Journal of Chromatography, 240 (1982) 81–94
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,612

SEPARATION OF FLAVONOIDS BY REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

KAREL VANDE CASTEELE
Laboratory of Plant Biochemistry, Rijksuniversiteit Gent, K.L. Ledeganckstraat 35, 9000 Ghent (Belgium)
HANS GEIGER
Institut für Chemie, Universität Hohenheim, Garbenstrasse, 30, D-7000 Stuttgart 70 (G.F.R.)
and
CHRISTIAAN F. VAN SUMERE*
Laboratory of Plant Biochemistry, Rijksuniversiteit Gent, K.L. Ledeganckstraat 35, 9000 Ghent (Belgium)
(Received December 10th, 1981)

SUMMARY

The ability of a reversed-phase high-performance liquid chromatographic system to separate flavonoids [LiChrosorb RP-18 and a combination of an isocratic and a gradient (5% aqueous formic acid and methanol) technique] has been studied. Retention times of some 141 flavonoids ranging from triglycosides to aglycones are reported. The correlations between structure and t_R values are discussed.

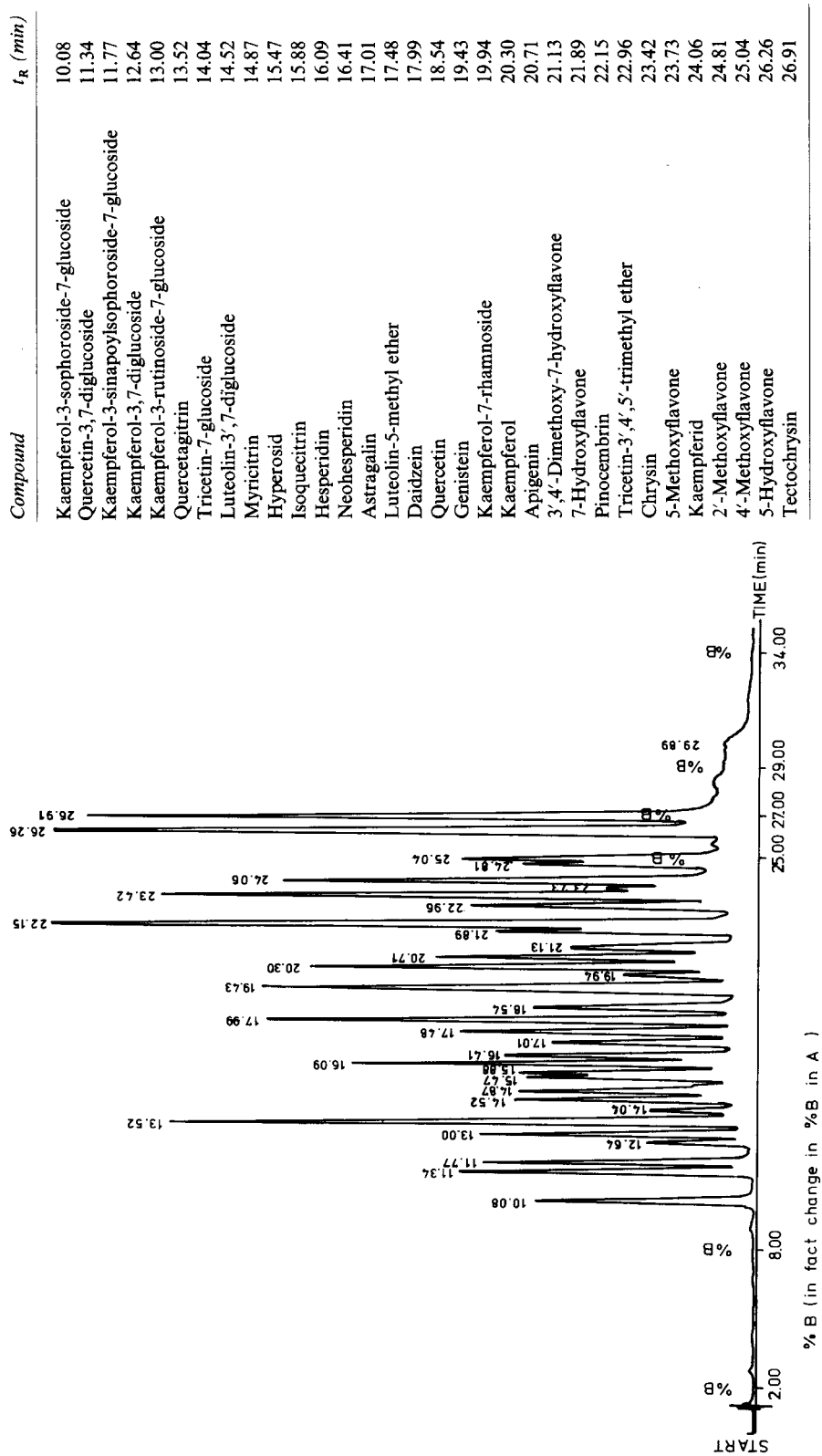
INTRODUCTION

For the separation and quantification of non-volatile compounds in complex mixtures, high-performance liquid chromatography (HPLC) is at present perhaps the method of choice. Indeed, in comparison with gas–liquid chromatography (GLC) there is no need for derivatization, and also detection of compounds can be much better adapted to each specific problem, *e.g.*, by variation of wavelength with photometric detection. In addition, quantification is as straightforward as with GLC.

