UNIVERSITY OF BUCHAREST
FACULTY OF CHEMISTRY
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PhD THESIS ABSTRACT

Thermodynamic aspects of separations in reverse phase high performance liquid chromatography

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The numbering of figures, tables and bibliographic references is the one in the thesis.

2. ORIGINAL CONTRIBUTIONS

In general, temperature plays an important role in HPLC, influencing the retention, chromatographic resolution, symmetry and efficiency of chromatographic peaks, the fundamental relationship describing the influence of temperature on chromatographic retention being given by the van’t Hoff equation. This equation has been widely used in the literature in calculating the thermodynamic parameters, ΔH°, ΔS° and ΔG° that characterize the transfer of analyte molecules from the mobile phase to the stationary phase. Most of the studies are based on van’t Hoff-type graphical representations, from whose slope and ordinate intercepts are calculated the thermodynamic quantities mentioned above.

There are quite a few situations, also reported by the literature, in which the dependence between ln k 'and 1 / T is not a linear one [147, 161, 163]. Several theories have already been advanced regarding this deviation from the linearity of the van’t Hoff equation, including the possibility of multiple interactions between the analyte and the stationary phase. Another explanation is the variation of phase ratio in the chromatographic column to temperature [20], which may influence the linearity of the van't Hoff equation. Therefore, the present paper aims to address the influence of compound structure on thermodynamic behavior in RP-HPLC, as well as the possibility of variation of the parameter Φ with the temperature of the chromatographic column which may lead to a deviation from the linearity of the van’t Hoff equation. For these purposes, several classes of chemical compounds with complex structures have been selected which can be present in several forms in the mobile phase by varying the pH of the aqueous component. These classes of compounds have been studied in comparison with a class of aromatic hydrocarbons whose structure is not influenced by the composition of the mobile phase.

2.1. The study of the thermodynamic behavior in reverse phase liquid chromatography respecting the van’t Hoff dependence

Considerations about the study

To begin with, the effect of temperature on the retention of compounds whose structure is not altered by the pH of the mobile phase has been studied. For this purpose, functionally simple compounds, easily detectable by UV and having acceptable chromatographic retention, were chosen. In RP-HPLC separations, temperature is an essential parameter that controls chromatographic retention and selectivity of separations, and the dependence of the retention factor of a chromatographic column temperature compound is described by the van’t Hoff equation:

$$\ln k' = -\frac{\Delta H^o}{RT} + \frac{\Delta S^o}{R} + \ln \Phi \tag{2.1.1}$$

where ΔH° and ΔS° represent the variation of standard enthalpy and standard entropy, respectively, which characterizes the process of transferring the analyte from the mobile phase to the stationary phase, R is the universal gas constant, T is the temperature in Kelvin degrees, and Φ represents the phase ratio ($\Phi = V_{fs} / V_{fm}$, where V_{fs} is the volume of the stationary phase and V_{fm} is the empty or free volume of a chromatographic column).

For this study, a series of simple alkylphenyl chemicals were chosen for which the influence of the chromatographic column temperature as well as the composition of the mobile phase on their retention was followed. From the experimentally retention data obtained, the important thermodynamic parameters of the chromatographic retention process were calculated. Thus, the homologous benzene-propylbenzene series was taken into account, this being chosen for the mentioned reasons, the most important being that these are chemical species whose structure does not undergo changes during the chromatographic experiments.

The experiments were performed using several commercial HPLC columns having as stationary phase modified silica gel with octyl or octadecyl groups. The mobile phase consisted of two components: the organic component - acetonitrile (ACN) or methanol (MeOH) and the aqueous component. All experiments took place in the temperature range 20°-50°C, the flow rate of the mobile phase was 1 mL/min, the working mode being isocratic. Chromatograms were recorded by measuring the absorbance at a wavelength of 254 nm.

As can be seen from the example below, the resolution of the separations is very high and the shape and efficiency of the peaks are acceptable, the chromatograms showing a clear separation of the four substances and an improvement in peak characteristics concomitant with increasing temperature.

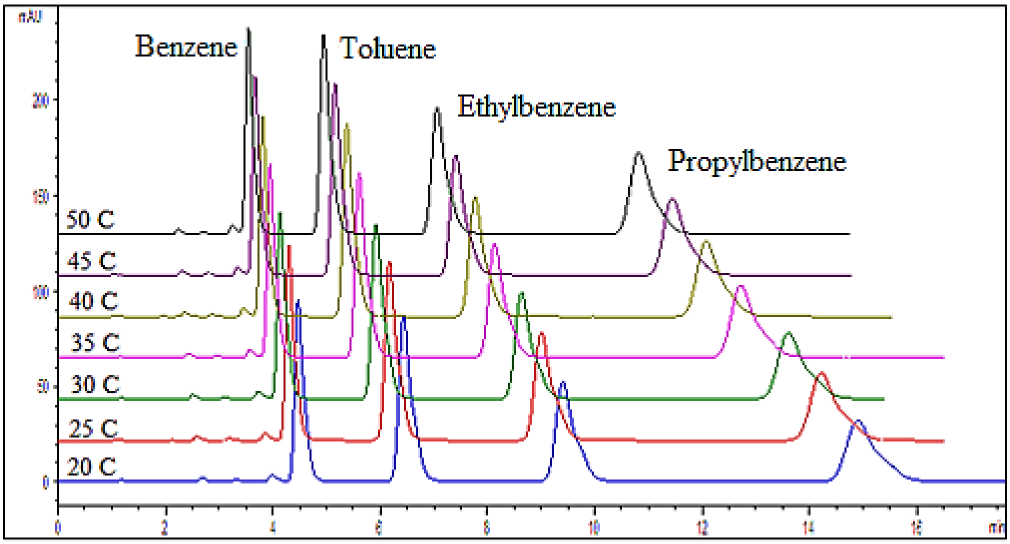

Figure 2.2. Overlapping chromatograms for the four hydrocarbons in the temperature range 20°-50°C obtained on the Gemini C18 column for the mobile phase 50% ACN (acetonitrile) and 50% H_2O (v:v).

2.1.1. Van't Hoff dependence for stationary octyl and octadecyl silica gel phases in various mobile phase compositions

Retention data processing and interpretation

The influence of the temperature of the chromatographic column on the retention of the four hydrocarbons was studied on four chromatographic columns and on several compositions of the mobile phase, for the two organic modifiers used.

The study of the chromatographic behavior of the four hydrocarbons in terms of the influence of temperature on the retention process has the same conclusion regardless of the column used or the composition of the mobile phase: the value of the retention time of the four hydrocarbons and therefore, implicitly, the value of the retention factor decreases with increasing temperature.

Also, the value of the retention time and, respectively, the value of the natural logarithm of the retention factor decreases with the increase of the percentage of organic modifier in the mobile phase, regardless of the organic modifier used.

The values of the natural logarithm of the retention factor calculated for all hydrocarbons were plotted against the inverse of the absolute value of the chromatographic column temperature. An example of these dependencies is shown in the following figure for an octadecyl silica gel stationary phase column (figure 2.4).

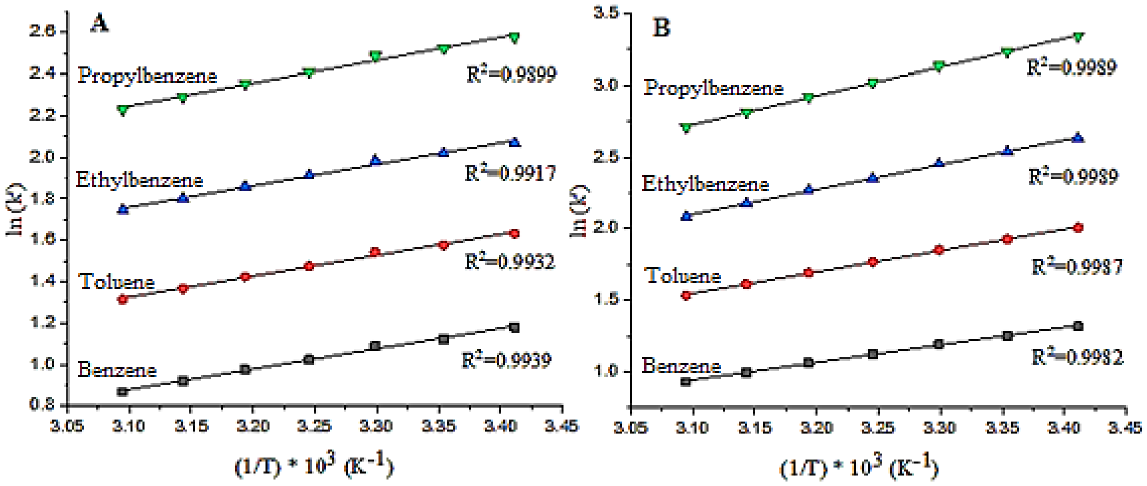

Figure 2.4. Dependence of the retention factor logarithm on the inverse of the absolute temperature value of the chromatographic column for the Gemini C18 column for the four hydrocarbons studied: A - mobile phase 50% CH_3CN / 50% H_2O; B - mobile phase 55% CH_3OH / 45% H_2O (v/v).

These dependencies can be mathematically modeled by a linear function, being fitted using a first degree equation, of the form:

$$\ln k' = a + b \cdot (1/T) \tag{2.1.2}$$

where a and b are linear regression parameters calculated using the Origin program.

