynamic quantities of this process (standard free enthalpy variation, standard enthalpy variation or the standard entropy variation corresponding to the transfer of the molecules of compounds (solutions) from the mobile phase to the stationary phase).

In this PhD work, all these three models were studied, on several classes of investigated compounds, on different types of stationary phases currently used in the reverse phase mechanism, for various mobile phases in which very organic solvents have been introduced. usual (methanol, acetonitrile, ethanol) or less studied in the specialized literature (organic solvents with hydrophobic medium). The obtained results allowed the elaboration of new models to explain the chromatographic partition process, being already published in the specialized journals.

2.1. Retention studies with hydrophobic additives in mobile phase

2.1.1. Introduction

Lipophilicity is one of the most important physico-chemical parameters that play a crucial role in pharmacological studies of drug activity, in particular on transport through biological membranes [5,6]. In the RP-LC the analyte retention is considered to be the result of the competition between hydrophobic interactions with the stationary phase hydrocarbon alkyl chains and the solvolithic interactions with the mobile phase [7]. According to the

solvophobic theory model adapted for RP-LC by Horvath et al [8], the interaction between the solute and the stationary phase is weak and non-selective. Moreover, the driving force that determines the phenomenon of chromatographic separation is the unfavorable interaction between the solute and the surrounding water molecules in the mobile phase. At present, it is accepted that the mobile phase plays a dominant role in the retention process [9]. In order to improve the understanding of the relationship between $\log K_{ow}$ (or different calculated lipophilicity parameters - logP) and $\log k_w$ the influence of different additives in the mobile phase was studied [10-12].

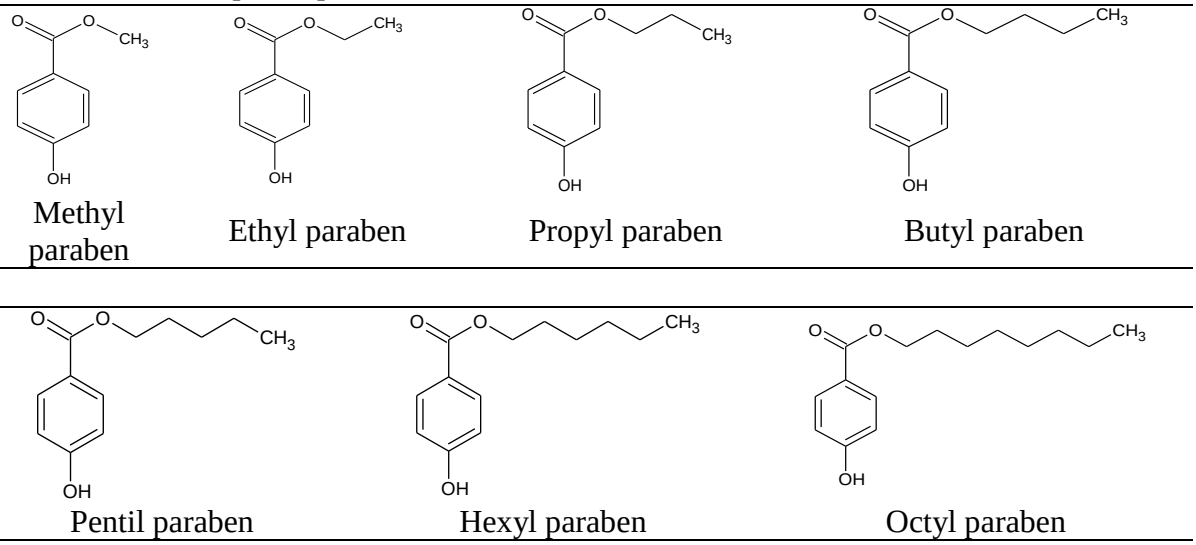

Figure 2.1.1. Structure of the analyzed analytes

The aim of the study was to investigate the influence of low and medium hydrophobicity alcohols as mobile phase additives on the chromatographic evaluation of the lipophilicity of a representative class of preservatives (parabens). The selected substances are a homologous series of 4-hydroxybenzoate alkyls. Parabens have an alkyl chain differing in the number of methylene groups. They have important anti-microbial effects and are used in cosmetics, pharmaceuticals and foods [13]. For some of these compounds $\log K_{ow}$ values have been reported in literature [14].

2.1.3. Results

All the chromatograms obtained showed a clear delimitation of each peak and also symmetries close to 1. The symmetry is measured as the ratio of the half-widths measured at the baseline.

The retention in RP-HPLC is influenced by the nature of the solvents used as organic modifiers in the mobile phase and by their concentrations. This influence is described by a general relationship of form:

$$\log k = \alpha_0 + \sum_{i=1}^{2} \alpha_i C_o^i \,, \qquad (2.1.1)$$

where α_0 and α_i are the regression parameters. In this case, a simpler dependency of order 1 was used: $\log k = \alpha_0 + \alpha_1 * C_o$.

All the correlation coefficients had values very close to 1 indicating a good correlation and the fact that the dependence can really be modeled as linear.

2.1.4. Modeling obtained data

2.1.4.1. Competitional model

For modeling the observed experimental results, a competitive model is considered where L represents the stationary phase hydrocarbon chains, S represents the hydrophobic organic additive and A represents the analyte. Thus the following equilibriums can be considered:

For analyte (A) :

L + A ⇄ LA

$$K_{LA}=\frac{[LA]_s}{[L]_s*[A]_m}=\frac{[A]_s}{[L]_s*[A]_m}=\frac{1}{[L]_s}*\gamma_1*K_{ow}^{A} \quad (2.1.1)$$

For organic additive (S) :

L + S ⇄ LS

$$K_{LS}=\frac{[LS]_s}{[L]_s*[S]_m}=\frac{[S]_s}{[L]_s*[S]_m}=\frac{1}{[L]_s}*\gamma_2*K_{ow}^{S} \quad (2.1.2)$$

where the ratio $\frac{[A]_s}{[A]_m}$ represents the partition coefficient of the analyte between the mobile phase and the stationary phase and is proportional to the partition coefficient water-octanol K_{ow}^{A}, noted with γ_1 the proportionality constant. The same relationship applies to S.

Substituting $[L]_s$ rom equation (2.1.2) into (2.1.1) results :

$K_{LA}=\frac{K_{LS}}{\gamma_2*K_{ow}^{S}}*\gamma_1*K_{ow}^{A}=\gamma*\frac{K_{ow}^{A}}{K_{ow}^{S}}$,

where γ is the constant resulting from the ratio $K_{LS}*\frac{\gamma_1}{\gamma_2}$

Taking into account the relation between k and $\frac{[A]_s}{[A]_m}$ and substituting in the relation (2.1.1) results :

$$k_A=\gamma*[L]_s*\frac{V_s}{V_m}*\frac{K_{ow}^{A}}{K_{ow}^{S}} \quad (2.1.3)$$

Since the concentration of L is much higher than the concentration of A and the ratio of volumes is constant for a given column (equal to 1/Φ), it follows from the relation (2.1.3), by logarithm, the following formula that links the main hydrophobic parameters involved in retention:

$$\log k_A=\Psi+\log K_{ow}^{A}-\log K_{ow}^{S} \quad (2.1.4)$$

This equation illustrates the dependence of the capacity factors of the studied compounds on the basis of the hydrophobicity of the analytes and of the hydrophobic additives.

2.1.4.3. Correlation of lipophilicity parameters

The most common chromatographic parameter for lipophilicity is the isocratic capacity factor ($\log k = \log \frac{t_r - t_0}{t_0}$, where t_0 is the retention time of an unretained compound, such as uracil) and the capacity factor extrapolated for a composition of 100% water ($\log k_w$). The $\log k_w$ parameter is determined by extrapolating the $\log k$ graph to the volume fraction of the organic modifier (φ) for a 100% water mobile phase composition. This extrapolation is based on the Snyder model which assumes a linearity between $\log k$ and φ, on a limited range of mobile phase compositions [15] :

$$\log k = \log k_w - S\,\varphi \qquad (2.1.6)$$

where S is a characteristic parameter of the solvent and φ is the volumetric fraction of the solvent in the mobile phase [12]. This parameter S is considered to be very important for the characterization of the compounds studied in a certain mobile phase / stationary system. A linear correlation between S and $\log k_w$ for a set of compounds indicates a similarity in the intermolecular interactions between the solute and the chromatographic system. Recently proposed lipophilicity parameters, such as the arithmetic mean of the retention parameters (mean of $\log k$ - $m \log k$) or scores ($PCI / \log k$) corresponding to the application of Principal Component Analysis (PCA) on retention data ($\log k$) have been used successfully for the analysis of different classes of compound.

