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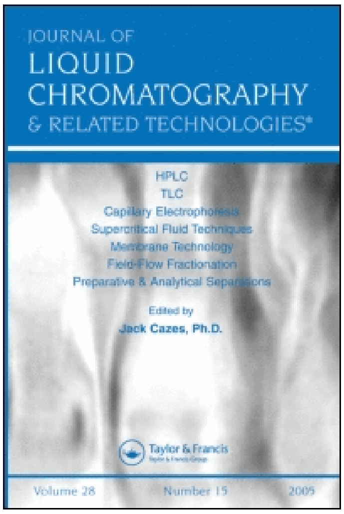

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HYDROPHOBICITY MEASUREMENTS BY HPLC: A NEW APPROACH TO π CONSTANTS

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ABSTRACT

A classical HPLC method of measuring log $P_{o/w}$ has been reevaluated and applied to 107 different mono- and disubstituted benzenes. The contribution of several functional groups has been estimated through multiple regression analysis, obtaining statistically significant mean π^*_m values.

Deviations of experimentally determined log P^* values for a set of ortho-, meta-, and para- disubstituted isomers from the "simple additive" model have been evaluated, and interpretations are suggested.

INTRODUCTION

Numerous papers have correlated biological effects of a substance with the logarithm of its octanol/water partition coefficient, log $P_{o/w}$, which has become a standard parameter for quantifying the hydrophobicity of a compound (1). log $P_{o/w}$ correlations are commonly used in structure-activity studies (QSAR) to optimize a specific biological response, thus substantially reducing drug development costs (2), and also in toxicological studies (3).

Clasically, determination of log P has been carried out by the "shake-flask" method (4), time-consuming, expemsive and sensitive to impurities of the sample. It involves numerous experimental difficulties and often affords different values among laboratories (5).

There have been numerous attempts of simplifying this determination, especially by means of chromatographic techniques, which can basically be divided into two groups:

a) octanol separation systems: TLC plates (6,7) or HPLC packing material (8-10) are covered with octanol; the eluent consists of octanol-saturated water or buffers.

b) "reversed-phase" separation systems: octadecylsilica is used as the stationary phase and binary mixtures of water (or buffer) and an organic modifier as eluents (11-14).

Some workers go a step further and suggest that HPLC retention data may prove to be a better correlate of biological activity than the o/w partition coefficient (15), arguing on the similarities between the HPLC process and the behaviour of a solute passing through a typical bilayer biomembrane system. In fact, although there is no guarantee that the variously determined HPLC data will better correlate with biological activities than n-octanol/water partition data, this has been described for some particular cases (16).

The use of log P values (determined by any technique) is sometimes preferred but the use of π values developed by Hansch and his co-workers (4) is a useful widespread approach, based on linear free energy relationships (LFER): each constitutive group in polysubstituted benzenes makes additive and constant contributions to hydrophobicity. Nevertheless, it is well known that some combinations of substituents show deviations from additivity which have been evaluated by multiple regression analysis (MRA) (17) or by principal components analysis (PCA) (18-20), in the case of meta- and para- disubstituted benzenes. All of these authors used data taken from different literature sources that may present a large variation range. We have developed our own method of determining log P, investigating a large series of different mono- and disubstituted benzenes (Table 1) with regard to their retention behaviour and correlating this property with log $P_{o/w}$ values described in the literature (21) (when more than one value was reported for a given compound, the same criteria as in ref. 18 were followed). A linear relationship was found between log $P_{o/w}$ and tha capacity factors (log k') for a great number of compounds (Fig. 1); others significantly deviated, especially those that ionized and could undergo hydrogen bonding with the unreacted silanol groups present on the C18 surface, very likely due to a dual retention mechanism involving solvophobic and silanophilic interactions (22,23).

Building up a bank of self-consistent log P^* data (see below), we have attempted to assess the validity of the "additive" model for ortho-, meta-, and para- disubstituted benzenes. By means of multiple regression analysis (MRA), mean hydrophobic contributions have been estimated for each of the 11 groups chosen for their frequent use in QSAR studies: F, Cl, Br, MeO, CHO, COMe, CO_2Me, CN, NO_2, Me and $CONH_2$. Deviations have been detected which can be accounted for by interactions between the different substituents, depending on their relative positions.

