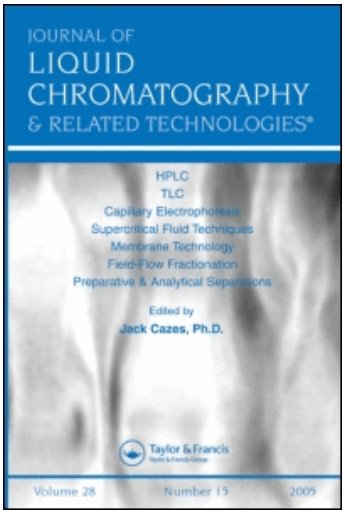

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Reversed-Phase Systems for the Separation of Coumarins and Furocoumarins by Thin-Layer and High-Performance Liquid Chromatography

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To cite this Article Glowniak, K. and Bieganowska, M. L.(1985) 'Reversed-Phase Systems for the Separation of Coumarins and Furocoumarins by Thin-Layer and High-Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 8: 16, 2927 — 2947
To link to this Article: DOI: 10.1080/01483918508076610
URL: http://dx.doi.org/10.1080/01483918508076610

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(16), 2927-2947 (1985)

REVERSED-PHASE SYSTEMS FOR THE SEPARATION OF COUMARINS AND FUROCOUMARINS BY THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The retention behaviour of eleven derivatives of coumarin was investigated by reversed-phase (RP) thin-layer and high performance liquid chromatography. The linear relationships between log k' or R_M values and the content of organic modifier in the aqueous mobile phase obtained for wide composition ranges indicate that the plots can be used to determine R_{MW} and log k'_W values by extrapolation to pure water. The effects of individual substituents on the retention and the correlation between TLC and HPLC data was analysed.

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INTRODUCTION

Coumarins and furocoumarins owing to their biological activity attract wide interest in research /1-4/. The isolation of pure compounds from plant material with definite therapeutic activity (i.e.bacteriostatic, anticoagulant, spasmolytic, dermatological) is useful /5-8/. In order to determine how to optimize the separation of investigated compounds, the effect of solvent composition on the retention was studied. The retention of a solute in reversed-phase systems is mainly controlled by the concentration and the molecular characteristics of the organic modifier in binary hydroorganic solvent eluents. The effect of the modifier on selectivity and on the overall chromatographic distribution equilibria of the solute has been demonstrated both in HPLC and in TLC systems /9-11/. Soczewiński and Wachtmeister /12/ derived the following simple equation describing quantitatively the relationships between retention and solvent composition:

$$\log k'_{w,org} = \varphi_w \log k'_w + \varphi_{org} \log k'_{org} \qquad (1)$$

where w and org denote components of the eluent-water and organic modifier and φ the volume fraction.

The R_M (and log k') value , easily determined in reversed-phase thin-layer and column chromatography

has been widely applied in structure-activity studies (in Hansch's approach) as a measure of the hydrophobicity of molecules /13-16/.

EXPERIMENTAL

Column HPLC was carried out using a liquid chromatograph (produced at the Institute of Physical Chemistry of the Polish Academy of Sciences, Warsaw) equipped with a 200 ml syringe pump, 5 µl sample injector valve and UV 254 nm detector. A stainlees steel column, 150 x 4 mm I.D., was packed with 7 µm LiChrosorb RP-18 (E.Merck, Darmstadt, FRG); the flow rate was 1.2 ml min^{-1}. The column dead volume was determined using an aqueous solution of sodium nitrate as the nonretained compound. The eluents were filtered and degassed in an ultrasonic bath. The temperature was kept at $20^{o} \pm 1^{o}C$. All values of the capacity factors presented are the mean of three determinations.

