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INFLUENCE OF ELUENT COMPOSITION ON LIPOPHILICITY MEASUREMENTS USING REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The influence of mobile phase composition on lipophilicities determined by reversed-phase thin-layer chromatography has been investigated by measurement of R_M values of homologous *n*-alkyl phenyl ketones (methyl- to *n*-heptyl-, *n*-decyl- and *n*-undecylketone) using liquid paraffin as the stationary phase and aqueous mixtures of acetone, methanol and N,N-dimethylformamide as the eluents. Of these, acetone appeared to be the least suitable because at high concentrations it appeared to induce perturbations of the stationary phase. All R_M values were made up of contributions from methylene groups, which were found to be almost independent of the type of organic co-solvent, and from the acetophenone moiety, which was strongly affected by the properties of the co-solvent. Extrapolation of the values for methylene groups to pure water showed a tendency for somewhat lower values than predicted from values in alkane–water mixtures.

INTRODUCTION

The use of chromatography in the field of quantitative structure–activity relationships (QSAR) has been extensively reviewed by Tomlinson[1] and Kaliszan[2]. Both reviews recognized the increasing interest in chromatographic parameters as rapidly measurable substitutes for "normal" shake-flask lipophilicities. Extrapolated R_M values from reversed-phase thin-layer chromatography (RP-TLC) should be particularly mentioned in this context. The practical advantages of this technique have been discussed[1–4]: several lipophilicities can be measured at once, only very small samples are required and the system is almost unaffected by impurities. However, there is a lack of agreement on the most suitable experimental conditions for the determination of lipophilicities by RP-TLC. Kaliszan[2], for example, describes the use of the following stationary phases: silicone oil, octan-1-ol, oleyl alcohol and liquid paraffin. A similar situation can be observed with regard to organic co-solvents in the mobile phase. Here, acetone seems to be the first choice but methanol, ethanol and acetonitrile are used as well.

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Obviously, the selection of optimum conditions should be based upon systematic studies of the quantitative influences of the various conditional parameters involved. The choice of a suitable stationary phase has been considered in a number of papers by Biagi and co-workers[5,6]. The selection of the best mobile phase, however, has hitherto not been studied in a systematic way. As a consequence, the decision to use a particular co-solvent is usually guided only by considerations of practical interest whilst the possibility of co-solvent dependent effects on the final results is tacitly neglected. The present study attempts to improve this situation by comparing three different co-solvents: acetone, methanol and N,N-dimethylformamide (DMF). Acetone was chosen because it seems to be the co-solvent most frequently used for the determination of RP-TLC lipophilicities. Similarly, methanol is first choice for reversed-phase high-performance liquid chromatography (RP-HPLC) applications. Inclusion of methanol seemed therefore useful for future comparisons between lipophilicities measured by RP-HPLC and RP-TLC. The selection of DMF was less obvious, but, among the solvents that are miscible with water in all proportions, DMF deviates the most from water, as far as its liquid structure is concerned[7,8]. DMF was thus included in order to create an extreme case which might improve the discussion of results obtained with the usual co-solvents methanol and acetone.

Apart from their practical advantages, reversed-phase chromatographic systems provide the attractive option of applying hydrophilic phases with a gradually changing water content. We have employed this feature to obtain information on the relative contributions of solute–water and solute–solvent interactions to RP-TLC lipophilicities. Homologous n-alkyl phenyl ketones (aceto- to octano-, undecano- and dodecanophenone) were used as solutes. These compounds were also used in earlier work[9–11] on the interactions between n-alkyl phenyl ketones and human erythrocyte membranes. Moreover, homologous series have the advantage of permitting the derivation of R_M contributions per methylene group. These methylene fragments, denoted $f(CH_2)$, are immediately comparable with methylene contributions to the capacity factor (k') in RP-HPLC[12] and the logarithm of the distribution coefficient (log K_D)[4,13,14] in shake-flask determinations, because any system dependence of either R_M, k' or K_D is likely to affect all homologues to the same degree. The $f(CH_2)$ values derived in the present study are thus suitable for investigating the occurrence of first-member anomalies exhibited by n-alkylbenzoates in RP-HPLC[12] (however, these were not observed for N-n-alkyltritylamines in RP-TLC by Boyce and Milborrow[3]). In addition, we have compared methylene contributions to K_D in alkane–water systems[13,14] with our extrapolated R_M values in pure water in order to test the reliability of lipophilicities measured, as usual, by extrapolation of a linear R_M versus composition plot. The results of this test will be discussed here as well.