MATERIALS

Mono- and disubstituted benzenes were of analytical grade and were collected from laboratory stock and from other colleagues All experiments were performed on a Series 10 Perkin-Elmer liquid chromatograph equipped with a fixed-wavelength detector at 254 nm. A 10µm C-18 Perkin-Elmer column (25 cm x 4.6 mm ID) was

TABLE 1. List of mono- (× in Fig. 1) and disubstituted (□) benzenes used in the initial correlation study.

No.	Subst.1	Subst.2	log P (21)	log k'
1	-Br	-H	2.99	0.195
2	-Cl	-H	2.84	0.143
3	-F	-H	2.27	-0.022
4	-NO$_2$	-H	1.85	-0.147
5	-H	-H	2.13	-0.021
6	-OH	-H	1.46	-0.468
7	-NH$_2$	-H	0.90	-0.494
8	-CCl$_3$	-H	2.92	0.436
9	-CF$_3$	-H	3.01	0.121
10	-CN	-H	1.56	-0.311
11	-N=C=S	-H	3.28	0.352
12	-CHO	-H	1.48	-0.312
13	-CH$_2$Cl	-H	2.30	0.049
14	-CONH$_2$	-H	0.64	-0.673
15	-OCONH$_2$	-H	1.08	-0.228
16	-CH$_3$	-H	2.69	0.171
17	-NHCSNH$_2$	-H	0.73	-0.725
18	-OCH$_3$	-H	2.11	-0.027
19	-CH$_2$OH	-H	1.10	-0.434
20	-CH$_2$NH$_2$	-H	1.09	0.325
21	-CH$_2$CN	-H	1.56	-0.371
22	-COCH$_3$	-H	1.58	-0.251
23	-COOCH$_3$	-H	2.12	-0.062
24	-CONHCH$_3$	-H	0.86	-0.530
25	-NHCOCH$_3$	-H	1.16	-0.467
26	-CH$_2$CH$_3$	-H	3.15	0.320
27	-CH$_2$CH$_2$OH	-H	1.36	-0.330
28	-N(CH$_3$)$_2$	-H	2.31	0.103
29	-CH=CHCHO	-H	1.90	-0.201
30	-COOCH$_2$CH$_3$	-H	2.64	0.106
31	-CH$_2$CH$_2$CH$_3$	-H	3.68	0.504
32	-C$_4$H$_4$N	-H	3.08	0.185
33	-COCH$_2$CH$_2$CH$_3$	-H	2.66	0.063
34	-N(CH$_2$CH$_3$)$_2$	-H	3.31	0.389
35	-N=N-C$_6$H$_5$	-H	3.82	0.606
36	-NHC$_6$H$_5$	-H	3.50	0.179
37	-COC$_6$H$_5$	-H	3.18	0.164
38	-CHOH-C$_6$H$_5$	-H	2.67	-0.165
39	-CH=CHCOC$_6$H$_5$	-H	3.08	0.347
40	-COOCH$_2$C$_6$H$_5$	-H	3.97	0.409
41	-Cl	p-Cl	3.38	0.261
42	-Cl	m-Cl	3.38	0.376
43	-Br	m-NO$_2$	2.64	0.091
44	-Cl	o-NO$_2$	2.24	-0.066
45	-Cl	m-NO$_2$	2.46	0.050

TABLE 1 (cont.)

