



*Dedicated to the memory of
Professor Victor-Emanuel Sahini (1927–2017)*

COMPARISON OF THE PHASE RATIO FOR C18 HPLC COLUMNS USING THREE DIFFERENT ORGANIC MODIFIERS (METHANOL, ETHANOL AND ACETONITRILE) IN MOBILE PHASE COMPOSITION

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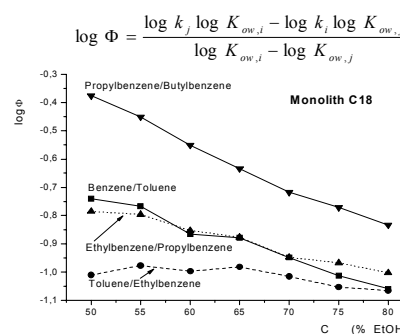
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A retention study for five aromatic hydrocarbons was performed on three C18 HPLC columns (two were packed columns and one was based on a monolithic stationary phase) using aqueous mobile phases containing one organic modifier (methanol, ethanol, or acetonitrile) at constant temperature (25°C). Linear dependences between the logarithm of the retention factor and organic modifier percentage in mobile phase were obtained and used to calculate the extrapolated value of the retention factor for mobile phase composed of only water. The value of phase ratio was calculated for each mobile phase composition using a theoretical approach previously developed and based on solvophobic theory (*J. Chromatogr. A*, **2015**, *1381*, 194-201). According to this model, the phase ratio depends not only on the nature of the stationary phase, but also on the nature and content of the organic modifier found in the composition of mobile phase. This study showed that the average values of phase ratio calculated over the four pairs of consecutive aromatic hydrocarbons from series benzene/butylbenzene varied in the order of organic modifier, methanol < ethanol < acetonitrile.



THEORETICAL CONSIDERATIONS

Phase ratio (denoted by Φ) is a fundamental parameter in characterizing a column in high performance liquid chromatography (HPLC) and it is defined as the ratio between the volume of the stationary phase V_{st} and the volume of mobile phase of the column, V_0 .^{1,2} This influences the retention and separation selectivity in HPLC,³ and it is included in van't Hoff equation as part of the entropic term.^{4,5} Its measurement is rather difficult

because usually there is no sharp boundary between the mobile phase and the stationary phase, and different mobile phases lead to different effective phase ratios.^{6,7} In this ratio, the main difficulty is to measure V_{st} , although several experimental attempts have been undertaken for this purpose.^{8,9} The value of V_0 is easier to calculate using the values of dead time (t_0) and flow rate of the mobile phase. Determination of t_0 can be performed using one of the following possibilities: *i*) accurate determination of the

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retention time value (t_R) for an unretained solute (polar organic molecule, e.g. uracil; organic or inorganic salt), when $t_R = t_0$; *ii*) the weight differences of the column sequentially filled with two solvents of different densities (so-called the pycnometric method); *iii*) the time for minor disturbance of the baseline in the chromatogram, when deuterated mobile phase or a mobile phase component is injected; *iv*) from the plot of t_R versus the homolog number of a series of compounds following by extrapolation to the unretained compound (i.e. zeroth homolog).²

Phase ratio and retention mechanism in reversed-phase HPLC are related as shown by the partition model, which is currently used for explaining the complex equilibria occurring during the elution process of the components from an injected sample into the hydrophobic column.¹⁰⁻¹² Recently, a new theoretical method has been proposed for the evaluation of phase ratio for a chromatographic column when a given mobile phase is used.^{13,14} A theoretical support for this method is based on the solvophobic theory of interactions in solution, which is one the most important models that describe the complex RP-HPLC process.¹⁵⁻¹⁷ It is the aim of this study to investigate this fundamental parameter for characterizing a chromatographic column, by using both packed and monolithic columns, for mobile phase containing methanol or acetonitrile as organic additive, and a less studied organic solvents, but with an important potential in various applications, due to many advantages emphasized in several studies, namely ethanol.^{18,19}

EXPERIMENTAL

The analytical purity standards of benzene, toluene, ethylbenzene, propylbenzen and butylbenzene were purchased

from Sigma-Aldrich (Steinheim, Germany). HPLC analytical grade methanol, ethanol, and acetonitrile were purchased from Merck (Darmstadt, Germany). Liquid chromatography grade water was produced within the laboratory by a Millipore Simplicity instrument and used as the component of the mobile phase.

