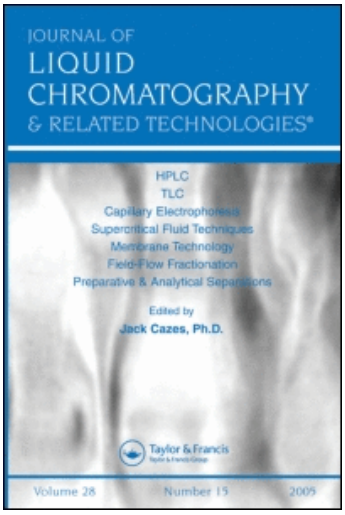

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Hydrophobicity Study for Some Pyrazolo-Pyridine Derivatives by RP-TLC and RP-HPLC

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Hydrophobicity Study for Some Pyrazolo-Pyridine Derivatives by RP-TLC and RP-HPLC

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Abstract: The hydrophobicity of thirteen 1*H*-pyrazolo[3,4-*b*]pyridine derivatives was studied by reversed-phase thin layer chromatography (RP-TLC) and reversed-phase high performance liquid chromatography (RP-HPLC). The partition coefficient log P value was measured for the unsubstituted derivative (**8**) using the classical shake flask method. The remaining compounds were the log P values calculated from **8** by Hansch-Fujita hydrophobicity fragmental constant, π. The R_M and log K parameters obtained by chromatography techniques were validated by comparison with those obtained from log P of **8** and, also, the log P calculated using the CLOGP computed software.

Keywords: Hydrophobicity, Retention parameters, Pyrazolo-pyridines

INTRODUCTION

The hydrophobicity is one of the properties which influence the partition of a substance in biological media. Hydrophobicity is usually expressed by the partition coefficient, $P_{o/w}$, of the organic compound between 1-octanol and water.[1,2] The partition coefficient can be experimentally measured by the classical shake flask method[1] and calculated using different methodologies.[3–7] The direct measurement of $P_{o/w}$ values by equilibrium between 1-octanol and water has several disadvantages, especially the laborious

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work leading to slow experiments, poor reproducibility, and it needs a reasonable quantity of the pure compound.

Alternative methodologies, such as chromatographic methods, have been utilized to overcome the difficulties, particularly reversed-phase thin layer chromatography (RP-TLC)[8] and reversed-phase high performance liquid chromatography (RP-HPLC).[9] These methods are commonly used to estimate partition coefficients and, therefore, have been the subject of many papers.[10–15] The main advantages of the chromatographic method have been the speed of experimental determinations, better reproducibility, because there are no great demands of the purity of the sample, and high quality correlations against the classical log P values and biological parameters in QSAR studies.

A linear relationship between the retention parameters and the concentration (φ) of organic modifier in the aqueous mobile phase has been established for a successful chromatographic measurement of hydrophobicity. The R_M and log K values are the retention time parameters measured by means of RP-TLC and RP-HPLC, respectively. Methanol is frequently used as an organic modifier in the aqueous mobile phase, due to its capacity to interact with different structural features of compounds and to its water like structure. The extrapolated values, R_{Mw} and log K_w, characterize the partition of the compound between the non-polar hydrocarbon stationary phase and water.[9–13]

