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Role of stationary phase and eluent composition on the determination of log *P* values of *N*-hydroxyethylamide of aryloxyalkylen and pyridine carboxylic acids by reversed-phase high-performance liquid chromatography

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Abstract

The partition coefficients, *P*, between *n*-octanol and water of a number of growth stimulating substances, *N*-hydroxyethylamide of aryloxyalkylen- and pyridine carboxylic acids were obtained from Pomona College (*C* log *P*), and Rekker's (log P_{Rekker}) revised fragmental constant system was used to calculate log *P* data sets. Both of these data sets were correlated with two different substance lipophilicity parameters, log k_w and φ_0. Log k_w was obtained by extrapolation of log retention factor (*k*) to 0% organic modifier measured in reversed-phase liquid chromatography (RPLC) systems. φ_0 values were obtained from the slopes and intercepts of these relationships. The RPLC experiments were performed on four commercially available reversed-phase columns. Binary mixtures of methanol–water, methanol–phosphate buffer (pH 7.0), methanol–tricine buffer (pH 7.0) and acetonitrile–water were used as mobile phases for the determination of log k_w values. For the methanolic eluents linear regression provided satisfactory correlations ($r>0.99$) for the relationships log *k* vs. organic modifier content in the eluent, while for the acetonitrile-containing eluents a second-degree polynominal regression was necessary. For all four RPLC columns, by linear regression satisfactory correlations ($r>0.99$) were obtained between log k_w and log *P* data using methanolic eluents. In such eluents φ_0 values were shown to be the second-best lipophilicity parameters. For acetonitrile-containing eluents the use of second-degree polynominal regression was necessary and, in contrast to methanol, significant influence of the applied column on regression results was observed. For acetonitrile-containing eluents the φ_0-index does not provide satisfactory results for our substances. No difference in regression results between the use of buffered and non-buffered eluents was observed.

Keywords: Stationary phases, LC; Mobile phases, LC; *N*-Hydroxyethylamide; Aryloxyalkylen carboxylic acid; Pyridine carboxylic acid

1. Introduction

The partition coefficient (log *P*) of a substance in a two-phase system consisting of *n*-octanol–water is

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often used to predict or to correlate its biological activity. Fujita et al. has proposed the *n*-octanol–water partition coefficient ($P_{o/w}$) as a standard measure of hydrophobicity of substances [1]. The determination of log *P* values of substances with potential biological activity by reversed-phase liquid chromatography (RPLC) can, in principle, overcome the difficulties of the conventional 'shake-flask' method. The direct measurement of $P_{o/w}$ values by the latter method faces different problems as the necessary high purity of substances, which must be available in reasonable quantities and the fact that the method is not applicable to very hydrophilic or very hydrophobic compounds. In addition, this method is rather time consuming too. Since the first research of Meyer [2] and Overton [3], several techniques have been developed to determine substance lipophilicity experimentally, by the 'shake-flask' method and by alternative approaches like, for example, chromatographic methods [4,5]. In a recent review these techniques were thoroughly reviewed [6].

For some time RPLC is proposed as an alternative method for log $P_{o/w}$ determination, showing distinct advantages as speed of determination and better reproducibility compared to conventional methods. Furthermore, only small amounts of even contaminated samples are sufficient to use RPLC for this purpose [7–12]. Many researchers have discussed the use of RPLC as an attractive alternative method for the determination of log $P_{o/w}$ values using correlations between chromatographic data and the corresponding log $P_{o/w}$ values from other sources, e.g. shake-flask experiments or calculations. At present a substantial number of papers can be found in the literature reporting the use of RPLC to establish octanol–water partition coefficients, with correlation coefficients in the range 0.5–0.999, depending on the applied column and compounds under investigation [13–19].

Still there is debate on and to what extent the various parameters in RPLC, like, for example, nature of the reversed-phase (RP) stationary phase and composition of the eluent, are of influence on log $P_{o/w}$ determination by RPLC. For instance, Brauman et al. [9] found that for neutral components the extrapolated to 100% water log k_w-data are generally not influenced by the specific nature of the RPLC phase. Conversely Abraham et al. [20] summarised and recalculated a substantial number of RPLC data for log *P* determination, observing a significant influence of the stationary phase nature on log *P* determination for non-congeneric classes of compounds.

In the same study, Abraham et al. also concluded that data obtained from certain RPLC packings were unsuitable for log $P_{o/w}$ determination and better correlated with other, e.g. log w/c (water, cyclohexane) partition coefficients. This conclusion is also supported by Helweg et al. [12] observing that for specific environmental pollutants a diol modified silica packing provided better log *k*–log *P* correlations compared to data obtained on a C_{18} silica. They concluded that by their hydrogen bonding potential such diol columns better mimics the octanol/water partition for azaarenes compared to C_{18} columns.

Valkó [21] found poor correlations too, especially for groups of structurally unrelated compounds, between log *P* and log k_w values determined by RPLC. Grouls [11] reports, however, for a rather unrelated group of compounds satisfactory correlations between log *P* and log *k* values in their experiments. In view of the often significantly different properties between RP-phases, these observations are easily explained. The number and nature of residual silanol groups, the length of the hydrocarbon chain (e.g. C_8 or C_{18}), the bonding chemistry and the technology involved to produce the silica or other substrates, which determine pore size distribution and specific surface area, have great impact on the final properties of RP packings [22,23].

In several papers it is shown that retention data obtained on RPLC columns from different sources under further identical experimental conditions are difficult to compare and to correlate with stationary phase properties [23–26]. The question whether RPLC and under which conditions reasonably reflects the octanol/water distribution process remains till now subject of dispute [27]. Another part of the discussion concerns which chromatographic parameters like, for example, the logarithm of retention factors or the value extrapolated to 100% water (log k_w) fit best to calculated or experimentally determined log *P* values. Recently Valkó et al. [28,29] introduced two other retention related parameters to measure solute's lipophylicities, namely an isocratic

chromatographic hydrophobicity index (φ_0) and CHI, a chromatographic hydrophobicity index obtained under gradient conditions. In these studies it is claimed that opposite to log k_w-measurements φ_0 values in the absence of secondary retention mechanisms are independent of the nature of the stationary phase. Good correlations between log P and φ_0 for acetonitrile and methanol-containing eluents were reported in these studies.

In many discussions on log P measurements by RPLC the impact of other experimental conditions, rather than the RP-phase like the composition of the eluent (e.g. buffered or non-buffered) and the nature of the organic modifier is somewhat underestimated. Especially, for polar and ionic substances this may cause a problem, since such compounds are prone to secondary interaction mechanisms to the RP-phase. In such cases the rate of (de)protonation of compounds may not be well defined and constant, and the same is true for residual silanols and other possible active groups at a stationary phase surface resulting in unreliable and irreproducible data.