MATERIALS AND METHODS

Experiments were performed on DC Fertigplatten Kieselgel 60 F_{254} (Merck) impregnated with liquid paraffin oil. Impregnation was carried out by a previously described standard method[3,5]: plates were immersed in a 5% (v/v) solution of liquid paraffin B.P.–light petroleum (40:60) followed by a drying procedure. The plates were always used immediately after drying. n-Alkyl phenyl ketones were purified as de-

scribed previously[9]. Water was doubly distilled, and acetone, methanol and DMF were from Baker, analytical grade, and used as such. Plates were developed in closed chromatography tanks at 21°C. The following aqueous volume fractions were employed throughout: 0.30, 0.35, 0.40, 0.45 and 0.50.

RESULTS AND DISCUSSION

Generally chromatograms showed compact spots. Very good separations of the various homologues were obtained throughout: R_M value derived from R_F values between 0.2 and 0.8[2] are summarized in Table I. Each entry in this table corresponds to the mean and standard deviation of 20 different determinations. From Table I it can be calculated that $f(CH_2)$ is a constant, independent of the position in the side chain. This observation is in agreement with previously reported[3] RP-TLC lipophilicities of homologous N-n-alkyltritylamines. However, methylene contributions to capacity ratios of n-alkylbenzoates in RP-HPLC were recently shown[12] to increase with increasing chain length for side chains smaller than five carbon atoms. Above this minimum length, constant $f(CH_2)$ values were observed as well. Differences in retention mechanism between RP-HPLC and RP-TLC are the most likely cause of this somewhat contradictory behaviour. In addition, the molecular geometry of n-alkyl phenyl ketones could also be important because the alkyl chain is not connected directly to the phenyl group but shielded from it by a carbonyl group. The influence of the side chain on the π-electron system formed by the phenyl group and the carbonyl group is virtually constant, as demonstrated for example by a constant molar extinction coefficient for all homologues[9].

According to the fragmental system[4], the lipophilicity contribution of a molecular fragment is a constant, irrespective of its position in a molecule or the properties of other fragments in its neighbourhood. A number of recent publications[15-17] have noted that a justification for the use of such a system cannot be derived from theoretical considerations alone and that empirical observations, such as the constancy of $f(CH_2)$ discussed above therefore constitute the only possible way of validating a fragmental system. In the present case, it is apparently justified to break down R_M into an appropriate number of $f(CH_2)$ values and a total contribution from the remaining fragments, which together form an acetophenone moiety:

$$R_M = N_C f(CH_2) + R_M(A) \tag{1}$$

where $R_M(A)$ represents the calculated R_M value of acetophenone and N_C gives the number of CH_2 groups. Linear regression analysis has been applied to the data in Table I to obtain the results presented in Table II.

Regressions of very high statistical quality were obtained throughout. For all three solvent systems a qualitatively similar increase of regression coefficients with increasing aqueous volume fraction, φ_w, is observed. The only exception to this trend is found in acetone–water where an increase of φ_w from 0.30 to 0.35 is accompanied by a small decrease of $f(CH_2)$. This observation is in agreement with the study of Boyce and Milborrow[3] on N-n-alkyltritylamines. The only difference between their results and those from the present study is the volume fraction of acetone reported in their study (0.91) compared with that of 0.70 in the present study which is required

TABLE I

R_M VALUES OF HOMOLOGOUS n-ALKYL PHENYL KETONES AS A FUNCTION OF THE WATER CONTENT OF MIXED ELUENTS CONTAINING VARIOUS CO-SOLVENTS

The stationary phase consisted of Kiesel gel 60 F_{254} impregnated with paraffin oil. Standard deviations are given in parentheses. Key: φ_w = volume fraction of water in the eluent, N_C = number of methylene groups in the side chain of a particular homologue.