Currently, a wide range of databases and a large number of programs are available that can provide experimental data for 1-octanol / water partition coefficients but also to calculate different lipophilicity ($\log P$) descriptors based on different algorithms. . Lipophilicity parameter values were calculated using Chem3D Ultra 8.0 ($\log P^C$-using the Crippen method, $\log P^V$-using the Viswanadhan method, $\log P^B$-using the Broto method and $\log P^{(1)}$), ALCHEMY 2000 ($\log P^{(2)}$ through SciLogP application version 2.2 and $\log P^{(3)}$ through SciQSAR application version 3.0). In addition, the module available online, ALOGPS (Virtual Computational Chemistry Laboratory), was used to calculate four other lipophilicity descriptors (ALOGPs, ALOGP, MLOGP, KOWWIN) and two solubility parameters (ALOGpS and AC logS).

The experimental lipophilicity parameters obtained from the retention data include the values $m \log k$ and $PCI / \log k$ but also the values $\log k_w$ and S (equation 2.1.6) obtained by extrapolating the $\log k$ values for a mobile phase composition 100% aqueous. The profiles of the investigated parameters showed a similar behavior of the compounds when using low lipophilic alcohols and slightly different values for high lipophilicity alcohols. In all cases, an increase in lipophilicity was observed with the increase of the alkyl chain of parabens.

According to the results obtained, it can be considered that the mobile water-hydrocarbon phase is better for determining the lipophilicity of the parabens than the water-hexanol and water-octanol mixtures, in the case of C18 columns. The correlation between $\log K_{ow} - \log k_w$ and different $\log P - \log k_w$ was investigated using Collander-type regression equations. Based on the best linear regression parameters, the value of $\log K_{ow}$ parameter for pentylparaben (PtyP) and octylparaben (OP) was estimated.

2.1.5. Conclusions

Hydrophobicity is the main parameter when chromatographic retention is discussed. If the complete change in the nature of the mobile phase is an obvious way to change its

hydrophobicity, it often brings uncertainty about the fundamental nature of the retention mechanism. The addition of small amounts of higher alcohols as additives of small and medium hydrophobicity offers the possibility to change the hydrophobicity of the mobile phase, in sufficiently small steps.

The results obtained in this section allow us to propose a competitive model between solute molecules and additives in the mobile phase to explain the observed trends. The obtained equation describes the trend of variation of $\log k_A$ for an analyte A in certain mobile phase-stationary systems. The retention of the compounds decreases with the decrease of their hydrophobicity and with the increase of the hydrophobicity of the additive used.

Methylene selectivity was another evaluated parameter. As the percentage of organic modifier increases, the methylene selectivity decreases, most likely due to the increase of the apparent hydrophobicity of the mobile phase. It is interesting to note that there is the possibility of creating correspondence databases for hydrophobic additives. These tables would allow the choice of the mobile phase depending on the desired methylene selectivity and the percentage of acceptable organic modifier in the mobile phase. Thus, the correct additive is chosen which allows the running of mobile phases with an organic modifier content lower than the conventional situations.

The lipophilicity parameters determined experimentally from the retention data showed a strong correlation with the reference values $\log K_{ow}$ but also with various lipophilicity parameters calculated. Using the experimental results, the log values $\log K_{ow}$ for two of the investigated parabens (pentyl paraben and butyl paraben), which were not reported in the literature, were accurately determined.

2.2. Thermodynamic parameters derived from retention data of hydrophobic additives

2.2.1. Introduction

One of the parameters that can influence the chromatographic retention is the thermostat temperature of the chromatographic column [16]. The retention-temperature relation is used to estimate the thermodynamic parameters related to the partitioning process of the substances studied in a certain system mobile phase / stationary phase. The dependence of temperature retention factors can provide information on the nature of the interactions between the analytes and the stationary phase. If this dependence is linear it could suggest the preponderance of hydrophobic interactions, whereas the deviations from the linearity may suggest complex retention mechanisms based on both hydrophobic and polar interactions [17].

The chromatograms obtained showed a clear separation of the selected compounds and a slight improvement in the shape of the peaks as the temperature increased. This effect is most likely due to the limitation of the longitudinal dispersions due to the shorter retention time.

After obtaining the chromatograms and calculating the retention times, the van Hoff curves were drawn and from the values of slopes and intersections the values of enthalpy and entropy were calculated

$$\log k = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} - \ln \Phi \qquad (2.2.1)$$

The calculated enthalpy and entropy values show some interesting variations. In previous studies, a relatively constant value of enthalpy was reported with the change in the percentage composition of the mobile phase. In the present case, a strong variation of the enthalpy can be observed depending on the nature of the alcohol used as additives although the composition remains constant at 50/50%. This can be explained if one considers that for a system with the same compounds but in different percentages, approximately the same analyte-solvent associations are formed and therefore the adsorption process is approximately the same each time. For systems with different components in the same percentage ratio different analyte-solvent associations are formed and therefore the adsorption process is different for each alcohol.

2.2.4.1 Enthalpy-entropy compensation

The enthalpy-entropy compensation was studied under RP-LC conditions for the set of 2 model compounds (propyl paraben and butyl paraben) and various hydrophobic additives (from ethanol to octanol). The following relationship describes the thermodynamic parameters :

$$\Delta H = T_c \Delta S + \Delta G_{T_c} \qquad (2.2.2)$$

where T_c is the compensation temperature, ΔG_{T_c} is the Gibbs free energy variation at T_c while ΔH and ΔS are the enthalpy and entropy variations.

In addition to the retention data presented above, tests were performed for a composition with 1.5% organic modifier and for a monolithic column (Chromolith RP-C18). Thus all the experiments used for the enthalpy-entropy compensation calculations are:

Zorbax XDB-C18 : 1% hydrophobic additive, 1 mL/min, 50/50, 15-50 ° C

Zorbax XDB-C18 : 1.5% hydrophobic additive, 1 mL/min, 50/50, 15-50 ° C

Chromolith RP-C18: 1% hydrophobic additive, 1 mL/min, 50/50, 15-50 ° C

Chromolith RP-C18: 1% hydrophobic additive, 1.5 mL/min, 50/50, 15-50 ° C

In order to investigate the possibility of an enthalpy-entropy compensation mechanism, the procedures suggested by Krug et al. [18] to confirm whether the nature of the enthalpy-entropy compensations observed is due to physico-chemical phenomena or due to variations in experimental data. The suggested procedures are statistical in nature and have as their main purpose the elimination of the hypothesis that the observed trends would be part of the natural variations that may occur in the experiments.

I. The first step to confirm the existence of an enthalpy-entropy compensation mechanism is the calculation of T_c. After calculating the thermodynamic parameters the graph $\Delta H = f(\Delta S)$ is represented and T_c is determined from the slope of these curves. The results are shown in the following table.

Table 2.2.4. The T_c values determined from the slopes of the curves $\Delta H = f(\Delta S)$

	Propylparaben 1 % modifier	Butylparaben 1 % modifier	Propylparaben 1,5 % modifier	Butylparaben 1,5 % modifier
Zorbax	397,69 K	395,41 K	417,39 K	425,96 K
	Propylparaben 1 mL/min	Butylparaben 1 mL/min	Propylparaben 1,5 mL/min	Butylparaben 1,5 mL/min
Monolithic	401,69 K	399,75 K	400,06 K	400,12 K

II. The next step is to compare the T_c value calculated from $\Delta H = f(\Delta S)$ with the T_c values obtained from $\Delta H = f(\Delta G_{T_{hm}})$ and with the value T_{hm}.

