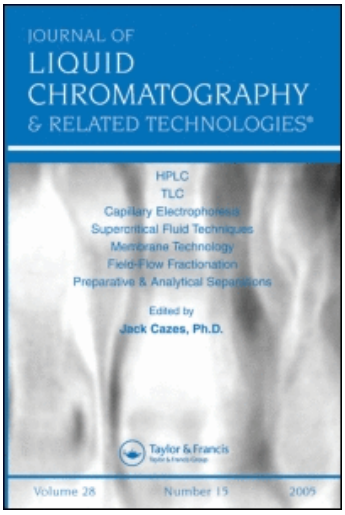

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SALTING-OUT AND SALTING-IN EFFECTS IN THE REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY OF DANSYLATED AMINO ACIDS. EFFECT OF ACIDS

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ABSTRACT

The retention of 15 dansylated amino acid derivatives was determined in aqueous solutions of formic, acetic, propionic and perchloric acid in reversed-phase thin-layer chromatography. The acids increased the retention of each derivatives at the low concentration range. This effect has been tentatively explained by the suppression of dissociation of polar groups in the solute molecules resulting in increased apparent lipophilicity (salting-out phenomenon). The higher concentrations of acid solutions decreased the retention (salting-in effect), the undissociated acid molecules probably act as an organic mobile phase. Both salting-in and salting-out phenomena can be simultaneously described by bilinear function. The polarity parameters of the amino acids, their hydrophobicity and the strength of the acid in the eluent simultaneously influence the retention.

INTRODUCTION

Reversed-phase thin-layer chromatography (RPTLC) has been extensively applied to determine the lipophilicity of various bioactive compounds (1-3). The RPTLC method to determine lipophilicity has some distinct advantages over the traditional partition method between water and n-octanol (4): it is rapid and relatively simple, uses very small quantities of compounds, and the compounds need not to be very pure. However, in RPTLC the actual value of lipophilicity (R_M value) depends on the chromatographic conditions. The supports partially retain their original adsorptive character even after impregnation (5), the R_M value changes with the quantity (7) and quality (8) of the impregnating agent. When the compound also contains one or more dissociable polar substituents the pH of the eluent (9,10) and its salt concentration (11-13) modify the lipophilicity. The characteristics of the organic mobile phase also influences the retention (14,15). To increase the accuracy of the lipophilicity determination the R_M value extrapolated to zero organic phase concentration (R_{MO}) has been calculated from the linear correlation between the actual R_M value and the organic phase concentration in the eluent (16,17). However, in the case of quaternary amino steroids (18), peptides (19) and crown ether derivatives (13), no linear correlation was found between the R_M value and the concentration of the organic mobile phase. The R_M value decreased with increasing organic phase concentration in the lower concentration range, reached a maximum, and then increased with further increase of the organic phase ratio.

This phenomenon was tentatively explained in terms of a silanophyl effect (20,21): at higher organic phase concentrations, the solute molecules have an enhanced probability of access to the silanol groups uncovered by the impregnating agent. The adsorptive side effect of free silanol groups can be eliminated or decreased by the addition of alkylamines (22) or salts (23) to the eluent. It was assumed that the ions of the dissociated salt bind to the remaining adsorption sites of the support decreasing their influence on the retention.

Dansylated amino acids have been frequently used in amino acid, peptide and protein analysis. Dansylated derivatives of amino acids have been applied in HPLC to separate D and L isomers (24) and in paper electrophoresis to separate amines and amino acids (25). Taurine was also determined as dansyl derivative in reversed-phase HPLC in eluent containing acetic acid (26). Dansylation has been recently applied in biochemical and biophysical studies to label porcine pancreatic colipase (27) and to synthetise human renin inhibiting peptides (28).

As dansylated amino acids contain a large hydrophobic and a highly polar hydrophilic moiety, it was of interest to study the effect of pH on their retention in RPTLC.

The objectives of our work were to study the effect of various acids on the reversed-phase retention of some dansyl amino acids and to correlate the retention behavior with the physicochemical parameters of the dansyl amino acids.

MATERIAL AND METHODS

Silufol UV_{254} silica plates (Kavalier, Czechoslovakia) were impregnated with an overnight predevelopment in paraffin oil:n-hexane 5:95 v/v and then dried at room temperature. Dansyl derivatives were prepared from amino acids of analytical purity as described in ref.29, they were dissolved in ethanol at a concentration of 0.5 mg/ml, and 2 μl of each solution was spotted onto the plates. To exclude the effect of organic mobile phase and that of the possible solvent demixing distilled water was used as eluent.