Studies on the retention process of these hydrocarbons can be found in the literature in different contexts: calculating the octanol/water partition coefficient, Kow, [15] calculating the phase ratio, Φ, [14] or in temperature influence studies on extrapolated values of water retention factor as mobile phase [172].

The values of the linear regression parameters can be used to calculate the thermodynamic parameters associated with the chromatographic retention process: enthalpy variation and entropy variation associated with the transfer process of the analyte from the mobile phase to the stationary phase, and the variation of Gibbs free energy. These thermodynamic parameters were calculated in this study of the behavior of the temperature retention process using the following equations:

$$\Delta H^o = -Rb \tag{2.1.3}$$

$$\Delta S^o = R(a - ln\Phi) \tag{2.1.4}$$

$$\Delta G^o = \Delta H^o - T \cdot \Delta S^o \tag{2.1.5}$$

The value of the natural logarithm of the phase ratio of the chromatographic column intervenes in the calculation of the standard entropy variation. Some approximations can be made to calculate the variation of entropy in van't Hoff graphical representations. Thus, considering that the temperature range used in this study is small, a correct approximation [13, 14, 173] of the phase ratio could be considered in the range of 0,20-0,25. The highest value of Φ, 0,25, was used in this study.

The values corresponding to the variation of the standard enthalpy follow the same trend, increasing from benzene to propylbenzene, regardless of the organic modifier used or the chromatographic column on which the experiment was performed. At the same time, there is a decrease in the value of the variation of the standard enthalpy with the increase of the percentage of organic modifier in the mobile phase. From a thermodynamic point of view, the negative values of the variation of the standard enthalpy for the transfer of analytes from the mobile phase to the stationary phase correlate with an exothermic process, ie with an energy release in the chromatographic system when this transfer is performed. When the temperature of the chromatographic column increases, the retention process is discouraged and thus the retention time decreases, a van’t Hoff-type behavior.

The values corresponding to the variation of the standard entropy follow the same trend as those corresponding to the variation of the standard enthalpy when the organic modifier is methanol, these increase from benzene to propylbenzene in accordance with the increase of the hydrophobic character of hydrocarbons and a decrease of the variation of the standard entropy as the percentage of methanol increases in the mobile phase. This indicates that the interactions become stronger between hydrocarbons and the stationary alkyl silica gel phase from benzene to propylbenzene as their hydrophobic character increases, the aliphatic chain contributing to the hydrophobic interactions that take place in the chromatographic system.

In the case of the use of acetonitrile as an organic modifier, the variation of the standard entropy does not follow a specific trend, although in some cases it is inverse to the variation of the standard enthalpy, decreasing from benzene to propylbenzene. Another difference is that when using methanol as an organic modifier the values of the variation of the standard entropy are negative regardless of the column used or the percentage of methanol in the mobile phase, while in the case of using acetonitrile as an organic modifier in the mobile phase some values of the variation of the standard entropy are positive. A negative value of ΔS^o correlates with the fact that the retention of the analyte in the stationary phase has as a consequence an ordering of the system being achieved through a strong interaction of it with the stationary phase.

Through the thermodynamic prism between the two parameters there is always a phenomenon of compensation, in this case the retention process being dictated by the enthalpy parameter, for which much more important values are obtained from the afferent calculations.

The values corresponding to the variation of Gibbs free energy at 25°C follow the same trend regardless of the organic modifier used or the chromatographic column used, they increase from benzene to propylbenzene according to the hydrophobic character of hydrocarbons and suffer a slight decrease with increasing percentage of organic modifier in the mobile phase. The variation of Gibbs free energy values are negative regardless of the organic modifier used or its percentage in the mobile phase, which indicates that the hydrocarbon transfer process between the two chromatographic phases is spontaneous, the interaction of the analytes being stronger with the stationary phase than with the mobile phase.

Study conclusions

The values obtained for the variation of the standard enthalpy and the variation of the standard entropy for the four hydrocarbons fall in the intervals [-10,3; -5,8] kJ mol for benzene, [-12,5; -7,3;] kJ/mol for toluene, [-14,4; -7,5] kJ/mol for ethylbenzene and [-16,7; -8,1] kJ/mol for propylbenzene (values for ΔH^o), respectively in the ranges [-13,2; -2,9] J/mol•K for benzene, [-14,9; 0,1] J/mol•K for toluene, [-16,2; 2,9] J/mol•K for ethylbenzene and [-18,7; 5] J/mol•K for propylbenzene (values for ΔS^o). These values are also consistent with some of the literature data from the HPLC studies presented in the theoretical part of the thesis in table 1.5 [149, 151, 153, 154].

2.1.2. Van't Hoff dependence for stationary phenyl silica gel phases in various mobile phase compositions

Retention data processing and interpretation

The present study aims to analyze the influence of the organic modifier present in the mobile phase, acetonitrile versus methanol, on the retention of the four previously studied hydrocarbons (benzene, toluene, ethylbenzene, propylbenzene) using as stationary phase an Eclipse XDB-Phenyl chromatographic column (Zorbax) (modified silica with phenyl groups). This time, in addition to the van der Waals interactions between the analyte molecules and the hydrocarbon chains in the stationary phase, there are also π-π type interactions. The influence of the organic modifier will be analyzed by calculating and comparing the values of some thermodynamic parameters specific to the chromatographic retention process. Three mobile phase compositions for acetonitrile or methanol were studied. The retention data for the four hydrocarbons were recorded in the same temperature range, 20°-50°C. The values of the natural logarithm of the retention factor calculated for all hydrocarbons were plotted against the inverse of the absolute value of the chromatographic column (van't Hoff type representations).

Examples of these dependencies, for two mobile phase compositions, are shown in the following figure (figure 2.6).

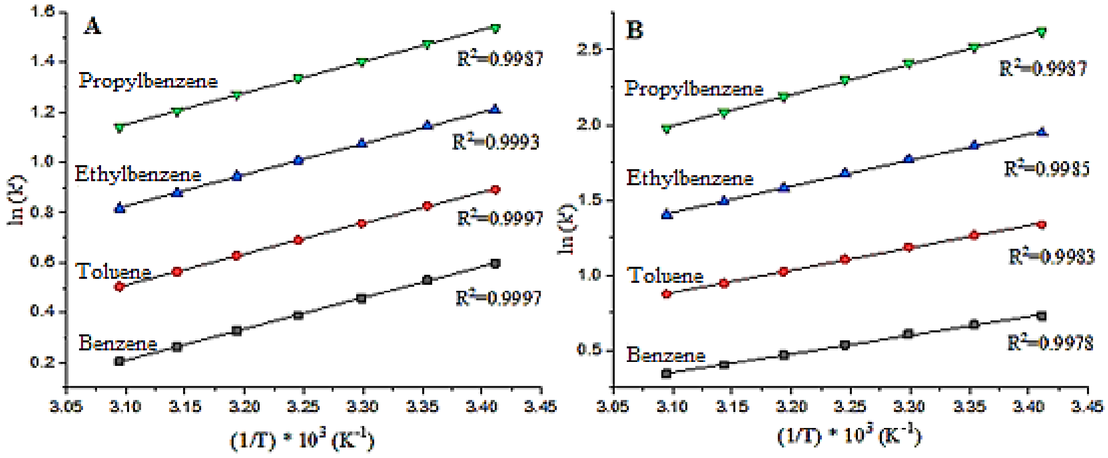

Figure 2.6. Dependence of the retention factor logarithm on the inverse of the absolute temperature value of the chromatographic column for the Eclipse XDB-Phenyl column for the four hydrocarbons studied: A - mobile phase 50% CH_3CN / 50% H_2O; B - mobile phase 50% CH_3OH / 50% H_2O (v / v).

These dependencies can be mathematically modeled by a linear function, the linear regression parameters for the four studied compounds, for the different mobile phases, being presented in table 2.17. The literature reports a similar study performed under other chromatographic separation conditions, used to compare the calculated thermodynamic data [175].

According to the current methodology, the slope values for the four hydrocarbons studied from the six mobile phase compositions were used to calculate the variation of the standard enthalpy associated with the process of transferring the analyte from the mobile phase to the stationary phase, while the intercept values were used to calculate the variation of the standard entropy, taking into account the approximations mentioned above [13, 14, 173] for the value of Φ and using the equations (2.1.3) and (2.1.4) presented above.

A graphical representation of the ΔH^o values as a function of the percentage of organic modifier in the mobile phase is shown in figure 2.7.

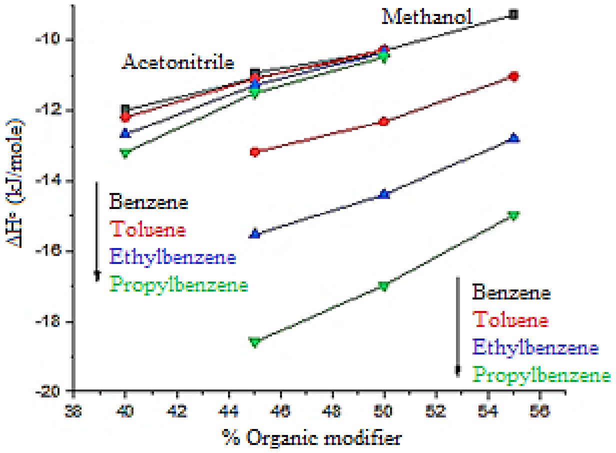

Figure 2.7. Dependence of the standard enthalpy variation (ΔH°) corresponding to the transfer of aromatic hydrocarbons from the mobile phase to the stationary phase with the content of the mobile phase in organic modifier (acetonitrile versus methanol).