Table 2.2.5. The values T_c (° K) and the ranges

Compound	Experimental conditions	T_c calculated from $\Delta H = f(\Delta S)$	T_c calculated from $\Delta H = f(\Delta G_{T_{hm}})$	Range	
				$T_c(min)$	$T_c(max)$
Propilparaben	Zorbax 1 % modifier	397,69	413,92	367,63	427,74
	Zorbax 1,5% modifier	417,39	481,46	352,61	482,17
Butilparaben	Zorbax 1 % modifier	395,41	409,67	367,52	423,31
	Zorbax 1,5% modifier	425,95	469,42	370,41	481,51
Propilparaben	Monolithic 1 mL/min	401,69	405,39	385,99	417,4
	Monolithic 1 mL/min	400,06	404,91	382,6	417,53
Butilparaben	Monolithic 1,5 mL/min	399,75	403,67	383,82	415,67
	Monolithic 1,5 mL/min	400,12	403,24	385,61	414,63

To calculate the values of ΔH and $\Delta G_{T_{hm}}$ (the values of the Gibbs free energy at the harmonic mean of the temperatures), the graph of $\ln k = f({}^{1}/_{T} - \langle {}^{1}/_{T} \rangle)$ is represented, where $\langle {}^{1}/_{T} \rangle$ represents the value of the harmonic mean of parameters ${}^{1}/_{T}$, for each alcohol used. The values of ΔH and $\Delta G_{T_{hm}}$ are calculated according to the relationships

$$\Delta H = -R(slope) \tag{2.2.3}$$

$$\Delta G_{T_{hm}} = -RT_{hm}(intercept) \tag{2.2.4}$$

By graphing $\Delta H = f(\Delta G_{T_{hm}})$ we calculate the value T_c according to the expression:

$$T_c = T_{hm}/(1 - \frac{1}{slope}) \tag{2.2.5}$$

After calculating the T_c values they must be compared with the T_{hm} values (harmonic mean of the temperatures used in the study) to verify the existence of physico-chemical phenomena. To make this comparison, compare the estimated T_c values with the minimum and maximum values calculated after the relations:

$$T_c(min) = T_c - t * [V(T_c)]^2 \tag{2.2.6}$$

$$T_c(max) = T_c + t * [V(T_c)]^2 \tag{2.2.7}$$

where t is the value of the Student coefficient (for the present situation with 8 degrees of freedom and a 95% confidence level its value is 1.86)

$$T_c = \frac{\sum(\Delta H - \langle \Delta H \rangle)(\Delta S - \langle \Delta S \rangle)}{\sum(\Delta S - \langle \Delta S \rangle)^2} \tag{2.2.8}$$

$$V(T_c) = \frac{\sum(\Delta H - \Delta G_{T_c} - T_c \Delta S)^2}{(m-2)\sum(\Delta S - \langle \Delta S \rangle)^2} \qquad (2.2.9)$$

where m is the number of pairs of experimental data, 8 in this case.

The calculated domain for T_c does not include the value T_{hm} (305,22° K for the set of temperatures used) and therefore indicates the existence of an enthalpy-entropy compensation phenomenon.

III. By representing the van't Hoff curves and tracing the trend curves, the area of intersection is studied.

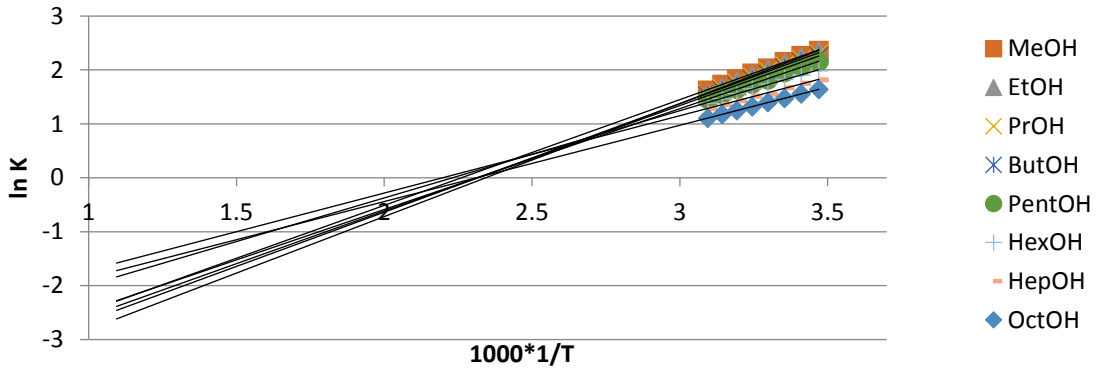

Figura 2.2.5. Van't Hoff curves for propylparaben and 1% modifier on the Zorbax column

For all situations, curves were obtained that intersect on an area and not at a point. This is most likely due to experimental errors. Visually you can estimate the area of intersection to estimate a domain of T_c. This domain in all situations did not include T_{hm}.

IV. The last test to confirm the existence of an enthalpy-entropy compensation mechanism is to compare the probability of the intersections of the Van´ Hoff curves with the probability of non-intersection. The ANOVA (analysis of variance) procedure was used to evaluate the probabilities. If the enthalpy-entropy compensation mechanism is present then the probability of intersection must be much higher than the probability of non-intersection.

Table. 2.2.6 ANOVA calculations for propylparaben and 1% modifier on Zorbax column

Source of variations	Degree of freedom	SS (Sum squared)		MS (Mean Square)	
Total	*63*	*SS t*	*5,920384*	MS t	*0,09397*
Rows (additive)	7	SS r	2,7335978	MS r	*0,39051*
Columns (temperature)	7	SS c	3,1227001	MS c	*0,4461*
Interactions	49	SS rc	0,0640864	MS rc	*0,00131*
Slopes	7	SS S	926572,42	MS s	*132367*
Concurrent	1	SS con	926569,82	MS con	*926570*
Noncurrent	6	SS noncon	2,5962365	MS noncon	*0,43271*
Residual	42	SS e	-926572,4	MS e	*-22061,2*
				MS con/MS noncon	2141337,631
				F(1, 6, 0,95)	234
				MS noncon/MS e	-1,96139E-05
				F(6, 42, 0,95)	3,78

For the case of propyl paraben and 1% modifier on the Zorbax column it is observed that the Mscon / Msnoncon ratio is approximately 10,000 times greater than the corresponding F-factor indicating that the probability of intersection is much higher than the probability of non-intersection. Also the value of the MSnoncon / MSe ratio is much lower than the value of the corresponding F-factor and therefore the probability of non-intersection is lower than the experimental errors.

The same types of values were observed for both compounds under all experimental conditions.

After these tests we can conclude that there is a correlation between enthalpy and entropy due to the existence of an enthalpy-entropy compensation mechanism..

2.2.5. Conclusions

Within this chapter the thermodynamic of chromatographic retention was studied. The values of enthalpy, entropy and free energy Gibbs were calculated after which their variation with the hydrophobicity of the organic additive was studied. The experimental results suggested a second degree dependence of the Gibbs free energy on the hydrophobicity of the additive, which suggests mechanisms involving molecular associations as the basis of the adsorption process.

Another aspect investigated was the existence of an enthalpy-entropy compensation mechanism. According to statistical procedures, the possibility that these experimental observations are the result of normal measurement errors was eliminated. By representing the van Hoff curves, trend lines with slopes of about 0.75 are obtained, suggesting that any enthalpy variation is offset by 75% of the associated entropy variation..

2.3. The ratio of the stationary phase / mobile phase volumes ("phase ratio") in the reverse phase mechanism

2.3.1. The premises of the chromatographic study

The characterization of HPLC columns is done using several parameters including the phase ratio (Φ). This represents the ratio of the volume of the stationary phase V_{st} and the volume of the mobile phase V_o in the column ($\Phi = V_{st}/V_o$) and influences the retention and selectivity of the separation in HPLC, being included, for example, in the van-Hoff equation as part of the entropic term. Measuring this parameter is difficult because there is no clear separation limit between the two phases and the different nature of the mobile phases causes different degrees of penetration of the mobile phase in the stationary phase.

The main difficulty in determining the phase volume ratio is the measurement of the V_{st} value. For the value V_o we can use the value of the flow rate for the mobile phase (D) and the dead time t_o ($V_o = D\ t_0$). Determination of the dead time t_o can be done by: i) accurate determination of the retention time t_r for a non-retained compound (polar organic molecule, such as uracil, an organic or inorganic salt); ii) measuring the weight difference of the chromatographic column when it is filled with two solvents of different densities; iii) measuring the time until a minor disturbance of the baseline in the chromatogram occurs in the case of injecting the deuterated mobile phase or a component of the mobile phase; iv) by extrapolating to the point of 0 the retention time graph t_r according to the homologous number of a series of compounds.

For the explanation of the separation mechanism on a RP chromatographic column, the partitioning model is currently used to explain the complex phenomena that occur within it. Within this model, the phase report and the retention mechanism are closely linked.