Chromatographic experiments were carried out with a PerkinElmer Flexar Quaternary liquid chromatograph system (PerkinElmer, Waltham, USA) equipped with a degasser, quaternary pump, autosampler, column thermostat, and UV detector. System control and data acquisition were made with the Chromera CDS 4.3. The chromatographic instrument was operationally qualified before starting the entire study.

The retention study was carried out on three different chromatographic columns: Phenomenex Luna 150 x 4.6 mm 5 μ m particle size; Phenomenex Gemini 150 x 4.6 mm 5 μ m, and Merck Chromolith RP-C18 100 x 4.6 mm. Detection was made at 254 nm. The retention time was measured at a constant flow rate of 1 mL/min, and a fixed column temperature (25°C).

Mobile phase composition given by water and single organic modifier (acetonitrile, methanol, or ethanol) varied with steps of 5% volumetric content of organic modifier, within the intervals indicated in next section for the discussion of the chromatographic results.

Column dead-time (t_0) was determined from the retention time of uracil, injected into the column for different mobile phase compositions. Retention time (t_R) for each hydrocarbon was determined in triplicate in order to ensure the accuracy of data, and the average value was used for calculating the retention factor k of analyte with equation $k = (t_R - t_0) / t_0$.

RESULTS AND DISCUSSION

Benzene, toluene, ethylbenzene, propylbenzen and butylbenzene can be used as probe compounds due to their hydrophobic character and the lack of polar functional groups. Some data related to their hydrophobic character, as given by octanol/water partition constant (K_{ow}), are indicated in Table 1 (where data about the used organic modifiers are also included).

Table 1

Experimental and theoretical values of $\log K_{ow}$ for studied hydrocarbons

Compound	$\log K_{ow}$ (experimental)	$\log K_{ow}$ (theoretic)*
Benzene	2.13	1.99
Toluene	2.73	2.54
Ethylbenzene	3.15	3.03
Propylbenzene	3.69	3.52
Butylbenzene	4.38	4.01
Methanol	-0.77	-0.63
Ethanol	-0.31	-0.14
Acetonitrile	-0.34	-0.15

* calculated with EPI Suite Software, Environmental Protection Agency, EPA (www.epa.gov)

The experimental retention study was performed for various compositions of the mobile phase, consisting in aqueous component and acetonitrile (AcCN), methanol (MeOH), or ethanol (EtOH), typically used as organic solvents in RP-HPLC. Dependences of the retention factor (k) on the percentage of the organic modifier in the mobile phase composition ($C_{o.m.}$) were firstly investigated from the retention data of the studied compounds obtained for different mobile phase compositions. These dependences are generally described by a linear equation between the logarithm of k (here as ten base logarithm, \log),²⁰ although polynomial dependences can also be obtained for particular situations.^{21,22} In this equation a main parameter is $\log k_w$, which corresponds to the logarithm of extrapolated value of the retention factor corresponding to a mobile phase consisting in only aqueous component:

$$\log k = \log k_w + \alpha \cdot C_{o.m.} \quad (1)$$

The parameters $\log k_w$ and α can be calculated for each studied compound from the linear regressions between $\log k$ and $C_{o.m.}$, and they are specific for each organic modifier used as component of the mobile phase (MeOH, EtOH, or AcCN).²⁵⁻²⁹ Fig 1 shows an example of these dependences obtained for the studied aromatic hydrocarbons, and the corresponding regression parameters are given in

Tables 2-4. The parameter denoted by $\log k_w$ represents the extrapolated value of the retention factor, corresponding to a mobile phase consisting in only water, which is usually related to the chromatographic hydrophobicity ($K_{ow}^{RP-HPLC}$).^{23,24}

As can be noticed from Tables 2-4, all dependences between $\log k$ and $C_{o.m.}$ were characterized by high correlation coefficients ($r^2 > 0.99$).