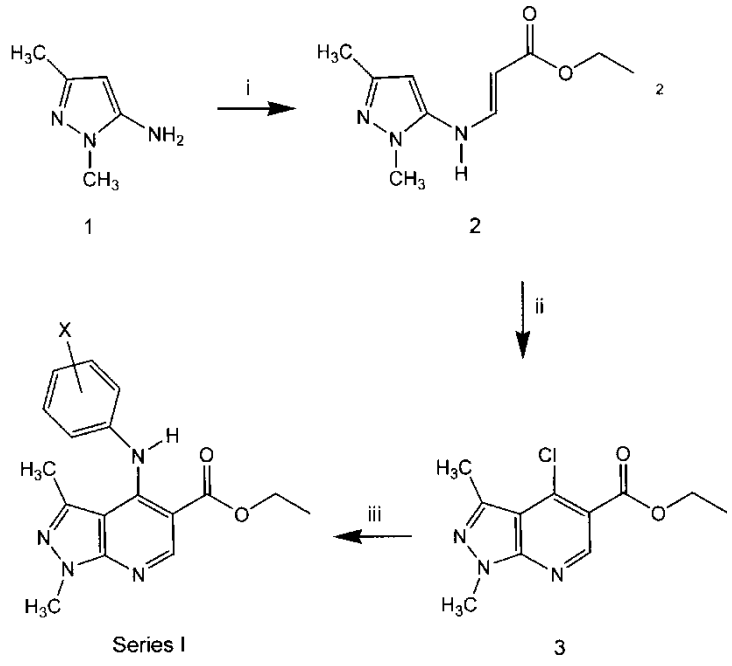
The purpose of this work is the determination and investigation of the linear correlations between R_{Mw}, log K_w and log $P_{o/w}$ values for a series of thirteen pyrazolo-pyridine derivatives. In addition, the comparison with the Clog P calculated using CLOGP computer programme,[4] was included. The pyrazolo-pyridine derivatives are known to possess remarkable and significant biological and medicinal importance.[16,17] The 4-(3′ or 4′-X-phenylamino)-5-carbethoxy-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*]pyridine, where X = H, Cl, Br, F, CH_3, OCH_3, OH, were prepared by the reported procedure[18] (Figure 1) with anti-leishmanial activity against *Leishmania amazonensis*.[19]

The log P_H value by the shake flask method was measured for the unsubstituted derivative (**8**). The remaining compounds were the log P_X values calculated from log P_H, by Hansch-Fujita hydrophobicity constant, π, in addictive-constitutive property using the following equation log P_X = log $P_H + \pi_X$.[5,7] The Hansch-Fujita hydrophobicity constant, π, was taken from the literature.[5]

EXPERIMENTAL

Chemicals

The 4-(3′ or 4′-X-phenylamino)-5-carbethoxy-1,3-dimethyl-1*H*-pyrazolo[3,4-*b*] pyridine derivatives were prepared by nucleophilic substitution reactions between the intermediate 4-chloro-1*H*-pyrazolo[3,4-*b*]pyridine and the appropriate 3 or 4-substituted anilines, following our previous procedure.[18,20]



4 X = H
5 X = 4'-NO_2
6 X = 4'-OH
7 X = 4'-Br
8 X = 4'-F
9 X = 4'-OCH_3
10 X = 4'-CH_3
11 X = 4'-OAc
12 X = 4'-Cl
13 X = 3'-NO_2
14 X = 3'-OCH_3
15 X = 3'-CH_3
16 X = 3'-Cl

Reaction conditions: (i) Diethyl ethoxymethylenemalonate, ethanol; (ii) $POCl_3$, Δ; (iii) substituted aniline, Δ.

Figure 1. Synthetic approach used for pyrazolo-pyridine derivative preparation.

The pKa determination was realized for all compounds using a classical potentiometric method,[21] and the values are listed in Table 1. Reagents and solvents were obtained from commercial sources and were used after appropriate treatment. Water was obtained from Millipore Milli-Q Water Purification System. All solutions used for HPLC were filtered through a membrane filter of 0.45 μm pore size from PRCOLA.

Chromatographic Hydrophobic Constant-R_M Determination

RP-TLC experiments were performed on 20 × 20 silica gel hydrocarbon impregnated plates with 254 nm fluorescent indicator (Uniplate Analthec, USA). The mobile phases were acetone-phosphate buffer (0.01 M;

Table 1. The dependence of R_f[a] data and R_M[b] for 1*H*-pyrazolo[3,4-*b*]pyridines derivatives as a function of modifier concentration, φ[c] and pKa's values