Since we are interested in the biological activity of a specific group of growth stimulating substances, we started a study to establish a rapid and reliable RPLC method for the log $P_{o/w}$-determination of these compounds and their future derivatives. To investigate the possible influence of the nature of organic modifier two eluents consisting of methanol and acetonitrile/water mixtures were used for log k_w and φ_0 determination. Since our substances contain nitrogen in their molecular structure we also included two aqueous–methanol pH 7.0 buffers in this study, viz. a phosphate and a tricine buffer. This, to investigate a possible influence of (de)protonation of the substances and silanol activity on log P-measurements by RPLC. Log k_w and φ_0 measurements were performed on four different commercially available RPLC phases.

We obtained C log P values from Pomona College and used Rekker's revised fragmental constant system too for the calculation of log P_{Rekker} data of the substances [30–32]. Log k_w values were obtained from the RPLC measurement by extrapolation to 100% water or buffer. The correlations between the experimentally obtained log k_w and φ_0 values, and the log P data are discussed and related to the nature of the applied stationary and mobile phases.

2. Experimental

The structures of the studied compounds, eleven *N*-hydroxyethylamide of aryloxyalkylen- and pyridine-carboxylic acids (F1–F11) are shown in Fig. 1. These are new compounds synthesised by the Organic Chemistry Department (Faculty of Chemistry and Chemical Engineering, Babes-Bolyai University, Cluj-Napoca, Romania) and have shown growth stimulating activity in the Moewus test with *Lepidium sativum* [33]. The substances were prepared as 0.1 mg/ml solutions in methanol.

All solvents were of gradient grade. Methanol was obtained from Merck (Darmstadt, Germany) and acetonitrile from Biosolve (Valkenswaard, Nether-

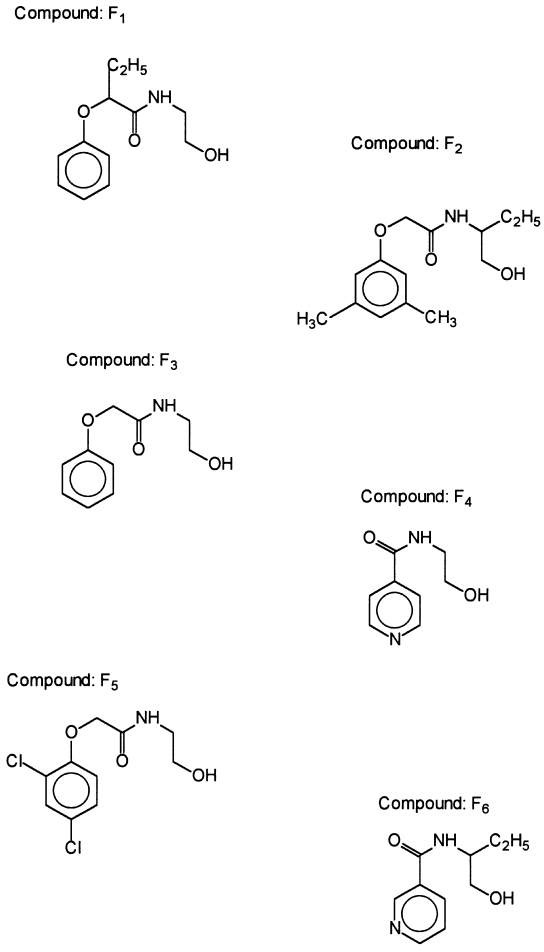

Fig. 1. Compound structures.

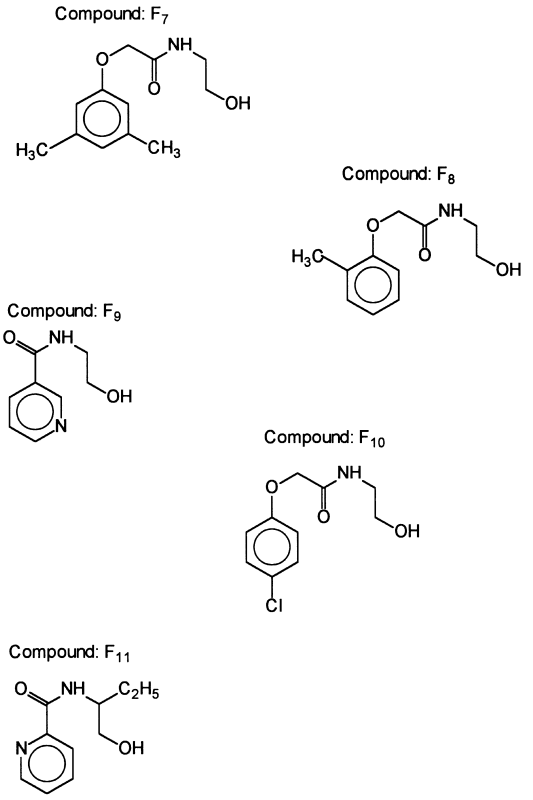

Fig. 1. (*continued*)

lands). Deionised water was prepared by a Milli-Q Water Purification System (Millipore, Bedford, MA, USA). 1-octanol for the experimental determination of partition coefficients, *P*, in octanol–water and octanol–phosphate buffer systems, was obtained from Merck (Darmstadt, Germany). Sodium dihydrogenophosphate ($NaH_2PO_4 \cdot 2H_2O$) from Vel (Leuven, Belgium) and Tricine (*N*-tris[hydroxymethyl]methylglycine) from Sigma (St. Louis, MO, USA) were used to prepare buffer pH 7.0 solutions by adjusting with NaOH.

2.1. Determination of the log k_w- and φ_0 values

For obvious reasons for these measurements it must be sure that the substances were not ionized. Therefore, *k*-values of the compounds vs. the pH of a series of eluents, comprising of phosphate buffers in the pH 2–9 range were measured according to a procedure described in [34]. The sigmoidal curves revealed that all compounds were unionized in the pH range 7.0–7.5. To preserve column lifetime, especially with respect to the phosphate buffers, pH 7.0 was selected for further studies.

Retention factors were determined for the F1–F11 substances by RPLC using the following binary solvent systems as mobile phases: methanol–water, methanol–phosphate buffer (20 m*M*, pH 7.0), methanol–tricine buffer (20 m*M*, pH 7.0) and acetonitrile–water. The measurements were performed starting with 60% methanol or 50% acetonitrile, respectively, gradually decreasing to 0% organic modifier, in cases where this was possible. For the less hydrophobic compounds, 8 measurements were performed with 60, 50, 40, 30, 20, 10, 5 and 0% organic modifier in the mobile phase. Each measurement was performed in triplicate and the mean value was used for calculations. The log k_w values were obtained by extrapolation to 0% organic modifier by determining linear or quadratic relationships between log *k* values and the concentration of the organic modifier (methanol or acetonitrile) in the eluent.

