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Note

High-performance thin-layer chromatography of selected flavonoid aglycones on ready-for-use layers of silanized silica gel

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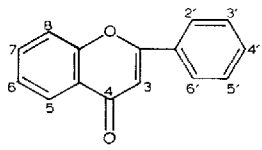
The flavonoids are widely distributed plant constituents. Because of this biological importance, many studies of their separation and determination have been made. High-performance liquid chromatography (HPLC) is the technique most often used[1–5], while little attention has been devoted to high-performance thin-layer chromatography (HPTLC)[6,7], notwithstanding the great simplicity of this technique and the good results which can be achieved with two-dimensional development.

The object of this study is to investigate the best elution conditions in order to separate complex mixtures of these compounds and to find a relationship between their chromatographic behaviour and the number and position of the substituent hydroxyl groups.

EXPERIMENTAL

Standard solutions of the flavonoid aglycones were prepared by dissolving the commercially available products (Roth and Sigma) in water–methanol (1:1). The amount deposited on the layer was between 0.2 and 0.5 μg. After the elution the flavonoids were visualized under UV light (254 and 360 nm). The compounds studied, except as indicated below, are hydroxy and methoxy derivatives of flavone:



The structures of taxifoline, naringenine and hesperetine are derived from that of flavonone:

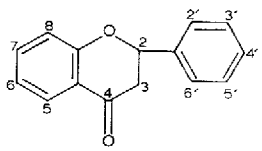


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TABLE I

R_F VALUES OF FLAVONOID AGLYCONES ON RP-18 AND Sil C_{18}-50 PLATES

Eluents: (a) 1 *M* acetic acid in 60% methanol; (b) 1 *M* acetic acid in 50% methanol; (c) acetic acid–methanol–water (3:3:4); (d) 0.1 *M* ammonia in 55% methanol; (e) hexane–ethyl acetate–acetic acid (72:27:1). e.s. = Elongated spot.

Compound	*RP-18* *(a)*	*Sil C_{18}-50*			
		(b)	*(c)*	*(d)*	*(e)*
1 Chrisine (5,7-dihydroxyflavone)	0.13	0.07	0.23	0.58	0.67
2 Apigenine (4′,5,7-trihydroxyflavone)	0.30	0.12	0.34	0.83	0.37
3 Luteoline (3′,4′,5,7-tetrahydroxyflavone)	0.42	0.14	0.40	0.85	0.19
4 Galangine (3,5,7-trihydroxyflavone)	0.11	0.06	0.21	0.49	0.68
5 Kaempferol (3,4′,5,7-tetrahydroxyflavone)	0.32	0.10	0.32	0.72	0.45
6 Fisetine (3,3′,4′,7-tetrahydroxyflavone)	0.50	0.20	0.46	0.85	0.17
7 Quercetine (3,3′,4′,5,7-pentahydroxyflavone)	0.48	0.16	0.43	e.s.	0.24
8 Miricetine (3,3′,4′,5,5′,7-hexahydroxyflavone)	0.63	0.24	0.54	0.93	0.11
9 Morine (2′,3,4′,5,7-pentahydroxyflavone)	e.s.	0.31	0.59	0.90	0.11
10 Acacetine (5,7-dihydroxy-4′-methoxyflavone)	0.07	0.05	0.20	0.51	0.54
11 Rhamnetine (3,3′,4′,5-tetrahydroxy-7-methoxyflavone)	0.09	0.08	0.28	0.10	0.35
12 Naringenine (4′,5,7-trihydroxyflavonone)	0.45	0.20	0.50	0.85	0.42
13 Taxifoline (3,3′,4′,5,7-pentahydroxyflavonone)	0.75	0.46	0.74	0.91	0.12
14 Hesperetine (3′,5,7-trihydroxy-4′-methoxyflavonone)	0.44	0.20	0.46	0.80	0.44

The chromatographic behaviour of flavonoid aglycones was studied on RP-18 (Merck) and Sil C_{18}-50 (Macherey and Nagel) layers.