The R_M value of dansyl amino acids having a reasonable mobility in distilled water as eluent was determined in aqueous solutions of formic, acetic and propionic acid in the concentration range of 0.125 mM - 2 M. To elucidate the differences between organic and inorganic acids, the same experiments were also carried out with perchloric acid. However, in this case only the dansyl derivatives of Ala, Asp, Gly, Gln, Glu and Ser were in-

vestigated. To compare the effect of free acids and their sodium salts, the same experiments were carried out using the sodium salts of the acids. After development the plates were dried at 105°C and the spots were detected under an UV lamp. Each experiment was run in quadruplicate.

The R_M values were calculated according to eq.1.

$$R_M = \log(1/R_f - 1) \qquad (1)$$

As the dependence of the R_M value on the acid concentration in the eluent showed a maximum, we tried to fit a bilinear function (30) to our experimental data, the R_M value and the logarithm of the acid concentration being the dependent and independent variables respectively:

$$R_M = a + b_1.\log C + b_2.\log(b_3.10^{\log C} + 1) \qquad (2)$$

To elucidate the individual effects of the physicochemical parameters of amino acids and those of the acids in the eluent on the retention, stepwise regression analysis (31) was applied to select the independent variables influencing the retention significantly. In the common multivariate regression analysis the presence of independent variables exerting no significant influence on the dependent variable lessens the significance level of the independent variables significantly influencing the dependent variable. To overcome this difficulty the stepwise regression analysis automatically eliminates of the selected equation the insignificant independent variables increasing in this manner the information power of the calculation.

The parameters of eq.2 (a, b_1, b_2 and b_3 value) as well as the logarithm of the acid concentration in the eluent causing maximum retention ($\log C_M$) were separately taken as dependent variables.

The $pK_{\alpha NH_2}$, $pK_{\alpha COOH}$, pK of tertiary polar group taken from ref.32 as well as the z_1, z_2 and z_3 values related to the hydrophobici- ty, side chain bulk and electrostatic properties of amino acids respectively (taken from ref.33) served as independent variables. The acceptance limit for the independent variables was set to 95% significance level. The calculations were sepa-

rately carried out for each acid in the eluent. To assess the role of strength of acids the same calculations were carried out with the modification that the parameter of each dansyl amino acid was simultaneously included as dependent variable and the equilibrium constant (taken from refs 34 and 35) of the acids was added to the independent variables. The other conditions of the calculations were as before. To lend support to the hypothesis that the lipophilicity of the undissociated acid plays a considerable role in the anomalous retention behavior of dansyl amino acids we intended to include the lipophilicity value of acids in the calculation. Unfortunately, we could not find any lipophilicity data for perchloric acid, therefore the lipophilicity parameter was excluded from the calculation.

RESULTS AND DISCUSSION

The dansylated derivatives of Ala, Asn, Asp, Citr, Gly, Gln, Glu, HyPro, Ile, Leu, Norleu, Pro, Ser, Thr and Val showed reasonable mobility in distilled water as eluent therefore only these derivatives were included in the experiments. The data were excluded from the calculation when the coefficient of variation of the parallel determinations was higher than 6%. Each dansylated amino acids showed irregular retention behavior in the presence of each acid (Figs 1 and 2). The R_M values increased at lower acid concentrations, reached a maximum, and then decreased with further increase of the acid concentration. It means that the same additive in the eluent firstly causes a salting-out and then a salting-in effect. The retention of dansyl amino acids very rapidly increased with growing acid concentration in the mM concentration range, the salting-in effect (decrease of retention) less steeply changed with the acid concentration. This finding suggests that the acids influence the retention of dansyl amino acids in minimally two different manners:

1. Very low acid concentrations are sufficient to suppress the dissociation of the polar groups increasing their apparent lipophilicity. The indisso-