According to Hsieh and Dorsey [35] for accurate log k_w measurements for the methanolic eluents linear regression between log *k* and volume percentage modifier was applied. This is in contrast to acetonitrile-containing eluents, where a second polynominal function much better describes such relationships. Therefore, to determine log k_w values by extrapolation we used Eqs. (1) and (2):

$$\log k = A + B\varphi \quad (1)$$

$$\log k = A + B\varphi + C\varphi^2 \quad (2)$$

where *A*, *B* and *C* are fitting constants and φ is volume portion of modifier.

Hydrophobicity values φ_0 were calculated from the slope (*S*) and the intercept of the straight line (*I*) according to [29].

$$\varphi_0 = -I/S \quad (3)$$

unlike the extrapolation of log k_w values, *I*- and *S*-values were calculated from the data points close to log $k=0$ in the plots, which is equivalent to retention times that are twice the column dead time. In this approach it was assumed that the absence of secondary retention mechanisms the φ_0-values are

rather independent of the nature of the reversed-phase column [28].

2.2. Instrumentation

The instrumentation included a Beckman pump Model 100A (Beckman, Fullerton, CA, USA), a Merck-Hitachi AS-2000A autosampler (Merck, Darmstadt, Germany), an UV-VIS Philips detector (ATI Unicam, Cambridge, UK) and a PE Nelson 900 Interface (Perkin Elmer, San Jose, CA, USA). The detection wavelength was 254 nm, the flow-rate 1 ml/min and the amount of sample solution was 10 μl/injection. Four different types of reversed-phase columns were used for the measurements of the log k and log k_w values under the same experimental conditions: LiChrosorb® RP-18 (5 μm, $L=125$ mm, I.D.=4 mm), LiChrospher® 60 RP-Select B (5 μm, $L=12.5$ cm, I.D.=4 mm) (Merck, Darmstadt, Germany), Zorbax® Rx-C_{18} (5 μm, $L=15$ cm, I.D.=4.6 mm) and Zorbax®-Eclipse® XDB-C_{18} (5 μm, $L=15$ cm, I.D.=4.6 mm) (Hewlett Packard, Newport, DE, USA). In this study the dead time, t_0, was measured for each experimental condition, using uracil as unretained compound.

3. Results and discussion

The calculated log P values (C log P) for the 11 (*N*-hydroxyethylamide of aryloxyalkylen- and pyridine-carboxylic acids) compounds were obtained from *P*omona College (Claremont, CA, USA) [32].

The log P values of these compounds were also calculated using Rekker's revised fragmental constant system (log P_{Rekker}) [30,31]. Each calculated log P value was obtained by the addition of the corresponding fragmental constants and the necessary corrections expressed as 'magic constant' $C_M=0.219$, as shown in Eq. (4)

$$\log P = \sum a_n f_n + mC_M \quad (4)$$

where, f is the hydrophobic fragmental constant type n characterising the lipophilicity contribution of a constituent part of a structure to the total lipophilicity, and a is a numerical factor indicating the incidence of a given fragment in the structure; m is the number of the necessary C_M corrections and was applied using the rules of adding 2 C_M for two polar groups separated by two aliphatic carbons, 3 C_M for two polar groups separated by an aliphatic carbon, 1 C_M for extra chain-branching or 'ortho' substituent and 1 C_M for intra-molecular hydrogen-bonding.

The calculated log P values, from Pomona College and from Rekker's system, do not take into account the substituent position in the aromatic ring regarding the N heteroatom. As a consequence, the calculated log P values for compounds F4 and F9 are identical: −0.453 (Pomona College) and −0.559 (Rekker's system), respectively. For similar reasons, compounds F1 and F7 should have the same log P value of 1.260 using Rekker's system having similar aliphatic substituents, but in different positions. However, an extra C_M was included in the log P value of compound F7 due to chain-branching. In Table 1 the C log P and log P_{Rekker} values of the compounds are summarised.

In order to quantify the relationships between both log P data sets, the mutual correlations between C log P and log P_{Rekker} values were determined for 95% confidence limits:

$$\log P_{\text{Rekker}} = 1.022(\pm 0.039)\, C \log P - 0.094(\pm 0.049)$$

$$s_{a0}=0.022,\ s_{a1}=0.017,\ =3546.34,\ r=0.998 \quad (5)$$

where s_{a0} and s_{a1} are the standard errors for the intercept and the slope, respectively, F is the parameter for F-distribution and r is the correlation coefficient. Excellent correlation was found for Eq. (5) indicating that both data sets are applicable in our study.

Especially for ionisable and polar compounds the use of non-buffered eluents in RPLC is a matter of discussion. This, because of the eventually undefined rate of dissociation of residual silanols of the stationary phase and compounds (de)protonation, and the consequent risk of local differences in chemical equilibrium in the case of non-buffered eluents. Some authors in this field, however, use non-buffered aqueous-organic eluents for log P measurements of such compounds [20]. Therefore, for our compounds we studied the use of buffered vs. non-buffered eluents for comparison reasons.

Table 1
Log P values for 11 N-hydroxyethylamide of aryloxyalkylene- and pyridine carboxylic acids

No.	Compound	C log P (calculated value obtained from Pomona College)	Log P_{Rekker} (value calculated using Rekker's system)
1	F1	1.333	1.260
2	F2	2.331	2.299
3	F3	0.495	0.440
4	F4	−0.453	−0.559
5	F5	1.943	1.897
6	F6	0.385	0.261
7	F7	1.493	1.479
8	F8	0.994	0.959
9	F9	−0.453	−0.559
10	F10	1.348	1.168
11	F11	0.735	0.699

For the methanol–water and methanol–phosphate buffered eluents the log k_w-data together with the slope of the relationships log k vs. φ and statistical data for the four investigated columns are presented in Tables 2–5. Results were obtained by linear regression, Eq. (1). Inspection of Tables 2–5 shows that for each of the columns the differences in log k_w values measured in buffered and non-buffered sys-

Table 2
Log k_w values obtained on the LiChrosorb® RP-18 column, with methanol–water and methanol–phosphate buffer (20 mM, pH 7.0) as eluents

