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Support matrix effects in the reversed-phase thin-layer chromatography of some peptides

Tibor Cserháti

Central Research Institute for Chemistry, Hungarian Academy of Sciences, P.O.B. 17, H-1525 Budapest (Hungary)

ABSTRACT

The retentions of 28 peptides in reversed-phase thin-layer chromatography (RPTLC) were determined on cellulose and on impregnated cellulose and alumina layers with 1-propanol as the organic component of the mobile phase. Each peptide showed a support matrix effect: their R_M values first decreased to a minimum, then increased with increasing 1-propanol concentration. On cellulose layers only the increasing phase was observed. The retention behaviour of peptides was adequately described with a quadratic or linear function, but the slope value of the linear function had a positive value. The results demonstrate that the support matrix effect can be observed on non-silica supports and it may occur in reversed-phase chromatography in the case of polar solutes and supports with free adsorptive centres on their surfaces. Both the intercept and slope values of the function are needed to describe the lipophilicity of peptides, but the correlation is not strong enough for the determination of the lipophilicity of peptides by RPTLC. Principal component analysis showed that the peptides form distinct clusters on the basis of their retention characteristics: peptides containing a basic amino acid, peptides with a ring structure in the amino acid side-chain and peptides containing uncharged amino acids.

INTRODUCTION

Reversed-phase thin-layer chromatography (RPTLC) has been extensively applied to determine the lipophilicity of bioactive molecules [1,2]. To increase the accuracy of the lipophilicity determination, linear correlations have been calculated between the R_M values and the concentration of the organic component of the mobile phase; the R_M value extrapolated to zero organic phase concentration (R_{M0}) was regarded as the most accurate estimate of lipophilicity [3,4]. However, in the case of peptides [5], quaternary amino steroids [6] and crown ether derivatives [7,8], no linear correlation was found between the R_M value and the concentration of the organic component of the mobile phase. The R_M value decreased with increasing organic concentration in the lower concentration range, reached a minimum and then increased with further increase of the organic phase ratio. This phenomenon was tentatively explained in terms of a silanophilic effect: at higher organic concentrations, the solute molecules have an enhanced probability of access to the silanol groups uncovered by the impregnating agent. The interaction with the free silanol groups results in an increased retention and an increased apparent lipophilicity [5]. The adsorptive side-effect of free silanol groups can be eliminated or decreased by the addition of alkylamines [9] or salts [10] to the eluent.

Recent research indicates that in RPTLC the adsorptive character of the support material has a considerable influence on retention, even after impregnation [11–13], because the adsorptive sites not covered with the impregnating agent also affect the binding of solutes. This indicates that the surface pH value and adsorption characteristics of the support may have some impact on the retention of polar compounds even after impregnation. It has recently been established that the surface pH of the silica influences the RPTLC retention of dansylamino acids [14] and of free amino acids [15].

So far as we are aware, the structural characteristics of solutes accounting for the silanophilic effect,

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and the influence of the surface pH of the support on it, have never been studied in detail. The term "silanophilic" effect becomes misleading when supports other than silica are used in the study of the polar (possibly hydrogen bonding) effects mediated by the matrix. We consider that the expression "support matrix effect" better describes the phenomena discussed above. We assumed that owing to their amphipathic character peptides are ideal test solutes to study the effects outlined above.

Reversed-phase chromatography has been extensively applied to separate peptides on both the analytical [16] and preparative scale [17,18]. The retention depended on the type [19] and density of the hydrophobic ligand [20]. Moreover, reversed-phase chromatography has been utilized as a physico-chemical tool for the study of peptide behaviour at hydrophobic liquid–solid interfaces which mimic biological lipid bilayers. This helped to identify and characterize both the hydrophobic interaction sites and the existence of conformational equilibria of peptides such as β-endomorphin [21,22], luteinizing hormone-releasing hormone [23], myosin kinase analogues [24] and human growth hormone related peptides [25,26].

The objectives of this work were to determine the contributions of the physico-chemical parameters of peptides and supports to the support matrix effect in the RPTLC of some peptides.