The main observation in figure 2.7 is the small difference in the ΔH° value for the four aromatic hydrocarbons studied when using acetonitrile as an organic modifier in the composition of the mobile phase. This influence of acetonitrile on the ΔH° values can be attributed to the suppressing effect of the π-π interactions between the aromatic hydrocarbons and the phenylene rings attached to the surface of the silica gel. Methanol and water can only be involved in weak hydrogen bonds with aromatic structures [177] and cannot suppress van de Waals forces between the solute and the stationary phase. The change of the stationary phase to phenyl silica gel from octadecyl or octyl silica gel has no influences in terms of system behavior, the retention process being also exothermic, governed by the enthalpy parameter.

Contrary to the variation of the standard enthalpy, the values corresponding to the variation of the standard entropy decrease with increasing hydrophobic or aliphatic chain attached to the benzene ring when the organic modifier used is acetonitrile and increase with increasing hydrophobic hydrocarbons when the organic modifier used is methanol, following the same trend as the variation of the standard enthalpy (figure 2.8).

The values of ΔG° for the whole temperature range (20°-50°C) were taking into account the parameter of the volume ratio of the two phases used to estimate the entropic contribution to the partitioning process. The value of ΔG° increases from benzene to propylbenzene for each composition of the mobile phase. On the other hand, the value of ΔG° is influenced by the composition of the mobile phase, which decreases as the content of the organic modifier increases. This is in line with other previous thermodynamic studies of the RP-HPLC retention process under similar elution conditions [17, 178-181].

Study conclusions

The chromatographic study presented aimed to determine the influence of the organic modifier on the retention process of a mixture of hydrocarbons (benzene-propylbenzene series) in reverse phase liquid chromatography for a stationary phase chromatographic column of phenyl silica gel type.

This experimental study showed that the studied hydrocarbons show a van't Hoff type behavior regardless of the mobile phase used, the van't Hoff type dependencies obtained being linear and characterized by very good coefficients of determination, being calculated the thermodynamic parameters associated with the process of transfer of the analytes from the mobile phase to the stationary phase (ΔH^o, ΔS^o and ΔG^o), as well as another important parameter of the retention process: the separation selectivity.

2.1.3. The influence of temperature on the extrapolated retention values for mobile phases with total water content

In addition to temperature, retention in RP-HPLC is influenced by the composition of the mobile phase. A general dependence observed in RP-HPLC for the variation of the retention factor (k') on the concentration of the organic modifier, φ, is given by the empirical equation between the logarithm of the retention factor k' and φ [10, 185, 186]:

$$log\ k' = a + b \cdot \varphi \qquad (2.1.16)$$

This dependence can be studied for any compound that has retention against a stationary C8, C18 or phenyl phase for a certain range of organic modifier concentration [187-190]. Linear regressions based on equation (2.1.16) can be developed using the set of experimental values (k', φ) and used to characterize the retention behavior of the studied analyzes on a stationary phase used [191-193]. This dependence is explained by the solvophobic theory and can be used to predict the retention behavior of nonpolar or weakly polar organic compounds by reverse phase mechanism [79, 86].

For φ = 0, the value of log k' corresponds to a mobile phase consisting only of water, and this is usually referred to as the extrapolated value of the decimal logarithm of the retention factor, log k'_w. It follows from equation (2.1.16) that log k'_w = a. On the other hand, the extrapolated value of the retention factor is correlated with the partition constant ($K_{w/s}$) of the solute distributed between the mobile (water) phase and the hydrophobic stationary phase, according to the known formula:

$$k'_w = \Phi \cdot K_{w/s} \qquad (2.1.17)$$

In addition to determining the parameter k'_w in the dependencies (2.1.16) applied to the experimental values (k', φ), the study aimed at the possibility that these extrapolated values k'_w in turn depend on the temperature of the column. In this case, the van't Hoff equation can be written as:

$$\log k'_w = -\frac{\Delta H^o}{2{,}303RT} + \frac{\Delta S^o}{2{,}303R} + \log \Phi \qquad (2.1.18)$$

Such a possibility could provide the opportunity to determine the thermodynamic parameters that would characterize the partition of the studied hydrocarbons between a totally aqueous mobile phase and the hydrophobic stationary phase. At the same time, the existence of this relationship would be a demonstration of the correctness of the experiments performed, affected by small systematic or accidental errors.

Retention data processing and interpretation

The influence of mobile phase composition on retention was studied over the temperature range 25°-50°C at five different percentages of the organic modifier used, acetonitrile and methanol. In the case of acetonitrile use, its percentages in the mobile phase were: 45%, 47,5%, 50%, 52,5% and 55%. In the case of using methanol as an organic modifier, its percentages in the mobile phase were: 50%, 52,5%, 55%, 57,5% and 60%. In both cases the mobile phase is two-component: organic-water modifier. This study was performed on two chromatographic columns: Gemini C18 (Phenomenex) and BDS Hypersil C18 (Thermo Scientific).

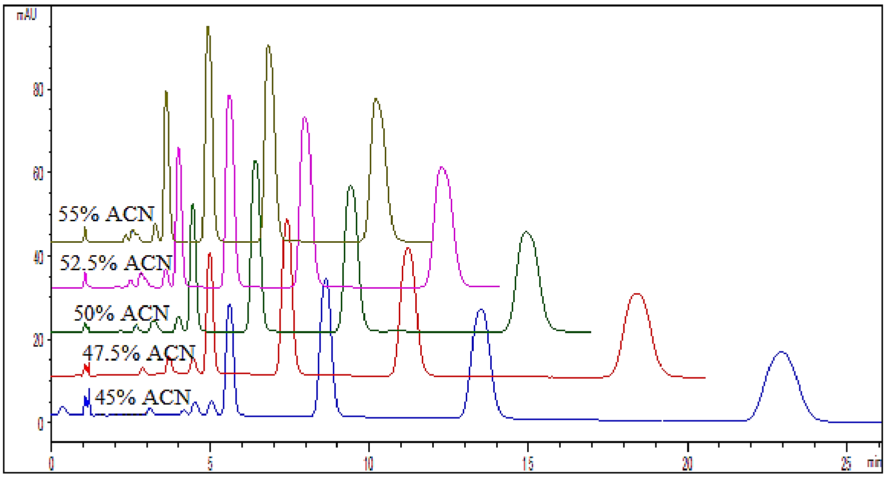

Figure 2.10. Overlapping chromatograms for the four hydrocarbons (elution order: benzene, toluene, ethylbenzene, propylbenzene) obtained at 25°C on the Gemini C18 column for the various mobile phase compositions (45-55% acetonitrile).

The retention factor (k') for the four aromatic hydrocarbons was measured using the different mobile phase compositions mentioned above, for both chromatographic columns, the linear regressions applied to the log k' versus φ dependencies leading to the regression parameters a and b. The extrapolated values of log k' for the mobile phase which consists only of water, namely log k'_w, depend on the temperature of the column according to the van't Hoff equation, with the regression parameters denoted this time a' and b'. Regardless of the organic modifier used, the differences between the regression parameters obtained for the two chromatographic columns are very small.

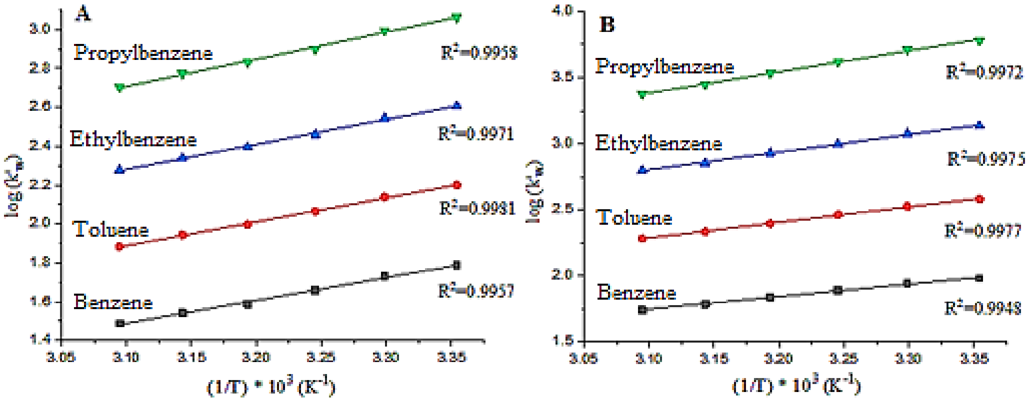

Figure 2.12. Dependence of the extrapolated values of log k'_w versus 1/T for the studied aromatic hydrocarbons obtained on the BDS Hypersil C18 column for organic modifier acetonitrile (A) and methanol (B), respectively.

Using equations (2.1.3) and (2.1.4), the values for ΔH^o and ΔS^o were calculated, values corresponding to a hypothetical hydrocarbon partition studied between water as mobile phase and stationary phase of modified silica gel type with C18 groups (table 2.26 and table 2.27).

These values are the same size as the values reported for other hydrophobic compounds and for mobile phases consisting of different mixtures of organic modifier (acetonitrile) and water [7, 178, 194]. A conclusion that can be drawn from the values listed in tables 2.26 and 2.27 is related to the hydrophobic character of the studied hydrocarbons: the more hydrophobic the character of the studied compound, the higher the value of the variation of the standard enthalpy for the transfer of the compound from mobile to stationary phase. According to the data in table 2.26 the variation of the standard entropy with the hydrophobic character of the aromatic hydrocarbons is inverse to the variation of the standard enthalpy, and according to the data from table 2.27 the values of the variation of the standard entropy increase from benzene to propylbenzene.