These correlations can be considered as reliable for further calculations of various chromatographic parameters. An important parameter is the chromatographic hydrophobicity ($K_{ow}^{RP-HPLC}$), which can be calculated with the known formula that links the extrapolated value of the retention factor, k_w , and phase ratio Φ :

$$\log K_{ow}^{RP-HPLC} = \log k_w - \log \Phi \quad (2)$$

Theoretically, the values of parameters $K_{ow}^{RP-HPLC}$ or $\log k_w$ should be correlated to the values of $\log K_{ow}$ given in Table 1. The dependences between $\log k_w$ obtained for the five aromatic hydrocarbons for each organic modifier and each stationary phase used in this study, and their values of $\log K_{ow}$ were highly correlated ($r^2 > 0.9$), for both experimental and theoretical values of $\log K_{ow}$ presented in Table 1.

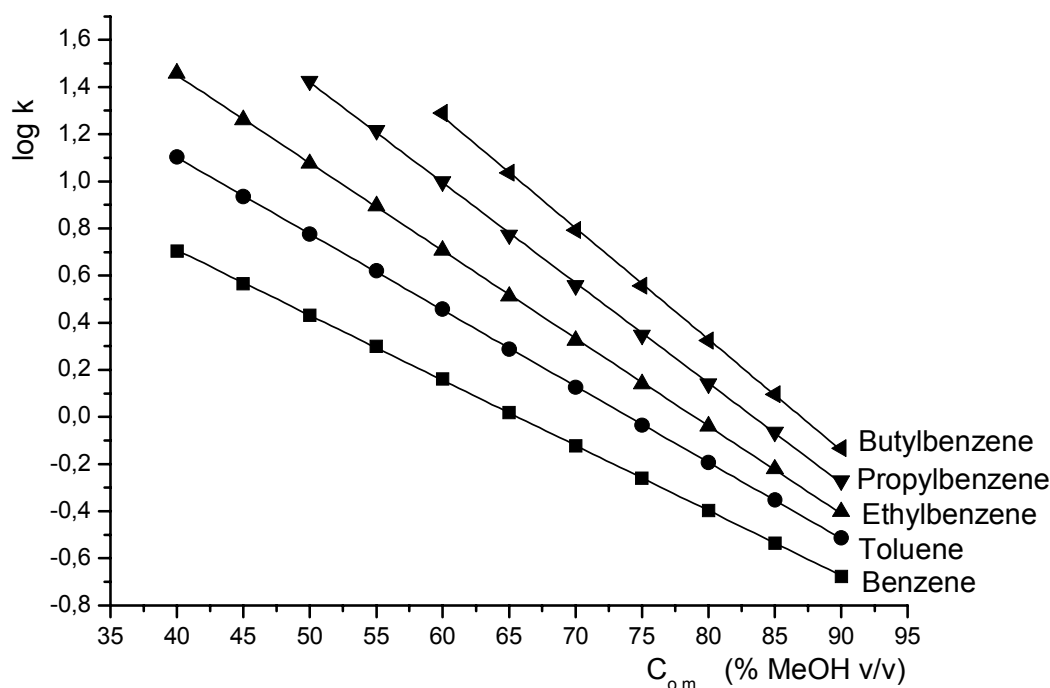


Fig. 1 – Typical linear dependences between $\log k$ and the organic modifier content in mobile phase, as volume percentage, exemplified for monolith column and MeOH used as organic modifier.

Table 2

Regression parameters calculated from eq. (1) applied to the retention data obtained for three studied C18 columns using acetonitrile as organic modifier in mobile phase composition

Compound	$\log k_w$	α	r^2	% AcCN interval
Luna C18 column				
Benzene	1.893	-0.0251	0.9969	40 - 70
Toluene	2.277	-0.0284	0.9950	40 - 70
Ethylbenzene	2.662	-0.0318	0.9935	40 - 70
Propylbenzene	2.974	-0.0337	0.9946	45 - 70
Butylbenzene	3.256	-0.0352	0.9952	50 - 70
Gemini C18 column				
Benzene	1.836	-0.0250	0.9962	40 - 70
Toluene	2.210	-0.0282	0.9944	40 - 70
Ethylbenzene	2.586	-0.0316	0.9929	40 - 70
Propylbenzene	2.888	-0.0333	0.9942	45 - 70
Butylbenzene	3.167	-0.0348	0.9939	50 - 70
Monolith C18 column				
Benzene	1.357	-0.0233	0.9978	40 - 70
Toluene	1.751	-0.0267	0.9969	40 - 70
Ethylbenzene	2.134	-0.0302	0.9943	40 - 70
Propylbenzene	2.561	-0.0339	0.9926	40 - 70
Butylbenzene	2.847	-0.0354	0.9952	45 - 70