No.	Subst.1	Subst.2	log P (21)	log k'
46	-NO$_2$	m-NO$_2$	1.49	-0.234
47	-NO$_2$	p-NH$_2$	1.39	-0.510
48	-NO$_2$	m-CN	1.17	-0.372
49	-NO$_2$	p-CN	1.19	-0.407
50	-Cl	o-CH$_3$	3.42	0.315
51	-Cl	p-CH$_3$	3.33	0.294
52	-CH$_3$	o-NO$_2$	2.30	-0.049
53	-CH$_3$	p-NO$_2$	2.37	0.003
54	-NH$_2$	o-CH$_3$	1.32	-0.338
55	-NH$_2$	p-CH$_3$	1.39	-0.335
56	-NH$_2$	m-OCH$_3$	0.93	-0.533
57	-Br	p-COCH$_3$	2.43	-0.005
58	-Cl	o-COCH$_3$	2.09	-0.138
59	-Cl	p-COCH$_3$	2.32	-0.033
60	-NH$_2$	p-COCH$_3$	0.41	-0.643
61	-OCH$_3$	p-CHO	1.59	-0.251
62	-CH$_3$	o-CH$_3$	3.12	0.318
63	-CH$_3$	m-CH$_3$	3.20	0.357
64	-OCH$_3$	o-OCH$_3$	2.21	-0.225
65	Naphtalen		3.37	0.286
66	-C$_6$H$_4$NH$_2$	p-NH$_2$	1.34	-0.616

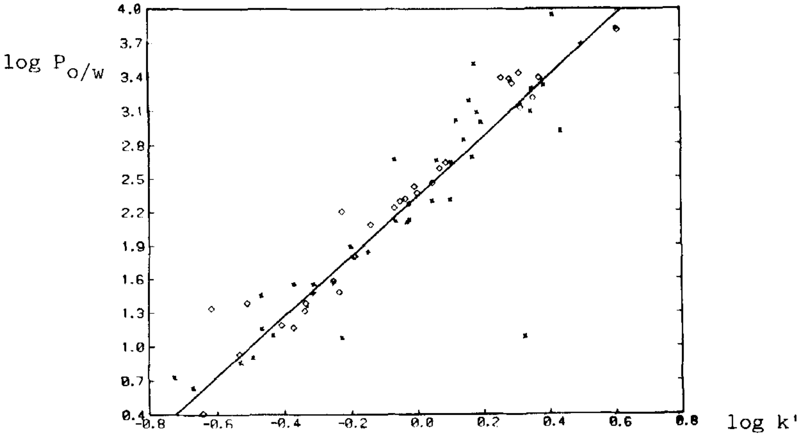

FIGURE 1: Correlation between log $P_{o/w}$ and log k' for a series of mono- (×) and disubstituted (□) benzenes (C_{18};MeOH/pH 7.0 phosphate buffer 75:25). Numbers 6, 8, 15, 20, 32, 36, 37, 38, 40, 41, 47, 64 and 66 were excluded from the regression analysis for deviating more than 10% from the expected value.

Equation: log $P_{o/w}$ = 2.328 + 2.655 log k' (n = 55; r = 0.988) used throughout. Retention times were computed either by a Perkin-Elmer Sigma 10 Data Station or by a Hewlett-Packard 3390A Integrator. Isocratic elution with a mixture of HPLC-grade methanol and pH 7.0 0.01 M sodium phosphate buffer (75:25 v/v) was maintained at a constant flow rate of 1.0 mL min^{-1} at room temperature. Temperature variations are not a major factor during a single run, but they can affect the log k' measurements and log P^* reproducibility. Available data suggest a consistent decrease in the intercept and a slight change in the slope as the temperature increases. For this reason, a calibration set was always used, as will be discussed later. Samples were dissolved in methanol at such a concentration that permitted their UV detection with injections of up to 20 µL (typically 10 µL) and yielded similar areas under the peaks. Column dead time, t_o, was determined by injection of 10 µL of a methanol solution of $NaNO_2$, which is expected to be unretarded, and by injection of 10 µL of pure methanol. Capacity factors appeared to be better correlated with log $P_{o/w}$ when this latter value was taken as a measure of t_o and it was consequently used in further calculations.

The NEMROD programs (24) were run in an Apple IIe computer.

METHODS

In view of the generally good correlation obtained between log k' and log $P_{o/w}$ we started the systematic evaluation of a larger number of disubstituted benzenes, namely 95, in order to study the contribution of each group to hydrophobicity and the validity of HPLC as a technique for the determination of log $P_{o/w}$ values. As a first approximation, no substituted phenols or anilines were included in the study in order to avoid the introduction of the term F_{HB} (18) (intramolecular hydrogen bonding effect) in the calculations.