Compound	% Methanol	Log k_w (e) (a_0)	S (a_1)	r	s_{a0}	s_{a1}	$\pm a_0$	$\pm a_1$
Eluent: methanol–water								
F1	60–10	2.205	−0.037	0.9987	0.037	0.001	0.102	0.003
F2	60–30	3.292	−0.047	0.9981	0.095	0.002	0.407	0.009
F3	50–0	1.695	−0.037	0.9959	0.042	0.001	0.109	0.004
F4	50–0	0.830	−0.037	0.9905	0.065	0.002	0.166	0.006
F5	60–30	2.804	−0.042	0.9979	0.089	0.002	0.385	0.008
F6	50–0	1.429	−0.038	0.9943	0.051	0.002	0.132	0.005
F7	60–20	2.626	−0.041	0.9982	0.059	0.001	0.187	0.004
F8	60–10	2.195	−0.038	0.9976	0.051	0.001	0.142	0.004
F9	50–0	0.851	−0.040	0.9896	0.073	0.003	0.189	0.007
F10	60–10	2.229	−0.038	0.9989	0.036	0.001	0.101	0.003
F11	60–5	1.881	−0.038	0.9907	0.083	0.002	0.213	0.006
Eluent: methanol–phosphate buffer								
F1	60–10	2.164	−0.037	0.9989	0.033	0.001	0.091	0.002
F2	60–30	3.321	−0.047	0.9987	0.079	0.002	0.342	0.007
F3	60–0	1.612	−0.034	0.9943	0.050	0.001	0.123	0.004
F4	50–0	0.775	−0.036	0.9913	0.059	0.002	0.153	0.005
F5	60–20	2.773	−0.041	0.9995	0.032	0.001	0.102	0.002
F6	50–0	1.337	−0.036	0.9918	0.058	0.002	0.149	0.005
F7	60–20	2.559	−0.040	0.9990	0.042	0.001	0.135	0.003
F8	60–10	2.151	−0.037	0.9976	0.050	0.001	0.140	0.004
F9	50–0	0.816	−0.039	0.9946	0.051	0.002	0.130	0.005
F10	60–10	2.154	−0.035	0.9967	0.057	0.001	0.157	0.004
F11	60–5	1.825	−0.036	0.9916	0.077	0.002	0.197	0.005

S = slope, r = regression coefficient. s_{a0} and s_{a1} are the standard errors; $\pm a_0$ and $\pm a_1$ are the 95% confidence limits; e = extrapolated.

Table 3
Log k_w values obtained using the Zorbax® Rx-C_{18} column, with methanol–water and methanol–phosphate buffer (20 m*M*, pH 7.0) as eluents

Compound	% Methanol	Log k_w (e) (a_0)	S (a_1)	r	s_{a0}	s_{a1}	$\pm a_0$	$\pm a_1$
Eluent: methanol–water								
F1	60–10	2.353	−0.039	0.9988	0.038	0.001	0.104	0.003
F2	60–30	3.577	−0.051	0.9990	0.074	0.002	0.319	0.007
F3	60–0	1.794	−0.036	0.9926	0.061	0.002	0.150	0.004
F4	40–0	0.541	−0.040	0.9924	0.056	0.002	0.154	0.007
F5	60–30	2.967	−0.044	0.9988	0.069	0.001	0.295	0.007
F6	40–0	1.447	−0.047	0.9911	0.070	0.003	0.195	0.009
F7	60–20	2.771	−0.043	0.9990	0.046	0.001	0.146	0.003
F8	60–10	2.281	−0.039	0.9987	0.048	0.001	0.153	0.004
F9	40–0	0.556	−0.040	0.9933	0.053	0.002	0.147	0.006
F10	60–10	2.261	−0.037	0.9994	0.026	0.001	0.071	0.002
F11	60–5	1.902	−0.038	0.9908	0.086	0.002	0.220	0.006
Eluent: methanol–phosphate buffer								
F1	60–10	2.348	−0.034	0.9981	0.047	0.001	0.130	0.003
F2	60–30	3.513	−0.049	0.9978	0.107	0.002	0.461	0.010
F3	60–0	1.768	−0.037	0.9912	0.067	0.002	0.165	0.005
F4	40–0	0.554	−0.040	0.9888	0.068	0.003	0.188	0.008
F5	60–30	2.930	−0.043	0.9978	0.094	0.002	0.403	0.009
F6	40–0	1.474	−0.048	0.9887	0.081	0.004	0.225	0.010
F7	60–20	2.727	−0.042	0.9986	0.054	0.001	0.170	0.004
F8	60–10	2.319	−0.040	0.9966	0.064	0.002	0.177	0.004
F9	40–0	0.575	−0.041	0.9893	0.067	0.003	0.186	0.008
F10	60–10	2.320	−0.039	0.9982	0.044	0.001	0.123	0.003
F11	60–5	1.916	−0.039	0.9887	0.096	0.003	0.246	0.007

S = slope, r = regression coefficient. s_{a0} and s_{a1} are the standard errors; $\pm a_0$ and $\pm a_1$ are the 95% confidence limits; e = extrapolated.

tems are rather small (maximally 7% for F6 on the Lichrosorb RP-18 column).

In order to check whether the nature of the buffer may have influence on the partition equilibrium of compounds between the stationary and mobile phase, additional experiments with a pH 7.0 tricine buffer were performed. Comparing these results (not shown) obtained on the Zorbax® Eclipse® XDB-C_{18} column with methanol–tricine buffer (20 m*M*, pH 7.0) with the results in Table 5, it could be concluded that both pH 7.0 buffer systems provided nearly the same log k_w results (maximal difference 5.3%). This shows that for this set of compounds under these conditions the use of buffered or non-buffered eluents is of minor, if at all, influence on log k_w-measurement by RPLC. The same is true for both buffers.

Since column stability and longevity can be significantly enhanced by organic buffers instead of using phosphate and other inorganic buffers [36], this was another reason to use tricine buffers in this study.

From Tables 2–5 it can further be concluded that for this set of substances significantly different log k_w values between these four RPLC columns are obtained. One could argue that these differences could be attributed to the different phase ratios of the columns. The variations in log k_w-values of the substances over the various columns do not support this assumption. In addition these variations strongly suggest that secondary retention interaction mechanisms are influencing the retention of certain substances. In most cases the largest differences in log k_w values were found between the Zorbax Eclipse XDB-C_{18} and the Lichrospher 60 RP-Select B columns. For the compounds F1, 2, 5, 7, 8, 10 and 11 differences in the range 10–16% were found. Compound F3 showed a difference in log k_w value

Table 4
Log k_w values obtained on the LiChrospher® 60 RP-Select B column, with methanol–water and methanol–phosphate buffer (20 m*M*, pH 7.0) as eluents

