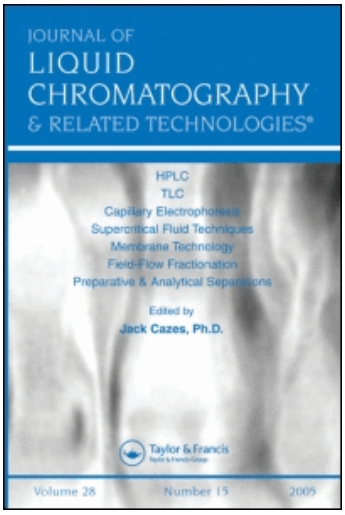

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CALCULATION OF PHASE RESIDENCE TIMES IN MICELLAR ELECTROKINETIC CHROMATOGRAPHY

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ABSTRACT

Method to calculate micellar phase residence time [$t_{mic} = t_{mc}\, k'''$, time spent by the analyte in the micellar phase] and aqueous phase residence time [$t_{aq} = t_0\,(1 - k''')$, time spent by the analyte in the aqueous phase] was developed, where t_m, t_o and t_{mc} are the migration time of the analyte, the flow marker and the micelles, respectively. It was proved that migration time of the analyte is the sum of the phase residence times [$t_m = t_{mic} + t_{aq} = t_0\,(1 - k''') + k'''\, t_{mc}$]. Two physico-chemical parameters characterizing hydrophobicity of the analyte, namely the ratio of the moles of the analyte in the micellar (n_{mc}) and aqueous (n_{aq}) phase ($n_{mc}/n_{aq} = t_{mic}/t_{aq}$) as well as distribution coefficient (K) have also been determined from the phase residence times. Good correlation was found between the micellar phase residence times and computer calculated

hydrophobicity values (S) in the case of hydroxy- and aldehyde benzene derivatives, benzene- and alkyl phenone homolog series and in the case of seven hydrophobic protected peptides. Comparison of hydrophobicity values and phase residence times showed that the higher the hydrophobicity of the analyte the longer the time spent in the micellar (hydrophobic) phase. The micellar phase residence time determinable experimentally in MEKC reflects the hydrophobicity of the molecule investigated.

INTRODUCTION

One of the approaches in drug design is to estimate hydrophobic nature of the molecules on the base of certain physico-chemical parameters, because hydrophobic properties of the molecules play an important role in the mechanism of their biological action as well as in the structure-biological activity relationships (1–3). Since the proposal of Hantsch the hydrophobicity of molecules has been typically characterized by the log 1-octanol/water partition coefficient (log P_{ow}) (4–7).

In the last decade, different reasons promoted the displacement of the shake flask method conventionally used to determine log P_{ow}. One of them is the experimental difficulty of this method, other drawbacks aroused by the invention of the combinatorial molecular libraries in the rational drug design. These aspects have been shortly discussed in our previous paper (8).

The latest developments of drug design triggered efforts to replace the conventional partition method to characterize hydrophobicity of great number of molecules by more easily executable techniques. One kind of approache is the application of different software to calculate hydrophobicity from structural data of groups or subunits of a molecule (6,7,9). The other possibility is the application of separation (including chromatographic and MEKC) methods for experimental characterisation of the hydrophobicity of the molecules (10–12). The aim is to estimate hydrophobicity of the molecules on the basis of the retention factor determined in the separation process. Expected biological activity of the constituents of a molecule library can be evaluated on the basis of their retention factors (10–12). The number of molecules to be biologically tested can be considerably reduced if biological activity of the compounds can be reliably predicted on the basis of these data. There is no need to perform biological characterisation of every single member of the library. It is widely accepted that constituents possessing reasonable biological activity could be selected for further biological investigations by determining their retention factors (13,14).

Micellar electrokinetic chromatography (MEKC), where micellar solution of anionic surfactant (SDS) was applied in combination with electrophoresis has

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been introduced by Terabe in 1984 (15). This new method extended the possibilities of capillary electrophoresis for the analysis of compounds exhibiting no electric charge and it was possible to differentiate between the analytes on the basis of their hydrophobicity.