Instead of using a retention index scale as that proposed by Baker (25), we created for more practical purposes a calibration system consisting of four compounds with well-known log $P_{o/w}$ values that best fitted our regression line (26) and to which the capacity factors of all the samples studied were referred, namely:

- Benzyl alcohol	log P = 1.10
- Nitrobenzene	log P = 1.85
- Toluene	log P = 2.69
- N,N-diethylaniline	log P = 3.31

The chromatographic determination of unknown partition coefficients for the system n-octanol/water was then performed as follows:

1) Retention times, t_R, were measured for the set of reference substances with well-known log $P_{o/w}$ values and their log k' calculated by using the equation:

$$\log k' = \log ((t_R - t_o)/t_o)$$

2) The relationship between log k' and log $P_{o/w}$ was determined by regression analysis (i.e. log $P_{o/w}$ = a + b log k'). For the set of calibration compounds the standard deviation of the mean value of log P was lower than 0.01.

3) t_R of the test substance was measured and its log k' calculated.

4) log $P_{o/w}$ of the test substance was calculated using the regression equation obtained under 2) and termed log P^*. Each compound was determined at least three times in different days and the mean value taken as its log P^* (max. st. dev. = 0.02 log P units).

RESULTS

log P^* were experimentally determined according to the method described. Their values for this series of 21 ortho-, 42 para-, and 23 meta- disubstituted isomers plus the eleven monosubstituted benzenes can be seen in Table 2. The objective was to calculate the contributions of these 10 groups to hydrophobicity with the aid of this sample and their possible interactions.

TABLE 2: Experimental log P* for the series of mono- and *ortho*, *meta* and *para* disubstituted benzenes.

	H	F	Cl	Br	OMe	CHO	COMe	COOMe	CN	NO_2	Me
H	2.17	2.14	2.62	2.76	2.15	1.40	1.59	2.11	1.41	1.85	2.69
F		2.07	2.72 2.55	2.83 2.68		1.50	1.61		1.35	1.74 2.00 1.78	2.63 2.59
Cl			2.99 3.24 3.04	3.34	2.38 2.68	1.90 1.97 1.96	1.89 2.15	2.11 2.74	1.99 1.86	2.03 2.46 2.26	3.13 3.07
Br				3.29	2.92 2.83	2.06	2.29 2.28			2.10 2.54 2.38	3.28 3.26 3.22
OMe					1.67 2.01	1.68 1.61	1.81 1.80 1.67	2.25	1.79	2.03	
CHO										1.06 1.27 1.29	1.89
COMe							1.31		1.16	1.58 1.53	2.05 1.98
COOMe								1.50 2.40		1.48 2.09 2.08	2.54 2.58
CN									0.83	1.23 1.33 1.22	1.86 1.92 1.85
NO_2										1.70 1.52	2.16 2.26
Me											3.07 3.17 3.19

o-	m-
p-	

The set of ortho- derivatives as well as nine substituted benzamides and benzamide itself were initially excluded, as a preliminary examination of the whole set of compounds revealed that they systematically deviated from their estimated values, which induced us to analyze them separately.

The approach involving functional group contributions to retention, introduced by Martin in 1950 (27), is based on LFER and has been applied by Tomlinson et al. (30) for the structural elucidation of biologically active substances by means of data obtained from HPLC.

Assuming that the presence of a given group at any one position in the molecule always changes log P^* by the same amount, a Free-Wilson model was used to study the contributions of these groups to the hydrophobicity of disubstituted benzenes. To perform the calculations, a matrix of indicator variables was prepared. The value 1 was assigned to the group when that substituent was present in the molecule, 0 if it was absent, and the value of 2 was given when both substituents were identical. This is the Fujita-Ban variant (29) that allows the calculation of the hydrophobicity contributions relative to that of hydrogen.