Compound	% Methanol	Log k_w (e) (a_0)	S (a_1)	r	s_{a0}	s_{a1}	$\pm a_0$	$\pm a_1$
Eluent: methanol–water								
F1	50–10	2.116	−0.037	0.9996	0.019	0.001	0.060	0.002
F2	50–30	3.322	−0.049	0.9993	0.073	0.002	0.9280	0.023
F3	50–0	1.529	−0.032	0.9969	0.032	0.001	0.082	0.003
F4	50–0	0.678	−0.034	0.9995	0.014	0.001	0.035	0.001
F5	50–20	2.734	−0.042	0.9996	0.029	0.001	0.126	0.003
F6	50–0	1.344	−0.035	0.9945	0.047	0.002	0.120	0.004
F7	50–20	2.563	−0.041	0.9996	0.029	0.001	0.125	0.003
F8	50–10	2.115	−0.037	0.9998	0.014	0.0005	0.045	0.001
F9	50–0	0.680	−0.031	0.9982	0.023	0.001	0.061	0.002
F10	50–10	2.116	−0.036	0.9999	0.010	0.0003	0.031	0.001
F11	50–5	1.772	−0.036	0.9985	0.029	0.001	0.081	0.003
Eluent: methanol–phosphate buffer								
F1	50–10	2.148	−0.037	0.9990	0.031	0.001	0.100	0.003
F2	50–30	3.248	−0.048	0.9999	0.014	0.0003	0.180	0.004
F3	50–0	1.510	−0.032	0.9969	0.032	0.001	0.082	0.003
F4	50–0	0.641	−0.031	0.9987	0.019	0.001	0.050	0.002
F5	50–20	2.661	−0.041	0.9999	0.004	0.0001	0.018	0.001
F6	50–0	1.320	−0.034	0.9947	0.045	0.002	0.115	0.004
F7	50–20	2.491	−0.040	0.9999	0.010	0.0003	0.045	0.001
F8	50–10	2.081	−0.037	0.9993	0.026	0.001	0.083	0.002
F9	50–0	0.643	−0.031	0.9969	0.030	0.001	0.077	0.003
F10	50–10	2.077	−0.035	0.9998	0.012	0.0004	0.038	0.001
F11	50–5	1.730	−0.035	0.9981	0.032	0.001	0.090	0.003

S = slope, r = regression coefficient. s_{a0} and s_{a1} are the standard errors; $\pm a_0$ and $\pm a_1$ are the 95% confidence limits; e = extrapolated.

up to 23% between these two columns and for compound F6 the largest difference was observed at 29% between the Eclipse and the Lichrosorb RP-18 columns. The compounds F4 and F9 showed the largest differences from 41 to 54% between the Lichrosorb RP18 and the Zorbax RX C_{18} column. These observations hold for buffered and non-buffered methanolic eluents as well, and no significant variations in log k_w values between these eluents exist. It was also observed that the differences in log k_w values between both the Zorbax packings were maximally 5%. The differences between both other stationary phases are much larger, e.g. up to more than 20% for the compounds F4 and F9.

Much more important is the impact of these observations on log P measurements by RPLC. It makes little difference whether or not the eluent is buffered confirming the non-ionized state found earlier for this group of compounds under these conditions. Next, the data of all extrapolated log k_w values obtained on buffered and non-buffered eluents were regressed vs. the C log P and log P_{Rekker}-data. In Fig. 2 as an example, the results and plots are shown for the C log P data regressed vs. the data obtained on the methanol–water eluents together with statistical significance. The other data are summarised in Table 6A–C. With one exception for all plots satisfactory correlation coefficients >0.99 were obtained.

From these data it is clear that no significant differences occur between the use of non-buffered vs. buffered eluents. Furthermore, it can be concluded that between the columns absolute differences of 0.2–0.6 log P units are obtained, emphasising the different chromatographic response of these stationary phases towards these compounds. All four col-

Table 5
Log k_w values obtained on the Zorbax® Eclipse® XDB-C_{18} column, with methanol–water and methanol–phosphate buffer (20 m*M*, pH 7.0) as eluents

Compound	% Methanol	Log k_w (e) (a_0)	S (a_1)	r	s_{a0}	s_{a1}	$\pm a_0$	$\pm a_1$
Eluent: methanol–water								
F1	60–10	2.421	−0.039	0.9998	0.017	0.004	0.053	0.001
F2	60–30	3.656	−0.051	0.9996	0.069	0.001	0.873	0.017
F3	60–0	1.841	−0.038	0.9949	0.052	0.001	0.128	0.004
F4	60–0	0.651	−0.035	0.9878	0.075	0.002	0.185	0.005
F5	60–30	2.064	−0.045	0.9995	0.047	0.001	0.201	0.004
F6	60–0	1.513	−0.039	0.9888	0.081	0.002	0.199	0.006
F7	60–30	2.859	−0.043	0.9996	0.042	0.001	0.182	0.004
F8	60–20	2.330	−0.039	0.9995	0.029	0.001	0.092	0.002
F9	60–0	0.660	−0.036	0.9846	0.117	0.003	0.324	0.009
F10	60–20	2.382	−0.039	0.9997	0.0220	0.0005	0.069	0.002
F11	60–10	1.898	−0.038	0.9961	0.065	0.002	0.181	0.005
Eluent: methanol–phosphate buffer								
F1	60–20	2.428	−0.039	0.9993	0.037	0.001	0.118	0.003
F2	60–40	3.588	−0.049	0.9981	0.152	0.003	0.932	0.038
F3	60–0	1.859	−0.038	0.9945	0.055	0.002	0.134	0.004
F4	60–0	0.646	−0.034	0.9816	0.091	0.003	0.222	0.006
F5	60–30	3.008	−0.044	0.9976	0.099	0.002	0.426	0.009
F6	60–0	1.523	−0.039	0.9878	0.085	0.002	0.207	0.006
F7	60–20	2.890	−0.044	0.9992	0.043	0.001	0.138	0.003
F8	60–20	2.357	−0.039	0.9989	0.045	0.001	0.144	0.003
F9	60–0	0.697	−0.036	0.9877	0.087	0.002	0.223	0.006
F10	60–20	2.413	−0.039	0.9994	0.033	0.001	0.105	0.002
F11	60–10	1.931	−0.039	0.9932	0.089	0.002	0.246	0.013

S = slope, r = regression coefficient. s_{a0} and s_{a1} are the standard errors; $\pm a_0$ and $\pm a_1$ are the 95% confidence limits; e = extrapolated.

umns, however, especially for the log k_w–log P_{Rekker} relationships show satisfying correlation coefficients indicating that each of these phases is a good candidate column for further log P-studies of our grow stimulating substances.

To investigate whether the correlation of the log k–log P relationships could be further improved, we followed the suggestions of Valkó [29] applying the isocratic hydrophobicity index φ_0 as another lipophilicity parameter. From the data points close to log $k=0$ in the log $k-\varphi$ relationships, $\varphi_{0,methanol}$ and $\varphi_{0,methanol,buffer}$ values were calculated. Since log φ_0-values were very similar in buffered and non-buffered methanol-containing eluents Table 7 only contains the values of the latter eluent system. This table also includes $\varphi_{0,max}$-values, representing the maximal percentual deviation in φ_0 for a specific compound between the four columns. In Table 8 the results of regression calculations of $\varphi_{0,methanol}$ and $\varphi_{0,methanol,buffer}$ values vs. C log P and log P_{Rekker} are summarised.