RESULTS AND DISCUSSION

Table I lists the R_F values of flavonoids on RP-18 plates eluted with 1 *M* acetic acid in 60% methanol. Under these conditions, the influence of the number and the position of the hydroxyl groups on the retention can be evaluated. Two series of compounds with an analogous behaviour can be characterized, that is crisine, apigenine and luteoline and the flavonols (3-hydroxyflavones) galangine, kaempferol, quercetine and miricetine. Within each series the retention decreases with increasing number of hydroxyl groups; the introduction of an hydroxyl group in the 3 position, on the contrary, involves small differences in the retention (for crisine R_F = 0.13 and for galangine R_F = 0.11; for luteoline R_F = 0.42 and for quercetine R_F = 0.48) probably owing to interactions between this hydroxyl group and the carbonyl group in the 4 position[1].

Fisetine and kaempferol, two isomers with four substituent hydroxyl groups, behave in quite a different way (R_F = 0.50 and 0.32, respectively) because of the hydrogen bond between the hydroxyl group in the 5 position in kaempferol and the carbonyl in the 4 position which causes a sharp decrease in the polarity of the molecule with respect to the isomer with an unsubstituted 5 position[5].

As in the case of phenolic acids[8], the introduction of a methoxyl group involves a retention increase. In fact acacetine (R_F = 0.07) and rhamnetine (R_F = 0.09) are more strongly retained than crisine (R_F = 0.13) and quercetine (R_F = 0.48) respectively.

The flavonones are less strongly retained than flavones owing to the higher polarity of their molecules[1].

The use of eluents with lower water contents[7] yields better separastions. On Sil C_{18}-50 plates a weaker retention is observed even if the above-mentioned sequences do not change. Very compact spots are obtained with 1 *M* acetic acid in 50% methanol as eluent (Table I) even in the case of morine. Morine (five hydroxyl groups) is remarkably less strongly retained than quercetine and miricetine (five and six hydroxyl groups, respectively). This behaviour can be explained on the basis that the interactions between morine and the polar solvent are strongest because of the presence of two hydroxyl groups in the meta position (2′ and 4′) which cannot mutually interact. Furthermore, on this layer, acacetine and rhamnetine can be separated. Eluting with methanol–acetic acid–water mixtures, with a given water content, the retention decreases as the acetic acid concentration (that is the more polar organic solvent) increases (see Table I).

On Sil C_{18}-50 layers, the effect of the pH of the eluent with a given organic solvent content has been examined. Table I lists the R_F values which refer to elution with 0.1 *M* ammonia in 55% methanol (apparent pH 10.6). With eluents at lower pH values there are no significant differences with respect to the elution in acidic solution. At this pH value, the retention is affected by the deprotonation of the hydroxyl groups which results in a remarkable increase in the R_F value; with this eluent, the pairs crisine and galangine and apigenine and kaempferol, which each differ in the presence of an hydroxyl group in the 3 position, may be separated.

TABLE II

SLOPES OF THE LINEAR PLOTS OF R_M *versus* THE NUMBER OF HYDROXYL GROUPS FOR THE COMPOUNDS GALANGINE, KAEMPFEROL, QUERCETINE AND MIRICETINE

Eluent	*Slope*
1 *M* Acetic acid in 50% methanol	−0.23
Acetic acid–methanol–water (3:3:4)	−0.19
Acetic acid–methanol–water (6:2:2)	−0.16
Hexane–ethyl acetate–acetic acid (72:27:1)	0.42
Hexane–ethyl acetate–acetic acid (65:34:1)	0.39

Table I also reports the R_F values of the flavonoids when using non-aqueous eluents, that is hexane–ethyl acetate–acetic acid (72:27:1). This kind of eluent cannot be used on RP-18 plates since elongated spots are obtained. The elution order is opposite to the one previously observed, since under these conditions the stationary phase behaves like a polar phase because of the presence of unsilanized hydroxyl groups[9]. On eluting with hexane–ethyl acetate–acetic acid (65:34:1) a general decrease in the retention is observed; however, this does not involve an higher selectivity.

Retention mechanism

In order to understand the retention mechanism for these compounds, the trends in R_M with the number of hydroxyl groups of homologous series of compounds have been studied.

In the cases of galangine, kaempferol, quercetine and miricetine, plots of the R_M values obtained in different eluents on Sil C_{18}-50 against the number of hydroxyl groups were linear. This demonstrates that a liquid–liquid partition mechanism is operating. Table II lists the slopes of these plots for different eluents.