MEKC has been gaining an ever increasing importance in characterisation of hydrophobicity of molecules in the last years (14–18). Besides the practical benefit of this rapid separation method to obtain hydrophobicity parameter on a relatively easy and quick way, MEKC offers other advantages over the shake-flask method. Properties of drugs controlling pharmacokinetic characteristics are determined both by the polar groups and the hydrophobic part of the molecule. The binding of a drug molecule to the receptor is mediated by several parameters, including ion-ion interactions, hydrogen bonding, dipole-dipole interaction, hydrophobicity (19). Biomembranes are much different from the 1-octanol/water system if one considers the rigidity, anisotropy, and amphiphilic properties of the membrane structure. The 1-octanol/water system is now considered to be a relatively poor model for investigation of the membrane/drug interactions and to characterize expected biological activity of the drugs (13). Therefore, application of alternative methods, e.g., liposomes (20), cells (21), and micellar systems are proposed to characterize drug-membrane interactions (13).

Micellar systems may offer both ionic and hydrophobic sites for interaction. The composition of the micellar (pseudo-stationary) phase can easily be varied by changing the type of the surfactant (e.g. by varying the hydrophobic tail, or hydrophilic head groups of the synthetic surfactants, or by using bile acids, or surfactants with structure similar to the phospholipids) (14,16–18). The physicochemical properties of the interior core of the micelles can be changed in a versatile way and MEKC offers an easily variable model for investigation of different types of interactions occurring in the biological systems (13,22,23).

Retention in MEKC is originated from the distribution (partition of analyte between the polar aqueous phase and the apolar micellar pseudophase). Migration times of the analysed components depend upon their partition between the two phases. Neutral analytes can be separated, providing they differ in their hydrophobicity. Since the solubilization (the interaction of solute with micelles) operates as a distribution process, the hydrophobicity of the analyte governs the retention. Retention data involves the information related to the interaction between the solute and micelles, and finally to the hydrophobicity of the analyte (24,25).

Retention of the analyte was characterized by the capacity factor

$$k'' = (t_m - t_0)/(t_0(1 - t_m/t_{mc})) \quad (1)$$

where t_m, t_o, and t_{mc} are the migration time of the analyte, the flow marker, and the micelles, respectively (15).

All these advantages of MEKC promote its application in the quantitative characterisation of the hydrophobicity (especially in the case of combinatorial

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libraries) and in modeling of drug/membrane interactions (13). The question is how to deduce a useful reliable characteristic parameter from the experimental (migration) data relevant to the hydrophobicity of the analysed molecule.

A normalised retention factor

$$[k''' = (t_m - t_0)/(t_{mc} - t_0) \quad (2)$$

was introduced in our earlier works to characterize retention of the analyte in MEKC (8,26).

In the present work, a method has been developed to determine phase residence times (time spent by the analyte in the aqueous- [mobile] and micellar [pseudo stationary] phases). Two physico-chemical parameters characterising the hydrophobic nature of the analyte, namely the distribution ratio (ratio of the number of moles in the micellar phase and those of in the aqueous phase), and the distribution coefficient (K) have also been determined from the phase residence times. Correlation between computer calculated hydrophobicity values and micellar phase residence times was investigated, too. It was demonstrated that physico-chemical characteristics (distribution ratio, distribution coefficient) of the analyte can be measured in MEKC by determining phase residence times. Micellar phase residence times of the analytes investigated here were compared with computer predicted (calculated) hydrophobicity values of the same analytes.

CALCULATION METHODS

Calculation of the Phase Residence Times

Residence times of the analyte (time spent by the analyte in the aqueous or in the micellar phase) can be calculated on the basis of the k'' value. The train of thought to obtain these parameters was as follows:

The analyte travels with the velocity of the micelles while staying in the micellar phase, and it travels with the velocity of the electroosmotic flow while staying in the aqueous phase. Let us suppose that "l" is the length of the capillary (to the detector), t_o is the migration time of the component travelling with the speed of the electroosmotic flow, t_{mc} is the migration time of the micelles, and t_m is the migration time of the analyte. In this case:

velocity of the electroosmotic flow: $v_{eof} = l/t_0$

velocity of the micelles: $v_{mc} = l/t_{mc}$

velocity of the analyte: $v_a = l/t_m$

Let us suppose that the analyte (having a migration time of t_m) spends X fraction of its whole migration time in the micelles and spends $(1 - X)$ portion of