This matrix was entered into a NEMROD program (24) and together with the vector of the corresponding log P^* values made up the input to the multiple regression calculation (MRC). The benzene ring (i.e. 0 in all the positions) was taken as the constant term and its calculated log P value was obtained from the intercept of the equation. Different isomers were given different numbers and treated separately: the whole set of compounds was divided into two subsets:

1) Mono- and meta di-substituted benzenes, and
2) Mono-, meta and para di-substituted benzenes.

Meta- disubstituted benzenes. When subset 1) was subjected to MRC, the squared multiple correlation coefficient, R^2, was 0.993. The statistical significance of the contribution of each individual variable was $p < 0.001$, except for F and COOMe, for which $p < 0.05$. The coefficients of the model, i.e. the contributions of each group to the retention and hence to hydrophobicity (π^{*}_{m}), can be seen in Table 3 a) and compared to the frequently used π values b) described in the literature (21). The differences observed between both are an expression of the chromatographic behaviour of these compounds (see Fig. 1) and also due to the different mathematical calculations employed in both cases. log P^{*} for this set of meta disubstituted benzenes were then estimated by adding the value of the coefficients for both groups to the constant term. These results can be seen in Table 4. When the residuals (experimental-calculated) were examined it was seen that they were very small (< 0.13 log P units), benzene (H in every position) being one of those that deviated most: the model assigned the value of 2.06 to the group C_6H_6 , which is the constant term in all the series and differs from the experimental value for benzene (2.17). This mean value is in accordance with the term r_k for H (20) and is a consequence of treating H as an ordinary substituent instead of using it as a reference (4).

As it was seen that this meta subset did not deviate from the calculated values, even when two groups with opposed electronic effects (e.g. MeO and NO_2) were present on the benzene ring, it was not necessary to introduce a correction of the type F_{σ} (18). Intramolecular interactions are presumed to be minimal in this meta series of derivatives.

Para- disubstituted benzenes. When subset 2) was examined the coefficients of the model resembled those previously obtained for subset 1), as can be seen in Table 3 c). The squared regression coefficient, R^2 , was 0.981. The contribution of the F group was not significantly different from 0, but for the rest $p < 0.001$, except for MeO and COOMe for which $p < 0.05$. The residuals were not found to be randomly distributed: they were either null or positive for all of the meta isomers (except for m-CN,Me benzene which showed a slightly negative residual

TABLE 3. Hydrophobic contributions: π and π^*_m.

	π^*_m a)	π b)	π^*_m c)
C_6H_6	2.06	2.13	2.09
F	0.08	0.14	0.002
Cl	0.58	0.71	0.51
Br	0.67	0.86	0.63
MeO	0.21	-0.02	0.08
CHO	-0.64	-0.65	-0.62
COMe	-0.43	-0.55	-0.42
COOMe	0.12	-0.01	0.10
CN	-0.60	-0.57	-0.63
NO_2	-0.15	-0.28	-0.21
Me	0.54	0.56	0.49

a) Subset 1: R^2 = 0.993; degrees of freedom = 23; 35 experiences

b) Hansch's values: ref. 21.

c) Subset 2: R^2 = 0.981; degrees of freedom = 65; 77 experiences

Equation: $\pi^*_m = -0.0023 + 1.0206\,\pi$

TABLE 4. Calculated log P* values for subset 1 (mono- and meta derivatives).

	H	F	Cl	Br	O Me	C HO	COMe	COOMe	CN	NO_2	Me
H	2.06	2.14	2.63	2.73	2.27	1.42	1.63	2.18	1.45	1.91	2.60
F		2.21	2.71	2.81	2.35	1.50	1.71	2.25	1.53	1.99	2.68
Cl			3.21	3.31	2.84	2.00	2.21	2.75	2.03	2.48	3.17
Br				3.40	2.94	2.09	2.30	2.85	2.13	2.58	3.27
OMe					2.48	1.63	1.84	2.38	1.66	2.12	2.81
CHO						0.78	1.00	1.54	0.82	1.27	1.96
COMe							1.21	1.75	1.03	1.48	2.17
COOMe								2.29	1.57	2.02	2.72
CN									0.85	1.30	1.99
NO_2										1.76	2.45
Me											3.14

Δ= -0.03), while most of the para isomers fell on the negative side of the distribution (Figure 2), i.e. their experimentally determined log P were lower than those calculated by the model, while the reverse was true for all but one of the meta isomers. The one that deviated most on the positive side was m-OMe,CN (Δ= +0.243) while on the negative side it was p-OMe,OMe that deviated by -0.251 log P units from the experimental value.