	$\varphi_{Acetone}(\%)$					
Cpd	70	55	50	45	40	pKa
1	0.820	0.493	0.440	0.247	0.213	11.05
	−0.658	0.012	0.105	0.484	0.568	
2	0.820	0.513	0.453	0.267	0.233	11.27
	−0.658	−0.023	0.082	0.439	0.517	
3	0.847	0.527	0.467	0.253	0.247	10.23
	−0.743	−0.046	0.058	0.471	0.485	
4	0.813	0.547	0.487	0.3	0.273	9.36
	−0.633	−0.077	0.027	0.373	0.430	
5	0.827	0.553	0.480	0.313	0.273	12.39
	−0.679	−0.092	0.035	0.341	0.425	
6	0.887	0.680	0.573	0.440	0.413	9.93
	−0.894	−0.326	−0.126	0.106	0.154	
7	0.9	0.707	0.607	0.473	0.427	10.37
	−0.954	−0.382	−0.188	0.047	0.128	
8	0.860	0.647	0.560	0.413	0.373	10.53
	−0.788	−0.263	−0.104	0.153	0.226	
9	0.867	0.640	0.547	0.387	0.353	9.35
	−0.809	−0.245	−0.077	0.205	0.268	
10	0.867	0.653	0.573	0.427	0.4	10.67
	−0.814	−0.274	−0.127	0.128	0.176	
11	0.873	0.660	0.593	0.427	0.406	10.11
	−0.836	−0.287	−0.163	0.129	0.166	
12	0.893	0.820	0.760	0.673	0.660	12.27
	−0.921	−0.659	−0.501	−0.313	−0.288	
13	0.88	0.747	0.753	0.540	0.513	12.02
	−0.865	−0.470	−0.07	−0.07	−0.023	

[a]First value of each row.
[b]Second data.
[c]Volumetric percentage, v/v.

pH = 7.4) mixtures with concentrations ranging from 40–70 (% v/v) in acetone.

Samples were prepared in acetone at a concentration of 0.15 mg/mL, and were applied onto the plate, 5 μL, 2.0 cm from the bottom edge. The sample spot was applied on the plate with aid of a Hamilton syringe of 10 μL, dried in a gentle air stream, and developed in normal chromatography chamber. The mobile phase migration distance was 15 cm in all cases. Each sample was applied in triplicate, and the R_f values were used in the calculations. After development, the plates were dried at room temperature and the spots were detected under UV light (254 nm). R_M values were calculated

according to the equation: $R_M = \log [(1/R_f) - 1)]$, where R_f is the ratio of the distance run by the analyte from the start point to the front marker.

Determination of Capacity Factor (K)

The RP-HPLC experiments were recorded on a Shimadzu instrument equipped with two LC-10AD pumps, UV detector SPD 10A set at 254 nm and Chromapac recorder 6R. The stationary phase was LC8 SUPELCO column (25.0 × 4.6 cm x 5 μM i.d.). The mobile phase consisted of a buffer of $NaH_2PO_4 \cdot H_2O$, 0.1 M and $Na_2HPO_4 \cdot 7H_2O$, 0.1 M (pH = 7.4) and acetonitrile as organic modifier.

The acetonitrile content in the mobile phase composition was in the range of 40–65 (% v/v). The flow rate of 1.5 mL/min was used throughout all the experiments. The capacity factor K, was determined from the equation $K = [(t_R - t_o)/t_o]$, where t_R is the retention time of the solute, and t_0 is the holdup time defined as the retention time of a nonretained compound (methanol).

Shake Flask Octanol-Water Partition Coefficients

The 1-octanol/water partition coefficient of pyrazolo-pyridine unsubstituted derivative (**8**) was determined by the shake flask technique using the conventional methodology.[1] Samples in a concentration range of 0.4 nM in 1,4-dioxane were partitioned between 100 mL of 1-octanol saturated with aqueous buffer ($Na_2HPO_4 \cdot 7H_2O$, 0.2 M and citric acid, 0.1 M) at pH = 7.4, and 200 mL of aqueous buffer saturated with 1-octanol. The compound was partitioned between 1-octanol and aqueous buffer with shaking for 1 hour. After separation, the two layers were centrifugated for 15 minutes (300 rpm). The relative proportion of compound in each layer was determined by UV absorption compared to a control solution. Three replicates were performed. The 1-octanol/water distribution ($P_{o/w}$) of **8** was taken according to the equation: $P_{oc\ t} = C_o/C_w$, where C_o and C_w corresponded to concentrations of the compound in 1-octanol and aqueous phase, respectively.