φ_w	$N_C = 0$	$N_C = 1$	$N_C = 2$	$N_C = 3$	$N_C = 4$	$N_C = 5$	$N_C = 6$	$N_C = 9$	$N_C = 10$
Methanol water									
0.30	−0.65 (0.07)	−0.42 (0.05)	−0.27 (0.03)	−0.09 (0.03)	0.05 (0.02)	0.27 (0.04)	0.49 (0.03)		
0.35	−0.53 (0.07)	−0.30 (0.04)	−0.12 (0.04)	0.07 (0.04)	0.29 (0.04)	0.51 (0.04)	0.72 (0.04)		
0.40	−0.43 (0.03)	−0.12 (0.04)	0.05 (0.03)	0.28 (0.05)	0.53 (0.04)	0.74 (0.05)			
0.45	−0.23 (0.05)	0.02 (0.04)	0.27 (0.04)	0.52 (0.04)	0.72 (0.04)				
0.50	−0.12 (0.04)	0.19 (0.03)	0.43 (0.03)	0.72 (0.04)					
Acetone water									
0.30				−0.73 (0.08)	−0.58 (0.06)	−0.41 (0.05)	−0.21 (0.05)	0.28 (0.02)	0.42 (0.02)
0.35		−0.69 (0.03)	−0.57 (0.03)	−0.43 (0.03)	−0.26 (0.03)	−0.10 (0.03)	0.07 (0.03)	0.54 (0.03)	0.73 (0.04)
0.40	−0.71 (0.03)	−0.52 (0.03)	−0.37 (0.03)	−0.18 (0.04)	0.02 (0.03)	0.24 (0.03)	0.46 (0.04)		
0.45	−0.58 (0.02)	−0.35 (0.02)	−0.15 (0.02)	0.07 (0.02)	0.27 (0.02)	0.51 (0.03)	0.75 (0.03)		
0.50	−0.47 (0.04)	−0.20 (0.05)	0.03 (0.04)	0.26 (0.04)	0.55 (0.06)	0.74 (0.04)			
DMF water									
0.30			−0.50 (0.03)	−0.29 (0.02)	−0.14 (0.02)	0.11 (0.02)	0.36 (0.02)		
0.35		−0.59 (0.03)	−0.38 (0.02)	−0.13 (0.03)	0.09 (0.02)	0.39 (0.02)	0.66 (0.02)		
0.40	−0.70 (0.05)	−0.43 (0.04)	−0.21 (0.03)	0.09 (0.02)	0.32 (0.03)	0.65 (0.03)			
0.45	−0.61 (0.06)	−0.33 (0.03)	−0.05 (0.03)	0.29 (0.02)	0.53 (0.02)				
0.50	−0.58 (0.05)	−0.19 (0.03)	0.12 (0.02)	0.45 (0.03)	0.71 (0.03)				

TABLE II

COEFFICIENTS $f(CH_2)$ AND $R_M(A)$ TOGETHER WITH THEIR 95% CONFIDENCE LIMITS OF REGRESSION EQUATIONS

$R_M = N_C f(CH_2) + R_M(A)$, where N_C = number of methylene groups, $f(CH_2)$ = fragmental value of a methylene group and $R_M(A)$ = calculated R_M value of acetophenone. Key: φ_w = volume fraction of water in the eluent, n = number of data points, r = correlation coefficient, s = standard error of estimate and F = Fisher's variance ratio.