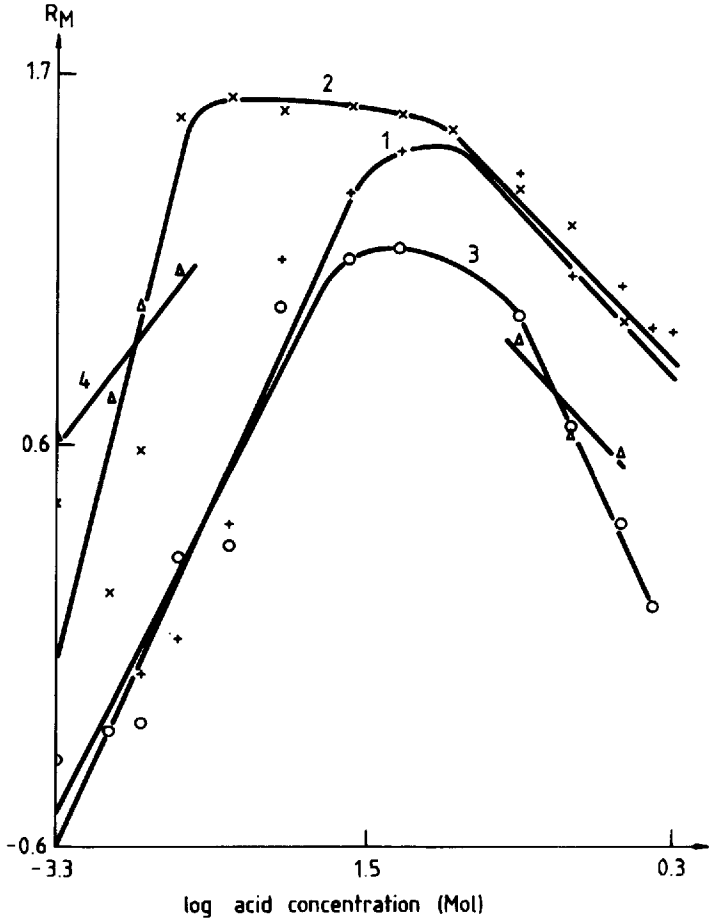

Figure 1. Effect of acid concentration in the eluent on the lipophilicity (R_M value) of dansyl aspartic acid.
1. Formic acid 2. Acetic acid 3. Propionic acid 4. Perchloric acid.

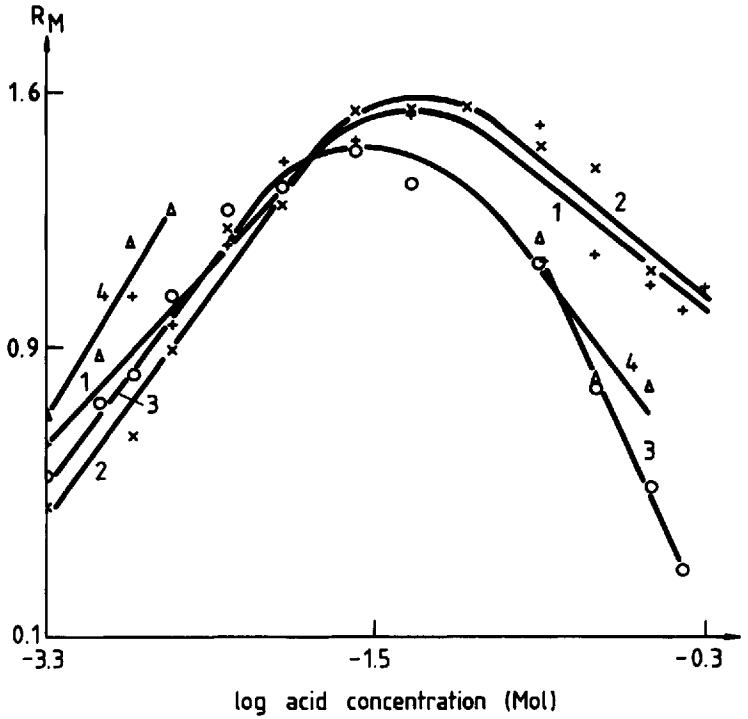

Figure 2. Effect of acid concentration in the eluent on the lipophilicity (R_M value) of dansyl serine. 1. Formic acid 2. Acetic acid 3. Propionic acid 4. Perchloric acid.

ciated form of dansyl amino acid binds more strongly to the hydrophobic surface of the sationary phase resulting in enhanced retention (salting-out effect).

2. At higher acid concentrations the ratio and number of the undissociated acid molecules increase in the eluent. The retention data can be explained by the assumption that the undissociated acid molecule acts as a simple organic modifier decreasing the retention in the same manner as methanol, acetone etc. do (salting-in effect).