RESULTS AND DISCUSSION

Reversed-Phase-Thin Layer Chromatography (RP-TLC)

The chromatographic hydrophobic constant, R_M values, were obtained for 1*H*-pyrazolo[3,4-*b*]pyridine derivatives **1**–**13** by RP-TLC using the acetone-phosphate buffer (pH = 7.4) in a gradient concentration. The R_f

and R_M values are shown in Table 1. The R_M values were corrected using the equation $R_M^{corr} = R_M^{app} + \log [1 + 10^{(pH-pKa)}]$ where the R_M^{corr} and the R_M^{app} are the corrected and the apparent values, respectively.

The linear correlations between the R_M values and the organic modifier concentration in the mobile phase, φ (v/v), are shown in Table 2. These correlations showed a good linearity, usually with $r \geq 0.96$. The intercepts, a_o, represent the theoretical R_{Mw}, and can be regarded as a measure of the compounds' partition between a nonpolar stationary and the aqueous polar mobile phase (at 0% organic solvent).

The linearity of the relationships $R_M = f(\varphi)$ was maintained even at low acetone concentration in the mobile phase, so permitting the reliable extrapolated R_M values at 0% acetone to afford the R_{Mw} values.

Reversed-Phase High Performance Liquid Chromatography (RP-HPLC)

RP-HPLC chromatographic conditions were found in an isocratic elution. In a similar way to RP-TLC, linear relationships between log K values (RP-HPLC) and the composition of the mobile phase can be seen in Table 3. The extrapolated log K values at 0% acetonitrile (log K_w) and their corresponding linear regressions with φ (v/v) were summarized in Table 4. All

Table 2. Linear correlation $R_M = a_0 + a_1\varphi$

Cpd	a_0	A_1	$\pm s_{\bar{a}o}$	$\pm s_{a1}$	P	s	r
1	2.282	0.042	0.174	0.003	0.00103	0.075	−0.99
2	2.167	0.040	0.159	0.003	0.00090	0.069	−0.99
3	2.277	0.043	0.224	0.004	0.00203	0.097	−0.99
4	1.932	0.037	0.161	0.003	0.00123	0.070	−0.99
5	1.974	0.038	0.123	0.038	0.00005	0.053	−0.99
6	1.687	0.037	0.131	0.037	0.00067	0.057	−0.99
7	1.590	0.036	0.054	0.036	<0.0001	0.024	−0.99
8	1.665	0.035	0.102	0.002	0.00036	0.044	−0.99
9	1.810	0.037	0.122	0.002	0.00051	0.053	−0.99
10	1.606	0.034	0.121	0.002	0.00063	0.053	−0.99
11	1.615	0.035	0.142	0.003	0.00098	0.062	−0.99
12	0.632	0.022	0.122	0.002	0.00200	0.053	−0.98
13	1.277	0.030	0.276	0.005	0.01009	0.120	−0.96

a_o is the intercept R_{Mw}; a_1 is the slope; φ is the organic modifier concentration in the mobile phase (v/v); s_{ao} and s_{a1} are the standard errors for the intercept and the slope; s is the fit standard error; r is the correlation coefficient for 95% confidence limits; P is the variance.

Table 3. The capacity factor K[a] and log K[b] values for 1*H*-pyrazolo[3,4-*b*]pyridines derivatives as a function of modifier concentration, φ[c]