Thin-layer chromatography was carried out using HPTLC loxlo cm plates of RP-18 F_{254} (E.Merck) and also glass plates 10 x 20 cm covered with a 0.25 mm layer of slurry of the support obtained by mixing 30.0 g of silanized silica gel Si 60 HF_{254} (E.Merck) with either 60 cm^3 of methanol-water mixture (1:2) or with benzene containing 4.5 g of oleic acid or with benzene containing 4.5 g of oleyl alcohol or with acetone containing

Table 1. The investigated compounds

1. Coumarin
2. 4-Hydroxycoumarin
3. 7-Hydroxycoumarin (umbelliferone)
4. 7-Methoxy-8-isopentenylcoumarin (osthol)
5. 4-Methyl-6,7-dihydroxycoumarin
6. 6-Methylcoumarin
7. 8-Hydroxypsoralen (xanthotoxol)
8. 8-Methoxypsoralen (xanthotoxin)
9. 5-Methoxypsoralen (bergapten)
10. 5,8-Dimethoxypsoralen (isopimpinellin)
11. 8-Isopentenyloxypsoralen (imperatorin)

3.0 g of tributylphosphate. The plates were dried in air, spotted with 2 µl samples (0.5 mg/ml in methanol) and placed in tanks with the appropriate eluent. The spots were localized in UV light.

RESULTS AND DISCUSSION

The separation of 11 coumarins (Table 1) was systematically studied by thin-layer and column chromatography. As the stationary phase, silanized silica gel was used alone and then impregnated with oleic acid, oleyl alcohol or tributylphosphate. As the mobile phases were used mixtures of water with organic modifier (methanol, ethanol, acetone, acetonitrile,

Table 2. RP-TLC and HPLC conditions for the determination of the lipophilicity ($\triangle R_M$ or $\triangle \log k'$) of some coumarins and furocoumarins

No of layer or column system	Organic solvent	Concentration in the eluent Vol.%
1. Kieselgel 60 F_{254}, silanized (Merck)	Acetonitrile	40
2. - " -	Acetone	40
3. - " -	Ethanol	40
4. - " -	Tetrahydrofuran	40
5. - " -	Methanol[a]	40
6. - " -	Methanol	50
7. - " -	Methanol[b]	50
8. - " -	Methanol[c]	50
9. Kieselgel 60 F_{254}, silanized (Merck) + 15% oleic acid	Methanol	50
10. - " -	Methanol[c]	50
11. Kieselgel 60 F_{254}, silanized (Merck) + 15% oleyl alcohol	Methanol	50
12. - " -	Methanol[c]	50
13. Kieselgel 60 F_{254}, silanized (Merck) + 10% tributyl phosphate	Methanol	50
14. RP-18 F_{254}, (Merck)	Methanol	60
15. LiChrosorb RP-18 7 µm, (Merck)	Methanol	60
16. LiChrosorb RP-18 7 µm, (Merck)	Methanol[b]	60

eluent contains: a. 0.01 M phosphate buffer, pH = 7,7; b. 3% acetic acid; c. 2% cetrimide.

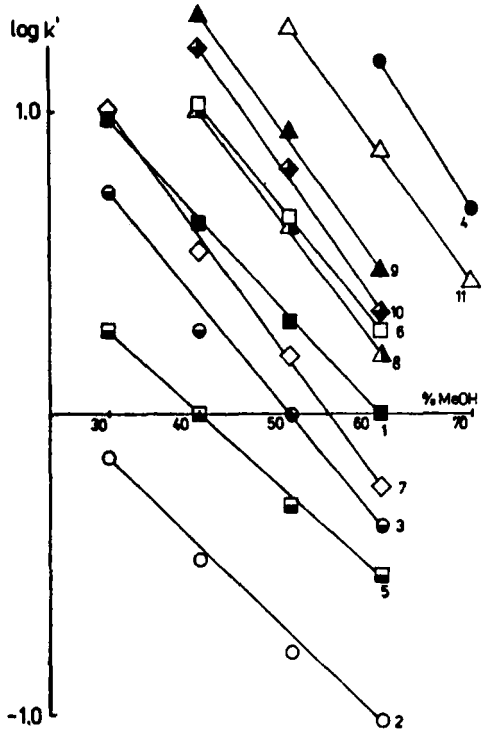

Fig. 1 log k' values of coumarins and furocoumarins plotted against % v/v concentration of methanol in the eluent /HPLC - RP-18/. Notation of solutes in all figures as in Table 1.

tetrahydrofuran) or mixtures of water with methanol, additionally containing acetic acid, cetrimide or phosphate buffer (Table 2).