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its whole migration time in the aqueous phase. In this case:

$$\text{micellar phase residence time: } t_{mic} = Xt_m \tag{3}$$

$$\text{aqueous phase residence time: } t_{aq} = (1 - X)t_m \tag{4}$$

Distances covered by the analyte while it stayed in the micellar or aqueos phase can be obtained by multiplying the velocities with the relevant times:

distance covered by the analyte while it stayed in the micellar phase: $X\,t_m\,(l/t_{mc})$

distance covered by the analyte while it stayed in the aqueous phase: $(1 - X)t_m\,(l/t_0)$

The sum of the two distances is equal to "l":

$$(1 - X)t_m(l/t_0) + Xt_m(l/t_{mc}) = 1$$

simplifying and rearranging the equation:

$$X = t_{mc}(t_m - t_0)/[t_m(t_{mc} - t_0)] \tag{5}$$

That means, ratio of the micellar phase residence time of the analyte and the whole migration time can be calculated on the basis of the migration times determined experimentally (if $t_m = t_0$, then $X = 0$, if $t_m = t_{mc}$, then $X = 1$). Multiplying by 100 micellar residence time can be given as percentage of the whole migration time(100 X), while the product 100 $(1 - X)$ gives the residence time in the aqueous phase in percentage.

Knowing the value of X, inserting equation (5) into the equation (3) and applying equation (2) the time spent by the analyte inside the micelle (t_{mic}, micellar residence time) can be expressed:

$$\begin{aligned} t_{mic} &= X\,t_m = t_m t_{mc}(t_m - t_0)/[t_m(t_{mc} - t_0)] \\ &= t_{mc}(t_m - t_0)/(t_{mc} - t_0) = t_{mc}k'' \end{aligned} \tag{6}$$

The time spent by the analyte in the aqueous phase (t_{aq}) can be expressed by substituting the expression $(1 - X)$ into the equation (4):

$$\begin{aligned} t_{aq} &= (1 - X)\ \ t_m = t_m - t_m\ \ t_{mc}\ \ (t_m - t_0)/[t_m(t_{mc} - t_0)] \\ &= t_m - t_{mc}\ \ (t_m - t_0)/(t_{mc} - t_0) = t_m - t_{mc}\ \ k''' \end{aligned} \tag{7}$$

Inserting k''' [from equation (2)] into the right side of the Equation (7) time spent by the analyte in the aqueous phase can be obtained:

$$\begin{aligned} t_{aq} &= t_m - t_{mc}\ \ k''' = t_m - t_{mc}\ \ [(t_m - t_0)/(t_{mc} - t_0)] \\ &= t_0\ \ [(t_{mc} - t_m)/(t_{mc} - t_0)]. \end{aligned}$$

Because $(t_{mc} - t_m)/(t_{mc} - t_0) = 1 - k''$, aqueous phase residence time (t_{aq}) can be expressed:

$$t_{aq} = t_0\ \ [(t_{mc} - t_m)/(t_{mc} - t_0)] = t_0\ \ (1 - k'') \tag{8}$$

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Sum of the phase residence times:

$$t_{aq} + t_{mic} = (1 - X)\ t_m + Xt_m = t_0(1 - k'') + t_{mc}\ k'' \quad (9)$$

Equation (2) can be written in the following form:

$$t_m = t_0(1 - k''') + t_{mc}\ k'' \quad (10)$$

or:

$$t_m = t_0 + k'''(t_{mc} - t_0) = t_0 + k''W \quad (11)$$

where W is the width of the migration window ($W = t_{mc} - t_o$).

From the equations (10) and (9) it follows, that

$$t_m = t_0(1 - k''') + k'''t_{mc} = t_{mic} + t_{aq} \quad (12)$$

which means, that

a) residence times of the analyte can be determined knowing the value of k'': micellar residence time of the analyte having a retention factor of k'' is

$$t_{mic} = t_{mc}\ k'', \quad \text{while aqueous residence time of this analyte is } t_{aq} = t_0(1 - k'');$$

b) sum of the residence times gives the whole migration time of the analyte.