Recently, a new theoretical method for evaluating the phase ratio for a given column and a certain mobile phase has been proposed [19]. The theoretical support for this method is based on solvophobic theory. Next, the phase ratio for different columns and different mobile phases was evaluated.

The phase report is defined by the expression :

$$\Phi = \frac{V_{st}}{V_0} \qquad (2.3.1)$$

where V_{st} is the volume of the stationary phase and V_0 s the dead volume of the column. This expression implies that retention in RP-HPLC separation is a process with a pure partition-based mechanism. This has been demonstrated in many works although other models have been proposed.

The retention factor k'_j for a compound j is dependent on the ratio of the phases of the respective column:

$$k'_j = K_j \Phi \qquad (2.3.2)$$

where K_j is the constant that describes the partition balance of compound j between the stationary phase and the mobile phase and depends on the nature of the analyte, the chromatographic column and the mobile phase while Φ is independent of the analyte.

Due to the importance of knowing Φ there are a considerable number of studies dedicated to measuring V_0 and estimating V_{st}. For HPLC columns the value V_0 can be obtained by using relatively simple procedures such as measuring the retention times of some nonretained compounds. Unlike V_0, V_{st} is difficult to calculate without knowing certain parameters (in the case of RP-HPLC:% of carbon, surface area, number of carbon atoms per mole of silane, surface area of silica, weight of stationary phase, density of alkyl groups, molecular mass of the silane used to make the stationary phase, etc.). Even knowing all the parameters, the calculation V_{st} cannot take into account the potential solvent molecules immobilized on the stationary phase and therefore the value of the parameter Φ cannot be estimated without taking into account the variation of the composition of the mobile phase.

Among the evaluation procedures for Φ a procedure is distinguished based on measuring the retention factors of hydrocarbons in a homologous series and using the values of the partition coefficients water / octanol $\log K_{ow}$ for the same compounds. The relationship that shows the dependence of these parameters is as follows :

$$\log k'_j = a \log K_{ow,j} + \log \Phi \qquad (2.3.3)$$

The linear dependence between the retention factors and the water / octanol partition coefficients has been observed frequently in the literature. This correlation has been studied and a possible explanation has been published. The solvophobic theory allows the estimation

of the Helmholtz free energies of the solute j in two immiscible environments A and B for the equilibrium $j_B \leftrightarrow j_A$. Based on this estimate, the equilibrium constant can be obtained from the formula:

$$\log K_{BA,j} = a'A_j + b'\left(V_j\right)^{-2/3}A_j + c'\mu_j^2 + d'\alpha_j \quad (2.3.4)$$

In formula (2.3.4) A_j is the van der Waals molecular surface, V_j is the molar volume, μ_j is the dipole moment and α_j is the polarizability, all sizes being characteristic for the j solute. The parameters a', b', c' and d' are constant for a given system. By simplifying the equation (2.3.4) we obtain:

$$\log K_{BA,j} = a''A_j - b'' \quad (2.3.5)$$

Within the expression (2.3.5) a'' depends only on the solvent system BA and b'' depends on the functionality of analyte j and the solvent system. The values b'' are transferable for a specific functional group for any compound j. In the case of hydrocarbons it has been shown that the value of this parameter is approximately 0. By applying the previously presented theory for two separate hydrocarbon equilibria j, in the case of a water / octanol system and a stationary / mobile phase system results:

$$\log K_{ow,j} = a''_j A_j \quad (2.3.6)$$

$$\log k'_j = a''_2 A_j + \log \Phi \quad (2.3.7)$$

Elimination of the term A_j between the two equations results in obtaining the equation (2.3.3) which is only valid for a hydrocarbon.

The four consecutive pairs of aromatic hydrocarbons were studied by this theoretical process for finding the value of $\log \Phi$, but which vary according to the chosen pairs. Thus the highest value for the $\log \Phi$ was found for the propylbenzene / butylbenzene pair and the lowest value for the toluene / ethylbenzene pair. These observations can be seen in Figures 2.3.3, obtained for ethanol as an organic modifier.

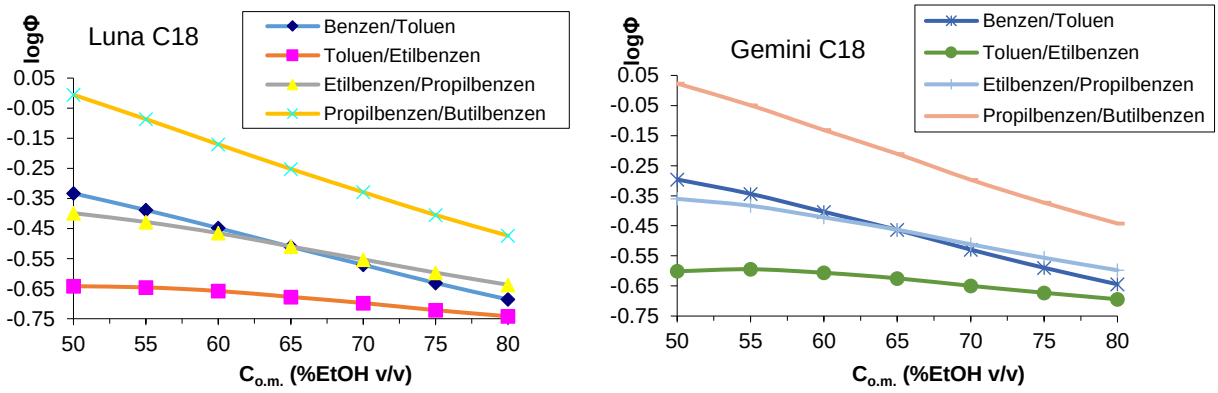

Figure 2.3.3. Phase profile comparison for different pairs of aromatic hydrocarbons using

ethanol as an organic modifier.

2.3.5. Phase report and equilibrium constants

2.3.5.1. The premises of the study

In the previous sections the parameter describing the phase ratio for a set of 3 columns, a set of homologous compounds and 3 compositions of the mobile phase was studied. In order to investigate in detail aspects regarding methylene selectivity and the phase ratio, a number of 7 chromatographic columns (of which 2 core-shell type), 4 sets of homologous compounds and 2 mobile phase compositions were studied.

Solvophobic theory has been successfully used to explain retention in reverse phase liquid chromatography [7,8,20]. Within this theory for the partition of a species j between two immiscible liquids denoted by A and B, one can write equation 2.3.4.

$$\log K_{BA,j} = aA_j + b\left(V_j\right)^{-2/3} A_j + c_j \mu_j^2 + d_j \alpha_j \qquad (2.3.4)$$

where A_j is the van der Waals molecular surface, V_j is the molar volume, μ_j is the dipole moment and α_j is the polarizability, all sizes being characteristic for solute j. Parameters a, b c_j and d_j depend on a lot of molecular parameters such as: molar volume, molecular diameters, critical pressures, Kihara parameters, ionization potentials, surface voltages and dielectric constants. For a certain number of systems these parameters are available in the literature [21-23], or can be calculated with certain software packages [24,25]. Even under these conditions, the direct calculation of the $\log K_{BA,j}$ is difficult. Fortunately the term $c_j \mu_j^2$ in equation 2.3.4 can be eliminated. The value of this term which depends on the dipole moment is very small and as such can be neglected for a high number of solvents and solutions. Equation 2.3.4 becomes thus:

$$\log K_{BA,j} = aA_j + b\left(V_j\right)^{-2/3} A_j + d_j \alpha_j \qquad (2.3.10)$$

Using some simplifications equation 2.3.10 becomes:

$$\log K_{BA,j} = \left(a + b\left(V_j\right)^{-2/3} + d^*\right) A_j \qquad (2.3.12)$$

where d^* is a direct coefficient proportional to d_j. Equation 2.3.12 suggests the possibility of calculating the distribution constant $\log K_{BA,j}$ based on the geometry of the molecule provided that the parameters a', b' and d^* are known. In addition, this equation suggests a potential linear correlation between $\log K_{BA,j}$ and the van der Waals surface A_j provided that the sum $a + b\left(V_j\right)^{-2/3} + d^*$ has only slight variations from compound to compound and depend only on solvents A and B. Equation 2.3.12 becomes as follows:

$$\log K_{BA,j} = a' A_j \qquad (2.3.13)$$

The linear dependence expressed by equation 2.3.13 was observed experimentally for the system A = octanol and B = water using a hydrocarbon as species j but also for A =

stationary phase C8 / C18 and B = polar mobile phase using also a hydrocarbon as species j [19].