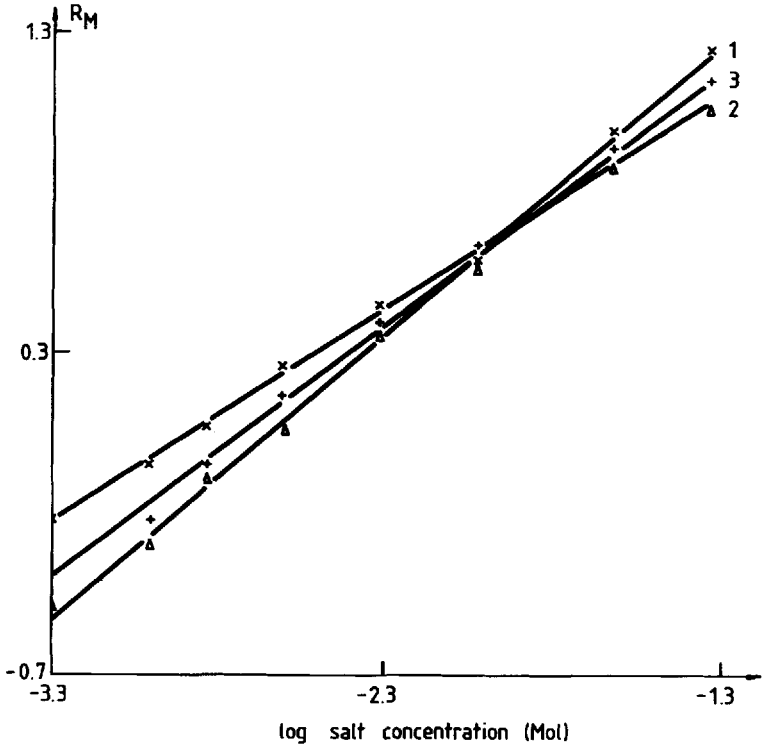

Figure 3. Effect of sodium formiate, acetate and propionate concentration in the eluent on the lipophilicity (R_M value) of dansyl aspartic acid.
1. Sodium formiate 2. Sodium acetate 3. Sodium propionate

The hypothesis outlined above accounts well for the effect of organic acids, their undissociated form being more hydrophobic than the water. However, our data prove that perchloric acid behaves similarly that is the undissociated perchloric acid molecule has to have a similar lipophilicity as for example the propionic acid molecule. This statement is probably not true but we have to admit that we do not have any valid explanation for this phenomenon.

TABLE 1.

Parameters of the Bilinear Correlations Between the Lipophilicity of Dansyl Amino Acids (R_M) and the Logarithm of Formic Acid Concentration (C) in the Eluent. n = 13, for Leu n = 9

$$R_M = a + b_1.\log C + b_2.\log(b_3.10^{\log C} + 1)$$

Parameters	Ala	Asn	Asp	Citr	Gly	Gln
a	72.23	56.00	-65.66	83.09	69.44	68.72
b_1	62.72	59.74	132.76	55.29	56.93	58.71
b_2	-112.6	-106.3	-209.8	-121.8	-120.7	-122.4
b_3	-2.10	-2.00	-2.07	-1.30	-2.13	-2.13
log C_M	-1.18	-1.24	-1.00	-1.08	-1.22	-1.20
r^2	0.9691	0.894	0.9591	0.9304	0.9389	0.9451
F	141.0	38.0	105.4	60.2	69.2	77.5

	Glu	HyPro	Leu	Ser	Thr	Val
a	-55.90	68.44	131.4	63.97	72.09	114.2
b_1	129.1	58.73	44.89	62.26	61.37	50.17
b_2	-210.6	-131.9	-98.97	-117.8	-140.0	-105.6
b_3	-2.10	-2.30	-1.64	-1.98	-2.30	-1.70
log C_M	-1.01	-1.10	-1.75	-1.27	-1.11	-1.65
r^2	0.9478	0.9596	0.7699	0.9172	0.9653	0.9179
F	81.7	106.9	10.4	49.8	125.1	50.3

TABLE 2.