Ratio of the micellar and aqueous residence times gives:

$$t_{mic}/t_{aq} = t_{mc}\ k''/[t_o(1 - k''')] = [t_{mc}\ (t_m - t_o)/(t_{mc} - t_o)]/[t_o(1 - (t_m - t_o)/(t_{mc} - t_o)] \quad (13)$$

simplifying we can obtain:

$$t_{mic}/t_{aq} = t_{mc}\ k''/[t_o(1 - k''')] = (t_m - t_o)/(t_o\ (1 - t_m/t_{mc})) \quad (14)$$

As it is proven in ref. (24):

$$(t_m - t_o)/(t_o(1 - t_m/t_{mc})) = n_{mc}/n_{aq} \quad (15)$$

therefore

$$t_{mic}/t_{aq} = n_{mc}/n_{aq} \quad (16)$$

where n_{mc} and n_{aq} are the number of moles of the analyte in the micellar and aqueous phases, respectively. This means, that distribution ratio (ratio of the number of analyte molecules in the micellar and aqueous phases) can also be deduced from the k'' values.

Distribution coefficient (K) of the analyte can also be calculated (24,27):

$$t_{mic}/t_{aq} = n_{mc}/n_{aq} = K\,V_{mc}/V_{aq} = K\,v(C_{sf} - CMC)/[1 - v(C_{sf} - CMC)] \quad (17)$$

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where K is the distribution coefficient, V_{mc} and V_{aq} are the volumes of the micellar and aqueous phases, respectively, C_{sf} is the total concentration of the surfactant in the background electrolyte, CMC is the critical micellar concentration of the surfactant, v is the partial specific volume of the micelle (24,27).

Application of the Calculation Method

Phase residence times (t_{mic} and t_{aq}) of hydroxy- and aldehyde derivatives of benzene and naphthalene, those of alkyl benzene and alkyl phenone homolog series, as well as those of seven protected peptides, has been calculated from MEKC experimental data with the method shown in Calculation Methods. Phase residence times, k'' values, migration times, and computer calculated hydrophobicity, of these compounds were summarised in Table 1. The calculation method of software predicted the hydrophobicity of the compounds investigated previously (8). These software calculated hydrophobicity values are designed in the following as S values in the text.

Phase residence time functions (t_{mic} and t_{aq}) derived in Calculation Methods. are shown in Figure 1. The higher the k'' value the longer the micellar phase residence time, and at the same time the shorter the aqueous phase residence time. Both the micellar- and aqueous phase residence times are linear function of the retention factor (k''). Micellar phase residence time values go from zero to t_{mc}; t_{mic} is equal to zero if $k'' = 0$ and it is equal to t_{mc} if $k'' = 1$. Aqueous phase residence time values go from t_0 to zero; t_{aq} is equal to t_0 if $k'' = 0$ and it is equal to zero if $k'' = 1$. It means (considering that the whole migration time of the analyte is the sum of the micellar- ad aqueous phase residence times ($t_m = t_{mic} + t_{aq}$, [equation 12]), that an analyte having k'' value of zero always stays (spends its whole migration time) in the aqueous phase, while an analyte having k'' value of 1 always stays (spends its whole migration time) in the micellar phase.

The phase residence times versus the k'' values for benzene derivatives are shown in Figure 2. The phase residence time values show, that the analytes prefer to stay in the hydrophobic micellar phase more and more as their k'' values increase. The higher the k'' value the longer the time spent by the analyte in the micellar (pseudo stationary) phase and at the same time the shorter the time spent by the analyte in the aqueous (mobile) phase. The sum of the phase residence times is always equal to the whole migration time of the same analyte ([$t_{aq} + t_{mic} = t_m$], section A in Table 1). Similar results were obtained in the case of the benzene- and phenone homolog series and in the case of the seven hydrophobic peptides analysed here (sections B, C, and D in Table 1).