Table 3

Regression parameters calculated from eq. (1) applied to the retention data obtained for three studied C18 columns using methanol as organic modifier in mobile phase composition

Compound	$\log k_w$	α	r^2	% MeOH interval
Luna C18 column				
Benzene	2.332	-0.0295	0.9996	50 - 90
Toluene	2.918	-0.0343	0.9993	50 - 90
Ethylbenzene	3.484	-0.0394	0.9991	50 - 90
Propylbenzene	4.013	-0.0438	0.9998	60 - 90
Butylbenzene	4.599	-0.0488	0.9998	65 - 90
Gemini C18 column				
Benzene	2.223	-0.0282	0.9999	50 - 90
Toluene	2.786	-0.0328	0.9999	50 - 90
Ethylbenzene	3.334	-0.0377	0.9998	50 - 90
Propylbenzene	3.908	-0.0427	0.9998	60 - 90
Butylbenzene	4.473	-0.0475	0.9998	65 - 90
Monolith C18 column				
Benzene	1.812	-0.0276	0.9999	40 - 90
Toluene	2.393	-0.0323	0.9999	40 - 90
Ethylbenzene	2.938	-0.0372	0.9999	40 - 90
Propylbenzene	3.546	-0.0425	0.9997	50 - 90
Butylbenzene	4.111	-0.0473	0.9996	60 - 90

Table 4

Regression parameters calculated from eq. (1) applied to the retention data obtained for three studied C18 columns using ethanol as organic modifier in mobile phase composition

Compound	$\log k_w$	α	r^2	% EtOH interval
Luna C18 column				
Benzene	1.803	-0.0252	0.9973	50 - 80
Toluene	2.236	-0.0290	0.9967	50 - 80
Ethylbenzene	2.650	-0.0329	0.9962	50 - 80
Propylbenzene	3.125	-0.0374	0.9962	50 - 80
Butylbenzene	3.537	-0.0411	0.9953	50 - 80
Gemini C18 column				
Benzene	1.824	-0.0252	0.9982	50 - 80
Toluene	2.252	-0.0289	0.9976	50 - 80
Ethylbenzene	2.662	-0.0329	0.9972	50 - 80
Propylbenzene	3.108	-0.0371	0.9966	50 - 80
Butylbenzene	3.537	-0.0411	0.9963	50 - 80

Table 4 (continued)

Monolith C18 column				
Benzene	1.433	-0.0251	0.9930	50 - 80
Toluene	1.888	-0.0290	0.9917	50 - 80
Ethylbenzene	2.311	-0.0331	0.9900	50 - 80
Propylbenzene	2.773	-0.0375	0.9892	50 - 80
Butylbenzene	3.218	-0.0416	-0.9887	50 - 80