Figs 1-7 represent the experimental data as relationships of R_M or log k' vs. percent concentration of methanol or acetonitrile in the eluent. In most instances linear relationships were obtained. The

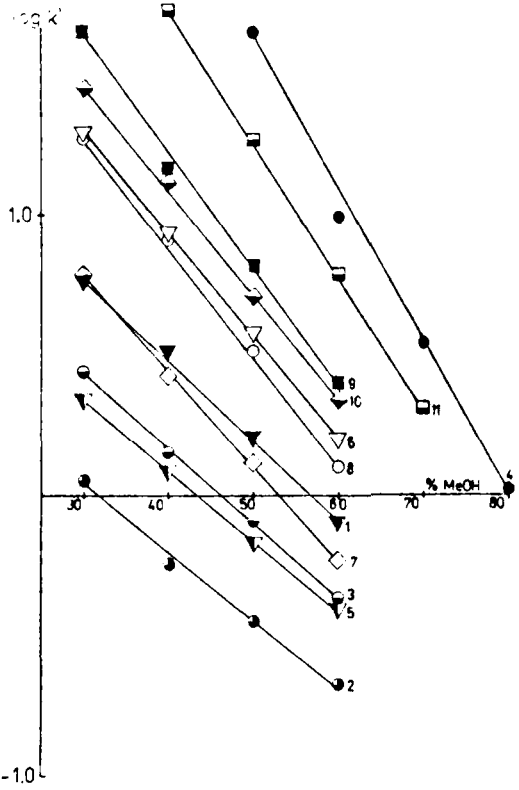

Fig.2. log k' vs % MeOH with 3% acetic acid /HPLC - RP-18/.

different correlation plots for each compound indicate selective effects upon the retention due to the solute-solvent and solute-stationary phase interactions. The eluent strength changes with the molecular structure of the solute. The specific properties of modifiers can be utilized to obtain desired selectivity for the separation of the investigated coumarins and furocoumarins. The selectivity of the compounds increases with the

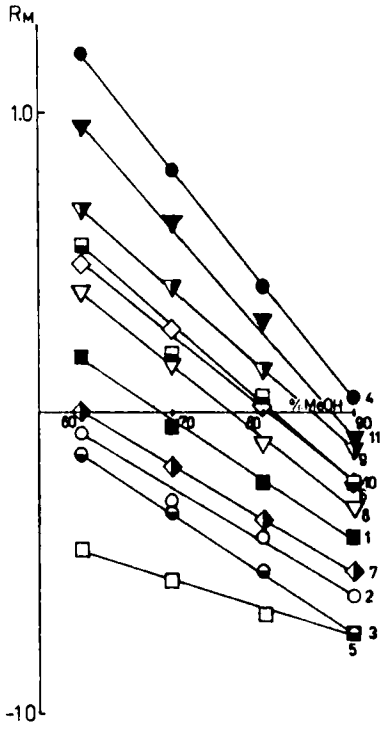

Fig.3. R_M vs % MeOH /HPTLC - RP-18/.

concentration of water in the eluent due to the hydrophobic interactions /17-18/.

The linear relationship $R_M = f(\varphi)$ permitted to estimate quantitative the effects of individual substituents on retention in terms of the group contribution values expressed as $\triangle R_{Mw}$ or $\triangle \log k'_w$ - analogous to the hydrophobic group constant π /19-20/.

In Table 3 R_M and log k' values and in Table 4 $\triangle R_M$ and $\triangle \log k'$ values are summarized in order to

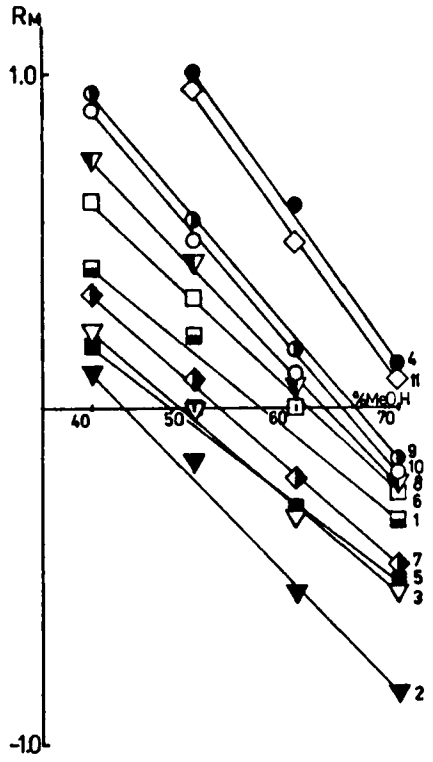

Fig.4. R_M vs % MeOH /TLC - RP-2/.