In order to be able to apply equation 2.3.13 and other compounds other than hydrocarbons, it is necessary to introduce corrections for the polar entities present in these molecules. For polar molecules the van der Waals surface A_j include atât componentele polare cât și pe cele nepolare. includes both polar and nonpolar components. Since equation 2.3.4 is based on hydrophobic interactions it is assumed that the polar part of the molecule dissolved in a polar solvent does not contribute to the solvophobic interactions, the ratio of the organic component to the van der Waals surface A_j must be subtracted from equation 2.3.13:

$$\log K_{BA,j} = a'\left(A_j - \sum_i b_i'\right) = a'A_j - \sum_i b_i'' \qquad (2.3.14)$$

where a' depends only on the solvent system A and B and b_i'' depends on the functional groups of the analyte j and on the solvent system A and B. The values b_i'' are ideally specific to the different functional groups and can be transferred from one compound to another for the same functional grouping. Equation 2.3.14 has been verified for the octanol / water system for a wide range of compounds. In this case equation 2.3.14 can be written simplified :

$$\log K_{ow,j} = a_1 A_j - b_1 \qquad (2.3.15)$$

where a_1 is a coefficient specific to the octanol / water system and $b_1 = \sum_i b_i''$

For the application of the theoretical partitioning model of a solute j between two immiscible liquids A and B in reverse phase liquid chromatography it is necessary that the chromatographic separation is described as partition and governed mainly by solvophobic interactions [19]. For a system that meets these conditions equation 2.3.14 leads to the following expression of the capacity factor :

$$\log k_j' = a'A_j - \sum_i b_i'' + \log \Phi \qquad (2.3.16)$$

There are literature data [82] and even software packages [24] for calculating van der Waals volumes and surfaces. Equation 2.3.16 can be rewritten

$$\log k_j' = a_2 A_j - b_2 \qquad (2.3.17)$$

where a_2 is a coefficient specific to the mobile phase and the chromatographic column used and $b_2 = \sum_i b_i'' - \log \Phi$. Expressions 2.3.15 and 2.3.17 can be combined to form:

$$\log k_j' = \frac{a_2}{a_1} \log K_{ow,j} + \left(\frac{a_2 b_1}{a_1} - b_2\right) \qquad (2.3.18)$$

Linear dependencies between $\log k_j'$ and $\log K_{ow,j}$ have been repeatedly experimentally reported [26]. For the special case of a hydrocarbon as species j, $\sum_i b_i'' = 0$ and noting $a = \frac{a_2}{a_1}$ the expression 2.3.18 becomes:

$$\log k_j' = a \log K_{ow,j} + \log \Phi \qquad (2.3.19)$$

For the partition balance of the species j between the mobile phase and the stationary phase can be written

$$k_j' = K_j \Phi \qquad (2.3.20)$$

By comparing equations 2.3.19 and 2.3.20 it can be concluded that :

$$\log K_j = a \log K_{ow,j} \qquad (2.3.21)$$

2.3.5.4. Phase ratio

Equation 2.3.19 was verified for benzene, toluene, ethylbenzene, propylbenzene and butylbenzene using different columns and compositions of the mobile phase. The $\log K_{ow}$ values were taken from table 2.3.7 and the $\log k`$ values were calculated according to the procedure described above. Figure 2.3.6 shows a very good linear correlation between $\log k`$ and $\log K_{ow}$ (r^2 values were between 0.9957 and 0.9985). The values of the slopes for $\log k` = f(\log K_{ow})$ for the other situations can be found in the following tables.

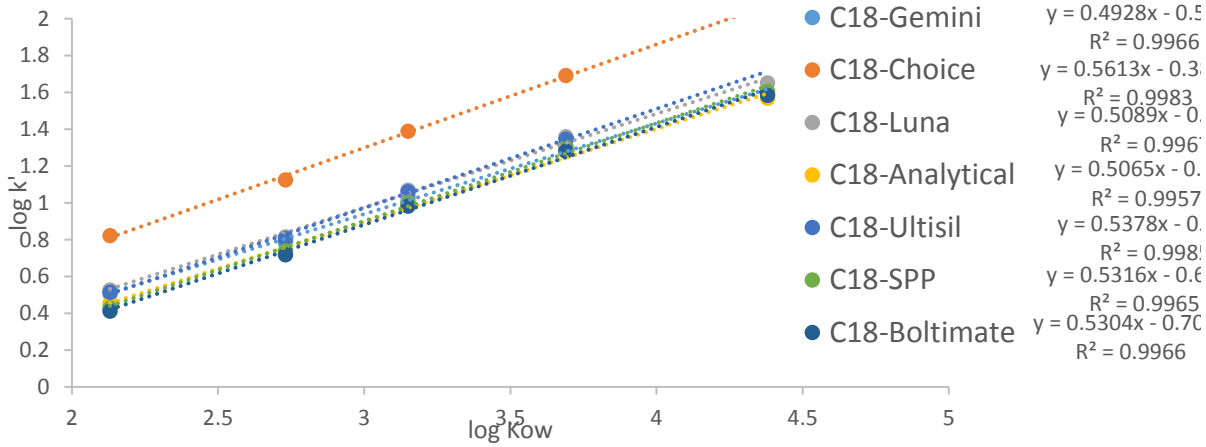

Figure 2.3.6. Example $\log k` = f(\log K_{ow})$ for the benzene-butylbenzene series, using MeOH as an organic modifier

Table 2.3.9 The $\log \Phi$ values for $\log k` = f(\log K_{ow})$ for the benzene-butylbenzene series and the mobile phase MeOH / Water with 0.1% H_3PO_4 and for the mobile phase ACN Water with 0, 1% H_3PO_4

% MeOH	C18-Gemini	C18-Choice	C18-Luna	C18-Analytical	C18-Ultisil	C18-SPP	C18-Boltimate
45	-	-	-	-	-	-0,6066	-0,6313
47,5	-	-	-	-	-	-0,6102	-0,6158
50	-	-	-	-	-	-0,6278	-0,6395
52,5	-	-	-	-	-	-0,6468	-0,6635
55	-	-	-	-	-	-0,6568	-0,6695
57,5	-	-	-	-	-	-0,6710	-0,6853
60	-0,5399	-0,3848	-0,5524	-0,6240	-0,6408	-0,6935	-0,7099
62,5	-0,5574	-0,3903	-0,5748	-0,6385	-0,5843	-	-
65	-0,5739	-0,4066	-0,5861	-0,6552	-0,6003	-	-
67,5	-0,5941	-0,4219	-0,6003	-0,6743	-0,6195	-	-
70	-0,6126	-0,3663	-0,6176	-0,6969	-0,6426	-	-
72,5	-0,6331	-0,4550	-0,6386	-0,7206	-0,6623	-	-

75	-0,6570	-0,4759	-0,6631	-0,7419	-0,6835	-	-
%ACN	C18-Gemini	C18-Choice	C18-Luna	C18-Analytical	C18-Ultisil	C18-SPP	C18-Boltimate
30	-	-	-	-	-	-0,3926	-0,3798
32,5	-	-	-	-	-	-0,3682	-0,3840
35	-	-	-	-	-	-0,3775	-0,3919
37,5	-	-	-	-	-	-0,3676	-0,4008
40	-	-	-	-	-	-0,3965	-0,4199
42,5	-	-	-	-	-	-0,4131	-0,4363
45	-	-	-	-	-	-0,4339	-0,4674
47,5	-	-	-	-	-	-	-
50	-0,2803	-0,1377	-0,2633	-0,3555	-0,2629	-	-
52,5	-0,2810	-0,0922	-0,2730	-0,3758	-0,2794	-	-
55	-0,2961	-0,1218	-0,2884	-0,3705	-0,2980	-	-
57,5	-0,3308	-0,1470	-0,3166	-0,4254	-0,3234	-	-
60	-0,3594	-0,1733	-0,3449	-0,4557	-0,3510	-	-
62,5	-0,3681	-0,2047	-0,3719	-0,4838	-0,3810	-	-
65	-0,4204	-0,2808	-0,4070	-0,5134	-0,4105	-	-

The obtained results illustrate a common linearity between $\log k`$ and $\log K_{ow}$ but also a certain similarity between K_j for a certain composition of the mobile phase and analyte and the different columns C18 (the slope being proportional to this parameter). The application of equation 2.3.21 allows the calculation of $\log K_j$ values using the average values of which can be found in table 2.3.8

The values a obtained for ACN are lower than those obtained for MeOH for the same % organic modifier because ACN is considered a stronger solvent than MeOH.