Parameters of the Bilinear Correlations Between the Lipophilicity of Dansyl Amino Acids (R_M) and the Logarithm of Acetic Acid Concentration (C, in the Eluent. n = 14

$$R_M = a + b_1.\log C + b_2.\log(b_3.10^{\log C} + 1)$$

Parameters	Ala	Asn	Asp	Citr	Gly
a	48.61	1.37	20.09	67.87	33.40
b_1	138.8	87.02	169.1	175.4	148.5
b_2	-222.8	-109.3	-244.1	-227.8	-237.1
b_3	-1.60	-2.35	-1.30	-1.00	-1.55
log C_M	-1.48	-1.10	-1.65	-1.78	-1.53
r^2	0.7930	0.8934	0.8134	0.7371	0.8009
F	21.1	46.1	24.0	15.4	22.1

	Gln	Glu	HyPro	Ser	Thr
a	34.19	34.86	37.77	27.17	38.70
b_1	164.1	169.4	75.1	77.8	83.4
b_2	-245.0	-243.3	-216.4	-235.2	-239.1
b_3	-1.39	-1.30	-2.60	-2.60	-2.60
log C_M	-1.60	-1.64	-0.97	-1.01	-0.97
r^2	0.7973	0.7950	0.9062	0.8901	0.9007
F	21.6	20.1	53.1	44.5	49.9

TABLE 3.

Parameters of the Bilinear Correlations Between the Lipophilicity of Dansyl Amino Acids (R_M) and the Logarithm of Propionic Acid Concentration (C) in the Eluent. n = 13, for Leu n = 12, for Pro n = 10.

$$R_M = a + b_1.\log C + b_2.\log(b_3.10^{\log C} + 1)$$

Parameters	Dansylated amino acid				
	Ala	Asn	Asp	Citr	Gly
a	81.04	55.00	-50.55	94.32	74.18
b_1	50.61	51.73	109.3	41.85	48.57
b_2	-219.1	-170.2	-262.7	-274.2	-216.4
b_3	-1.60	-2.30	-2.30	-2.90	-2.60
log C_M	-1.22	-1.36	-1.15	-1.14	-1.24
r^2	0.9874	0.9831	0.9486	0.9879	0.9904
F	290.9	92.2	407.3	513.5	369.7
	Gln	Glu	HyPro	Ile	Leu
a	78.78	-31.75	65.89	131.2	126.4
b_1	47.09	93.47	63.34	35.36	51.12
b_2	-219.8	-298.4	-203.6	-168.3	-179.3
b_3	-2.60	-2.60	-2.30	-2.30	-2.09
log C_M	-1.26	-1.04	-1.35	-1.58	-1.61
r^2	0.9867	0.9436	0.9999	0.9819	0.9837
F	369.7	83.6	1222.9	271.5	271.7
	NorLeu	Pro	Ser	Thr	Val
a	146.5	108.2	54.95	72.07	112.2
b_1	25.01	94.30	70.31	63.41	51.90
b_2	-149.4	-221.1	-187.6	-212.2	-160.1
b_3	-2.30	-1.95	-2.05	-2.35	-1.99
log C_M	-1.70	-1.48	-1.48	-1.33	-1.63
r^2	0.9819	0.9846	0.9884	0.9896	0.9801
F	270.7	223.8	425.3	476.7	246.2

Sodium salts showed only salting-out effect, at higher salt concentrations the dansyl amino acids remained on the start (Fig.3). This observation indicates that the sodium salts at low concentrations exert similar effect on the retention of dansyl amino acids as the free acids, namely the suppression of dissociation resulting in increased retention. The salts did not decrease the retention in the higher concentration range that is the effect of undissociated salts and acids is markedly different.

The parameters of bilinear correlations between the lipophilicity of dansyl amino acids and the acid concentration in the eluent (see eq.2) are compiled in Tables 1 - 4. The calculated F values clearly show that

TABLE 4.

Parameters of the Bilinear Correlations Between the Lipophilicity of Dansyl Amino Acids (R_M) and the Logarithm of Perchloric Acid Concentration (C) in the Eluent. n = 10

$$R_M = a + b_1.\log C + b_2.\log(b_3.10^{\log C} + 1)$$

Parameters	Dansylated amino acid					
	Ala	Asp	Gly	Gln	Glu	Ser
a	94.06	63.12	80.06	81.18	81.44	70.21
b_1	91.14	83.84	83.36	82.43	79.04	102.5
b_2	-136.3	-140.4	-141.9	-151.2	-144.3	-175.9
b_3	-1.13	-1.30	-1.30	-1.44	-1.41	-1.39
log C_M	-4.86	-1.83	-1.85	-1.79	-1.81	-1.77
r^2	0.9158	0.9412	0.9924	0.8880	0.9022	0.9692
F	38.1	56.0	26.3	27.8	32.3	110.5

Table 5.