EXPERIMENTAL

The structures of the peptides are given in Table I. DC Fertigplatten Cellulose, DC Fertigplatten Aluminiumoxid 60 and DC Alufolien Kieselguhr F_{254} (Merck, Darmstadt, Germany) plates were impregnated by overnight predevelopment in *n*-hexane–paraffin oil (95:5, v/v). As it had been demonstrated previously that non-impregnated cellulose may behave as a reversed-phase sorbent under appropriate conditions [4], non-impregnated cellulose plates were also used. The peptides were dissolved in water–1-propanol (2:1, v/v) at a concentration of 2 mg/ml, and 2 μl of each solution was spotted on the plates. 1-Propanol was applied as the organic component of the mobile phase in the concentration range 0–90 vol.% at 5% (cellulose and impregnated cellulose) and 10% intervals (alumina and diatomaceous earth). After development, the peptides were detected with ninhydrin. For each experiment, five independent parallel determinations were carried out.

TABLE I

STRUCTURE OF PEPTIDES

All amino acids had the L-configuration. β-Abu = β-aminobutyric acid; γ-Abu = γ-aminobutyric acid; γ-Ape = γ-aminopentanoic acid; γ-Amh = γ-amino-δ-methylhexanoic acid.

Compound No.	Structure	Compound No.	Structure
1	β-Abu–Ala	15	Pro–Thr–Ile–Pro
2	Gly–Gly	16	Trp–Ser–Tyr–Gly
3	Phe–Ala	17	Trp–Ala–Ile
4	Ala–Ala	18	Phe–Leu–Glu–Glu–Val
5	β-Abu–β-Abu	19	Phe–Gly–Glu–Glu–Leu
6	γ-Amh–γ-Amh	20	Arg–Thr–Asn–Thr–Gly
7	γ-Ape–γ-Abu	21	Leu–Ala–Ala–Ala
8	γ-Abu–γ-Abu	22	Lys(Ala)–Pro–Arg
9	Ala–β-Abu	23	Leu–Lys–Pro–Arg
10	Ala–Thr	24	Lys–Ala
11	Gly–Leu–Gly	25	Ala–Lys–Pro–Lys
12	Gly–β-Abu–Gly	26	Val–His–Asn
13	Glu–Cys–Gly	27	Reduced glutathione
14	Thr–Ile–Pro	28	Oxidized glutathione

MATHEMATICAL METHODS

When in a given RPTLC system the peptide spot remained at the start, or was very near to the front (deformed spot shape), or the relative standard deviation of the five parallel determinations was higher than 8%, the data were omitted from the calculations. Linear correlations between the R_M values of the peptides and the concentration of 1-propanol (C) in the eluent were calculated:

$$R_M = R_{M0} + b_1 C \quad (1)$$

where b_1 is the slope. The calculation was carried out separately for each peptide and for each layer. In some instances eqn. 1 did not give a good fit to the experimental data. Hence a quadratic correlation was calculated for each peptide–1-propanol pair exhibiting an irregular (non-linear) dependence of the retention on the organic phase ratio:

$$R_M = R_{M0} + b_1 C + b_2 C^2 \quad (2)$$

Application of eqn. 2 was motivated by the fact, that the irregular retention behaviour of peptides on impregnated silica layers has been successfully described with a quadratic function [27].

To find the relationships between the retention behaviour of peptides and their lipophilicity, linear correlations were calculated between the parameters of eqn. 2 (independent variables) and the lipophilicity values of the peptides taken from ref. 28 or calculated accordingly. As the inclusion of non-significant independent variables in the equation lessens the information power of the equation, stepwise regression analysis [29] was applied to overcome this difficulty. The number of accepted variables was not limited; they were accepted above 95% significance level. The calculation was carried out separately for both cellulose and alumina supports. As the peptides did not show any retention on the diatomaceous earth support these data were omitted from the calculations.