compare the selectivity of various investigated systems. $\triangle R_M$ and $\triangle \log k'$ values were estimated relative to coumarin (No. 1) and xanthotoxol (No. 7). Each pair of compounds can be easily separated and the best modifier and adsorbent can be chosen. The best separations were obtained for octadecyl silica gel (TLC and HPLC) and for silanized silica gel impregnated with 15% oleic acid or 15% oleyl alcohol. The addition of 2% cetrimide to the eluent significantly changed the selectivity of

Table 3. R_M values of coumarins and furocoumarins

Systems as in Table 2	No of compound as in Table 1										
	1	2	3	4	5	6	7	8	9	10	11
1	0.35	-0.21	0.18	1.01	-0.01	0.50	0.27	0.58	0.66	0.55	0.91
2	0.18	-0.48	0.00	0.91	-0.09	0.27	0.09	0.37	0.48	0.45	0.75
3	0.23	-0.31	0.05	1.19	0.31	0.45	0.18	0.45	0.60	0.55	1.06
4	0.14	-0.33	0.02	0.55	-0.07	0.25	0.23	0.31	0.48	0.37	0.72
5	0.41	-0.10	0.14	1.19	0.07	0.66	0.21	0.75	0.95	0.86	1.12
6	0.21	-0.18	0.02	1.01	0.00	0.33	0.09	0.43	0.55	0.50	0.95
7	-0.03	-0.07	0.23	0.83	0.31	0.18	-0.14	0.23	0.41	0.31	0.72
8	0.09	-0.18	0.05	0.95	-0.14	0.35	0.02	0.41	0.45	0.43	0.95
9	0.18	-0.50	0.25	1.20	-0.33	0.55	0.09	0.58	0.72	0.66	1.12
10	0.29	0.00	0.14	1.19	-0.21	0.52	-0.31	0.57	0.62	0.69	1.12
11	0.10	-0.55	0.14	1.06	-0.21	0.36	0.03	0.37	0.63	0.55	0.90
12	0.07	-0.60	0.27	1.06	-0.31	0.33	-0.03	0.33	0.66	0.48	0.95
13	0.27	-0.52	0.31	1.28	0.07	0.43	0.27	0.55	0.75	0.60	0.95
14	0.18	-0.07	0.14	1.19	0.45	0.54	0.00	0.39	0.66	0.48	0.95
15[a]	0.02	-1.04	0.34	1.15	0.53	0.27	0.24	0.19	0.47	0.10	0.86
16[a]	-0.10	-0.66	0.36	0.99	-0.40	0.19	0.23	0.11	0.39	0.33	0.77

a - log k′ values

Table 4. ΔR_M values relative to coumarin (No 1) or xanthotoxol (No 7 for compounds No 8 - 11)