The φ_0-values in Table 7 reveal that for seven substances the $\varphi_{0,max}$ is maximally +6%. This confirms that in the absence of secondary retention mechanisms φ_0-values for different RPLC columns are rather similar [29]. For the substances 6 and 11 the $\varphi_{0,max}$-values are substantially higher, up to 26.8% for substance 11. For the compounds 4 and 9 $\varphi_{0,max}$ is again much larger, up to +66% for substance 4. The data from Table 7 reveal that the nature of the RPLC column in many but not all cases is arbitrary to obtain reliable φ_0-results. Therefore, column selection remains a delicate matter in that sense.

The correlation coefficients in Table 8 ($r>0.95$) are somewhat less compared to the results obtained

	LiChrosorb RP-18	Zorbax Rx-C_{18}	LiChrospher 60 RP-Select B	Zorbax Eclipse XDB-C_{18}
a_0	1.214 ± 0.110	1.081 ± 0.143	1.069 ± 0.116	1.156 ± 0.125
a_1	0.857 ± 0.087	1.041 ± 0.114	0.912 ± 0.092	1.040 ± 0.099
standard error				
s_{a0}	0.048	0.063	0.051	0.055
s_{a1}	0.037	0.050	0.041	0.044
F-parameter				
F	490.88	424.84	499.97	557.83
correlation coefficient				
r	0.991	0.990	0.991	0.992

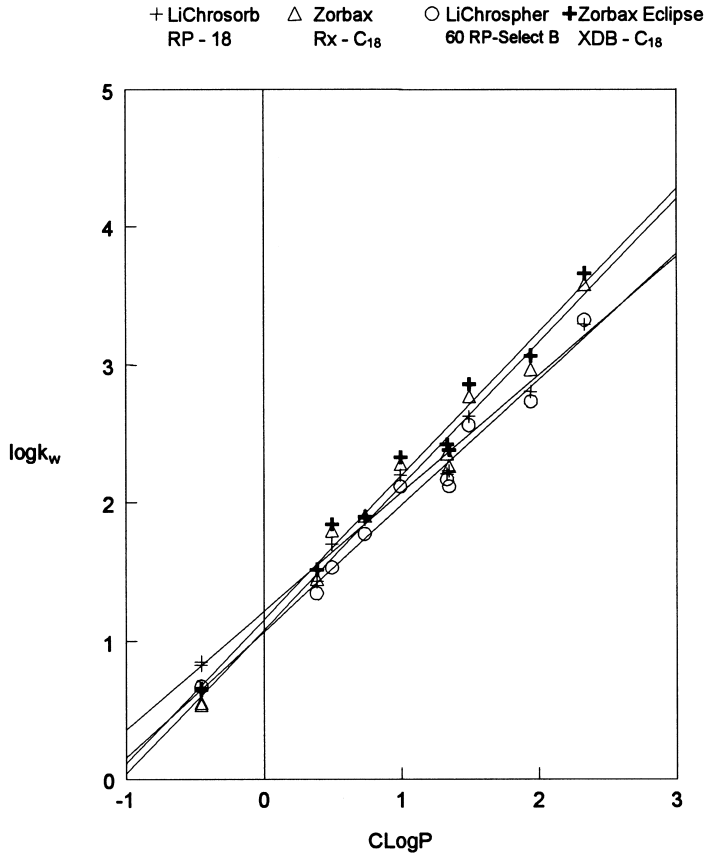

Fig. 2. Regression data and plot log k_w vs. C log P for the four HPLC columns (95% confidence limits). Eluent system: methanol–water; log $k_w = a_0 + a_1$ C log P.

Table 6
Regression and statistical data for 95% confidence limits, for the four HPLC columns

	LiChrosorb RP-18	Zorbax Rx-C_{18}	LiChrospher 60 RP-Select B	Zorbax Eclipse XDB–C_{18}
(A) Log $k_w = a_0 + a_1$ *C* log *P*; eluent: methanol–phosphate buffer (20 m*M*, pH 7.0)				
a_0	1.148±0.122	1.100±0.132	1.040±0.109	1.183±0.133
a_1	0.873±0.097	1.018±0.105	0.897±0.087	1.017±0.106
Standard error				
s_{a0}	0.054	0.058	0.048	0.059
s_{a1}	0.043	0.046	0.038	0.047
F-parameter				
F	411.08	478.33	542.70	473.77
Correlation coefficient				
r	0.989	0.991	0.992	0.991
(B) Log $k_w = a_0 + a_1$ log P_{Rekker}; eluent: methanol–water				
a_0	1.290±0.078	1.174±0.103	1.151±0.082	1.250±0.090
a_1	0.839±0.064	1.021±0.085	0.895±0.068	1.019±0.074
Standard error				
s_{a0}	0.035	0.046	0.036	0.040
s_{a1}	0.028	0.037	0.030	0.033
F-parameter				
F	869.89	742.39	894.51	966.69
Correlation coefficient				
r	0.995	0.994	0.995	0.995
(C) Log $k_w = a_0 + a_1$ log P_{Rekker}; eluent: methanol–phosphate buffer (20 m*M*, pH 7.0)				
a_0	1.226±0.090	1.192±0.100	1.121±0.077	1.275±0.101
a_1	0.856±0.074	1.998±0.082	0.856±0.063	1.997±0.083
Standard error				
s_{a0}	0.040	0.044	0.034	0.044
s_{a1}	0.033	0.036	0.028	0.036
F-parameter				
F	687.67	761.69	991.44	742.83
Correlation coefficient				
r	0.993	0.994	0.993	0.994

Table 7
φ_0-measurements for the data of Tables 2–5 calculated from Eq. (3) for aqueous methanol eluents, together with the maximal deviation in φ_0 calculated as $\varphi_{0,max} = (\varphi_{0,largest} - \varphi_{0,smallest})/\varphi_{0,smallest}$

Compound	φ_0 values				$\varphi_{0,max}$
	Lichrosorb RP18	Zorbax Rx C_{18}	Lichrosorb 60 RP Select B	Zorbax Eclipse XDB-C_{18}	(%)
F1	59.6	60.3	58.5	62.1	6.1
F2	70.0	70.1	67.8	71.7	5.7
F3	46.8	49.8	47.8	48.4	6.4
F4	22.4	13.5	19.9	18.6	65.9
F5	66.8	67.4	65.1	68.1	4.6
F6	37.6	30.8	38.4	38.8	26.0
F7	64.0	64.4	62.5	66.5	6.4
F8	57.8	58.5	57.2	59.7	4.4
F9	21.3	13.9	21.9	18.3	57.6
F10	58.1	61.1	58.8	61.1	5.2
F11	39.5	50.1	49.2	49.9	26.8