To elucidate the similarities and dissimilarities between the retention behaviour of the peptides and the parameters of eqns. 1 and 2, principal component analysis (PCA) was applied [30]. The peptides were taken as observations, and the parameters (R_{M0}, b_1 and b_2 values separately for both celluloses and alumina) served as variables. Peptides 6, 16–23, 25–26 and 28 were omitted from the PCA because their retention behaviour cannot be significantly modelled by eqn. 2 on one or both supports. Two-dimensional non-linear mapping of the PC loadings and variables was also carried out [31]. The iteration was carried out to the point when the difference between the two last iterations was less than 10^{-8}.

RESULTS AND DISCUSSION

The peptides did not show any measurable retention on the diatomaceous earth support, the spots being very near to the front both with pure distilled water and water–1-propanol (1:9, v/v) mobile phases. This finding shows again that the hydrophobic impregnating agent does not entirely cover the active adsorption centres on the support surface and the presence of adsorption centres uncovered with the hydrophobic impregnation agent is necessary for retention.

The peptides were not retained on the cellulose surfaces at lower 1-propanol concentrations; at higher concentrations they showed the opposite retention behaviour to that expected (Fig. 1).

The retention of solutes generally decreases with increasing mobile phase ratio, but the retention of peptides increased with increasing concentration of 1-propanol in the mobile phase. This means that the peptides showed typical support matrix effects even

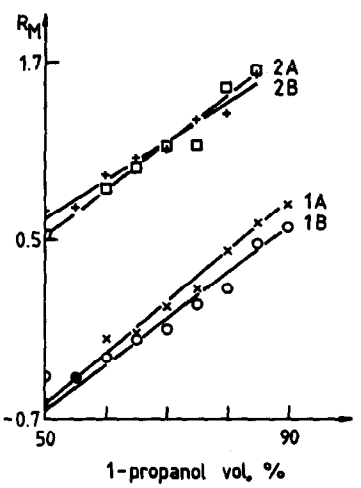

Fig. 1. Dependence of R_M value of (1) Ala–Thr and (2) Lys(Ala)–Pro–Arg on the 1-propanol concentration in the mobile phase. (A) Unimpregnated cellulose; (B) impregnated cellulose.

on cellulose and impregnated cellulose surfaces, where the silanol groups were definitely absent.

Two types of peptide retention behaviour were observed on impregnated alumina (Fig. 2). The retention behaviour of each peptide exhibited typical support matrix effects. In some instances the R_M value decreased with increasing 1-propanol concentration in the lower concentration range, and then increased with further increase in 1-propanol concentration. In other instances the retention of the peptides increased monotonically with increasing 1-propanol concentration. These results also indicate that the support matrix effect can be observed on non-silica layers, that is, the presence of silanol groups is not a prerequisite for the support matrix effect.

The parameters of the equations describing the dependence of R_M values on the 1-propanol concentration on impregnated and non-impregnated cellulose layers are given in Table II. In most instances a significant linear correlation was found between the R_M value of peptides and the 1-propanol concentration in the mobile phase. The r^2 values (ratio of the change in a dependent variable determined by the change in an independent variable or variables) indicate that the change in the 1-propanol concentration explained 92–99% of the change in peptide

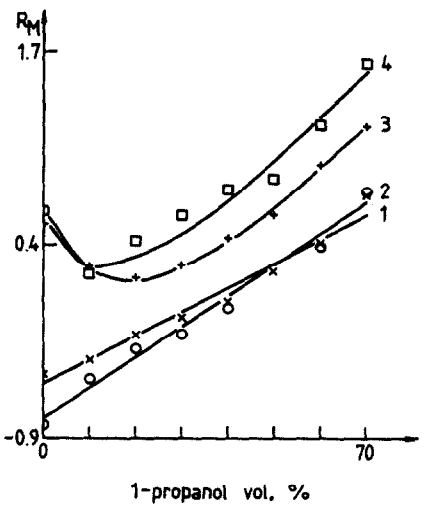

Fig. 2. Dependence of the R_M value of (1) β-Abu–Ala, (2) γ-Ape–γ-Abu, (3) Gly–β-Abu–Gly and (4) Lys–Ala on the 1-propanol concentration in the mobile phase. Impregnated alumina layer.

retention. We should stress that the equations are valid only for the higher concentration range (50–90 vol.%) of 1-propanol; at lower concentrations the peptides were at the front, showing no measurable retention.