Next, we estimated the differences between the experimentally determined log P* values (Table 2) of the series of para-disubstituted benzenes and the calculated values for the meta isomers (taken from Table 4) and tried to correlate these new residuals with some parameters of the molecules such as the field effect (F) and the resonance effect (R) (30), using the

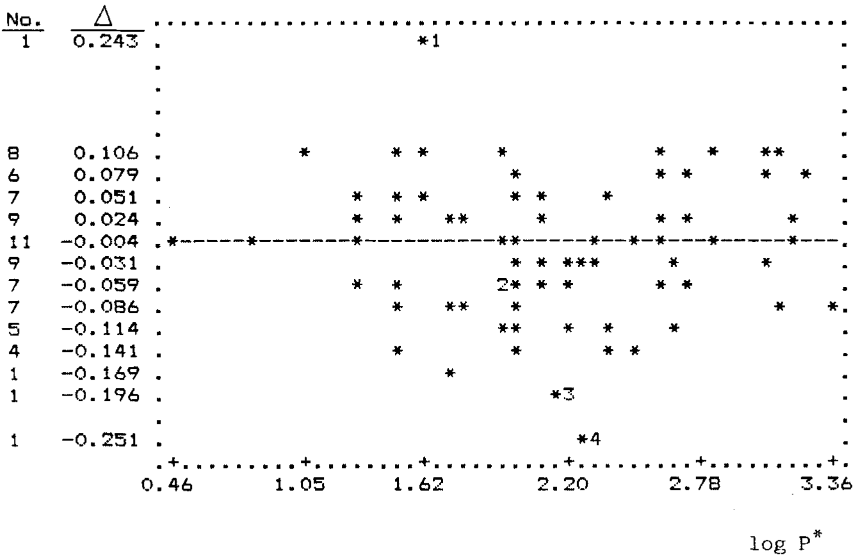

FIGURE 2: Distribution of Residuals (Δ) for the Set of mono-, meta, and para Disubstituted Benzenes.

Key: 1 m-OMe,CN; 2 m-CN,Me; 3 p-OMe,OMe; 4 p-OMe,OMe same program. Although F and R appeared to be significant ($p < 0.05$ and $p < 0.001$, respectively), the correlation found in this analysis was poor ($R^2 = 0.37$), showing that apparently none of the parameters studied accounted for all the differences observed. The approach followed by Leo (18), i.e. $F_\sigma = \rho\sigma$ ("responder"-"inducer"), did not account for the deviations from the model of this *para-* subset.

Ortho- disubstituted benzenes. When the series of *ortho* derivatives was subjected to the same calculation, trying to correlate the differences with several steric (MR = molar refractivity) and electronic parameters (30), a poor but significant correlation was found ($R^2 = 0.531$): MR ($p<0.01$), F ($p<0.05$) and R ($p<0.05$). The greatest residual corresponded to o-dimethoxybenzene ($\Delta = -0.81$). The quality of the regression improved if all the o-methoxy derivatives were taken out ($R^2 = 0.678$), but R lost significance and no rationale would have accounted for this elimination.

"Negative ortho effect": The application of the model proposed by Leo (18), i.e. ortho effect = $-0.29\ F_o$ (F_o = 0,1,2,3), in this instance led us to the following F_o values, in some cases different from the Leo values (in brackets):
o-F,NO_2 :1; o-Cl,Cl:0 (0); o-Cl,MeO:2(1); o-Cl,CHO:0; o-Cl,COMe:1(1); o-Cl,COOMe:2(1); o-Cl,NO_2 :1(1); o-Cl,Me:0(0); o-Br,NO_2 :1; o-OMe,OMe:2(0); o-OMe,COMe:0(1); o-CHO,NO_2:1(0); o-COMe,Me:0(0); o-COOMe,COOMe:3(3); o-COOMe,NO_2:2(1); o-COOMe,Me:1(0); o-CN,NO_2:0; o-NO_2,Me:1(0); o-Me,Me:0.
This difference, when observed, is ±1, except for o-OMe,OMe, for which $\Delta = 2$.