Systems as in Table 2	No of compound as in Table 1								
	2	3	4	5	6	8	9	10	11
1	-0.56	0.17	0.66	-0.36	0.15	0.31	0.39	0.28	0.64
2	-0.66	0.18	0.73	-0.27	0.09	0.28	0.39	0.36	0.66
3	-0.54	0.18	0.96	0.08	0.22	0.27	0.42	0.37	0.88
4	-0.47	0.12	0.41	-0.21	0.11	0.17	0.34	0.33	0.58
5	-0.51	0.27	0.78	-0.34	0.25	0.54	0.74	0.65	0.91
6	-0.39	-0.23	0.80	-0.21	0.12	0.37	0.46	0.41	0.86
7	-0.04	-0.20	0.86	-0.28	0.21	0.37	0.55	0.17	0.86
8	-0.27	-0.14	0.86	-0.23	0.26	0.39	0.43	0.41	0.93
9	-0.68	-0.43	1.02	-0.51	0.37	0.49	0.63	0.57	1.03
10	0.29	-0.43	0.90	-0.50	0.23	0.88	0.93	1.00	1.43
11	-0.65	-0.24	0.96	-0.31	0.26	0.34	0.60	0.52	0.87
12	-0.67	-0.34	0.99	-0.38	0.26	0.36	0.69	0.51	0.98
13	-0.79	-0.04	1.01	-0.20	0.16	0.28	0.48	0.33	0.68
14	-0.25	-0.34	1.01	-0.63	0.36	0.39	0.66	0.48	0.95
15	1.06	-0.36	1.13	-0.55	0.25	0.43	0.71	0.34	1.10
16	0.56	-0.26	1.19	-0.30	0.29	0.33	0.62	0.56	1.00

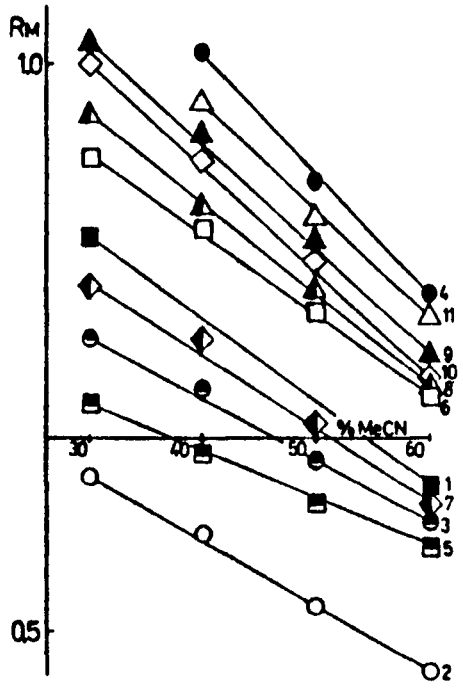

Fig. 5. R_M vs. % MeCN /TLC -RP-2/

separation of furocoumarins but for coumarins it was negligible. The longest retention times were obtained for osthol and imperatorin because the large isopentenyl radical caused large increases of hydrophobic properties. The shortest retention times were obtained for hydrophilic solutes with one or two hydroxy groups in the molecule. In some instances a considerable improvement in the resolution of pairs of solutes upon alteration of binary-additive composition was observed. (Figs.1,2,5). On the contrary, the separation of solu-

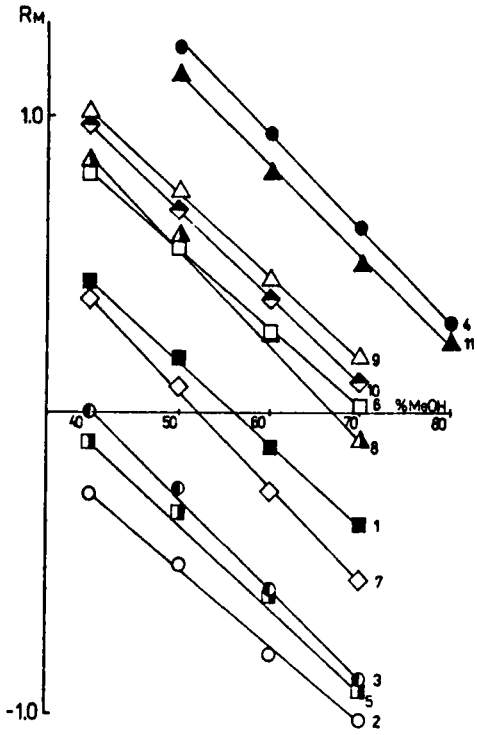

Fig.6. R_M vs % MeOH /TLC - with 15% oleic acid/

tes No.6 and No.8 was consistently poorer for all investigated systems. The structural effects were generally in accordance with those observed in liquid-liquid systems /21,22/. For samples containing solutes with wide differences in polarity gradient elution was frequently employed /23/.