Relationship Between the Physicochemical Parameters of Amino Acids and the Parameters of eq.2. Propionic Acid in the Eluent. Results of Stepwise Regression Analysis. n = 15

I. $a = a_1 + b_4.x_1 + b_5.x_2 + b_6.x_3$
II. $b_1 = a_1 + b_4.x_2 + b_5.x_4$
III. $b_2 = a_1 + b_4.x_2$
IV. $\log C_M = a_1 + b_4.x_3$

$x_1 = pK_{\alpha NH_2}$; x_2 = pK tertiary polar group; $x_3 = z_1$ (hydrophobicity); $x_4 = pK_{\alpha COOH}$

Parameter	Number of equation			
	I	II	III	IV
a_1	-303.4	265.4	-388.4	-1.37
b_4	13.91	-12.11	27.96	$7.67.10^{-2}$
s_{b4}	6.29	3.69	6.79	$1.36.10^{-2}$
b_5	37.31	-57.29	-	-
s_{b5}	2.35	22.18	-	-
b_6	-10.74	-	-	-
s_{b6}	1.18	-	-	-
F	170.8	13.5	17.0	32.0
r^2	0.9809	0.7105	0.5855	0.7273
b_4·%	8.19	55.98	-	-
b_5·%	56.63	44.02	-	-
b_6·%	35.18	-	-	-

Table 6.

Influence of the Physicochemical Characteristics of Amino Acids and the Strength of the Acids in the Eluent on the Parameters of eq.2. Results of Stepwise Regression Analysis. n = 43

I. $a = a_1 + b_4.x_2 + b_5.x_3 + b_6.x_5$
II. $b_1 = a_1 + b_4.x_2 + b_5.x_3$
III. $b_2 = a_1 + b_4.x_2 + b_5.x_5$
IV. $b_3 = a_1 + b_4.x_5$
V. $\log C_M = a_1 + b_4.x_5$

x_2 = pK tertiary polar group; x_3 = z_1 (hydrophobicity); x_5 = equilibrium costant of acids

Parameters	Number of equation				
	I	II	III	IV	V
a_1	-12.26	162.8	183.8	-0.99	-2.53
b_4	21.49	-13.30	17.92	-0.24	0.26
s_{b4}	3.40	4.43	5.30	0.06	0.07
b_5	-14.21	5.88	-28.00	-	-
s_{b5}	2.24	2.90	5.83	-	-
b_6	-12.84	-	-	-	-
s_{b6}	3.71	-	-	-	-
F	34.3	8.38	15.2	16.1	12.2
r^2	0.7251	0.2952	0.4325	0.2818	0.2299
b_4·%	39.14	59.66	41.30	-	-
b_5·%	39.58	40.34	58.70	-	-
b_6·%	21.28	-	-	-	-

eq.2 fits well to the experimental data, the significance level is generally over 99%. The coefficient of determination (r^2 values) indicates that the changes in the acid concentration in the eluent account for the the overwhelming majority of the change of retention.

The numerical values of the parameters vary according to the type of acid in the eluent and also according to the type of dansyl amino acid that is the retention is influenced equally by the character and concentration of the acid in the eluent and by the structure of the dansyl amino acid.

The results of the stepwise regression analysis showed that each parameter of eq.2 depended significantly on the physicochemical characteristics of amino acids (Table 5). As the effect of each acid depended similarly on the physicochemical parameters of amino acids, only the results concerning the propionic acid are shown.

Both the polarity and the hydrophobicity of the amino acids have a significant impact on the numerical value of the majority of parameters in eq.2. The intercept (a value) showed the best correlation with the physicochemical characteristics of amino acids (I.eq. in Table 5). The three parameters explained about the 98% of the total variance (see r^2 value) that is the change of the slope value can be well predicted by the change of the corresponding molecular characteristics of amino acids. The impact of polar and hydrophobic parameters is commensurable (see b·% values).

The strength of the acids in the eluent has a high effect on the parameters of eq.2 (Table 6).

In this case the pK value of the tertiary polar group and the hydrophobicity of the amino acids also influence the intercept (a) value, however, the strength of the acids exerts a considerable impact on the retention behavior of the dansyl derivatives (compare b· value).

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