Shortening of retention times for the ortho- subset of derivatives seems to be due to the impossibility of a given group of being desolvated to its maximum potential because of the physical effect of the adjacent group. The net effect is a reduction in the capacity factor of the molecule having o-interaction in comparison to its meta isomer.

TABLE 5. Experimental and Calculated log P^* Values for the Set of Substituted Benzamides.

	m-NO_2	p-NO_2	p-Cl	m-Cl	p-COMe	m-OMe	p-OMe	m-Me	p-Me
ELP	0.73	0.72	1.23	1.18	0.20	0.65	0.63	0.95	0.90
CLP	0.31	0.31	1.04	1.04	0.03	0.67	0.67	1.00	1.00
Δ	0.42	0.41	0.19	0.14	0.17	-0.02	-0.04	-0.05	-0.10
σ(26)	0.71	0.78	0.23	0.37	0.50	0.12	-0.27	-0.07	-0.17

$\Delta = -0.005 - 0.497\,\sigma$; $R^2 = 0.864$ (n = 9)

ELP = Experimental log P^* ; CLP = Calculated log P^* (coefficients from Table 2a added to the value of log P^* of benzamide).

Benzamides. A similar study was performed on a set of nine substituted benzamides. Adding the value of the corresponding coefficient to that of benzamide experimentally determined (log $P^* = 0.46$) we obtained the calculated log P of this set. The differences between the experimental log P^* and these calculated values were in this case highly correlated with Hammett electronic σ constant ($R^2 = 0.851$). These results can be seen in Table 5. We find it is better in this case to use σ_p and σ_m rather than the σ values proposed by Leo (18), although our results are in agreement with the existence of the term $F_\sigma = \rho\sigma$.

DISCUSSION

The results presented provide a bank of self-consistent hydrophobic data of mono- and disubstituted benzenes that subjected to multiple regression analysis permit the calculation of mean functional group contributions, analogous to Hansch's π values, for which the term π^*_m is proposed. Statistically significant fits of these molecules to the Free-Wilson model were observed: this means that the effect of a particular substituent on hydrophobicity is constant for the subset of meta- disubstituted benzenes. The excellent R^2 also means that the hydrophobicity of the remaining meta isomers can be predicted by using these π^*_m values with reasonable accuracy. Attempts to explain deviations from this "simple additive" model for the para and ortho isomers based on electronic effects were successful only for the latter. The set of benzamides was treated separately and the differences between the experimental and calculated log P* values could be adequately explained with the introduction of the electronic parameter σ.

Testa and his group (31) have tried to explain substituent interactions in a series of 43 disubstituted benzenes in terms of a hydration factor, ω, proportional to the volume of a water molecule in an ice-like hydration sphere. This constant turns out to be equal to one fourth of Rekker's "magic constant" (32). For each molecule it is necessary to multiply ω by an integer ranging from -3 (p-Cl,NO_2) to 18 (o-OH,NO_2) in order to account for the differences between experimental and calculated values. This multiplicity factor varies considerably and its magnitude has not yet been adequately explained, although it has been proposed (31) that it is related to the number of water molecules acquired by, or expelled from, the hydration sphere of a monosubstituted benzene ring when a second substituent is attached to it. Nevertheless, this hypothesis requires further experimental confirmation.

The simple procedure we present in this paper is also of interest for the obtention of log P^* values for mono- and polysubstituted aromatic heterocycles, under suitable chromatographic conditions, which otherwise would be very difficult to obtain. By means of MRC it should be possible to derive π^*_m constants for the substituents and coefficients for the different heteroaromatic rings in a similar fashion to the case of benzene derivatives. Work in order to test this hypothesis is currently under way.

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