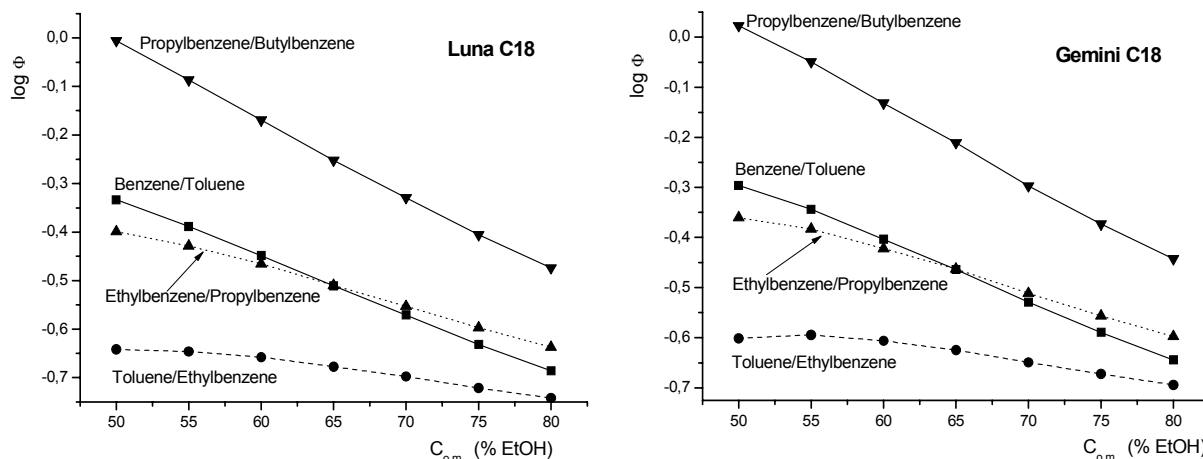


Fig. 2 – Comparison of phase ratio profiles for the two packed HPLC columns using various pairs of probe hydrocarbons, and ethanol as organic modifier in mobile phase composition.

Calculation approach of phase ratio Φ for a RP-HPLC column has been previously developed based on solvophobic theory,¹³ and requires the experimental retention data for at least two different probe hydrocarbons.¹⁴ If we denote these hydrocarbons by i and j , which are characterized by their $\log K_{ow,i}$ and $\log K_{ow,j}$, respectively, the following expression for calculation of $\log \Phi$ can be obtained for the characterization of a C18 chromatographic column:

$$\log \Phi = \frac{\log k_j \log K_{ow,i} - \log k_i \log K_{ow,j}}{\log K_{ow,i} - \log K_{ow,j}} \quad (3)$$

In this formula, the values of $\log k_i$ and $\log k_j$ are experimental values for the logarithm of retention factor measured for a certain mobile phase composition and a stationary phase. Thus, by selecting two hydrocarbons in the series benzene to butylbenzene, then the value of $\log \Phi$ for the columns can be calculated for different mobile phase compositions.

The four pairs of consecutive aromatic hydrocarbons were studied by this theoretical approach leading to $\log \Phi$ values, which depends however on the chosen pair. Thus, the highest value of $\log \Phi$ was obtained for the pair propylbenzene/butylbenzene, while the lowest value was obtained for toluene/ethylbenzene.

These remarks can be seen in Figures 2 and 3, obtained for instance for ethanol used as organic modifier.

Comparison between the values of $\log \Phi$ is given in Figures 4 and 5, and the regularity of decreasing this parameter in order from acetonitrile to methanol seems to be explained by the decrease of hydrophobic character of these organic modifier. This may influence their adsorption on stationary phase surface at the level of hydrocarbon chains C18, although the interaction between organic modifier and residual silanols onto the surface of stationary phase can also contribute to the value of phase ratio.³⁰⁻³² In this case, acetonitrile has less ability to interact with silanol group. Either way, they can play an important role in the RP retention mechanism as emphasized by other studies reported by literature.^{33,34}

Accuracy of the retention experimental data can influence tremendously the calculated values of $\log \Phi$, and this may explain the significant differences between the values of $\log \Phi$ calculated for the four pairs of aromatic hydrocarbons, as can be observed in Figures 2 and 3. A possibility to overcome this situation is to use the entire set of $\log \Phi$ for the four consecutive pairs of aromatic hydrocarbons, and then to calculate an average value of this parameter. This was carried out for the three

stationary phases and the retention data obtained for the three organic modifiers. These average values of Φ and $\log \Phi$ ($\bar{\Phi}$, and $\log \bar{\Phi}$ respectively) for mobile phase compositions that

allowed to measure the retention time for all aromatic hydrocarbons are given in Table 5. These calculated values are of the same magnitude as the few given values found in literature.^{35,36}