The parameters of the equations describing the dependence of R_M values on the 1-propanol concentration on impregnated alumina layers are given in Table III. In most instances a significant correlation was found between the R_M value of the peptides and the 1-propanol concentration in the eluent. In contrast to the cellulose layers, the correlation was not linear in each instance. In some instances a typical support matrix effect was observed, which can be well described with a quadratic function. The equations were similar to those calculated for impregnated silica layers. The r^2 values indicate that the change in the 1-propanol concentration explained 74–98% of the change in peptide retention.

The results suggest that the irregular retention behaviour of peptides (and probably other amphiphilic compounds) cannot be simply ascribed to the presence and availability of free silanol groups. We assume that the phenomenon is the result of at least two effects: (a) the free adsorption centres of the support (silanol groups or not) may influence the retention in eah instance when they are not totally covered (which is momentarily impossible) or masked with appropriate eluent additives; and (b) the dissociation of polar groups is suppressed in mobile phases with lower dielectric constants (higher organic phase ratio). As in reversed-phase chromatography, the retention is governed by the lipophilicity of the solute in the given mobile phase system and the apparent lipophilicity of a solute with dissociable substituents depends strongly on the mobile phase composition, and the variation in the apparent lipophilicity may contribute to the irregular retention behaviour. We believe that with the increasing application of modified alumina [32] and various cellulose derivatives [33–35] as HPTLC sorbents, the problem will increase in importance and its study will help in the elaboration of more efficient separations and a better understanding of the underlying retention mechanisms.

The parameters of the correlations between the chromatographic parameters and the calculated lipophilicity values for various layers are listed in Table IV. The significance level was over 99.9% with

TABLE II

PARAMETERS OF CORRELATIONS BETWEEN THE R_M VALUE OF PEPTIDES AND THE 1-PROPANOL CONCENTRATION IN THE MOBILE PHASE ON CELLULOSE LAYERS

See eqn. 1.

Compound No.[a]	Cellulose support							
	Impregnated				Unimpregnated			
	n^b	R_{M0}	$b_1 \cdot 10^2$	r^2	n^b	R_{M0}	$b_1 \cdot 10^2$	r^2
1	8	−1.95	2.88	0.9493	8	−2.87	4.12	0.9328
2	8	−1.89	3.72	0.9698	8	−2.30	4.08	0.9829
3	9	−2.30	2.91	0.9384	9	−2.19	2.74	0.9700
4	9	−1.78	2.87	0.9440	9	−2.09	3.16	0.9667
5	9	−2.15	2.98	0.9685	8	−2.95	4.28	0.9557
6		n.s.[c]			7	−1.84	1.64	0.9645
7	9	−2.70	4.00	0.9649	8	−2.70	3.77	0.9426
8	9	−2.97	4.37	0.9750	8	−3.18	4.81	0.9673
9	8	−2.07	3.34	0.9520	8	−2.94	4.56	0.9665
10	9	−2.13	2.99	0.9769	8	−2.26	3.33	0.9872
11	9	−2.40	3.35	0.9262	8	−2.79	3.87	0.9791
12	9	−2.26	3.46	0.9663	8	−2.45	3.98	0.9738
13	9	−2.40	4.23	0.9655	7	−2.91	5.04	0.9849
14	9	−1.71	1.78	0.9677	7	−1.71	1.61	0.9671
15	9	−1.75	1.95	0.9347	7	−2.05	2.25	0.9685
16	9	−2.62	3.57	0.9781		n.s.		
17		n.s.			7	−2.00	1.84	0.9299
18	7	−3.62	4.97	0.9696	8	−1.64	1.63	0.9516
19	4	−1.40	1.68	0.9397	6	−2.07	3.22	0.9584
20	7	−1.81	4.01	0.9326	8	−2.19	4.54	0.9746
21		n.s.			8	−1.92	2.13	0.9549
22	8	−0.62	2.56	0.9744	7	−1.06	3.15	0.9612
23	9	−2.57	4.24	0.9604	8	−3.16	4.77	0.9333
24	8	−0.99	2.43	0.9355	9	−1.56	3.17	0.9720
25	8	−4.02	5.28	0.9706	7	−2.83	3.80	0.9736
26	6	−0.69	2.33	0.9724	8	−2.59	4.88	0.9732
27	6	−2.39	4.14	0.9571	8	−2.40	4.10	0.9912
28	6	−2.34	4.94	0.9910	7	−2.82	5.43	0.9775