In addition from equation 2.3.19 it can be concluded that the intersection of linear regressions is in fact $\log \Phi$, this values can be found in table 2.3.9. It can be observed from this table that the phase ratio Φ varies with the composition of the mobile phase.

The variation of the ratio of the phases with the composition of the mobile phase and its nature is the result of changing the boundaries between the mobile phase and the stationary phase. Acetonitrile is a solvent that interacts more strongly with the stationary phase and therefore the C18-Acetonitrile systems are characterized by a higher phase ratio than the C18-Methanol systems, which can be seen from table 2.3.9

In order to determine the phase ratio it is necessary to minimize any interactions except hydrophobic ones. For this reason it is recommended to use hydrocarbons or compounds with nonpolar groups to determine the phase ratio. Figure 2.3.7 shows the variation of parameter a for the benzene-butylbenzene series on the seven columns and the two organic modifiers depending on the percentage of organic component.

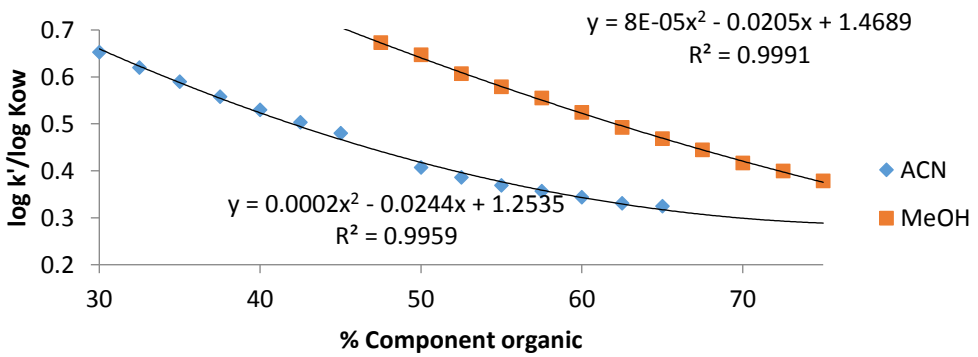

Figure 2.3.7. Representation $\log k` / \log K_{ow}$ depending on the % organic component for the benzene-butylbenzene series

2.3.5.5 Hydrophobic compounds with polar groups

In the case of hydrophobic compounds containing polar groups, equation 2.3.18 can be written as :

$$\log k'_j = a \log K_{ow,j} + b \tag{2.3.22}$$

$$\text{where b} = \frac{a_2}{a_1} \sum_i b''_i - \sum_j b'_j + \log \Phi \tag{2.3.23}$$

In equation 2.3.23 the term $\sum_i b''_i$ is for the octanol / water system and the term $\sum_j b'_j$ is for the stationary / mobile phase system.

The expression 2.3.22 cannot be used to calculate the values of K_j or Φ because the values of parameter b are unknown. This is why the evaluation of the phase ratio of a chromatographic column is done using a homologous series of hydrocarbons. In addition, the presence of polar groups results in the influence of other types of interactions besides the hydrophobic ones on the chromatographic separation. In practice, deviations of the parameter value from the measured values for the hydrocarbon series will be observed.

2.3.6 Methylene selectivity

2.3.6.1 The premises of the study

Methylene selectivity is a parameter that characterizes the hydrophobicity of a stationary phase and is defined as

$$\alpha(CH_2) = \frac{k(X-CH_2-Y)}{k(X-Y)} \tag{2.3.30}$$

where $\alpha(CH_2)$ is the methylene selectivity, and k is the retention factor for compounds $X-Y$ and $X-CH_2-Y$.

The value of methylene selectivity is experimentally determined to be constant for a homologous series for a given column and composition of the mobile phase, so the $\log k$ dependence on the number of methylene groups can be considered as linear. For this reason the value of methylene selectivity can also be obtained from the calculation of the slope β of the trend line related to the $\log k$ graph, depending on the number of methylene groups.

The constant value of methylene selectivity can be justified if one considers the contribution of the methylene group to the Gibbs free energy change associated with the transfer of a molecule from the mobile phase to the stationary phase as constant.

A theoretical justification for $\log k$ linearity can be obtained using solvophobic theory. Within this theory for the partition of a species j between two immiscible liquids denoted by A and B, one can write equation 2.3.4

$$\log K_{BA,j} = aA_j + b\left(V_j\right)^{-2/3} A_j + c_j \mu_j^2 + d_j \alpha_j \qquad (2.3.4)$$

where A_j is the van der Waals molecular surface, V_j is the molar volume, μ_j is the dipole moment and α_j is the polarizability, all sizes being characteristic for solute j. Using the simplifications used in the previous chapter we arrived at equation 2.3.16

$$\log k_j' = a' A_j - \sum_i b_i'' + \log \Phi \qquad (2.3.16)$$

The van der Waals areas and molecular volumes are geometrical properties and can be calculated based on the structure of the molecule and the van der Waals radii. Currently, there are multiple sources available with these sizes, but also various software packages capable of calculating them. One of the van der Waals area calculation methods uses the following equation with a good approximation [3]:

$$A = \sum_{atom} A_{atom} - \sum_{legături} C_{legături} - 13.75(m-1) \qquad (2.3.31)$$

where the term $\sum_{atom} A_{atom}$ refers to the sum of the areas per individual atoms, and the term $\sum_{legături} C_{legături}$ refers to the sum of the areas of the bonds and the term m is the number of atoms in the molecule. Taking into account the expression 2.3.31 the introduction of an additional methylene group ($-CH_2-$) into compound j induces a constant variation of the van der Waals area denoted by k. Thus the following equation can be written :

$$A_{j+n(CH2)} = A_j + nk \qquad (2.3.32)$$

The linear dependence of the van der Waals area according to the number of methylene groups is supported by different theoretical methods of calculation. By combining equation 2.3.32 with equation 2.3.16 it results:

$$\log k'_{j+n(CH2)} = a' A_j - \sum_i b_i'' + \log \Phi + na'k \qquad (2.3.33)$$

Equation 2.3.33 is equivalent to

$$\log k'_{j+n(CH2)} - \log k_j' = na'k \qquad (2.3.34)$$

Equation 2.3.34 indicates a constant increase in the $\log k_j'$ value for a number of homologous compounds. Although the increase is constant the exact value according to equation 2.3.33 is difficult to calculate because the values of the different parameters are difficult to know. In any case, this equation provides a theoretical justification for the observed linear dependence of $\log k_j'$ based on the number of methylene groups without using assumptions equivalent to $\Delta(\Delta G_{CH2}) = constant$.

In this model, it is not necessary to use a specific series of homologous compounds to measure $\alpha(CH_2)$. In practice, it is preferable to use a series of homologous aromatic hydrocarbons as they exhibit almost exclusively solvophobic interactions.

Equation 2.3.34 written for a value n = 1 is equivalent to

$$\log \alpha(CH_2) = \log k'_{j+(CH2)} - \log k'_j = a'k \qquad (2.3.35)$$

Although equation 2.3.35 is a simplification of equation 2.3.34 it is not useful for calculating $\log \alpha(CH_2)$ values because of the difficulty of calculating the values of $a'k$. For this reason, equation 2.3.35 cannot be used to predict the variations of $\log \alpha(CH_2)$ when changing the column or the mobile phase.