	$\varphi_{\text{Acetonitrile}}(\%)^c$				
Cpd	65	60	50	45	40
1	5.8	7.3	13.3	20.6	35.6
	0.248	0.407	0.751	0.974	1.254
2	5.7	7.2	13.3	20.5	36.2
	0.201	0.356	0.702	0.920	1.190
3	6.1	7.8	14.6	22.9	41.7
	0.202	0.357	0.70	0.921	1.191
4	5.7	7.2	13.2	20.5	36.3
	0.230	0.370	0.708	0.930	1.185
5	4.0	5.7	9.4	13.6	22.2
	0.215	0.365	0.703	0.922	1.181
6	4.6	5.5	9.3	13.6	22.4
	0.043	0.177	0.498	0.892	0.947
7	4.4	5.3	8.5	12.3	19.8
	0.0	0.150	0.457	0.662	0.904
8	4.6	5.5	9.1	19.3	21.6
	0.057	0.202	0.515	0.715	0.959
9	4.0	4.8	7.9	11.4	18.5
	0.046	0.181	0.514	0.719	0.968
10	4.0	4.8	7.9	11.4	18.5
	−0.086	0.072	0.414	0.621	0.870
11	5.9	7.3	13.3	20.7	35.5
	−0.085	0.073	0.414	0.622	0.871
12	2.9	3.2	4.3	5.5	7.7
	0.495	0.347	0.022	0.176	0.308
13	3.8	4.5	7.1	10.2	16.3
	−0.137	0.021	0.348	0.561	0.807

[a]First value of each row.
[b]Second data.
[c]Volumetric percentage, v/v.

the log K values were corrected using the equation $\log K^{\text{corr.}} = \log K^{\text{app.}} + \log [1 + 10^{(\text{pH}-\text{pKa})}]$, where the $\log K^{\text{corr.}}$ and the $\log K^{\text{app.}}$ are the corrected and the apparent values, respectively.

For all pyrazolo-pyridine derivatives, linear correlations ($r \geq 0.98$) provided the corresponding extrapolated log K_w values. The log K_w values increased while the acetonitrile perceptual in eluent decreased. The methanol has been the most suitable organic solvent for RP-HPLC lipofilicity measurements. However, the use of acetonitrile instead of methanol permitted to obtain sharp peak shapes and shorter retention times.

Table 4. Linear correlation $\log K = a_0 + a_1\varphi$

Cpd	a_0	a_1	s_{ao}	s_{a1}	P	s	r
1	2.769	0.039	0.131	0.002	0.00054	0.051	−0.99
2	2.689	0.039	0.120	0.002	0.00043	0.047	−0.99
3	2.689	0.039	0.123	0.002	0.00047	0.048	−0.99
4	2.643	0.038	0.121	0.002	0.00049	0.048	−0.99
5	2.650	0.038	0.117	0.002	0.00043	0.046	−0.99
6	2.522	0.039	0.222	0.004	0.00274	0.087	−0.98
7	2.272	0.035	0.108	0.002	0.00042	0.042	−0.99
8	2.326	0.035	0.107	0.002	0.00042	0.042	−0.99
9	2.376	0.036	0.112	0.002	0.00043	0.044	−0.99
10	2.332	0.038	0.096	0.002	0.00025	0.038	−0.99
11	2.330	0.038	0.097	0.002	0.00025	0.038	−0.99
12	1.777	0.035	0.080	0.002	0.00017	0.031	−0.99
13	2.245	0.037	0.103	0.002	0.00032	0.04	−0.99

a_o is the intercept log K_w; a_1 is the slope; φ is the organic modifier concentration in the mobile phase (v/v); s_{ao} and s_{a1} are the standard errors for the intercept and the slope; s is the fit standard error; r is the correlation coefficient for 95% confidence limits; P is the variance.

log P Calculations

The partition coefficient was measured for unsubstituted derivative (log $P_H = 0.96$, **8**) using the classical shake flask method[1] under optimized conditions, as described on Experimental Section. The remaining compounds (**1**–**7** and **9**–**13**) had the log P_X values calculated from log P_H of **8** by Hansch-Fujita hydrophobic parameters[5] (π) using the equation $\log P_X = \log P_H + \pi_X$. The great insolubility of pyrazolo-pyridine derivatives in the water complicated the partition coefficients experimental measurement by the shake flask method.

In addition, with comparative purposes, the log P values for pyrazolo-pyridine derivatives (**1**–**13**) were calculated by the CLOGP program.[4] The log P_{CLOGP} values are shown in Table 5.