The quantitative relationships between capacity factor and solvent composition in reversed phase systems for the rational optimization of both isocratic

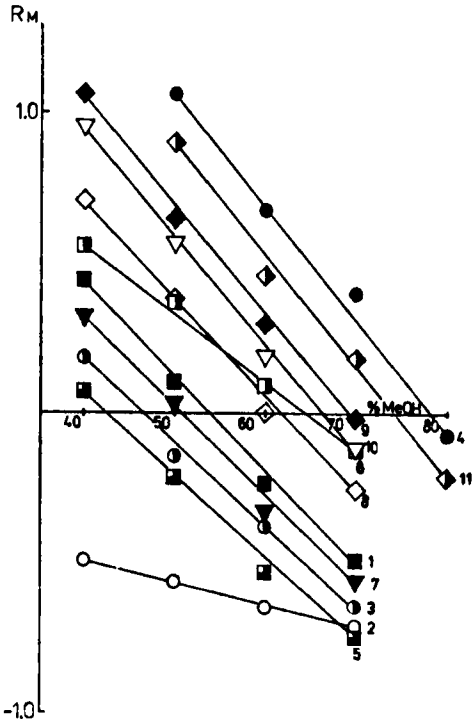

Fig.7. R_M vs % MeOH /TLC - with 15% oleyl alcohol/

and gradient elution, are given by the simple semiempirical equation (2).

$$\log k'_{w,org} = \log k'_w - b\varphi_{org} \qquad (2)$$

It is a modification of eq.(1). In the equation φ_{org} represents the volume fraction of the organic modifier (0.01 x vol %) and b is a constant, empirically between R_M or log k' values of coumarins and furophase: $\log k' = R_M = a + b\varphi$ (eq.2); a = $\log k'_w$ or R_{Mw};

derived for the system used ($b = \log k'_w - \log k'_{org}$). The R_{Mw} and $\log k'_w$ values, that is, the theoretical capacity factors (summarized in Table 5) are obtained by extrapolation from the experimental straight linear relationships of $R_M = f(\varphi)$ to pure water medium. They are suitable for the elimination of the selective effects of modifiers, and thereby useful for quantitative description of the hydrophobic nature of solutes. R_{Mw} and $\log k'_w$ are thus closely related to the partition coefficient (log P) from the standard n-octanol-water system used in QSAR studies /13,24-31/ and can be directly correlated wtih the biological activity in agreement with the equation (3) /32,33/

$$- \log C = a + b \log k' \qquad (3)$$

In this equation C represents the quantitative term of biological effect, a is intercept and b is the slope of the least squares straigth line.

Preliminary investigation of cytostatic activity of the coumarins and furocoumarins showed that an increase in the hydrophobicity is related to an increases in their activity. Osthol and imperatorin, the compounds possessing the strongest hydrophobic properties, have also the greatest cytostatic activity. The detailed results of these investigations will be published in the next paper.

Table 5. Equations describing the linear relationships coumarins and the composition of the mobile b = slope; r = correlation coefficient.

No of compound	Mobile phase: methanol - water						methanol - 3% acetic	
	RP-2		RP-18-HPTLC		RP-18-HPLC		RP-2	
	a	b	a	b	a	b	a	b
1	1.43 r=0.955	-2.45	1.33 r=0.998	-1.95	1.88 r=0.999	-3.14	0.78 r=0.998	-1.62
2	1.39 r=0.988	-3.22	0.94 r=0.994	-1.71	0.72 r=0.998	-2.97	0.89 r=0.998	-1.91
3	1.25 r=0.997	-2.59	1.02 r=0.999	-1.93	1.76 r=0.995	-3.60	0.49 r=0.998	-1.43
4	3.14 r=0.999	-4.24	3.46 r=0.999	-3.80	4.43 r=0.997	-5.40	2.60 r=0.998	-3.52
5	1.13 r=0.994	-2.45	0.09 r=0.993	-0.92	1.03 r=0.996	-2.64	0.38 r=0.995	-1.35
6	0.80 r=0.954	-0.96	2.02 r=0.998	-2.55	2.62 r=0.999	-3.93	1.34 r=0.998	-2.34
7	1.51 r=0.996	-4.91	1.03 r=0.999	-1.73	2.21 r=0.998	-4.09	0.90 r=0.975	-2.00
8	2.14 r=0.999	-3.42	1.80 r=0.999	-2.36	2.79 r=0.998	-4.36	1.48 r=0.999	-2.51
9	2.45 r=0.999	-3.78	2.23 r=0.999	-2.61	2.91 r=0.999	-4.04	1.93 r=0.999	-3.00
10	2.39 r=0.998	-3.76	1.92 r=0.999	-2.38	3.16 r=0.990	-4.96	1.77 r=0.997	-2.85
11	3.00 r=0.998	-4.15	3.03 r=0.998	-3.44	3.87 r=0.999	-4.95	2.40 r=1.00	-3.36