Table 2.3.14 $\log \alpha(CH_2)$ values for MeOH as an organic modifier

	% MeOH	60	62,5	65	67,5	70	72,5	75
C18-Gemini	Alkylbenzenes	0,271	0,257	0,244	0,231	0,218	0,205	0,193
	Benzoic esters	0,267	0,254	0,241	0,229	0,216	0,204	0,193
	Hydroxyesters	0,270	0,259	0,247	0,236	0,225	0,214	0,203
	Hydroxyketones	0,285	0,273	0,263	0,252	0,242	0,232	0,222
C18-Choice	Alkylbenzenes	0,283	0,266	0,253	0,240	0,235	0,214	0,202
	Benzoic esters	0,284	0,271	0,259	0,245	0,232	0,220	0,208
	Hydroxyesters	0,290	0,278	0,266	0,253	0,242	0,231	0,220
	Hydroxyketones	0,305	0,293	0,281	0,270	0,260	0,249	0,239
C18-Luna	Alkylbenzenes	0,280	0,267	0,253	0,238	0,224	0,211	0,199
	Benzoic esters	0,275	0,262	0,249	0,236	0,224	0,211	0,199
	Hydroxyesters	0,277	0,266	0,254	0,242	0,230	0,219	0,208
	Hydroxyketones	0,290	0,279	0,268	0,258	0,246	0,236	0,226
C18-Analytical	Alkylbenzenes	0,281	0,263	0,250	0,237	0,225	0,213	0,201
	Benzoic esters	0,276	0,263	0,251	0,239	0,226	0,214	0,202
	Hydroxyesters	0,287	0,275	0,263	0,251	0,239	0,228	0,217
	Hydroxyketones	0,301	0,290	0,280	0,269	0,258	0,247	0,237
C18-Ultisil	Alkylbenzenes	0,270	0,266	0,253	0,240	0,228	0,215	0,204
	Benzoic esters	0,276	0,263	0,251	0,240	0,229	0,215	0,204
	Hydroxyesters	0,279	0,268	0,257	0,245	0,235	0,224	0,213
	Hydroxyketones	0,292	0,281	0,271	0,261	0,251	0,241	0,231
	% MeOH	45	47,5	50	52,5	55	57,5	60
C18-SPP	Alkylbenzenes	0,385	0,370	0,356	0,332	0,317	0,303	0,290
	Benzoic esters	0,369	0,354	0,340	0,329	0,316	0,329	0,288
	Hydroxyesters	0,367	0,354	0,340	0,330	0,315	0,303	0,290
	Hydroxyketones	0,376	0,364	0,352	0,342	0,329	0,317	0,305
C18-Boltimate	Alkylbenzenes	0,390	0,371	0,356	0,335	0,317	0,303	0,290
	Benzoic esters	0,378	0,353	0,342	0,326	0,313	0,299	0,288
	Hydroxyesters	0,370	0,356	0,349	0,329	0,316	0,304	0,292
	Hydroxyketones	0,380	0,367	0,364	0,343	0,332	0,319	0,308

From tables 2.3.14 and 2.3.15 it can be observed that the variations of $\log \alpha(CH_2)$ from one column to another with the constant maintenance of the mobile phase composition is minimal, this can be explained by the similar nature of columns: all columns used have C18 groups. In addition it can be observed for the C18-Choice column, which has a high carbon content (27%), only a slight increase of $\alpha(CH_2)$. For the Boltimate core-shell column with a low carbon content (9%), $\alpha(CH_2)$ values relatively similar to the rest of the columns are observed. Because the values $\alpha(CH_2)$ vary strongly with the composition and not with the column used, the average of the methylene selectivity values was used to study its variation with the mobile phase composition.

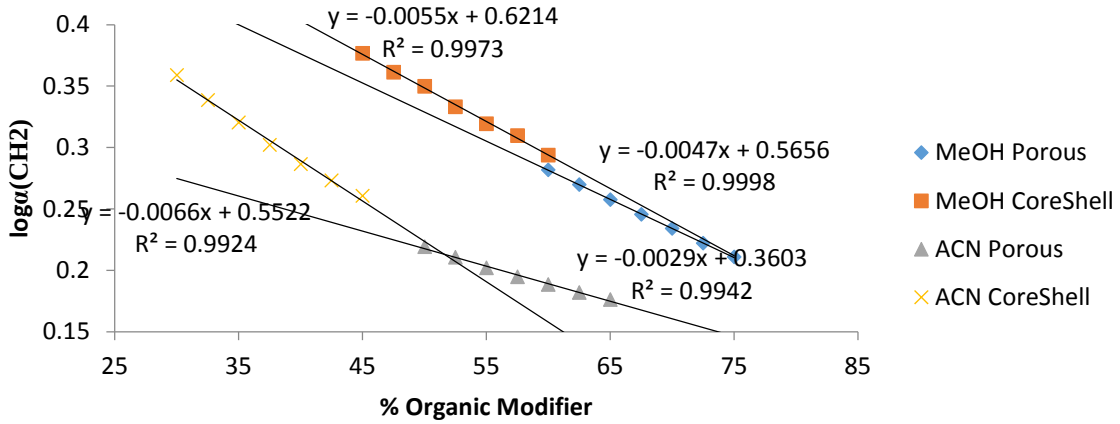

Figure 2.3.11. Variation of $\log \alpha(CH_2)$ for the columns studied according to the content of organic modifier in the mobile phase.

These results show a strong variation of the methylene selectivity with the mobile phase composition. Together with the small variation associated with the chromatographic column change, methylene selectivity is proving to be a relatively insensitive parameter to quantify differences in the ability to separate the different chromatographic columns. Specifically, a slight modification of the mobile phase (ex 2.5%) generates a variation of methylene selectivity equal to or greater than the use of other columns.

In practice, it is the nature of the stationary phase that generates differences between the selectivities of the different columns. According to the results presented in this study, the different selectivity between the different C18 columns is the result of interactions different from the hydrophobic ones, usually with the residual silanol groups. The residual silanol groups participate in polar interactions and justify the observed selectivity difference for C18 columns and compounds with polar groups.

By definition, methylene selectivity does not require the use of hydrocarbons to be measured experimentally and according to the theory set forth above any homologous series can be used provided the existence of hydrophobic interactions is the main factor of separation. Reducing the interactions that can occur in liquid chromatographic separations only at hydrophobic interactions is not easy to do. For this reason, it is preferable to use homologous series of hydrocarbons to limit further interactions. Even in these situations, additional interactions of sterile or π-π type may occur in the case of hydrocarbons with aromatic structure. Due to the difficulties of hydrocarbon detection in HPLC systems, the use of aromatic hydrocarbons is preferred.

The experimental results suggest that the premises of the theory that methylene selectivity does not depend on the series of homologous compounds used is partially confirmed. Only the existence of hydrophobic interactions can be confirmed in the case of C18 columns. The results of methylene selectivity are close for different homologous series with differences in the field of experimental errors (4-5%). It is confirmed that the nature of the mobile phase drastically influences the methylene selectivity while the nature of the stationary phase has a practically negligible influence.

2.4. The influence of π-π interactions on chromatographic retention

2.4.1. The premises of the study

For the separation of aromatic compounds using stationary phases with phenyl or cyano groups, the π-π interactions play an important role [27]. The π-π interactions also play an important role in the LC retention processes of the investigated compounds with aromatic structures, based on the use of acetonitrile as an organic modifier of the mobile phase.

The affinity of aromatic compounds for stationary phases with phenyl groups can be used to control the selectivity of separation [28], but also to increase the sensitivity in HPLC analysis based on the application of large injection volumes [29]. Some studies have concluded that π-π interactions are conditioned by certain geometric requirements and in fact π-π interactions are in fact the result of π-σ attractions that compensate for π-π repulsions. In general, the energies involved in these interactions are comparable to those involved in van der Waals interactions (8-30 KJ / mol).

The purpose of these sections is to compare the retention of a group of five aromatic hydrocarbons on three columns with phenyl-type stationary phase, using two types of mobile phase (water / methanol and water / acetonitrile).

It can be seen that the highest retention is the Phenomenex Gemini C6-Phenyl column. This phenomenon is predictable because this column has the highest percentage of carbon in the series of studied columns.

After obtaining the chromatograms and calculating the retention times, the $\log k$ curves were plotted according to the percentage of organic modifier, according to the equation (Soczewinski-Snyder):

$$\log k = \alpha + \beta * C_{m.o.} \qquad (2.4.1)$$

where $\log k$ is the logarithm of the capacity factor, $C_{m.o.}$ is the volume concentration of the organic modifier in the mobile phase, and the parameters α and β are the regression parameters.