Relationship Between log K_w and R_{Mw}

Linear correlation was observed between log K_W and R_{Mw} values with $r = 0.964$ (Equation 1, Figure 2). This correlation verifies the self consistency of RP-TLC and RP-HPLC methods and justifies the confidence in the chromatographic data as hydrophobic parameters.

$$\log K_W = 1.420\ (\pm 0.086) + 0.585\ (\pm 0.048) R_{Mw} \quad n = 13;$$
$$r = 0.964; \quad s = 0.075; P < 0.0001 \qquad (1)$$

Table 5. Calculated log $P_{Hansch\text{-}Fujita}$ and log P_{CLOGP} values of 1*H* -pyrazolo[3,4-*b*]pyridines derivatives

Compound	log $P_{Hansch\text{-}Fujita}$[a]	log P_{CLOGP}
1	1.82	5.30
2	1.67	5.12
3	1.67	5.14
4	1.52	4.92
5	1.52	4.92
6	0.94	4.34
7	0.94	4.34
8	0.96	4.42
9	1.10	4.57
10	0.68	4.24
11	0.68	4.24
12	1.03	3.75
13	0.32	3.77

[a]log $P_H = 0.96$.

Correlations Between Different Techniques

The linear regression curves described by equations (2–6) show log P relationships between the chromatographic values, those obtained from log P_H (log $P_{Hansch\text{-}Fujita}$) and the theoretical ones (log P_{CLOGP}). The log P of **12** (X = OH) was omitted from equations (2–5), because it presented a large

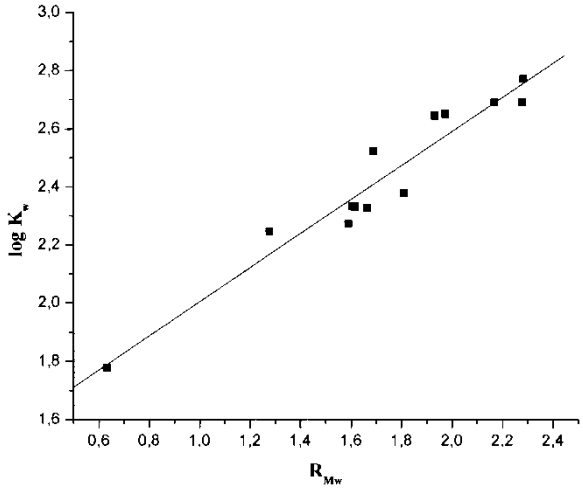


Figure 2. Correlation of the log K_w and R_{Mw} values, as described by equation (1).

deviation, which might suggest a stronger interaction of this compound with the stationary phase in the RP-TLC and RP-HPLC methods.

$$\log P_{CLOGP} = 1.918\ (\pm 0.150) + 1.476\ (\pm 0.081) R_{Mw} \quad n = 12;$$
$$r = 0.985; \quad s = 0.084; \quad P < 0.0001 \qquad (2)$$

$$\log P_{Hansch-Fujita} = -1.576\ (\pm 0.226) + 1.496\ (\pm 0.122) R_{Mw} \quad n = 12;$$
$$r = 0.968; \quad s = 0.126; \quad P = 0.0001 \qquad (3)$$

$$\log P_{CLOGP} = -0.953\ (\pm 0.729) + 2.237\ (\pm 0.292) \log K_W$$
$$n = 12; \quad r = 0.924; \quad s = 0.0186; \quad P < 0.0001 \qquad (4)$$

$$\log P_{Hansch-Fujita} = -4.612\ (\pm 0.729) + 2.317\ (\pm 0.293) \log K_W$$
$$n = 12; \quad r = 0.928; \quad s = 0.186; \quad P < 0.0001 \qquad (5)$$

Thereby, the log P and log P_{CLOGP} values were correlated against R_{Mw} (Equations 2 and 3, Figure 3) and against log K_w (Equations 4 and 5, Figure 4) to verify the alternative utilization of these hydrophobic parameters. The correlation coefficients for the relationship with log K_w (Equations 4 and 5 are slightly lower than for R_{Mw} (Equations 2 and 3). These results can be explained by differences in retention mechanisms between RP-TLC and RP-HPLC. The interactions between the compounds, and the residual free silanol groups on the C_8 non-polar stationary phase in the RP-HPLC column, are stronger than on the C_8 silica surface used in RP-TLC.