Micellar phase residence times of the analytes determined experimentally by the MEKC method have been compared with computer predicted (calculated) hydrophobicity (S) values of the same analytes. Good linear relationship has been

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Table 1. Name or Structure, Aqueous- and Micellar Phase Residence Times (t_{aq}, t_{mic}), Retention Factors (k'''), Computer Calculated Hydrophobicty (S) and Migration Time (t_m) of the Compounds Investigated

No.	Compounds	t_{aq}[5] (min)	t_{mic}[6] (min)	k'''[7]	S[8]	t_m (min)
A[1]	**Benzene Derivatives**					
1	Benzene	2.43	2.61	0.262	1.99	5.04
2	Naphthalene	0.51	8.41	0.841	3.17	8.92
3	Toluene	1.63	5.02	0.502	2.54	6.65
4	Benzaldehyde	2.37	2.78	0.279	1.71	5.15
5	Phenol	2.77	1.57	0.157	1.51	4.34
6	Rezorcine	2.97	0.96	0.096	1.03	3.93
B[2]	**Benzene Homolog Series**					
7	Benzene	2.73	2.96	0.231	1.99	5.69
8	Toluene	1.89	5.99	0.467	2.54	7.88
9	Ethylbenzene	1.11	8.83	0.687	3.03	9.94
10	Propylbenzene	0.47	11.17	0.869	3.52	11.64
C[3]	**Phenone Homolog Series**					
11	Acetophenone	2.19	4.13	0.361	1.59	6.33
12	Propiophenone	1.53	6.35	0.554	2.08	7.88
13	Butyrophenone	0.87	8.54	0.745	2.57	9.41
14	Valerophenone	0.40	10.13	0.884	3.06	10.53
D[4]	**Hydrophobic Protected Peptides**					
15	$(Z\text{-}Pro)_2$-Lys-Ome	4.26	9.61	0.239	3.78	13.87
16	[BOC-Asp(OBzl)]-Lys(Z)-OtBu	4.11	10.69	0.266	5.58	14.80
17	$[BOC\text{-}Pro\text{-}Glu(OBzl)]_2$-Lys-Ome	3.93	11.98	0.298	5.75	15.91
18	$[BOC\text{-}Pro\text{-}Pro\text{-}Glu(OBzl)]_2$-Lys-OMe	3.90	12.18	0.303	6.60	16.08
19	$[BOC\text{-}Glu(OBzl)]_2$-Lys-Ome	3.87	12.42	0.309	4.99	16.29
20	$[BOC\text{-}Asp(OBzl)\text{-}Glu()Bzl)_2$-Lys-OMe	3.82	12.78	0.318	6.66	16.60
21	FMOC-Glu[Lys(Z)OtBu]$_2$	2.40	22.99	0.572	9.82	25.39

[1]t_m values of benzene- and naphtalene derivatives were published by Muijselaar and coworkers in ref. 28; $t_0 = 3.29$ min, $t_{mc} = 9.96$ min; RSD value of the migration time data was less, than 2%. [2]t_m values of alkylbenzenes were published by Muijselaar and coworkers in ref. 28; $t_0 = 3.55$ min, $t_{mc} = 12.85$ min; RSD value of the migration time data was less, than 2%. [3]t_m values about phenones were published by Muijselaar and co-workers in ref. 28; $t_0 = 3.42$ min, $t_{mc} = 11.46$ min. [4]t_m values were determined in: ref. 29; $t_0 = 5.6$ min, $t_{mc} = 40.2$ min, RSD value of the migration time data was less, than 2%. [5]t_{aq}: time spent by the analyte in the aqueous (mobile) phase were calculated by the Equation (8). [6]t_{mic}: time spent by the analyte in the micellar (pseudo stationary) phase were calculated by the equation (6). [7]k'' values calculated with calculated by the equation (2). [8]computer calculated hydrophobicity (S) values were determined according to the method given in ref. 8.