[a] See Table I.
[b] Number of observations.
[c] Not significant.

cellulose supports and over 95% for impregnated alumina. Eqns. I and II in Table IV support the results of ref. 36 that the intercept (R_{M0}) and slope (b) values of the correlation between the retention value of a compound and the concentration of the organic component in the mobile phase are equally descriptors of the lipophilicity and both are needed for the exact determination of lipophilicity. This finding is supported by the result that both chromatographic parameters have a similar impact on the lipophilicity (see b' values). The r^2 values demonstrate that the predictive power of the equations, although significant, is fairly low. They explain only 50–72% of the lipophilicity change, which from a practical point of view is unacceptable. Our data indicate that the traditional RPTLC method is probably not suitable for the determination of the lipophilicity of peptides and the results obtained by similar methods for similar compounds have to be treated with caution.

The results of PCA are given in Table V. The first and second principal components explain most of

TABLE III

PARAMETERS OF CORRELATIONS BETWEEN THE R_M VALUE OF PEPTIDES AND THE 1-PROPANOL CONCENTRATION IN THE MOBILE PHASE ON AN ALUMINA SUPPORT

See eqn. 2.

Compound No.[a]	n	R_{M0}	$b_1 \cdot 10^2$	$b_2 \cdot 10^3$	r^2
1	8	−0.53	1.62	0	0.9675
2	8	0.04	1.57	0	0.9620
3	7	1.47	−11.14	1.32	0.9374
4	8	−0.26	1.77	0	0.9530
5	6	−0.52	1.54	0	0.9471
6	7	1.18	−8.58	1.02	0.8252
7	7	−0.78	2.07	0	0.9819
8	7	−0.56	2.16	0	0.9767
9	8	−0.26	1.54	0	0.9700
10	8	0.52	−5.15	0.78	0.7403
11	7	1.30	−7.13	0.95	0.8299
12	8	0.48	−2.01	0.45	0.9430
13	5	1.23	1.70	0	0.9249
14	7	1.36	−10.24	1.28	0.8369
15	8	1.33	−10.45	1.32	0.7628
16–19		n.s.			
20	7	−0.72	2.80	0	0.9526
21	8	1.41	−12.40	1.54	0.8172
22, 23		n.s.			
24	8	0.51	−2.01	0.45	0.8808
25, 26		n.s.			
27	7	1.09	1.03	0	0.9600
28		n.s.			

[a] See Table I.

TABLE IV

PARAMETERS OF CORRELATIONS BETWEEN THE LIPOPHILICITY VALUE (y) AND CHROMATOGRAPHIC PARAMETERS (R_{M0}, b_1 AND b_2) OF PEPTIDES ON VARIOUS LAYERS[a]

Results of stepwise regression analysis.

Unimpregnated cellulose: $y = a + b_3R_{M0} + b_4b_1$ (I)

Impregnated cellulose: $y = a + b_3R_{M0} + b_4b_1$ (II)

Impregnated alumina: $y = a + b_4b_1$ (III)

Parameter[a]	No. of equation (layer type)		
	I	II	III
n	23	23	17
a	3.69	1.62	1.58
b_3	−2.03	−2.41	–
s_{b_3}	0.63	0.47	–
b_4	−1.77	−1.45	−0.16
s_{b_4}	0.28	0.34	0.04
b'_3 (%)	34.0	54.9	–
b'_4 (%)	66.0	45.1	–
r^2	0.7203	0.5693	0.5016
F	27.04	13.22	–

[a] b_1–b_4 = Slopes; n = number of observations; a = intercept; b' (%) = normalized slope values (path coefficients); F = calculated value of the F-test.

the total variance, the former explaining about 67% of it. The loadings of each variable are high in the first component. As the slope values (variables 2, 4, 6 and 7) have the highest loadings in the first component and the slope values are related to the hydrophobic surface area of solutes [37], this PC can be regarded as the hydrophobic surface area of the peptides.