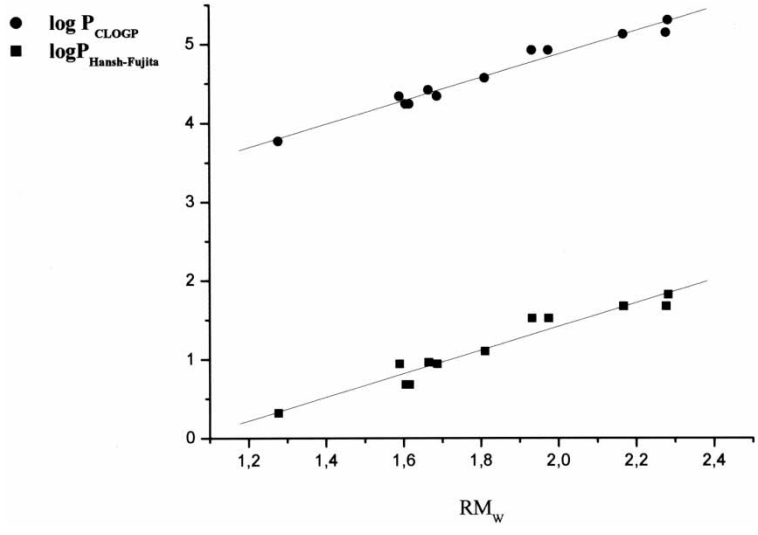


Figure 3. Correlations between R_{Mw} values and the log P_{CLOGP} and log $P_{Hansch-Fujita}$, as described by equations (2) and (3), respectively.

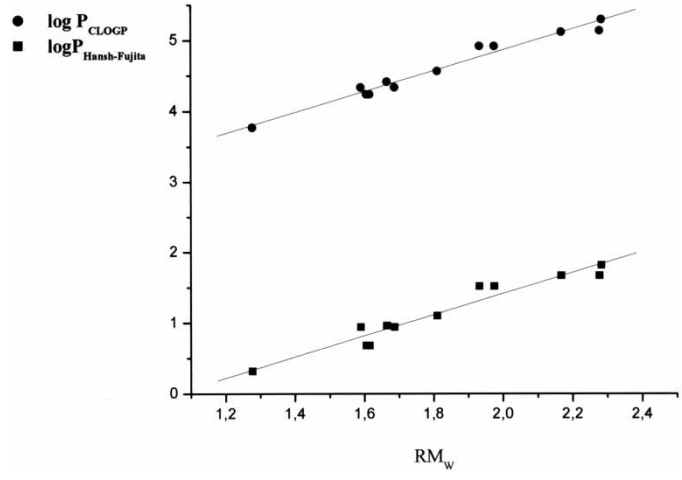


Figure 4. Correlations between K_w values and the log P_{CLOGP} and log $P_{Hansch\text{-}Fujita}$, as described by equations (4) and (5), respectively.

The plot of Figure 5 shows a good linear correlation between the slopes (a_1) and the intercepts (R_{Mw}) of the **1**–**13** RP-TLC equations (Table 2), as described by equation (6).

$$\text{Intercept} = 0.015\ (\pm 0.001) + 0.012\ (\pm 0.0007)\text{Slope} \quad n = 13;$$
$$r = 0.984; \quad s = 0.001; \quad P < 0.0001 \tag{6}$$