Study conclusions

The values obtained for the parameters ΔH^o and ΔS^o in this study are significantly higher than those obtained on the same columns in the study presented in subchapter 2.1.1, for both organic modifiers. Thus, the ΔH^o values obtained for the hypothetical partition of hydrocarbons studied between water and modified silica gel with C18 groups fall in the range [-30,7; -18] kJ/mol for the Hypersil BDS column and in the range [-36,2; -16,7] kJ/mol for the Gemini column compared to the intervals [-16,6; -8,2] kJ/mol for the Gemini column and [-13,6; -7,4] kJ/mol for the BDS Hypersil column. This correlates with a more pronounced exothermic effect associated with the transfer of analytes when the mobile phase is theoretically composed only of water.

Also, the entropic contribution is significantly higher, from the value ranges [-13,7; -0,7] J mol•K for the Hypersil BDS column and [-17,4; 1,8] J/mol•K for the Gemini column, values obtained in the study presented in subchapter 2.1.1, at the value ranges [-29,5; -10,8] J/mol•K for the BDS Hypersil column and [-35,1; 2,6] J/mol•K for the Gemini column obtained in this study.

2.2. The temperature dependence of the "phase ratio" parameter for octadecyl silica gel stationary phase columns

Considerations about the study

The ratio of phase volumes, Φ, defined as the ratio between the volume of the stationary phase, V_{fs}, and the empty or free volume of the column, V_{fm}, is a fundamental parameter in the modeling of chromatographic retention. Together with the equilibrium constant K of the separation process, it is part of the retention factor k' by the basic relation in HPLC:

$$k' = K \cdot \Phi \qquad (2.2.1)$$

Recently, using the concepts of solvophobic theory discussed in subchapter 1.2.3, it has been shown that the ratio of phase volumes Φ can be estimated using this model [12, 13, 196]. This procedure is based, to a large extent, on the dependence between the retention factor k' and the octanol/water partition constant K_{ow} for a homologous series of test compounds, such as the series of mononuclear aromatic hydrocarbons. This dependency can be expressed by the following relation:

$$\log k' = a \log K_{ow} + \log \Phi \qquad (2.2.2)$$

Another possibility [14, 15], which results from equation (2.2.2), is based on the study of the retention of two of the compounds of the series, denoted by A and B, which elute in the order $t_{r,A}<t_{r,B}$, and the relation above leads to the following formula for calculating the log Φ parameter, according to the relation:

$$\log\Phi = \frac{\log k'_B \log K_{ow,A} - \log k'_A \log K_{ow,B}}{\log K_{ow,A} - \log K_{ow,B}} \qquad (2.2.3)$$

This relationship is strongly affected by errors, so the most correct procedure is to use a homologous series of compounds and apply the linear regressions described by equation (2.2.2). This procedure was used in the studies performed in this thesis, dedicated to the temperature variation of the parameter Φ. To investigate the dependence of the parameter Φ on temperature, the values of log k' and log K_{ow} at various temperatures are required. These values introduced in linear regression relations, described by equation (2.2.2), will lead to the regression parameters that will depend on the temperature at which the values log k' and log K_{ow} are expressed. The log k' value can be measured experimentally from chromatographic retention studies performed at a chosen temperature. Instead, the value of the log K_{ow} parameter must be estimated at the temperature at which the retention factor k' was measured. These data are already reported for the compounds studied [197] and were used in this study.

In this part of the study, the potential variation of the value of the parameter Φ with the change of temperature was investigated. Retention data were obtained for three different columns containing stationary phase of modified silica gel type with C18 groups, these columns being Gemini C18 (Phenomenex), Ultisil XB-C18 (Welch) and BDS Hypersil C18 (Thermo Scientific). Two water/acetonitrile or water/methanol mobile phase compositions were used to study the retention behavior of the analytes in the temperature range 20°-50°C.

Retention data processing and interpretation

Graphical representations of the logarithm values of the retention factor as a function of log K_{ow} at the corresponding temperature were obtained, the linear dependence between log k' and log K_{ow} being verified for all three columns and seven temperature values.

The van’t Hoff-type graphical representations of these values (log K_{ow} versus 1/T) are shown in figure 2.14. As can be seen, these trends are characterized by a polynomial function of degree two, with acceptable R^2, with a maximum point located on the temperature range used.

Next, the log k' dependence on the log K_{ow} at various temperatures of the chromatographic column was studied. The obtained graphs clearly show a linear dependence of these quantities. As an example, the graph corresponding to the log k’ dependence as a function of log K_{ow} at different temperatures for the Gemini C18 column with mobile phase 50% H_2O / 50% CH_3CN is shown in figure 2.15.

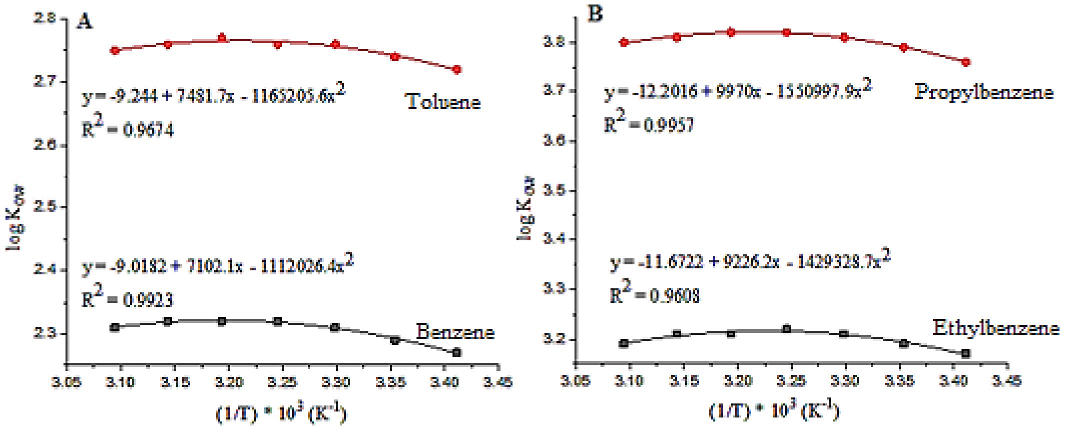

Figure 2.14. Van't Hoff type graphs for log Kow versus 1 / T dependencies for hydrocarbons analyzed over the studied temperature range: A - comparative benzene and toluene; B - comparative ethylbenzene and propylbenzene.

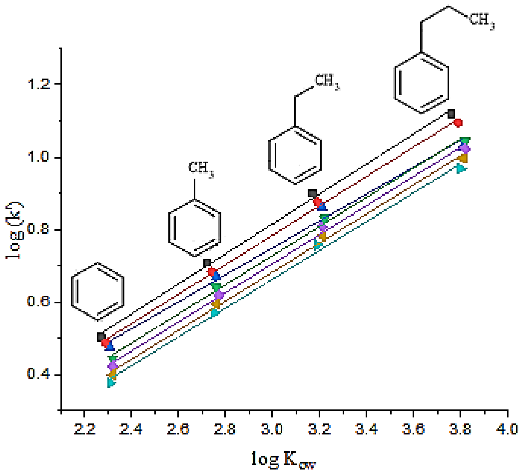

Figure 2.15. Graphical representation of log k' values as a function of log K_{ow} at different temperatures (20°C-50°C) for the Gemini C18 column with mobile phase 50% H_2O / 50% CH_3CN.

2.2.1. Log Φ dependence on T for mobile phases containing acetonitrile

According to the formula (2.2.2) presented above, the intercept of these linear regressions represents the corresponding values for log Φ, calculated at different temperatures (table 2.32). This procedure is applied to all columns and mobile phase compositions evaluated in this study.

The results in table 2.32 indicate that the value of the phase volume ratio decreases with temperature. This finding is in agreement with the results indicating that the adsorption of acetonitrile in the stationary phase decreases with increasing temperature [18, 19]. An additional finding is that for the narrow range of aqueous/organic components in the mobile phase evaluated in this study, the increase in the organic component in the mobile phase decreases the value Φ. The influence of mobile phase composition on the value of Φ for wider ranges of aqueous/organic components has been previously evaluated [13-15] and it has been reported that the phase volume ratio may increase or decrease with increasing organic component, depending on the solvent and the concentration.

The following were the graphical representations of the van't Hoff type for the parameter Φ, for all the mobile phases studied and for the three chromatographic columns used in this study.

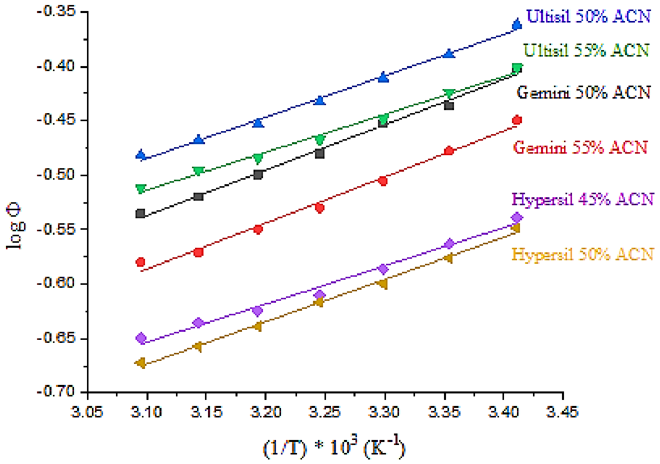

Figure 2.17. Graphical representation of the log depend dependence as a function of 1/T for the three chromatographic columns used when the organic modifier used is acetonitrile.