water + acid		methanol + 0.01M phosphate buffer		methanol - water			
RP-18-HPLC		RP-2		RP-18 + 15% oleic acid		RP-2 + 15% oleyl alcohol	
a	b	a	b	a	b	a	b
1.60	-2.82	1.17	-1.95	1.52	-2.70	1.64	-3.06
r=0.998		r=0.998		r=0.999		r=0.998	
0.72	-2.33	0.14	-0.60	0.80	-2.66	0.20	-0.71
r=0.996		r=0.995		r=0.999		r=0.997	
1.20	-2.61	0.78	-1.59	1.19	-2.94	1.22	-2.66
r=0.999		r=0.998		r=0.998		r=0.997	
4.18	-5.23	2.35	-2.88	2.73	-3.04	2.89	-3.66
r=0.998		r=0.997		r=0.997		r=0.996	
1.06	-2.45	0.67	-1.50	1.00	-2.70	1.13	-2.68
r=0.999		r=0.997		r=0.997		r=0.996	
2.36	-3.62	1.60	-2.40	1.83	-2.59	1.47	-2.28
r=0.999		r=0.997		r=0.999		r=0.997	
1.73	-3.27	0.86	-1.57	1.63	-3.12	1.48	-2.94
r=0.999		r=0.996		r=0.998		r=0.996	
2.48	-3.94	1.71	-2.49	2.11	-3.12	1.96	-3.19
r=0.999		r=0.993		r=0.997		r=0.997	
2.80	-4.02	2.12	-2.99	2.09	-2.75	2.46	-3.58
r=0.999		r=0.997		r=0.999		r=0.997	
2.58	-3.75	1.95	-2.77	2.08	-2.84	2.36	-3.58
r=0.999		r=0.998		r=0.999		r=0.998	
3.56	-4.66	2.34	-3.01	2.59	-2.98	2.67	-3.60
r=0.999		r=0.996		r=0.998		r=0.995	

Linear relationships obtained in the investigated systems permitted the application of the equation:

$$\log k'_{RP-18} = a + b R_M \quad (4)$$

to determine the quantitative relationships between column liquid chromatography and thin-layer chromatography data, by plotting the log k' values vs. the R_M values.

A good correlation was obtained for all systems studied but the best correlation was obtained for the RP-18 sorbent used in the pilot TLC and HPLC experiments. The correlation permitted the preliminary determination of programs for gradient elution. The slope, close to unity, indicates a close similarity of the separation processes in HPLC and TLC systems. A obtained slope $>$ 1 shows that the HPLC system is

Table 6. Regression equations for correlation of log k'_w against R_{Mw} values.

No system as in Table 2	Constant of equation (4) a	b	n	r	s
5	0.46	1.42	11	0.948	1.08
6	0.28	1.18	11	0.825	0.67
7	0.61	1.38	11	0.922	0.48
9	-0.69	1.79	11	0.987	0.13
11	0.13	1.33	11	0.940	0.42
14	0.63	1.08	11	0.938	0.43

generally more selective. High correlation coefficients were also observed for R_M values obtained from silanized silica gel impregnated with oleic acid or oleyl alcohol. These solvents of low toxicity, very suitable for the separation of coumarins and furocoumarins (Figs.6,7), were also used to determine the partition coefficients of drugs for QSAR analysis /34/. The systematic study of the selectivity of investigated systems permits one to choose the best condition for preparative extraction and isolation of those compounds from plant material.

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