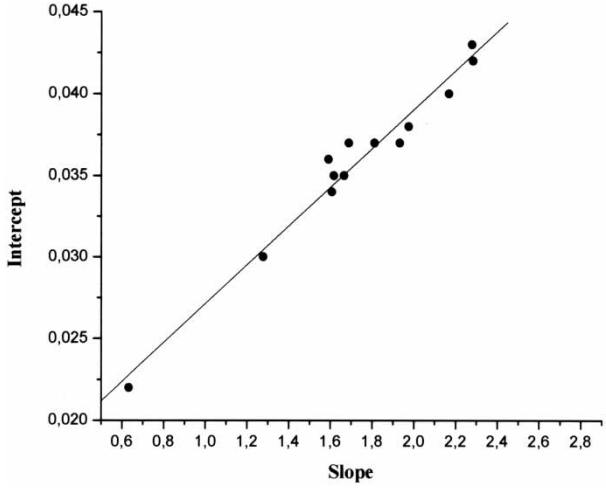


Figure 5. Correlation between intercept (R_{Mw}) and slope (a_1) values as described by equation (6).

The chromatographic assays of hydrophobicity can be expressed as the linearity between the intercept (a_o) and the slope (a_1) of $R_M = a_o + a_1\varphi$ (where φ is the concentration of the organic modifier in the mobile phase).[11] The slopes indicate the rate in which the solubility of the compounds increase in the mobile phase with the percent of organic solvent rise ($a_1 = 0.022$ to 0.043). The most lipophylic compound, **1** (X = Br), exhibits the higher R_{Mw} (2.282), because it is more sensitive to a decrease in the polarity of the mobile phase and, thereby, with a higher slope (0.042). The most hydrophobic compound, **12** (X = OH), with lower slope (0.022) and sensibility to a decrease in mobile phase polarity, has less R_{Mw} (0.632). The linearity of relationship between R_{Mw} and the a_1 shows similar chromatographic behavior.

The plot of Figure 6 shows a relatively poor correlation obtained for the relationship between slope, a_1 (Table 4) and the log K_w.

$$\text{Intercept} = -2.386\ (\pm 1.204) + 128.889\ (\pm 32.176)\text{Slope} \quad n = 13;$$
$$r = 0.77; \quad s = 0.179; \quad P = 0.0001 \tag{7}$$

This result can be explained by the differences in retention mechanisms between RP-HPLC and RP-TLC under the described experimental conditions. The stronger interaction can occur between the pyrazolo[3,4-*b*]pyridines derivatives and the free silanol group used in an RP-HPLC column.

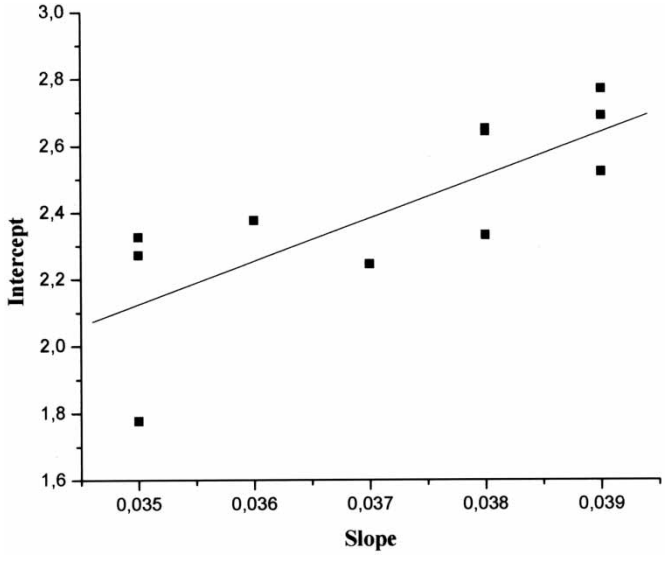


Figure 6. Correlation between intercept (log K_w) and slope (a_1) values as described by equation (7).

CONCLUSION

Although the shake flask method has become the standard technique for quantifying the hydrophobicity of organic compounds, it is particularly problematic when the compound is highly insoluble in one of the solvent phases. The pyrazolo-pyridine derivatives are slightly water soluble and their log P could not be determined by shake flask method.

But, reverse phase chromatographic methods (RP-TLC and RP-HPLC) have proven to be reliable and accurate methods to describe the hydrophobical nature of 1*H*-pyrazolo[3,4-*b*]pyridines derivatives. However, stronger interactions between the compounds and the residual silanol groups occur in the RP-HPLC measurements.

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