For the mobile phase 50% CH_3CN / 50% H_2O (v/v) we could make a correlation between the value of the parameter Φ and the percentage of carbon corresponding to each chromatographic column used, on the whole temperature range studied. This correlation is transposed in the form of a graphical representation and is shown in figure 2.18.

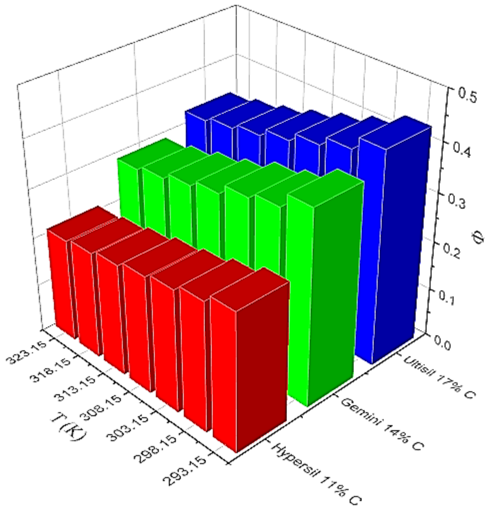

Figure 2.18. Correlation between the percentage of carbon corresponding to each chromatographic column used and the value of the parameter Φ.

According to this graph, the value of the parameter Φ increases simultaneously with the increase of the carbon percentage corresponding to the stationary phase, which correlates with a higher density of alkyl groups grafted on the surface of silica gel and decreases with increasing temperature, absolutely logical behavior considering that with increasing The desorption process of the organic modifier molecules adsorbed on the surface of the grafted alkyl groups is encouraged.

2.2.2. Log Φ dependence on T for mobile phases containing methanol

For the mobile phases in which the organic modifier is methanol, work in the same way as in section 2.2.1. Thus, according to equation (2.2.2) the intercept of linear regressions will represent the corresponding values for log Φ, calculated at the corresponding temperatures. This procedure is applied to all columns and mobile phase compositions evaluated in this study, the values for log Φ and parameter Φ, respectively, being presented in table 2.34 when the mobile phase is of water-methanol type.

Unlike the values obtained for the "phase ratio" parameter when the organic modifier in the mobile phase was acetonitrile, these show a decrease in the value of the parameter with increasing temperature, the values obtained for this parameter when using methanol as an organic modifier in the composition of the mobile phase they cannot be classified as having a certain tendency, be it ascending or decreasing, with increasing temperature. This can probably be explained by the differences between acetonitrile and methanol, but further studies are needed in this regard.

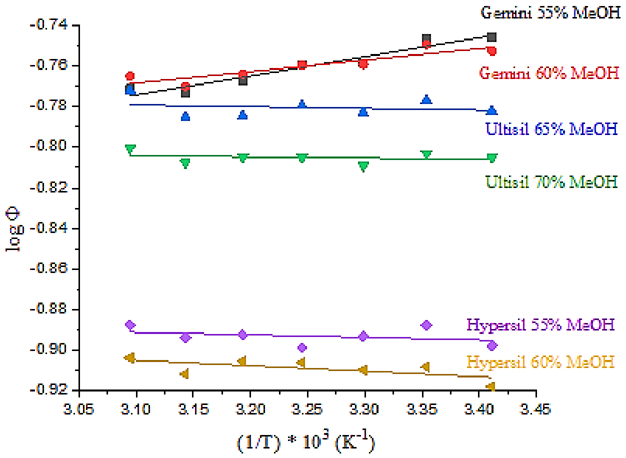

Figure 2.19. Graphical representation of the log depend dependence as a function of 1/T for the three chromatographic columns used when the organic modifier used is methanol.

In a way identical to the one for the water-acetonitrile mobile phases, the van't Hoff type graphical representations were obtained for the parameter Φ and for the water-methanol type mobile phases, these graphical representations being presented in figure 2.19 in the form of linear regressions.

In the case of using methanol as an organic modifier, the results obtained cannot be fitted using in all cases a linear function, in the case of the Ultisil column a fitter using a second degree polynomial function being more suitable for this dependence.

2.2.3. Dependence of the equilibrium constant of the separation process as a function of T

In addition to the evaluation Φ, the results obtained from the log k’ as a function of log K_{ow} allow the calculation of K, the equilibrium constant of the separation process (equations (2.2.2) and (2.2.1)). Thus, for the three columns and the two water-acetonitrile and water-methanol mobile phase compositions, respectively, the values of the equilibrium constant of the separation process were obtained.

As expected, K (log K) also depends on the temperature, its value following a decreasing trend when the temperature increases in the case of the mobile phases consisting of water and methanol, while for the mobile phases of water-acetonitrile type. the same behavior is not maintained, the only common observation that can be attributed to each chromatographic column used in this study is that the log K value is higher when the mobile phase contains more water. Examples of the 1/T log K dependency are shown in the following figure.

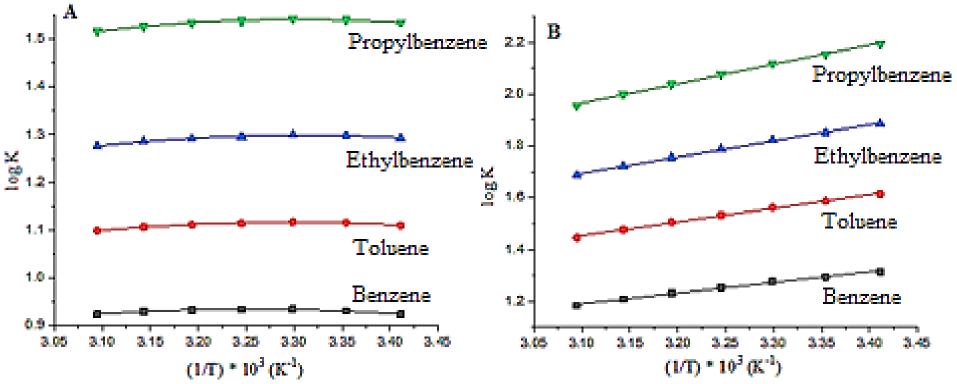

Figure 2.21. Representation of log K dependence according to 1/T for the series of benzene-propylbenzene hydrocarbons studied on the Gemini column with mobile phase water-acetonitrile 50% H_2O / 50% CH_3CN (A) and with mobile phase water-methanol 45% H_2O / 55% CH_3OH (B).

Study conclusions

In this study, the values of the “phase ratio” parameter and the constant corresponding to the K partition equilibrium constant were calculated for three chromatographic columns with modified silica gel stationary phase with octadecyl groups and binary mobile phases of water-organic modifier type (acetonitrile and methanol) and the influence of temperature on the parameters Φ and K was investigated.

The values of the parameter Φ obtained using the concepts of solvophobic theory decrease with increasing temperature, which correlates with the intensification of the desorption phenomenon of organic modifier molecules adsorbed on the surface of grafted alkyl groups which is encouraged by the increase in temperature.

Higher values of the parameter Φ were obtained when acetonitrile is used in the mobile phase as an organic modifier compared to those obtained for methanol, this observation being explained by the different behavior of the two solvents in terms of their interaction with the stationary phase. Given that acetonitrile achieves up to five layers of adsorption on the surface of the grafted hydrocarbon chains of the stationary phase compared to the monolayer made of methanol, then we can assume a higher volume of the stationary phase obtained by using acetonitrile and therefore a value higher phase ratio in this case.

The dependence of the parameter Φ as a function of 1/T cannot be bounded using the same function for both organic modifiers used: in case of using methanol as organic solvent the results obtained cannot be bounded in all cases using a linear function, in the case of Ultisil column a fitted using a second degree polynomial function being more suitable for this dependence, while in the case of the use of acetonitrile all the data obtained can be fitted using a linear function.

The values of the equilibrium constant of the partitioning process also depend on the temperature: when the organic solvent is methanol they show a decreasing trend with increasing temperature, and when acetonitrile is used as an organic modifier the values cannot be included in a certain trend.

2.3. The study of the thermodynamic behavior in reverse phase liquid chromatography with deviations from the van't Hoff dependence

Introduction regarding the choice of the studied compounds

This study was conducted to observe the behavior of polycyclic chemicals that are part of the class of active ingredients called phosphodiesterase type 5 inhibitors, which have vasodilating action [204]. The influence of certain parameters such as temperature or pH of the mobile phase on the retention of the investigated substances was followed and the important parameters of the chromatographic retention process were calculated.

Substances used were sildenafil (patented under the name Viagra by Pfizer), vardenafil (patented under the name Levitra by Bayer) and tadalafil (patented under the name Cialis by Eli Lilly) [205], the most commonly used and sildenafil is known. These pharmaceutical formulations are mainly used to treat erectile dysfunction in males, sildenafil is also recommended in pulmonary arterial hypertension (patented under the name Revatio by Pfizer) [206], or in altitude sickness with an effect on altitude pulmonary edema [207, 208]. Sildenafil is also the subject of several research groups whose main objective is to verify its potential, alone or in combination, in the treatment of various cancers and type 2 diabetes or insulin-independent [206]. The chemical structures of the three investigated substances are shown in figure 2.22.