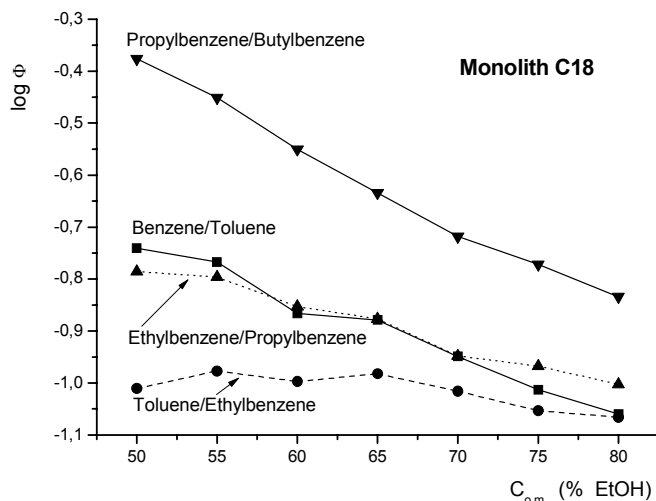


Fig. 3 – Comparison of phase ratio profiles for the monolithic column using various pairs of probe hydrocarbons, and ethanol as organic modifier in mobile phase composition.

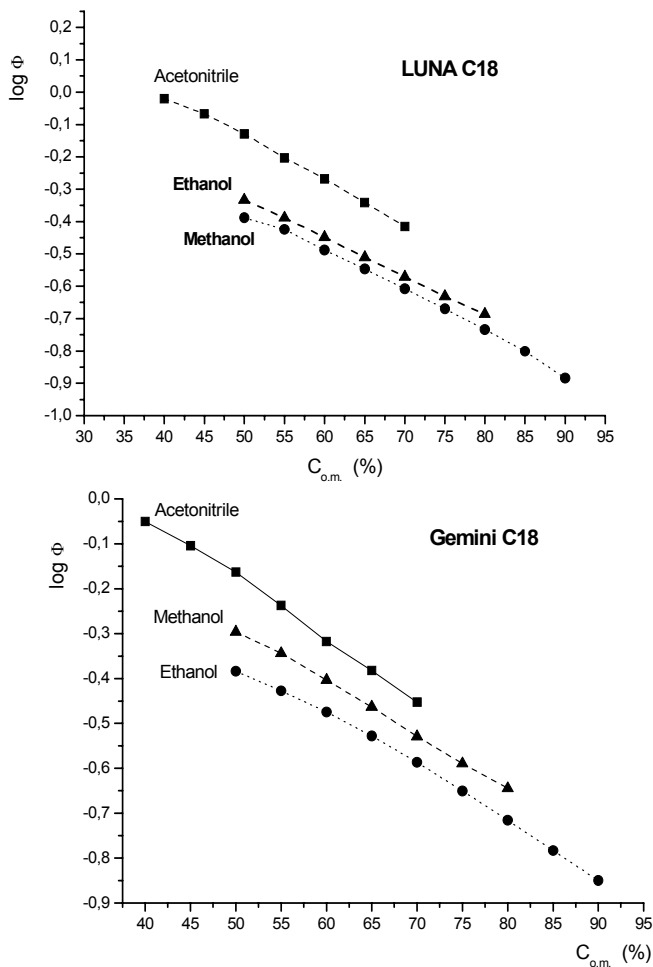


Fig. 4 – Comparison of phase ratio profiles for Luna C18 column and Gemini C18, using the benzene/toluene pair, and indicated organic modifiers in mobile phase composition.

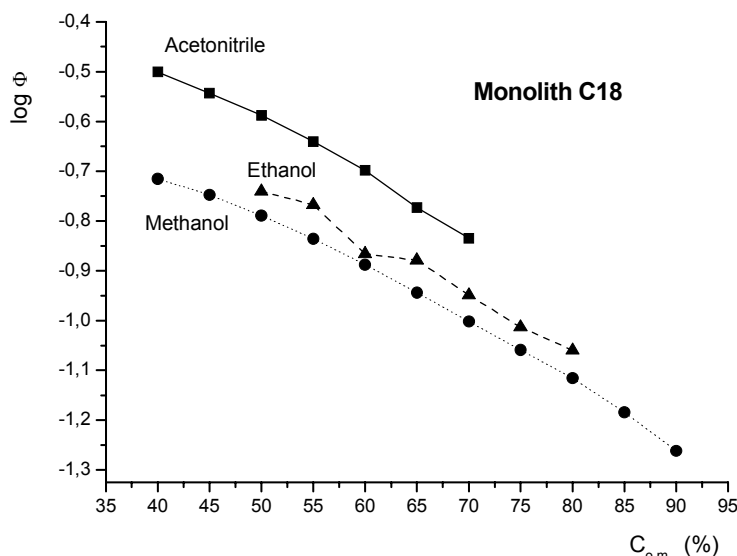


Fig. 5 – Comparison of phase ratio profiles for Monolith C18 column, using the benzene/toluene pair, and indicated organic modifiers in mobile phase composition.