φ_w	$f(CH_2)$	$R_M(A)$	n	r	s	F
Acetone–water						
0.30	0.166 ± 0.006	−1.225 ± 0.041	6	0.9993	0.019	3000
0.35	0.158 ± 0.005	−0.880 ± 0.027	8	0.9993	0.021	4310
0.40	0.193 ± 0.010	−0.731 ± 0.036	7	0.9982	0.027	1400
0.45	0.218 ± 0.005	−0.579 ± 0.018	7	0.9997	0.013	7500
0.50	0.243 ± 0.010	−0.456 ± 0.030	6	0.9992	0.083	2510
Methanol–water						
0.30	0.181 ± 0.011	−0.632 ± 0.039	7	0.9976	0.030	1040
0.35	0.206 ± 0.006	−0.528 ± 0.022	7	0.9994	0.017	4130
0.40	0.228 ± 0.015	−0.398 ± 0.043	6	0.9980	0.030	1000
0.45	0.240 ± 0.012	−0.221 ± 0.029	5	0.9992	0.018	1820
0.50	0.274 ± 0.017	−0.107 ± 0.031	4	0.9993	0.016	1470
DMF water						
0.30	0.211 ± 0.021	−0.936 ± 0.042	5	0.9967	0.031	457
0.35	0.249 ± 0.015	−0.868 ± 0.058	6	0.9983	0.031	1140
0.40	0.264 ± 0.014	−0.708 ± 0.043	6	0.9986	0.029	1410
0.45	0.289 ± 0.014	−0.611 ± 0.033	5	0.9997	0.020	2050
0.50	0.320 ± 0.025	−0.538 ± 0.062	5	0.9980	0.037	735

to produce a deviation. In order to investigate this phenomenon, which seems to be unique to acetone alone, we have estimated the stationary phase concentrations of the various co-solvents employed in the present work. To do so, it was assumed that partition coefficients of acetone, methanol and DMF in paraffin–water can be cal-

TABLE III

ESTIMATED CO-SOLVENT CONCENTRATIONS IN THE AQUEOUS AND ORGANIC PHASE OF A PARAFFIN–WATER SYSTEM

Key: c_m = co-solvent concentration in the aqueous phase, c'_m = co-solvent concentration in the organic phase, φ_m = volume fraction of co-solvent in the aqueous phase, φ'_m = volume fraction of co-solvent in the organic phase.

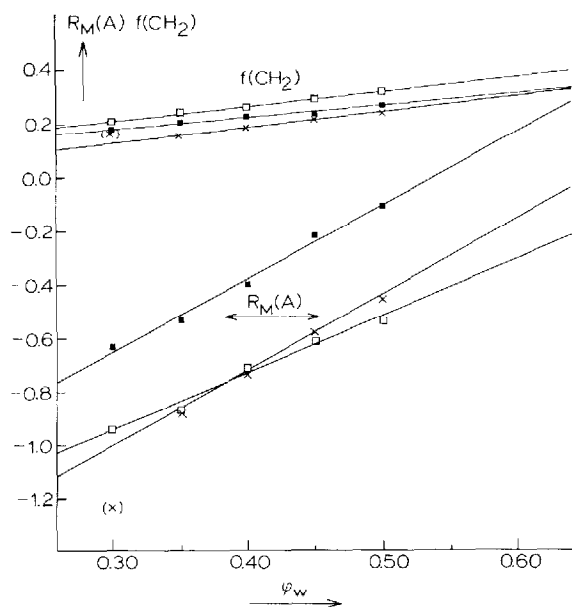| Co-solvent	c_m (mole/l)	c'_m (mole/l)	φ_m	φ'_m
Methanol	7.4	0.014	0.30	$5.8 \cdot 10^{-4}$
	17.3	0.033	0.70	$1.3 \cdot 10^{-3}$
Acetone	4.1	0.90	0.30	0.07
	9.5	2.09	0.70	0.15
Dimethyl-formamide	3.9	$5.1 \cdot 10^{-4}$	0.30	$3.9 \cdot 10^{-5}$
	9.1	$1.2 \cdot 10^{-3}$	0.70	$9.2 \cdot 10^{-5}$

Fig. 1. Methylene fragments, indicated by $f(CH_2)$ (top) and calculated R_M values of acetophenone, indicated by $R_M(A)$ (bottom) as a function of the aqueous volume fraction, φ_w, in mixed eluents containing water and one of the following organic co-solvents methanol (■), acetone (×) and DMF (□). Data points in acetone–water at $\varphi_w = 0.70$ are given in parentheses.