These substances are chemical species of interest, being some of the most counterfeited and altered active pharmaceutical ingredients worldwide, the market value for the segment corresponding to drugs aimed at treating erectile dysfunction reaching an amount of about 3-4 trillion dollars per the current moment [209]. For this reason, a sphere of interest has been created with the main subject of determining them, singly or in mixture with other substances, using various techniques including electrochemical techniques [210, 211], spectroscopic and spectrophotometric techniques [205, 212-218], thermal analysis techniques [219], liquid chromatography coupled or not with mass detection [205, 220-223], combination of solid phase extraction and liquid chromatography coupled with mass detection [224, 225].

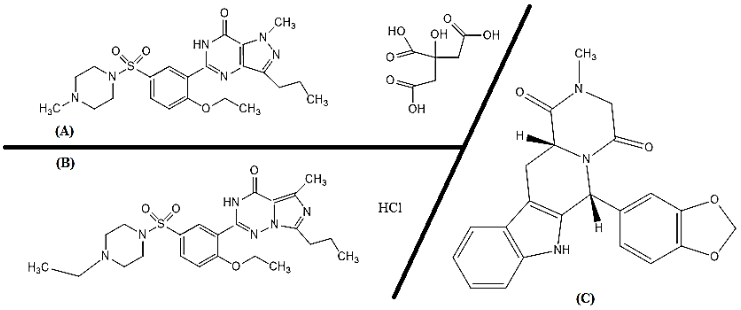

Figure 2.22. Chemical structure of chemicals used: (A) sildenafil citrate; (B) vardenafil hydrochloride; (C) tadalafil.

2.3.1. The influence of temperature on retention for mobile phases without pH control

Retention data processing and interpretation

The values for the natural logarithm of the retention factor k' ($k' = (t_r - t_o)/t_o$) were obtained for each substance investigated at a specific temperature and for a certain binary mobile phase composition. The calculated values of the natural logarithm of the retention factor were plotted against the inverse of the absolute value of the chromatographic column temperature.

Sildenafil citrate and vardenafil hydrochloride have a similar behavior: when the organic modifier used is acetonitrile the two active ingredients have an anti van't Hoff behavior, the value of the retention time and the natural logarithm of the retention factor increasing simultaneously with increase in the temperature value of the chromatographic column, while when methanol is used as an organic modifier the two substances have a van't Hoff type behavior which is described as a decrease in retention time with increasing temperature value. A discordant note is made by tadalafil, which is characterized by a van’t Hoff-type behavior regardless of the organic modifier used in the composition of the mobile phase.

The values of the polynomial regression coefficients can be used to calculate the thermodynamic parameters associated with the chromatographic retention process: enthalpy change, entropy change associated with the process of transferring analyte from mobile to stationary phase, and Gibbs free energy variation. These thermodynamic parameters were calculated in this study of the behavior of the temperature retention process using the following equations:

$$\Delta H = -R \cdot \left(b + \frac{2c}{T}\right) \tag{2.3.2}$$

$$\Delta S = R \cdot \left(a - ln\Phi - \frac{c}{T^2}\right) \tag{2.3.3}$$

The thermodynamic parameters corresponding to sildenafil citrate and vardenafil hydrochloride follow the same trend. Thus, although the use of acetonitrile as an organic modifier shows that the values for the entire temperature range for the variation of the enthalpy and entropy are positive, the process of transferring the analyte from the mobile phase to the stationary phase takes place, testimony is the increasingly negative value of Gibbs free energy. When methanol is used as an organic modifier, the value of the variation of the enthalpy is negative for the entire range of, and the value of the variation of the entropy is positive for the first two values in the temperature range used, then becoming more and more negative. And this time the analyte transfer process takes place, the value of Gibbs free energy being negative over the entire temperature range.

And this time tadalafil makes a discordant note compared to the other two substances analyzed: for both organic modifiers the values obtained for the variation of the enthalpy and, respectively, the variation of the entropy are negative over the entire temperature range studied. The same is true for the Gibbs free energy values corresponding to the transfer of the analyte from the mobile phase to the stationary phase, with the temperature value increasing the Gibbs free energy value decreasing in absolute value.

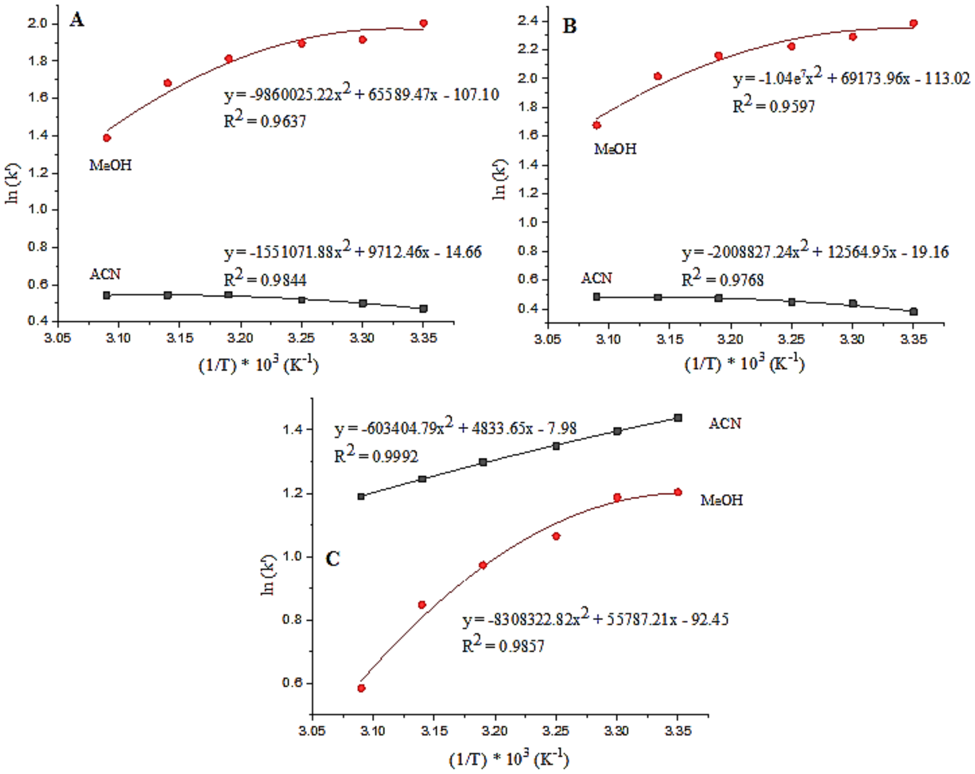

Figure 2.26. Graphical representation of the natural logarithm dependence of the retention factor as a function of 1/T for the three investigated substances and the two organic modifiers used in the mobile phase: A-sildenafil citrate; B-vardenafil hydrochloride; C-tadalafil.

Study conclusions

This experimental study showed that the active substances studied behave differently although they belong to the same class of substances, so tadalafil has a van't Hoff type behavior regardless of the mobile phase used in the retention process, while in the case of sildenafil citrate and vardenafil hydrochloride their behavior depends on the organic modifier present in the mobile phase composition. For all three substances the dependence of the natural logarithm of the retention factor on the inverse of the absolute value of the temperature of the chromatographic column shows deviations from linearity.

The thermodynamic parameters associated with the transfer process of the analytes from the mobile phase to the stationary phase were calculated: the variation of the enthalpy, the variation of the entropy and the variation of the Gibbs free energy, respectively.

2.3.2. The influence of the pH of the mobile phase on the retention

The structure of compounds with ionizable groups is influenced by the pH value of the mobile phase through its aqueous component. It is possible that at certain pH values the compounds participating in the chromatographic elution process may be present in several forms, which will have different partition constants between the mobile phase and the stationary phase. As this process of interphase partitioning is characterized by species-specific thermodynamic quantities, it is possible that the overall linearity of the van’t Hoff dependence may be affected. This remark is the subject of this study, using the mentioned compounds for which the chromatographic behavior of various compositions of the mobile phase and temperatures of the chromatographic column were studied.

Retention data processing and interpretation

The values of the natural logarithm of the retention factor corresponding to the three pharmaceutically active principles in the temperature range studied for the three columns, for all pH values and the two organic modifiers used were calculated. These values of the natural logarithm of the retention factor were then plotted against the inverse of the absolute temperature value of the chromatographic column (example figure 2.30).

As can be seen, sildenafil citrate does not have a universal behavior for all pH values of the mobile phase, especially when the organic modifier is acetonitrile.

For the mobile phase with aqueous component having pH 2,5, sildenafil has a van't Hoff type behavior with a deviation from linearity, the ln k' versus 1/T dependencies being characterized by second degree functions, for all the chromatographic columns used and those two organic modifiers except for the Ultisil XB-C18 column, an end-capped stationary phase column, which exhibits an anti van't Hoff behavior when the organic modifier in the mobile phase is acetonitrile.

