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Note

Reversed-phase high-performance liquid chromatography of 5-hydroxyflavones bearing tri- or tetrasubstituted A rings

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In the last few years, high-performance liquid chromatography (HPLC) has been applied to flavonoid aglycones both with silica columns[1–4] and with reversed-phase columns[5–10]. However, to our knowledge no work on the HPLC of 5-hydroxyflavones with a tri- or tetrasubstituted A ring has been published.

In the course of our research on the flavonoid aglycones from Labiatae species, we have isolated and identified more than thirty 5-hydroxyflavones, most of them bearing a tri- or tetrasubstituted A ring. These compounds have been used in this work as a model to study the effect of hydroxy or methoxy groups located at different positions on the flavone nucleus on the reversed-phase HPLC behaviour of these interesting compounds.

EXPERIMENTAL

HPLC analyses were carried out with a Perkin-Elmer liquid chromatograph, equiped with a 2/2 pump module, a Model LC85B UV–visible variable-wavelength detector and a Sigma 15 data station.

A Perkin-Elmer C_{18} reversed-phase column with 3-μm particles was used (10 cm × 2.7 mm I.D.). Working solutions contained approximately 1 mg of flavone per 2 ml of methanol. Runs were carried out for 25 min. The elution solvents were water–formic acid (19:1) from pump B (formic acid was added to prevent "tailing") and acetonitrile from pump A. The flow-rate was 2 ml/min with pump A providing 23% and pump B 77% isocratically for 11 min. A gradient increasing at the rate of 2%/min of acetonitrile was then applied for 25 min. Samples of 6 μl were injected, and peaks were detected at 340 nm.

RESULTS AND DISCUSSION

The retention times (t_R) and capacity factors (k') of the different 5-hydroxyflavones analysed are shown in Table I. The elution sequence of the individual compounds can be interpreted by assuming that the hydrophobic interaction increases the retention times and the formation of hydrogen bonds with the mobile phase decreases them.

As a general rule, the lower hydroxy/methoxy group ratio, the higher is the

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TABLE I

RETENTION TIMES AND CAPACITY FACTORS OF FLAVONES

Column, C_{18} (3 μm); solvent system, acetonitrile/water–formic acid (19:1); flow-rate, 2 ml/min.

Common name	*Systematic name*	*OH/OCH₃*	*t_R (min)*	*k'*
6-Hydroxyluteolin	5,6,7,3',4'-Pentahydroxyflavone	5:0	1.01	1.02
Hypolaetin	5,7,8,3',4'-Pentahydroxyflavone	5:0	1.10	1.20
Scutellarein	5,6,7,4'-Tetrahydroxyflavone	4:0	1.73	2.46
Isoleucanthogenin	5,6,3',4'-Tetrahydroxy-7,8-dimethoxyflavone	4:2	2.20	3.40
Luteolin	5,7,3',4'-Tetrahydroxyflavone	4:0	2.44	3.88
Nepetin	5,7,3',4'-Tetrahydroxy-6-methoxyflavone	4:1	2.58	4.16
Leucanthogenin	5,8,3',4'-Tetrahydroxy-6,7-dimethoxyflavone	4:2	2.84	4.70
–	5,6,4'-Trihydroxy-7,3'-dimethoxyflavone	3:2	3.99	6.98
Apigenin	5,7,4'-Trihydroxyflavone	3:0	4.25	7.50
Thymusin	5,6,4'-Trihydroxy-7,8-dimethoxyflavone	3:2	4.44	7.88
Hispidulin	5,7,4'-Trihydroxy-6-methoxyflavone	3:1	4.70	8.40
Chrysoeriol	5,7,4'-Trihydroxy-3'-methoxyflavone	3:1	4.97	8.94
Isothymusin	5,8,4'-Trihydroxy-6,7-dimethoxyflavone	3:2	5.09	9.18
Thymonin	5,6,4'-Trihydroxy-7,8,3'-trimethoxyflavone	3:3	5.55	10.10
Cirsiliol	5,3',4'-Trihydroxy-6,7-dimethoxyflavone	3:2	5.71	10.42
Isothymonin	5,8,4'-Trihydroxy-6,7,3'-trimethoxyflavone	3:3	6.05	11.10
Sideritoflavone	5,3'.4'-Trihydroxy-6,7,8-trimethoxyflavone	3:3	7.79	14.58
–	5,6-Dihydroxy-7,3',4'-trimethoxyflavone	2:3	9.10	17.20
Cirsimaritin	5,4'-Dihydroxy-6,7-dimethoxyflavone	2:2	10.87	20.74
Eupatorin	5,3'-Dihydroxy-6,7,4'-trimethoxyflavone	2:3	13.62	26.84
Cirsilineol	5,4'-Dihydroxy-6,7,3'-trimethoxyflavone	2:3	13.86	26.72
–	5,6-Dihydroxy-7,8,3',4'-tetramethoxyflavone	2:4	13.92	26.84
Xanthomicrol	5,4'-Dihydroxy-6,7,8-trimethoxyflavone	2:3	14.64	28.28
Ladanein	5,6-Dihydroxy-7,4'-dimethoxyflavone	2:2	15.23	29.46
Acacetin	5,7-Dihydroxy-4'-methoxyflavone	2:1	16.17	31.34
Gardenin D	5,3'-Dihydroxy-6,7,8,4'-tetramethoxyflavone	2:4	16.23	31.46
Genkwanin	5,4'-Dihydroxy-7-methoxyflavone	2:1	16.85	32.70
8-Methoxycirsilineol	5,4'-Dihydroxy-6,7,8,3'-tetramethoxyflavone	2:4	16.95	32.90
–	5,6-Dihydroxy-7,8,4'-trimethoxyflavone	2:3	16.98	32.96
–	5-Hydroxy-6,7,3',4'-tetramethoxyflavone	1:4	19.07	37.14
5-Demethylnobiletin	5-Hydroxy-6,7,8,3',4'-pentamethoxyflavone	1:5	20.35	39.70
Salvigenin	5-Hydroxy-6,7,4'-trimethoxyflavone	1:3	20.72	40.44
Gardenin B	5-Hydroxy-6,7,8,4'-tetramethoxyflavone	1:4	22.25	43.50