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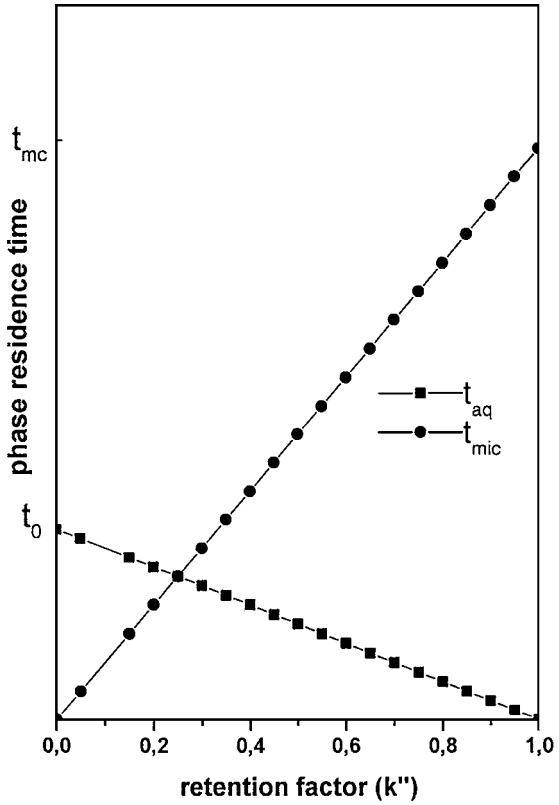

Figure 1. Micellar- (t_{mic}) and aqueous (t_{aq}) phase residence time functions versus the k''' values. Abscissa: k''', ordinate: phase residence time (min). t_{mic} is equal to zero if $k''' = 0$, and it is equal to t_{mc} if $k'' = 1$. t_{aq} is equal to t_0 if $k''' = 0$ and it is equal to zero if $k''' = 1$.

found between the computer calculated hydrophobicity (S) and the micellar phase residence time (t_{mic}) of the analytes investigated. The higher the calculated hydrophobicity of the analyte, the longer the micellar phase residence time. Figure 3 shows the calculated hydrophobicity—micellar phase residence time (S–t_{mic}) correlation in the case of benzene derivatives (correlation factor $r = 0.9684$, section A in Table 1). Similar to the S value, the micellar phase residence time reflects the structural changes of the molecule. Substitution of the $-CH_3$ group of the toluene by aldehyde- or hydroxy group diminishes the hydrophobicity (S) of the molecule, and because of this substitution, the micellar phase residence time (t_{mic}) also diminished (see data of toluene, benzaldehyde and phenol in section A, Table 1). Building of another hydroxy group into the molecule reduces further the

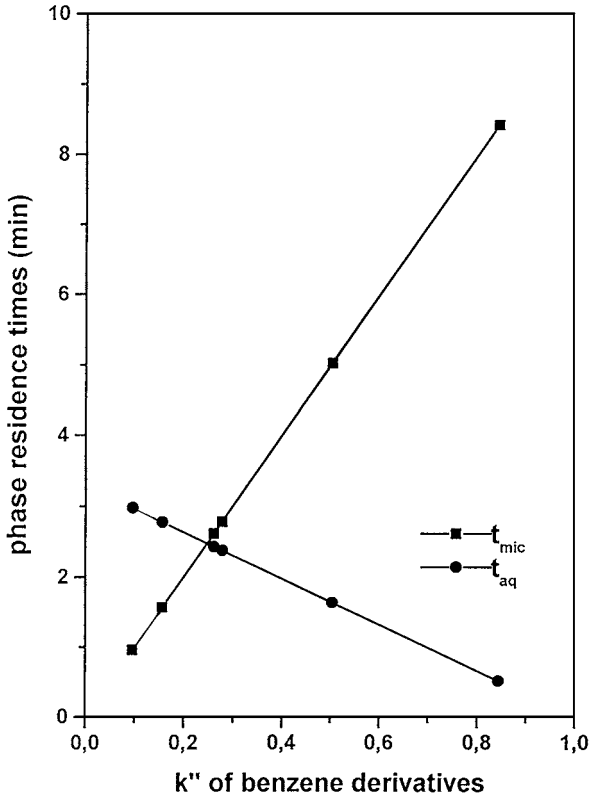

Figure 2. Micellar (t_{mic}) and aqueous (t_{aq}) phase residence times versus the k‴ values for the benzene derivatives (section A in Table 1). Abscissa: k‴ values determined by MEKC (for experimental details see Table 1). Ordinate: phase residence times in minutes (for data see Table 1), t_{mic} and t_{aq} values were calculated according to the equation (6) and (8), respectively).

hydrophobicity and, hence, the micellar phase residence time of the molecule, too (see rezorcin, Table 1).