For the mobile phase with aqueous component having pH 5,3, sildenafil behaves differently depending on the columns used, it has a certain chromatographic behavior on the columns with the stationary phase of silica gel modified with octadecyl groups and another chromatographic behavior on the column with the stationary phase of modified silica gel type with octyl groups. Thus, on C18 type columns sildenafil has an anti van't Hoff type behavior when the organic modifier used is acetonitrile and a van't Hoff type behavior when using methanol, while for the C8 type column when using acetonitrile ln k' dependence on 1/T has a convex allure, the value of retention time decreasing in the first half of the temperature range and then increasing for the rest of the range, and in the case of methanol use ln k' dependence on 1/T has a concave appearance, the value of the retention time increasing in the first half of the temperature range and then showing a decrease for the rest of the studied temperature range.

When the pH of the aqueous component in the mobile phase is 6,3, sildenafil has an anti van't Hoff-like behavior that deviates from linearity for acetonitrile used as an organic modifier in the mobile phase, but there are small discrepancies between the three columns (in the case of the Ultisil and Luna columns the value of the retention time increasing over the temperature range 25^{o}-40^{o}C and then decreasing, while in the case of the Gemini column the value of the retention time increasing over the whole temperature range studied), respectively a van't behavior Hoff when the organic modifier is methanol.

For the mobile phase with the aqueous component having a pH value of 7,5, sildenafil has a different behavior for each column used when using acetonitrile as an organic modifier. Thus, it presents a van't Hoff type behavior on the Gemini C18 column, it presents an identical behavior on the Ultisil C18 column as in the case of pH 6,3 being characterized by a concave allure dependence, and in the case of the Luna C8 column the dependence it has a convex appearance, the value of the retention time decreasing in the first half of the temperature range and then increasing for the rest of the range. For methanol as an organic modifier, the behavior of sildenafil citrate is identical for all columns used, this being van’t Hoff type.

Differences in chromatographic behavior of sildenafil citrate at different pH values of the mobile phase may be attributed to a complex retention mechanism, based on several types of interactions, or may be due to conformational changes in the stationary phase or the substance different retention behaviors as suggested in the literature [147, 194]. The following figure shows possible forms of sildenafil citrate depending on the pH value.

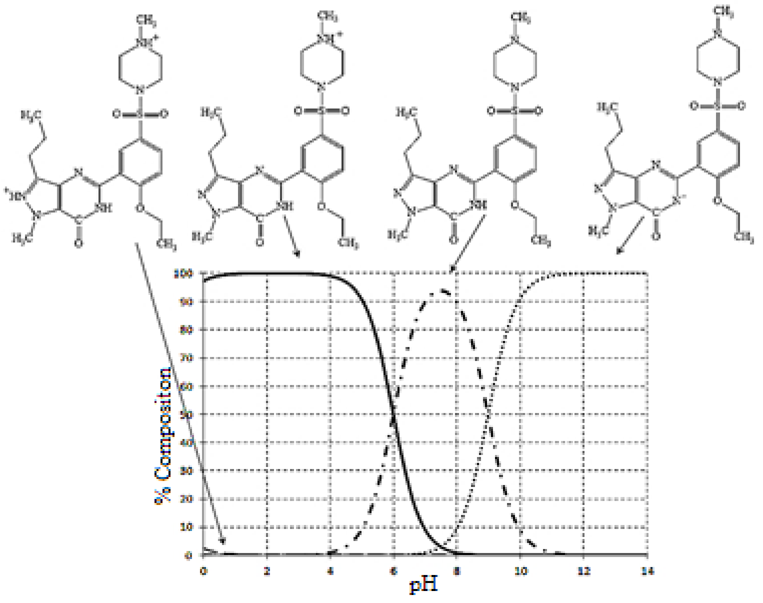

Figure 2.31. pH variation of different forms for the sildenafil molecule in aqueous solution. Reference adaptation [201].

As already mentioned, sildenafil can take many forms depending on the pH of the mobile phase, forms that interact differently with the two phases in contact, and the thermodynamic parameters calculated in this case are a result of these different interactions. An example of these statements can be found in figure 2.32.

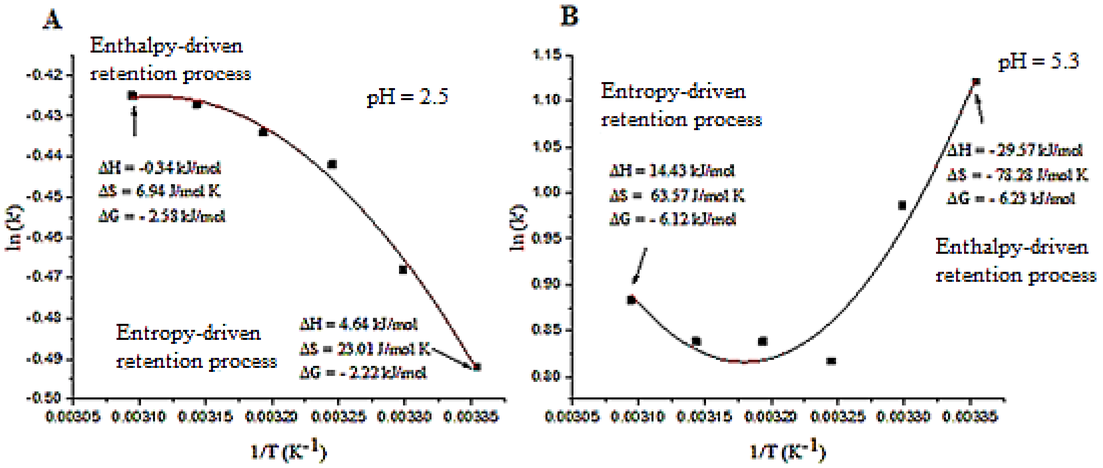

Figure 2.32. Examples of deviations from van’t Hoff-type behavior on the Ultisil XB-C18 column (A) and on the Luna C8 column (B) when the organic modifier is acetonitrile.

Sildenafil may also have several tautomeric forms (figure 2.34) which in turn interact in a specific way with the two phases and which may be associated with the reason for the deviation from van't Hoff behavior and with the presence of multiple retention mechanisms.

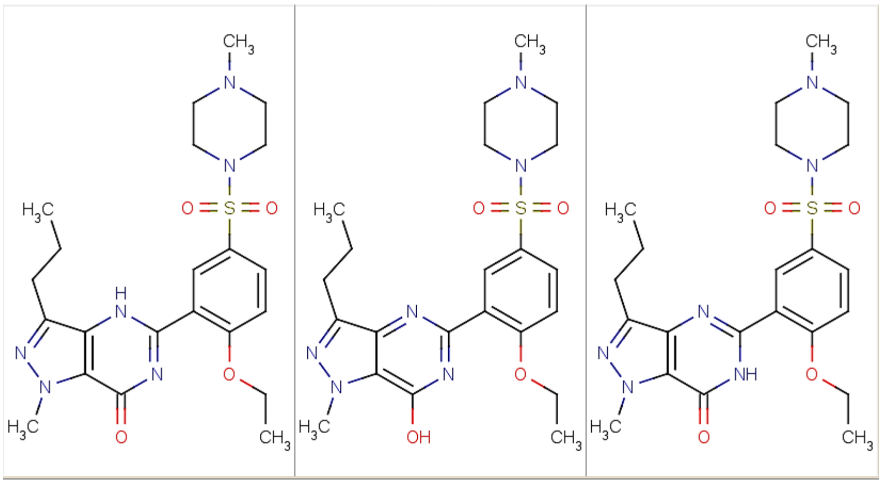

Figure 2.34. Tautomeric forms of sildenafil according to ChemAxon 5.4.0.0.

Similar studies were performed for the other active ingredients in the class of phosphodiesterase inhibitors: vardenafil hydrochloride, tadalafil and papaverine.