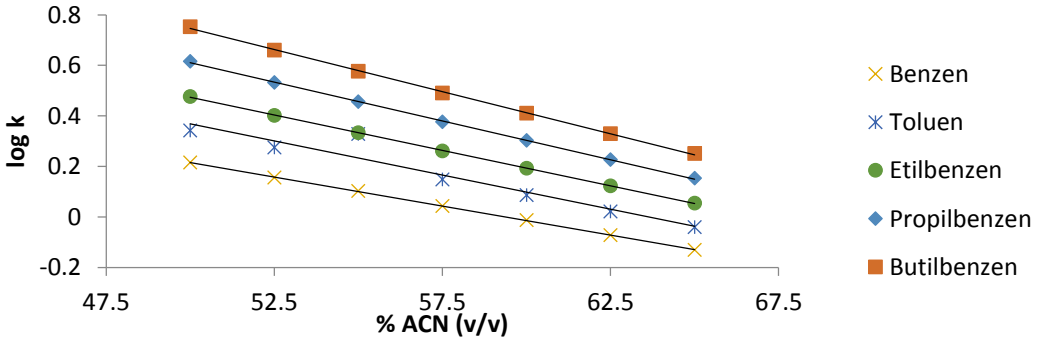

Figure 2.4.3. Linear regression $\log k = f(C_{o.m.})$ exemplified for the column Zorbax Phenyl and ACN as a modifier

Parameters α and β were calculated by linear regressions applied to the correlations $\log k = f(C_{m.o.})$. These regressions can be found in table 2.4.3 and show a very good correlation between $\log k$ and $C_{m.o.}$. Figure 2.4.2 shows the linear dependencies for the hydrocarbon series studied on the Zorbax Phenyl column with organic acetonitrile modifier.

From the presented data it is observed that the retention of the studied hydrocarbons is higher for methanol compared to acetonitrile, used in the same percentage in the mobile phase. This difference becomes evident by comparing the retention data under identical conditions (Figure 2.4.4)

In previous studies, it has been suggested that the use of acetonitrile as an organic modifier in such systems leads to a hindrance of the π-π interactions between the analytes and the stationary phase [30]. The difference between the observed acetonitrile and methanol could confirm this hypothesis, given that the studied analytes do not contain functional groups and are therefore not involved in other interactions with the mobile phase components.

The chromatographic behavior of the three studied columns differed under the same experimental conditions and even the similarity between the stationary phases. This can be attributed to the different phase ratio for each column:

$$k = \Phi_{coloană} * K \qquad (2.4.2)$$

where $\Phi_{coloană}$ represents the ratio of the volumes of the stationary phase and the mobile phase, and K represents the partition constant for the hydrocarbon studied in the mobile phase - stationary phase system and is considered to be equal for each column.

The ratio of the phases can be correlated by approximation with the percentage carbon content (% C) of the stationary phase. A stationary phase with a higher degree of carbon is equivalent to more frequent hydrocarbon chains and therefore with a larger volume of the stationary phase. Using% C we can sort the three columns in the order Phenomenex> Zorbax Eclipse> Brownlee Phenyl PE. This order also describes the order of the extrapolated parameter $\log k_w$, which is respected for each hydrocarbon and organic modifier.

2.4.5. Conclusions

Within this chapter, the retention of chromatographic liquid for analytes with aromatic structures was studied using phenyl type stationary phases. The higher retention was observed for methanol used as an organic modifier, compared to acetonitrile, suggesting a possible interference of acetonitrile on the π-π type interactions between analytes and the stationary phase.

The extrapolated $\log k_w$ values showed a very good correlation with the $\log K_{ow}$ despite the use of phenyl-type stationary phases, suggesting a fundamental similarity of the chromatographic partition mechanism.

3. Final conclusions

Within this thesis, aspects of the retention mechanism within the RP-LC were studied. The study started by modifying the hydrophobic character of the mobile phase by adding higher alcohols, resulting in the proposal of a competitive model of retention, where the analyte molecules are in competition with the solvent molecules and expressing this dependence by a equation similar to :

$$\log k_A = \Psi + \log K_{ow}^A - \log K_{ow}^S \qquad (2.1.4)$$

The obtained equation describes the trend of variation of $\log k_A$ for an analyte A in certain mobile phase-stationary systems. The retention of the compounds decreases with the decrease of their hydrophobicity and with the increase of the hydrophobicity of the additive used. In addition to l $\log k_A$ retention was studied through the perspective of methylene selectivity and different lipophilicity parameters. By extrapolating the obtained data, different parameters are deduced without carrying out practical experiments (ex choosing another mobile phase with similar selectivity, estimating the hydrophobicity parameters for unknown compounds, etc.).

From a thermodynamic point of view, retention is described by a Gibbs enthalpy, entropy and free energy just like any other process. The addition of an organic additive in the mobile phase produces a variation of these parameters, the experimental results suggesting a second degree dependence of the Gibbs free energy on the hydrophobicity of the additive. Retention mechanisms involving molecular associations are theorized to be the basis of the adsorption process.

The measured thermodynamic quantities showed a correlation that can be attributed to the experimental errors. According to statistical procedures, the possibility that these experimental observations are the result of normal measurement errors was eliminated. By representing the van Hoff curves, trend lines with slopes of about 0.75 are obtained, suggesting that any enthalpy variation is offset by 75% of the associated entropy variation.

The study of the retention from the perspective of the mobile phase cannot give a complete picture and the influence of the stationary phase was further studied. By using the different chromatographic columns with stationary phases of type C18 and different classes of compounds, the influence on the retention has been studied in terms of the ratio of the phases, the methylene selectivity and the resolution using the solvophobic model.

$$\log K_{BA,j} = aA_j + b\left(V_j\right)^{-2/3} A_j + c_j \mu_j^2 + d_j \alpha_j \qquad (2.3.4)$$

The phase report is a parameter that uniquely characterizes each stationary-mobile phase system. In the case of studies using the alkylbenzene series, it was observed that with the increase of the organic component ratio the phase ratio decreases. Thus, within the volume defined as the interior of a chromatographic column, a larger proportion is occupied by the mobile phase. The interface between the stationary phase and the mobile phase is a dynamic barrier that changes as the percentage of organic modifier changes. In essence, the degree of penetration of the hydrocarbon chains by the mobile phase increases with the amount of organic modifier and has the effect of reducing the volume occupied by the stationary phase.

The ratio of the phases varies both from one composition to another but also from one column to another. The variation between columns is much smaller than the variation between compositions suggesting a much greater influence of the mobile phase. The differences that appear between columns can also be justified taking into account the different physical parameters of the supports of the stationary phases (porosity, surface, etc.).

For methylene selectivity the experimental results suggest that it does not depend on the series of homologous compounds. Regardless of the series of homologous compounds used or their functional groups, the results of methylene selectivity are close with differences in the field of experimental errors (4-5%). It is confirmed that the nature of the mobile phase drastically influences the methylene selectivity while the nature of the stationary phase has a virtually negligible influence.

The use of phenyl columns provided a perspective on the potential π-π type interactions between the analytes and the stationary phase in the context of the solvophobic model. The extrapolated $\log k_w$ values showed a very good correlation with $\log K_{ow}$ values, despite the use of phenyl stationary phases, suggesting a fundamental resemblance of the retention mechanism even in the presence of additional interactions.

4. Published papers

4.1 List of papers published in the doctoral thesis:

1. E.Caiali, V.David, *Retention behaviour of aromatic hydrocarbons in reversed-phase HPLC based on phenyl-silica stationary phase*. **Revue Roumaine de Chimie**, 64 (4), 367-372 (2019).

2. S.C.Moldoveanu, E.Caiali, V.David, *Results from solvophobic theory applied on methylene selectivity in reversed-phase HPLC*. **Journal of Liquid Chromatography and Related Technologies**, 41 (1), 24-32 (2018).

3. E.Caiali, S.C. Moldoveanu, V.David, *Comparison of the phase ratio for C18 HPLC columns using three different organic modifiers (methanol, ethanol, and acetonitrile) in mobile phase composition*. **Revue Roumaine de Chimie**, 62 (8-9), 629-636 (2017).

4. S.C. Moldoveanu, E.Caiali, V.David, *Phase ratio and equilibrium constant in RP-HPLC obtained from octanol/water partition constant through solvophobic theory*. ***Chromatographia***, 80 (10), 1491-1500 (2017).

5. E.Caiali, V.David, H.Y. Aboul-Enein, S.C. Moldoveanu, *Evaluation of the phase ratio for three C18 high performance liquid chromatographic columns*. **Journal of Chromatography A**, 1435, 85-91 (2016).

6. E.Caiali, D.Casoni, P. Ionita, V.David, C. Sârbu, *Parabens lipophilicity determination with mobile phases containing low and medium hydrophobic alcohols*. **Journal of Liquid Chromatography and Related Technologies**, 37 (16), 2287-2301 (2014).

4.2. Scientific communications

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2. Sesiunea de Comunicări Științifice Studențești, prezentare "Thermodynamic study of the reversed-phase mechanism in liquid chromatography for mobile phases containing hydrophobic additives", 14 Iunie 2014, București, Romania

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