culated from alkane–water fragmental constants[13]. Results are presented in Table III. These results indicate that in systems with a co-solvent volume fraction of 0.70 in the mobile phase, the acetone concentration in the stationary phase will be at least two orders of magnitude higher than the concentration of either methanol or DMF, both of which are virtually negligible. As a consequence, stationary phases of systems containing methanol or DMF are expected to be essentially free of co-solvent, irrespective of the amount used to make up the elution mixture. In contrast, the stationary phase of an acetone-containing system is essentially mixed; volume fractions of acetone up to 0.15 can be expected at a volume fraction of 0.70 in the mobile phase. The data of Table III thus provide strong evidence for the hypothesis that the deviations observed in the acetone–water (70:30) system could arise from alterations of the lipophilic properties of the stationary phase caused by the presence of high amounts of acetone. We conclude that experimental studies on the partitioning of acetone in paraffin and other currently used lipoid stationary phases are necessary. As long as data of this type are not available, it seems appropriate to consider acetone as a less suitable co-solvent than methanol or DMF. In the following discussion of the quantitative dependence on eluent composition of fragmental values, the acetone–water (70:30) system is not considered. Furthermore, it is assumed that, at lower concentrations of acetone, the acetone water data were not significantly perturbed by alterations of the lipophilic behaviour of the stationary phase.

Values for $f(CH_2)$ and $R_M(A)$ obtained from Table II are given as a function of eluent composition in Fig. 1. The lines in this figure correspond to the regression equations given in Table IV. As the statistical quality of these regressions is very

TABLE IV

COEFFICIENTS a AND b OF REGRESSION EQUATIONS

Equations are of the form $f(CH_2) = a \varphi_w + b$ (upper part) or $R_M(A) = a \varphi_w + b$ (lower part), where the meaning of the symbols $f(CH_2)$, $R_M(A)$ and φ_w is given in Table II. Key: see Table II.

Elution mixture	a	b	n	r	s	F
$f(CH_2) = a\varphi_w + b$						
Acetone–water	0.560 ± 0.082	-0.035 ± 0.035	4	0.9962	0.007	261
Methanol–water	0.440 ± 0.073	0.050 ± 0.030	5	0.9910	0.005	164
DMF–water	0.516 ± 0.080	0.060 ± 0.032	5	0.9922	0.006	191
$R_M(A) = a\varphi_w + b$						
Acetone–water	2.848 ± 0.221	-1.872 ± 0.095	4	0.9989	0.011	921
Methanol–water	2.714 ± 0.287	-1.463 ± 0.117	5	0.9963	0.021	407
DMF–water	2.106 ± 0.355	-1.575 ± 0.144	5	0.9907	0.026	160

high, it can be concluded that the functional relationship between $f(CH_2)$ or $R_M(A)$ and the composition of the eluents used for their determination is very well represented by a straight line. A theoretical basis for this type of behaviour has been presented by several authors[18–20] who showed that application of regular solution theory[21] indeed gives retention as a linear function of eluent composition, expressed as a volume fraction. However, an extended regular solution approach was recently observed to predict parabolic retention *versus* composition plots[22]. It is therefore questionable to what extent extrapolation of the regression equations of Table IV far outside the experimentally accesible range of compositions can be representative for the actual partition behaviour in the extrapolated region. In a forthcoming paper[23] this question will be studied using a local composition theory[10]. Here, the validity of linear extrapolations can be investigated from an emperical point of view, by testing for the following conditions:

(1) Extrapolations to pure water should result in a unique value, irrespective of the type of co-solvent used,

(2) Assuming R_M to be fully determined by liquid–liquid partitioning alone, then extrapolated $f(CH_2)$ values in pure water should be virtually identical with fragmental constants for CH_2 groups derived from alkane–water systems.

Relevant extrapolation results are summarized in Table V. As shown by this table, all extrapolated $f(CH_2)$ values in pure water lie within an interval of 0.1 log

TABLE V

EXTRAPOLATED VALUES FOR $f(CH_2)$ AND $R_M(A)$ IN PURE WATER AND PURE CO-SOLVENT TOGETHER WITH THEIR 95% CONFIDENCE LIMITS