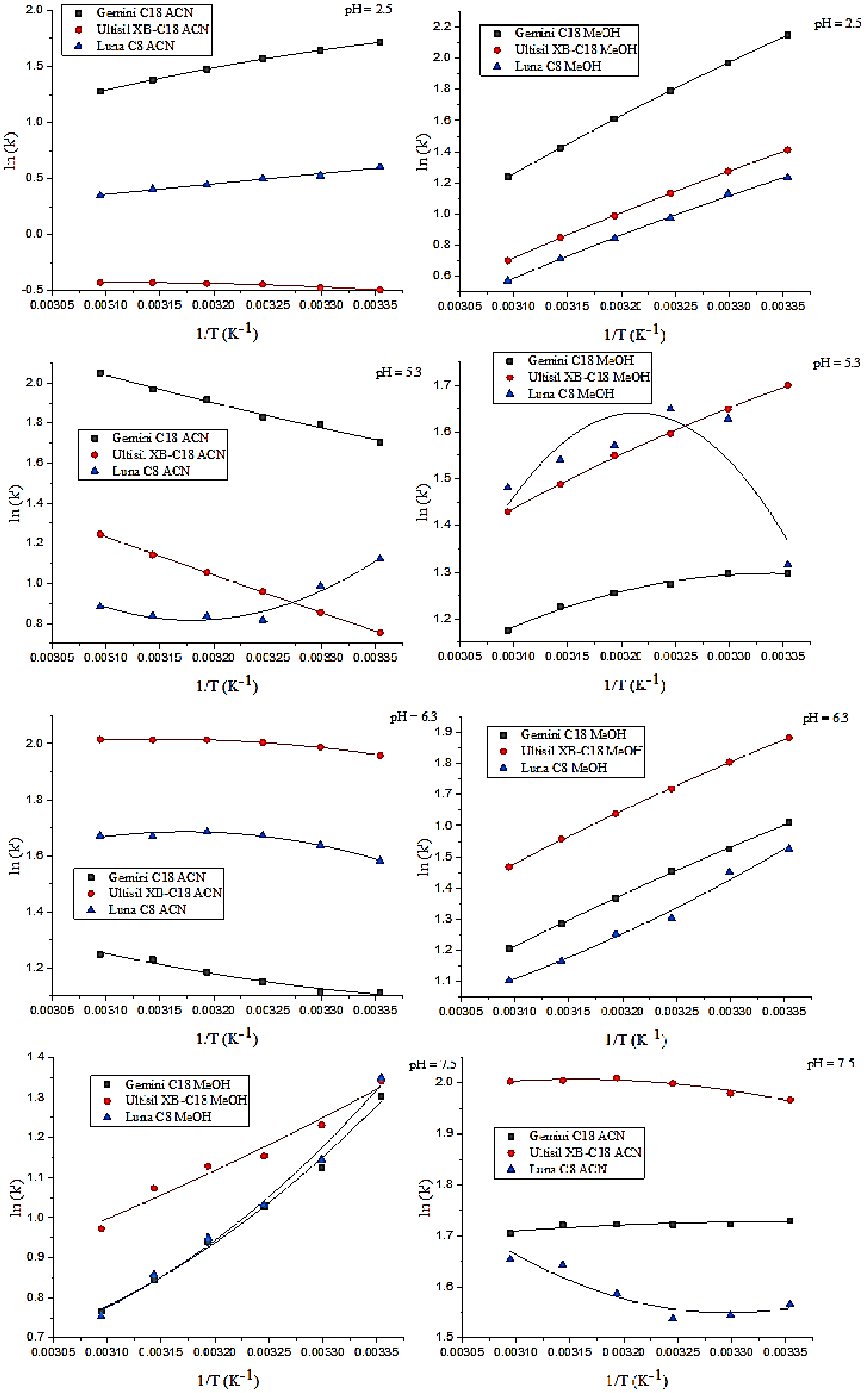

Figure 2.30. The ln k' versus 1/T dependencies for sildenafil citrate obtained on the three chromatographic columns used and for all pH values of the studied mobile phases.

Study conclusions

This experimental study showed that the active substances studied have different behavior even though they belong to the same class of substances, so tadalafil has a van't Hoff-like behavior regardless of the pH of the mobile phase used in the retention process or the modifier. In the case of sildenafil citrate and vardenafil hydrochloride, the behavior depends on both the organic modifier present in the mobile phase composition and the pH value of the aqueous component.

For all three substances the dependence of the natural logarithm of the retention factor on the inverse of the absolute value of the chromatographic column temperature deviates from linearity, these dependencies being processed using polynomial functions whose variables were used in calculating the thermodynamic parameters associated with the process. transfer of analytes from the mobile phase to the stationary phase (the variation of the enthalpy, the variation of the entropy and the variation of the Gibbs free energy, respectively).

In the literature, the identification of the causes of the deviation from the van't Hoff-type behavior of substances is a topic still unexplained, there are various theories that try to explain this behavior by the presence of secondary equilibria, tautomeric interconversions or the presence of multiple mechanisms retention [147, 161, 194, 201, 231-238].

2.4. General conclusions

HPLC chromatographic partition by reverse phase mechanism is generally influenced by temperature, with the variation of the retention factor measured experimentally as a function of column temperature being described by the van’t Hoff equation. This shows a linear dependence between ln k' for an analyte and the inverse of the absolute temperature of the chromatographic column (1/T), this linear dependence being observed in most situations where the partition mechanism is unique and no other secondary equilibria occur like dissociation or isomerization.

Studies of the chromatographic behavior of a series of aromatic hydrocarbons from benzene to propylbenzene have shown that the van’t Hoff linearity is observed regardless of the mobile or stationary phases used. From the van’t Hoff type representations it was possible to calculate the fundamental thermodynamic quantities that characterize the chromatographic partition of the mentioned compounds, estimating at the same time a variation range of [-16,7; -5,8] kJ/mol for the values of the standard enthalpy ΔH^o and of [-18,7; 5] J/mol•K for those of standard entropy ΔS^o. These values are consistent with the literature data from the HPLC studies for the compounds analyzed, and are also consistent with the values considered normal for van der Waals interactions between two hydrocarbon protein chains.

Also, using the same series of hydrocarbons, the influence of the composition of the mobile phase on the retention was determined, obtaining linear regressions using the set of experimental values (k', φ) and respectively the extrapolated values of log k' for the mobile phase consisting only of water and namely log k'_w. These extrapolated values also depend on the column temperature according to the van't Hoff equation, the values corresponding to the thermodynamic parameters ΔH^o and ΔS^o obtained for the hypothetical partition of hydrocarbons studied between water and modified silica gel with octadecyl groups being significantly higher than those obtained on the same columns in the studies previous.

A fundamental question that has been tried to be answered in this thesis is whether the ratio of phase volumes (known in the literature as "phase ratio", Φ) that characterizes a chromatographic column depends on its temperature. To date, the literature does not report studies on the temperature dependence of the "phase ratio" parameter, but only estimates of this parameter for various mobile phase compositions. In general, most thermodynamic studies on reverse phase HPLC partitioning consider a constant Φ, regardless of the temperature at which the separation takes place.

The present study shows that this dependence can be established for common mobile phase organic modifiers, such as methanol and acetonitrile. For this purpose, the same compounds with stable structure and literature data on the temperature dependence of the water-octanol K_{ow} partition constant used in modeling the chromatographic distribution of a compound between the hydrophilic mobile phase and the hydrophobic stationary phase were used.

In the last part of the thesis, some situations of chromatographic behavior were studied in which the variation between ln k' and 1/T shows deviations from the linearity of van’t Hoff. The selected compounds were sildenafil, vardenafil, tadalafil and papaverine, active pharmaceutical ingredients of interest, which may be involved in secondary dissociation or isomerization equilibria that complicate the global partitioning process, equilibria presented by diagrams describing the dependence of structures on the pH of the environment solvent. The study of these linearity variations was performed under various elution conditions, varying the pH of the aqueous component used in the composition of the mobile phase. Both deviations from linearity and abnormal behavior were observed by increasing chromatographic retention with increasing temperature. The study showed that for situations of linearity or nonlinearity the retention is controlled by the enthalpy of the process and in situations where the behavior is abnormal the retention is apparently controlled by the entropy.

3. PUBLISHED SCIENTIFIC PAPERS

1. The influence of column temperature on the extrapolated values of the retention factor in reversed-phase liquid chromatography for water as mobile phase, **Andreia-Cristina SOARE**, Toma GALAON, Serban C. MOLDOVEANU, Victor DAVID, *Revue Roumaine de Chimie*, 64(8), 2019, 727-731, DOI: 10.3224/rrch.2019.64.8.09

2. Thermodynamic study of the retention of aromatic hydrocarbons on phenylsilicagel based stationary phase under reversed-phase liquid chromatographic mechanism, **Andreia-Cristina SOARE**, Viorica MELTZER, Mihaela CHEREGI, Victor DAVID, *Revue Roumaine de Chimie*, 64(12), 2019, 1083-1089, DOI: 10.33224/rrch.2019.64.12.08

3. Sources of nonlinear van't Hoff temperature dependence in high-performance liquid chromatography, Maria TANASE, **Andreia SOARE**, Victor DAVID, Serban C. MOLDOVEANU, *ACS Omega*, 4, 2019, 19808−19817, DOI: 10.1021/acsomega.9b02689

4. Does phase ratio in reversed phase high performance liquid chromatography vary with temperature?, **Andreia-Cristina SOARE**, Victor DAVID, Serban C. MOLDOVEANU, *Journal of Chromatography A*, 1620, 2020, 461023, DOI: 10.1016/j.chroma.2020.461023

5. Variation with temperature of phase ratio in reversed phase HPLC for a methanol/water mobile phase, **Andreia-Cristina SOARE**, Victor DAVID, Serban C. MOLDOVEANU, *Chromatographia*, 84, 2021, 581–587, DOI: 10.1007/s10337-021-04038-7

4. SCIENTIFIC COMMUNICATIONS

1. Thermodynamic parameters obtained for the retention of aromatic hydrocarbons on phenylsilica stationary phase in reversed-phase liquid chromatography, **Andreia-Cristina SOARE**, Mihaela Carmen CHEREGI, Victor DAVID, *9th International Conference of the Chemical Societies of the South-Eastern European Countries (ICOSECS9), «Chemistry a Nature Challenger»*, 2019 – **Poster**

2. The temperature effect on the retention of sildenafil under reversed-phase liquid chromatography, **Andreia-Cristina SOARE**, Viorica MELTZER, Mihaela Carmen CHEREGI, Victor DAVID, „Priorităţile Chimiei pentru o dezvoltare durabilă – PRIOCHEM'', ediţia a XV-a, 2019 – **Poster**

3. Comparative thermodynamic study of retention on various stationary phases in reversed-phase liquid chromatography, **Andreia-Cristina SOARE**, Elena PINCU, Mihaela-Carmen CHEREGI, Victor DAVID, International Conference CHIMIA 2020 <<New Trends in Applied Chemistry>>, 2021 – **Poster**

4. Temperature dependence of the phase ratio parameter in reversed phase high performance liquid chromatography, **Andreia-Cristina SOARE**, Victor DAVID, Sesiunea de Comunicări Stiinţifice Studenţeşti, Facultatea de Chimie, Universitatea din Bucureşti, 2021 – **Oral presentation**

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