Table 8
Correlations between hydrophobicity index φ_0, C log P and log P_{Rekker}

Eluent: methanol–water Column	a_0	a_1	s_{a0}	s_{a1}	s	F	r
$\varphi_0 = a_0 + a_1$ C log P							
LiChrosorb RP 18	32.285±4.596	18.595±3.658	2.032	1.617	4.573	132	0.968
Zorbax RX C_{18}	28.990±6.867	21.772±5.466	3.036	2.416	6.833	81	0.949
LiChrospher Select B	33.263±4.611	17.851±3.670	2.038	1.622	4.588	121	0.965
Zorbax Eclipse XDB C_{18}	32.464±5.205	20.303±4.143	2.301	1.832	5.179	123	0.965
$\varphi_0 = a_0 + a_1$ log P_{Rekker}							
LiChrosorb RP 18	34.013±4.337	18.168±3.561	1.917	1.574	4.557	133	0.968
Zorbax RX C_{18}	30.965±6.332	21.327±5.199	2.799	2.298	6.654	86	0.951
LiChrospher Select B	34.899±4.262	17.467±3.499	1.884	1.547	4.479	127	0.966
Zorbax XDB C_{18}	34.324±4.806	19.867±3.946	2.125	1.744	5.050	130	0.967

s_{a0} and s_{a1} are the standard errors; $\pm a_0$ and a_1 are the 95% confidence limits; $F = F$-test value, r = correlation coefficient and s = standard error of fit.

in the log k_w–log P studies. Not surprisingly for our group of structurally related compounds the correlations from Table 8 are much better than reported in [28], where a much larger group of structurally unrelated compounds was investigated. These results confirm the conclusion of Valkó et al. [28,29] that also $\varphi_{0,\text{methanol,(buffer)}}$ values can be used in lipophilicity studies.

In order to investigate the role of the organic modifier nature in the RPLC determination of log P, two columns were also tested using acetonitrile instead of methanol as the modifier in the hydro-organic eluents. The results of the linear correlation calculations, Eq. (1) between the extrapolated log k_w values obtained from the acetonitrile–water eluent system and C log P and log P_{Rekker} values are shown in Table 9 for 95% confidence limits. For these nitrogen-containing compounds these findings are not surprising, since acetonitrile shows properties much different from methanol, e.g. it has poor hydrogen bonding or donating properties and can wet the organic ligands better compared to methanol [37], making the substances more susceptible for secondary stationary phase interactions.

We speculate that the less satisfactory correlations obtained on the LiChrosorb C_{18} column may be attributed to secondary interaction mechanisms. Clearly for this latter column this effect is somewhat more evident. As reviewed by Hsieh and Dorsey [35] this also confirms earlier reports on the findings of less pronounced linear log k vs. percentage modifier relationships for acetonitrile compared to methanol. Clearly however, this effect also depends on the nature of the applied RP-phase. Next, the data from the acetonitrile aqueous eluents were also calculated using Eq. (2). The results are summarised in Tables 10 and 11.

The results show that for both columns a significant improvement of the correlations between C log P and log P_{Rekker} and log k_w values has been obtained calculating the results by a second-degree polynominal function compared to the results of Table 9. Both columns show satisfying (LiChrosorb

Table 9
The linear correlation calculations between the extrapolated log k_w values obtained from the acetonitrile–water eluent system and C log P and log P_{Rekker}, for 95% confidence limits

Parameter	LiChrosorb RP-18	Zorbax Rx-C_{18}
Log $k_w = a_0 + a_1$ C log P		
a_0	1.035 (±0.336)	0.712 (±0.191)
a_1	0.597 (±0.267)	0.795 (±0.152)
s_{a0}	0.148	0.084
s_{a1}	0.118	0.067
F	25.58	139.74
r	0.860	0.969
Log $k_w = a_0 + a_1$ log P_{Rekker}		
a_0	1.089 (±0.316)	0.789 (±0.192)
a_1	0.585 (±0.259)	0.774 (±0.158)
s_{a0}	0.139	0.085
s_{a1}	0.115	0.069
F	26.01	122.72
r	0.862	0.965

s_{a0}, s_{a1} are standard errors, F is the distribution and r is the correlation coefficient.

Table 10
Regression calculations for the acetonitrile–aqueous eluents using Eq. (2)

Log $k=a_0+a_1\varphi+a_2\varphi^2$				
Compound	a_0	a_1	a_2	r
Column: LiChrosorb RP-18				
F1	2.252	−0.087	0.001	0.9993
F2	2.617	−0.073	0.0004	0.9999
F3	1.745	−0.083	0.001	0.9987
F4	0.747	−0.056	0.0005	0.9509
F5	3.358	−0.123	0.001	0.9991
F6	1.326	−0.091	0.001	0.9916
F7	2.761	−0.099	0.001	0.9994
F8	2.351	−0.098	0.001	0.9991
F9	0.892	−0.101	0.001	0.9960
F10	2.358	−0.086	0.001	0.9990
F11	1.592	−0.068	0.001	0.9978
Column: Zorbax Rx-C_{18}				
F1	2.455	−0.100	0.001	0.9997
F2	3.7325	−0.129	0.001	0.9997
F3	1.854	−0.095	0.001	0.9976
F4	0.666	−0.094	0.001	0.9590
F5	3.336	−0.124	0.001	0.9998
F6	1.561	−0.122	0.002	0.9866
F7	3.013	−0.115	0.001	0.9998
F8	2.304	−0.093	0.001	0.9987
F9	0.674	−0.107	0.001	0.9824
F10	2.521	−0.100	0.001	0.9999
F11	1.717	−0.092	0.001	0.9989

C_{18}) and excellent (Zorbax RX-C_{18}) correlations between the retention and the log P-data indicating that especially the latter column can be used too with acetonitrile as the organic modifier.