Elution mixture	Pure water		Pure co-solvent	
	$f(CH_2)$	$R_M(A)$	$f(CH_2)$	$R_M(A)$
Acetone–water	0.53 ± 0.12	0.98 ± 0.32	-0.04 ± 0.04	-1.87 ± 0.10
Methanol–water	0.49 ± 0.10	1.25 ± 0.40	0.05 ± 0.03	-1.46 ± 0.12
DMF–water	0.58 ± 0.11	0.53 ± 0.50	0.06 ± 0.03	-1.58 ± 0.14

units and must therefore be considered identical when their 95% confidence intervals are taken into account. A similar situation is encountered with respect to methylene fragments in alkane water systems. Davis[14] reported methylene contributions varying from 0.57 for values derived from alkanoic acids and alkanols in octane and dodecane–water to 0.67 for alkylpyridines in octane–water. Rekker[4] published a value of 0.65 for $f(CH_2)$ in cyclohexane–water, and a mean value of 0.62 resulted from later work[13]. Comparison of these data with the first column of Table V shows the DMF–water value to be the only one within the 0.57–0.67 interval. The extrapolated values in acetone–water and methanol water of 0.53 and 0.49, respectively, are too low although the occurrence of extrapolation errors of *ca.* 0.1 log units prevents the drawing of decisive conclusions.

Extrapolations of $f(CH_2)$ to pure co-solvent are given in the third column of Table V. Very small and almost identical values can be observed, the DMF value of only 0.06 being at maximum. As a consequence, all homologues should have very similar R_M values in case pure cosolvent is employed as the eluent. This hypothesis has been confirmed by a linear regression analysis whereby the data of Table I were used to fit R_M increased of $f(CH_2)$ as a function of φ_w. The results are not reproduced in detail but, for example, a maximum variation of only 0.1 log units was found in intercepts of regression equations for propio- ($N_C = 1$) to heptanophenone ($N_C = 5$) in DMF–water. We conclude that, to a very good approximation, the numerical value of a methylene contribution is directly proportional to the volume fraction of water. A similar conclusion has been drawn for shake-flask lipophilicities by Davis[14] who found that methylene fragments in alcohol–water systems correlated very well with the aqueous mole fraction at saturation in the alcohol phase. As far as methylene groups are concerned, our data thus support the view that "hydrophobic"[14–16] or "solvophobic"[12,24] interactions with water are responsible for the transfer from an aqueous to a lipoid environment. In this particular case, the choice of the co-solvent is apparently of secondary importance.

Extrapolated R_M values of acetophenone in pure water and pure co-solvent are given in the second and fourth column of Table V, respectively. In contrast to $f(CH_2)$, $R_M(A)$ values in pure water are not comparable with alkane–water distributions owing to the presence of a system-dependent factor[25] in the intercepts of the equations in Table II. The first of the above conditions is therefore not applicable but the second condition, regarding the uniqueness of extrapolations in pure water, remains valid. As shown by the second column of Table V, the latter condition is however not satisfied, the extrapolation in DMF–water being *ca.* 50% lower than the results for acetone water and methanol–water. Extrapolated $R_M(A)$ values in pure co-solvent also differ. Here, an outlying extrapolation result is observed in pure acetone. Together with a much larger variability as a function of composition of $R_M(A)$ as compared with $f(CH_2)$, these data lead to the conclusion that $R_M(A)$ depends to a much larger extent on the choice of the co-solvent than does $f(CH_2)$. Literature evidence on normal[26,27] and ion-pair[28] RP-HPLC reveals that this behaviour might be typical for certain combinations of polar groups and co-solvents.

CONCLUSIONS

The influence of three different organic co-solvents on lipophilicities of homol-

ogous n-alkyl phenyl ketones measured by RP-TLC has been compared. Acetone appears to be less suitable than methanol or DMF owing to irregularities observed at high concentrations. Evidence is presented that these deviations can be ascribed to acetone-induced perturbations of the stationary phase. The type of co-solvent is relatively unimportant for methylene fragments but, on the other hand, calculated R_M values of acetophenone are significantly affected by the properties of the co-solvents. To a very good approximation, methylene fragments are in all instances directly proportional to the water content of the elution mixture, expressed as a volume fraction. Extrapolated methylene fragments in pure co-solvents are thus negligible whereas those in pure water are very similar for all three co-solvents studied. Within experimental error, the latter values fall within a range predicted by methylene fragments from alkane–water distributions, although a tendency for too low values might exist.

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