retention time. Hence the highly hydroxylated flavones 6-hydroxyluteolin, luteolin, nepetin, etc., show shorter t_R and the highly methylated 5-desmethylnobiletin, gardenin B, salvigenin, etc., elute with higher t_R values. However, when pairs of compounds are studied, some surprising t_R can be found. These results can be explained on the basis of the capacity for the formation of hydrogen bonds between the flavonoid phenolic hydroxy groups and the mobile phase. Therefore, internal hydrogen bonding usually decreases the capacity for interaction with the solvent and increases retention times.

The strongest hydrogen bond acceptor in a flavone is the carbonyl group at C-4, which, owing to resonance, bears a partial negative charge. If a hydroxy group is present at position 5 (as in all the flavones studied in this work), a strong internal hydrogen bond is formed between this group and the carbonyl group, and therefore

the latter can no longer interact with the solvent[5,7]. In this work, we observed that 6-hydroxy compounds elute with shorter t_R than the 8-hydroxy isomers. This could be explained by internal hydrogen bonding between the hydroxy groups at C-6 and C-5, which decreases the interaction described above between the latter hydroxy and the 4-keto group, and so decreases t_R (Fig. 1). This also could explain the fact that 5,6-dihydroxy-7,8,3′,4′-tetramethoxyflavone and 5,6-dihydroxy-7,3′,4′-trimethoxyflavone elute with shorter t_R than 8-methoxycirsilineol (5,4′-dihydroxy-6,7,8,3′-tetramethoxyflavone) and cirsilineol (5,4′-hidroxy-6,7,3′-trimethoxyflavone), respectively. This is the only case in which an internal hydrogen bond between two hydroxy groups decreases t_R.

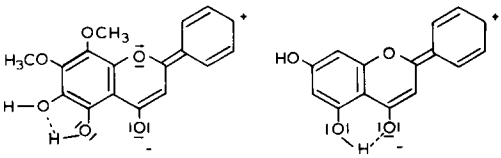

Fig. 1. Structures of 5-hydroxyflavones showing internal hydrogen bonding.

On the other hand, the internal interaction between hydroxy groups at C-3′ and C-4′ increases t_R when compounds bearing such groups are compared with those bearing the same substitution pattern and OH/OCH_3 ratio but with isolated hydroxy groups. Therefore, sideritoflavone and cirsiliol elute with higher t_R than isothymonin and 5,6,4′-trihydroxy-7,3′-dimethoxyflavone, respectively.

In addition, it has been observed that the size of the molecule affects t_R. This is important in the hydrophobic interaction, as the smallest molecules can interact easily with the C_{18} branches of the stationary phase. Thus, flavones bearing a single methoxy group on ring B (5,6-dihydroxy-7,8,4′-trimethoxyflavone, ladanein, gardenin B, salvigenin) interact more strongly with the stationary phase than their counterpart bearing two methoxy groups on ring B, although the latter compounds have a higher number of methoxy groups, which should increase t_R. The 4′-methoxy compounds, with their small size, penetrate more easily into the C_{18} matrix than do the 3′,4′-dimethoxy compounds, and therefore, the former can interact more strongly with the stationary phase (Fig. 2). These results are in accord with those reported previously for permethylated flavones[6]. This size effect is not observed when a hydroxy group is present at C-4′ (xanthomicrol and cirsimaritin elute faster than 8-methoxy cirsilineol and cirsilineol, respectively), possibly owing to the fact that hydrogen bond formation, which is the main effect in these compounds, is less affected by the molecular size than the hydrophobic interaction effect.

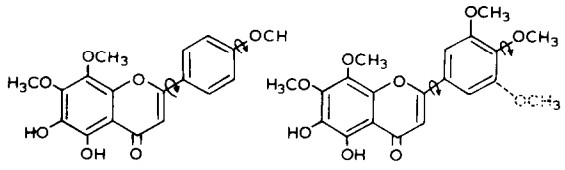

Fig. 2. Structures of 5-hydroxyflavones methoxylated on the B ring.

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