Owing to the high intercorrelation between the intercept and slope values, the R_{M0} values also have fairly high loadings in the first component. The two-dimensional non-linear map of PC variables (Fig. 3) shows the clustering of peptides, taking into consideration simultaneously all seven variables. The peptides form distinct clusters according to their structure and polarity. Cluster I contains the peptides with a linear side-chain. This cluster includes

TABLE V

RESULTS OF PRINCIPAL COMPONENT ANALYSIS

Parameter	No. of principal component		
	1	2	3
Eigenvalue	4.72	1.40	0.53
Sum of variance explained (%)	67.45	87.45	94.97
Principal component loadings			
Variable:			
1	0.84	0.36	0.26
2	−0.93	−0.08	0.20
3	0.62	0.72	0.22
4	0.86	−0.34	0.29
5	0.68	−0.51	0.50
6	−0.88	0.44	0.13
7	0.89	−0.42	−0.14

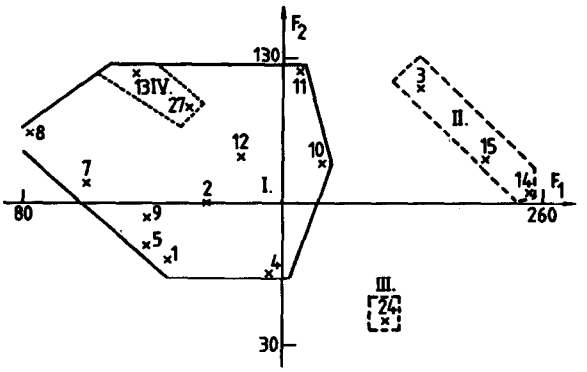

Fig. 3. Two-dimensional non-linear map of peptides. Number of iterations, 59. Error of mapping, $1.04 \cdot 10^{-2}$. Numbers are peptide numbers in Table I.

cluster IV, containing dicarboxylic amino acids. This finding indicates that the acidity of the peptide has a negligible effect on the retention under our experimental conditions. This cluster includes both di- and tripeptides, that is, the number of amino acids in the peptides does not influence the retention characteristics appreciably. The peptides with one or two ring structures are well separated from the others (cluster II). Peptide 24 with a net basic charge forms the third cluster. In contrast to the effect of the net acid charge, the basicity of the peptide markedly influences the retention. This clustering suggests that the polarity and the dimensions of the amino acid side-chain (bulkier ring structure) mainly influence the retention behaviour of peptides in RPTLC even on non-silica supports.

The two-dimensional non-linear map of PC loadings shows the similarities and dissimilarities of the seven variables (Fig. 4). The R_{M0} (points 1, 3 and 5) and linear b (slope) values (points 2, 4 and 6) form distinct clusters independent of the type of support. This finding suggests that the structural characteristics and polarity parameters of the peptides have a greater impact on their retention than the adsorptive character of the supports.

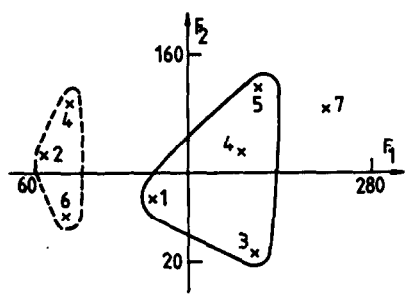

Fig. 4. Two-dimensional non-linear map of parameters of eqn. 2. Number of iterations, 31. Error of mapping, $7.60 \cdot 10^{-3}$. 1 and 2 = R_{M0} and b_1 values of eqn. 2 (unimpregnated cellulose); 3 and 4 = R_{M0} and b_1 values of eqn. 2 (impregnated cellulose); 5, 6 and 7 = R_{M0}, b_1 and b_2 values of eqn. 2 (impregnated alumina).

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