Similar as in the experiments also here the data were used to calculate the $\varphi_{0,ACN}$ hydrophobicity indexes for both columns (Tables 12 and 13). The correlations found for acetonitrile-containing eluents are significantly less than found for the corresponding methanol-containing eluents (Table 8). This finding is opposite to the results from [28], where for a large group of structurally unrelated compounds for acetonitrile-containing eluents higher correlation coefficients (0.88) were observed than for methanolic compounds (0.79). Obviously, opposite to the satisfactory $\varphi_{0,methanol}$ values found in this study the $\varphi_{0,ACN}$ values are not suitable to measure the lipophilicity of our substances. The results of the φ_0-measurements for methanol (Table 7) and acetonitrile (Table 12) aqueous eluents on the LiCh-

Table 11
Regression and statistical data of the log k_w vs. C log P and log P_{Rekker} regression calculations for the LiChrosorb RP-18 and Zorbax RX-C_{18} columns with acetonitrile–water eluents

Column	LiChrosorb RP-18	Zorbax RX-C_{18}
Log $k_w=b_0+b_1$ C log P		
b_0	1.219±0.314	1.148±0.149
b_1	0.846±0.250	1.104±0.112
s_{b0}	0.139	0.062
s_{b1}	0.110	0.050
F	58	491
r	0.931	0.991
s	0.312	0.141
Log $k_w=b_0+b_1$ log P_{Rekker}		
b_0	1.296±0.293	1.250±0.125
b_1	0.828±0.241	1.079±0.012
s_{b0}	0.130	0.055
s_{b1}	0.106	0.045
F	60	566
r	0.993	0.992
s	0.308	0.131

s_{b0} and s_{b1} are the standard errors; b_0 and b_1 are the 95% confidence limits. $F=F$-test value, $r=$correlation coefficient and $s=$standard error of fit.

Table 12
Measurements of $\varphi_{0,ACN}$ from Eq. (3) for aqueous–acetonitrile eluents

%ACN	Compound	I	S	r	φ_0
Eluent: ACN–water; Column: LiChrosorb RP-18					
50–20	F1	1.503	−0.038	0.981	39.6
50–30	F2	1.939	−0.038	0.996	51.0
50–20	F3	0.956	−0.033	0.982	29.0
60–40	F4	0.144	−0.016	0.987	9.0
50–20	F5	2.184	−0.048	0.980	45.5
50–20	F6	0.078	−0.013	0.937	6.0
50–20	F7	1.929	−0.045	0.982	42.9
50–20	F8	1.381	−0.035	0.959	39.5
50–20	F9	−0.341	−0.018	0.929	−18.9
50–20	F10	1.702	−0.043	0.984	39.6
50–20	F11	0.873	−0.022	0.956	39.7
Eluent: ACN–water; Column: Zorbax Rx-C_{18}					
50–20	F1	1.475	−0.038	0.980	38.8
50–20	F2	2.523	−0.052	0.982	48.5
50–20	F3	0.849	−0.029	0.983	29.3
50–20	F4	0.028	−0.025	0.834	1.1
50–20	F5	2.142	−0.048	0.980	44.6
50–20	F6	−0.131	−0.013	0.954	−10.1
50–20	F7	1.899	−0.045	0.980	42.2
50–20	F8	1.452	−0.039	0.983	37.2
50–20	F9	−0.696	−0.010	0.984	−69.6
50–20	F10	1.594	−0.040	0.979	39.9
50–20	F11	0.605	−0.023	0.953	26.3

Table 13
Regression and statistical data of the φ_0 vs. C log P and log P_{Rekker} regression calculations for the LiChrosorb RP-18 and Zorbax RX-C_{18} columns with acetonitrile–water eluents

Column	LiChrosorb RP-18	Zorbax RX-C_{18}
$\varphi_0 = d_0 + d_1 C \log P$		
d_0	9.811±10.820	−8.746±21.702
d_1	21.158±8.612	31.964±17.273
s_{d0}	4.783	9.594
s_{d1}	3.807	7.636
F	30	17
r	0.880	0.813
s	10.766	21.594
$\varphi_0 = d_0 + d_1 \log P_{\text{Rekker}}$		
d_0	11.657±9.993	−5.884±20.378
d_1	20.812±8.205	31.355±16.731
s_{d0}	4.418	9.009
s_{d1}	3.627	7.397
F	32	17
r	0.880	0.816
s	10.501	21.413

s_{d0} and s_{d1} are the standard errors; d_0 and d_1 are the 95% confidence limits; $F = F$-test value, r = correlation coefficient and s = standard error of fit.

rosorb C_{18} and Zorbax Rx C_{18} columns are correlated in Eqs. (6) and (7), respectively:

$$\varphi_{0,\text{MeOH}} = 28.596(\pm 9.897) + 0.711(\pm 0.276)\varphi_{0,\text{ACN}}$$

$$\text{where } s_{a0} = 4.375,\ s_{a1} = 0.122,\ s = 8.308,\ F = 34,\ r = 0.887,\ n = 11 \quad (6)$$

$$\varphi_{0,\text{MeOH}} = 38.366(\pm 8.063) + 0.517(\pm 0.204)\varphi_{0,\text{ACN}}$$

$$\text{where } s_{a0} = 3.584,\ s_{a1} = 0.090,\ s = 10.054,\ F = 33,\ r = 0.885,\ n = 11 \quad (7)$$

The results from the Eqs. (6) and (7) clearly show that apart from the column selection also the nature of the organic modifier influences the quality of φ_0-data. From the Tables 2–5,12 obviously for our substances methanol is the preferred organic modifier.

Summarising, the results from this work confirm earlier conclusions of other authors, e.g. Braumann and Miyake [9,38], that log P measurements by RPLC are only valid for a specific group of compounds under defined chromatographic conditions and are of limited general significance. This is also supporting their conclusions that standardisation of log P-measurement systems is a prime interest in this field.

4. Conclusions

From the present work for the investigated compounds the following conclusions can be drawn:

(1) The four investigated RP-phases respond chromatographically different to the test substances; all columns, however, show satisfactory log P–log k_w relationships ($r > 0.99$) under aqueous–methanol eluent conditions and are acceptable candidates for further log P-studies; for acetonitrile as the organic modifier of both tested columns the Zorbax RX-C_{18} provided comparable regression results ($r > 0.99$), while the LiChrosorb RP-18 column ($r > 0.93$) is less useful.

(2) For methanol-containing eluents linear regression of log k vs. φ provides satisfactory results, while for aqueous–acetonitrile eluents a second-degree polynominal regression must be applied.

(3) No significant differences in the regression results are observed between the use of buffered vs. non-buffered methanol-containing eluents.

(4) From the tested lipophilicity parameters log k_w values showed in all cases best regression results ($r > 0.99$) for methanol-containing eluents followed by the hydrophobicity index $\varphi_{0,\text{methanol}}$, which show somewhat lesser correlations ($r = 0.96$) compared to log k_w; φ_0 values obtained from acetonitrile-containing eluents are of limited use under these conditions ($r < 0.90$).

(5) The results of this study confirm earlier conclusions of other workers [9,37,38] that standardisation of log P-measurement protocols by RPLC, more particularly defining the applied column and organic modifier nature, would contribute to a wider general significance of such data.

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