Similarly, a good connection was found between the hydrophobicity and micellar phase residence time in the case of alkylbenzene homolog series (regression coefficient r = 0.9984, section B in Table 1). The longer the alkyl chain connected to the aromatic ring, the higher the hydrophobicity of the molecule and, consequently, the longer the micellar phase residence time.

A good linear relationship was found in the case of phenone homolog series (regression coefficient r = 0.9981, section C in Table 1) and, also, for the protected peptides (regression coefficient r = 0.9263, section D in Table 1).

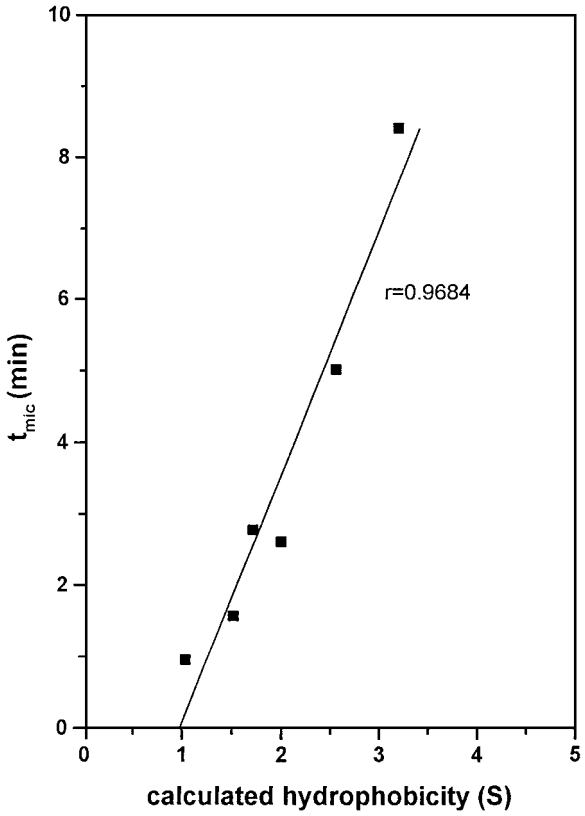

Figure 3. Micellar phase residence times (t_{mic}) versus the calculated hydrophobicity (S) values for the benzene derivatives (section A in Table 1). Abscissa: computer calculated hydrophobicity (S) values. Ordinate: micellar phase residence time in minutes; t_{mic} values were calculated according to the equation (6) and (8), respectively.

CONCLUSIONS

These results showed that micellar electrokinetic chromatography is an experimental method suitable to characterize the hydrophobic nature of the molecules investigated. Phase residence times (times spent by the analyte in the aqueous- and in the micellar phase [t_{aq} and t_{mic}, respectively]) can be determined in MEKC. The migration time of the analyte (t_m) can be combined from the migration times characteristic for the MEKC system (t_0 and t_{mc}), as it is given in equation (10), and the migration time of the analyte is the sum of the phase residence times (equation 12). The ratio of the moles of the analyte in the micellar and aqueous phase (n_{mc}/n_{aq}) can be determined by the expression t_{mic}/t_{aq} (equation 16).

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Knowing this ratio, the distribution coefficient (K) of the analyte can also be determined (equation 17). Micellar phase residence times change, as expected, considering the chemical structure of the analytes; they also correlate well with the calculated hydrophobicity values (S) of the same analytes. The micellar phase residence time (t_{mic}) is an experimental parameter obtained from MEKC which characterizes the hydrophobicity of the analyte in a scale extending from zero to t_{mc}. The higher the t_{mic}, the higher the hydrophobicity of the analyte. It means, that applying MEKC, a parameter (micellar phase residence time) characterizing hydrophobicity of the analyte can be experimentally determined.

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