Table 5

Average values of phase ratio calculated from retention data of aromatic hydrocarbons for the three columns used in this study and organic modifiers used as additive in mobile phase composition

$C_{o.m.} (\%)$	Luna C18		Gemini C18		Monolith C18	
	$\log \bar{\Phi}$	$\bar{\Phi}$	$\log \bar{\Phi}$	$\bar{\Phi}$	$\log \bar{\Phi}$	$\bar{\Phi}$
Methanol						
90	-0.7996	0.158	-0.7747	0.168	-1.1549	0.070
85	-0.7261	0.187	-0.7189	0.191	-1.0915	0.081
80	-0.6716	0.212	-0.6635	0.217	-1.0409	0.091
75	-0.6191	0.240	-0.6073	0.247	-0.9914	0.102
70	-0.5672	0.270	-0.5544	0.279	-0.9431	0.114
65	-0.5165	0.304	-0.5045	0.313	-0.8928	0.128
60	-	-	-	-	-0.8446	0.143
Ethanol						
80	-0.6226	0.238	-0.5839	0.260	-0.9797	0.104
75	-0.5727	0.267	-0.5333	0.292	-0.9371	0.115
70	-0.5165	0.304	-0.4773	0.333	-0.8918	0.128
65	-0.4598	0.346	-0.4142	0.385	-0.8230	0.150
60	-0.3995	0.398	-0.3567	0.439	-0.7834	0.164
55	-0.3404	0.456	-0.2979	0.503	-0.7043	0.197
50	-0.2843	0.519	-0.2505	0.561	-0.6656	0.215
Acetonitrile						
70	-0.4520	0.353	-0.4873	0.325	-0.8668	0.135
65	-0.3822	0.414	-0.4211	0.379	-0.8074	0.155
60	-0.3124	0.487	-0.3584	0.438	-0.7367	0.183
55	-0.2490	0.563	-0.2848	0.519	-0.6782	0.209
50	-0.1772	0.665	-0.2111	0.614	-0.6255	0.236

As can be noticed from the data given in Table 5, there is a similar trend for the three organic modifiers. By averaging, the values of Φ close to an expected value although there are a few experimental data for being compared. For example, the reported Φ value for Zorbax C8 manufactured column is 0.226, which was also computed, but based on the % C load supplied by the column manufacturer.³⁶ Other studies report

different values, ranging from 0.079 for a Zorbax Eclipse XDB-C8 column 5 μm particle size³⁵ to a value of 0.665 for an in-house made C18 column with a large stationary phase volume.³⁷ However, due to the complexity of the retention mechanism in RP-HPLC, the volume contribution of the C8 or C18 chains to the volume of stationary phase can not be assumed as only one, and the adsorption of the organic modifier molecules from mobile phase

onto the surface of stationary phase can contribute to the increase of phase ratio, and this was shown by means of solvophobic theory in this study.

CONCLUSIONS

Some of the main conclusions of this theoretical study focused on experimental retention data of several aromatic hydrocarbons can be grouped as following:

i) phase ratio depends on the nature of the organic modifier due to its adsorption into stationary phase as a result of interactions with hydrocarbon chains found on the surface of stationary phase; this is in agreement to other reported studies showing that methanol molecule can interact with residual silanol group by H-bonds from the surface of stationary phase, which is stronger than dipole-dipole interactions between acetonitrile molecule and the same silanol.

ii) the average values of phase ratio calculated over the four pairs of consecutive aromatic hydrocarbons from series benzene/butylbenzene varied in the order of organic modifier, methanol < ethanol < acetonitrile.

iii) for the same organic modifier the phase ratio value depends on its content in mobile phase, and increases with its decrease content in the mobile phase.

iv) the monolith column has the lowest value of phase ratio compared to the column based on packed stationary phases, which is in agreement with the known data showing a higher porosity for monolithic stationary phases compared to the packed stationary phases.

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