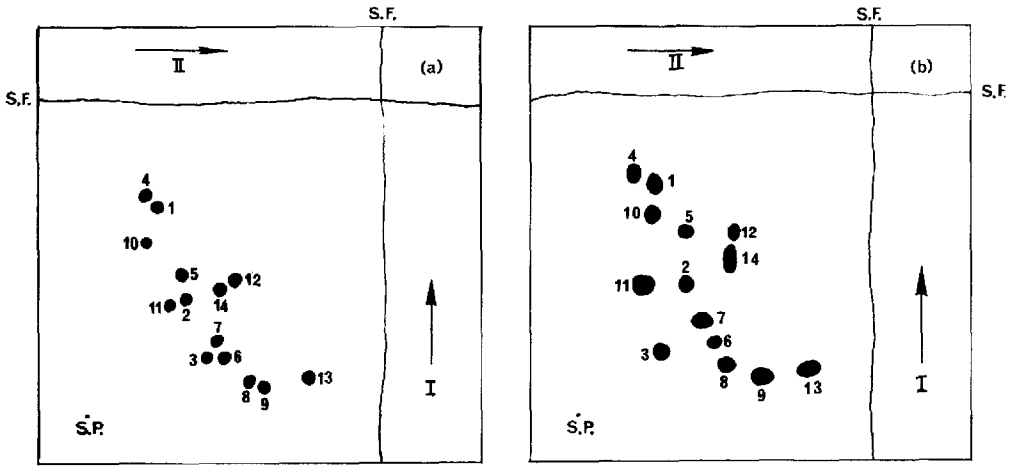

Fig. 1. (a) Theoretical and (b) experimental two-dimensional chromatogram on Sil C_{18}-50 plates. Eluents: first direction, hexane–ethyl acetate–acetic acid (72:27:1); second direction, acetic acid–methanol–water (3:3:4). Flavonoids as in Table I. S.P. = Starting point; S.F. = solvent front.

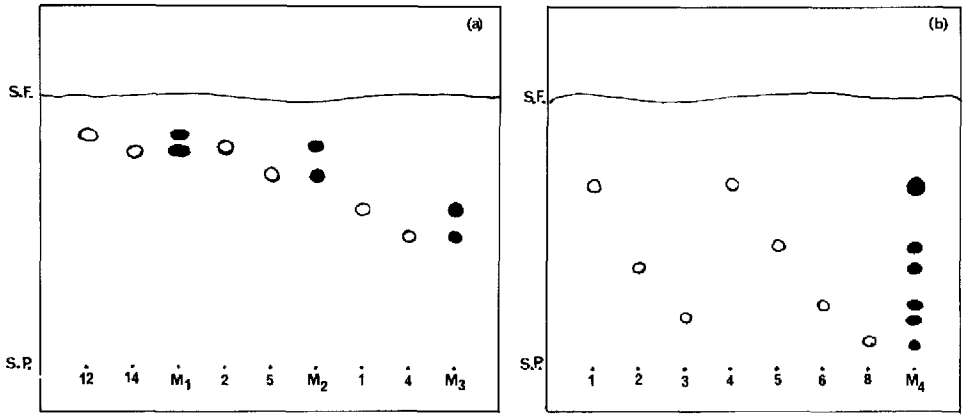

Fig. 2. Thin-layer chromatogram on Sil C_{18}-50 plates. Eluents: (a) 0.1 *M* ammonia in 55% methanol; (b) hexane–ethyl acetate–acetic acid (72:27:1). Flavonoids as in Table I. M_1 = Mixture of compounds 12 and 14; M_2 = mixture of 2 and 5; M_3 = mixture of 1 and 4; M_4 = mixture of 1–6 and 8. S.P. = Starting point; S.F. = solvent front.

By contrast, in the cases of crisine, apigenine and luteoline non-linear trends are obtained, indicating that adsorption phenomena must also be considered in addition to the partition process.

On RP-18 layers no linear trends are observed; in agreement with the fact that the spots on this layer were less compact (more diffuse).

Analytical applications

Two-dimensional chromatography was carried out using the eluents which give rise to the best separations, that is hexane–ethyl acetate–acetic acid (72:27:1) in the first direction and acetic acid–methanol–water (3:3:4) in the second direction. Fig. 1a reports the theoretical separation and Fig. 1b the experimental one. Fig. 2 shows the most interesting separations which can be achieved with the different eluents considered.

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