For polar substances, *e.g.*, flavonoids, the reversed-phase (RP) technique is far superior to the normal technique, since there is no danger that some highly polar substance(s) may be retained irreversibly, with the result that the separation characteristics of the column could be gradually changed. The RP column employed throughout this work has been in constant use for about 2 years without any significant change in its separation characteristics.

Most papers published on the HPLC separations of flavonoids have dealt with only a limited number of compounds[1–18]. However, we have successfully separated a whole array of substituted cinnamoyl- and benzoylamino acids and peptides by a reversed-phase HPLC system, consisting of a LiChrosorb RP-18 column and a combination of isocratic and linear gradient elution[19]. We therefore decided to investigate

0021-9673/82/0000–0000/$02.75 © 1982 Elsevier Scientific Publishing Company



Compound	t_R *(min)*
Kaempferol-3-sophoroside-7-glucoside	10.08
Quercetin-3,7-diglucoside	11.34
Kaempferol-3-sinapoylsophoroside-7-glucoside	11.77
Kaempferol-3,7-diglucoside	12.64
Kaempferol-3-rutinoside-7-glucoside	13.00
Quercetagitrin	13.52
Tricetin-7-glucoside	14.04
Luteolin-3′,7-diglucoside	14.52
Myricitrin	14.87
Hyperosid	15.47
Isoquecitrin	15.88
Hesperidin	16.09
Neohesperidin	16.41
Astragalin	17.01
Luteolin-5-methyl ether	17.48
Daidzein	17.99
Quercetin	18.54
Genistein	19.43
Kaempferol-7-rhamnoside	19.94
Kaempferol	20.30
Apigenin	20.71
3′,4′-Dimethoxy-7-hydroxyflavone	21.13
7-Hydroxyflavone	21.89
Pinocembrin	22.15
Tricetin-3′,4′,5′-trimethyl ether	22.96
Chrysin	23.42
5-Methoxyflavone	23.73
Kaempferid	24.06
2′-Methoxyflavone	24.81
4′-Methoxyflavone	25.04
5-Hydroxyflavone	26.26
Tectochrysin	26.91

Fig. 1. The retention time of flavonoids on a Knauer prepacked column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm). For the eluting system see Experimental. The t_R values differ only by 1–2% RTW (retention time identification window) from the values shown in Tables I–V. These differences, which are within the experimental error, are fully acceptable.

the capacity of the foregoing system to separate a relatively large set of flavonoids ranging from triglycosides to permethylated aglycones.

EXPERIMENTAL

Apparatus

A Hewlett-Packard 1084B liquid chromatograph equipped with a variable-wavelength Pye-Unicam LC3 UV detector and a Knauer prepacked analytical column (250 × 4.6 mm) of LiChrosorb RP-18 (10 μm) was used throughout this work.

Elution

Two solvents were used: A, formic acid–water (5:95 v/v); B, methanol. The elution profile was: 0–2 min, 7% B in A (isocratic); 2–8 min, 7–15% B in A (linear gradient); 8–25 min, 15–75% B in A (linear gradient); 25–27 min, 75–80% B in A (linear gradient); 27–29 min 80% B in A (isocratic). The temperature of the oven thermostat was set at 35°C. The flow-rate was 2.5 ml/min and the column pressure 80–100 bar.

Detection

The UV detector was set at 280 nm (optical bandwidth 8 nm).

Samples

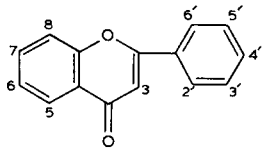
Samples of 0.0025–0.025% solutions in aqueous methanol were applied to the column by means of a 20-μl loop valve. The sources of all the flavonoids examined are given in Tables I–V. Most compounds came from our own laboratories, in which case reference is made either to a paper by one of us, or if the substance has been isolated from a known source, to the name of that plant, and the paper, which describes the first isolation, although the actual isolation might have been performed by present-day methods. Substances marked as synthetic have been synthesized in our laboratories by standard methods, and their identity has been confirmed by mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. (Details of these analyses will be published elsewhere.) For substances donated by colleagues, reference is made to the names of those who kindly provided them.

RESULTS AND DISCUSSION

Tables I–V show the retention times of some 141 flavonoids and isoflavonoids and Fig. 1 shows the separation in a single run of a set of 32 different flavonoids. In Table V the t_R values of protogenkwanin (141) and its 4′-glucoside (140) are reported. These two compounds have been included because they must be considered as being very acid labile[24]. However, as they are eluted unchanged it may be concluded that the formic acid-containing solvent system can be safely used with most flavonoids. Further, the t_R values of all compounds are located between 10.05 and 27.39 min; therefore only mixtures of about 40 substances can be separated, because good separations require that the t_R values of the compounds should differ by at least 0.4–0.5. However, 40 flavonoids do not usually occur in a single organism, although in certain

TABLE I

RETENTION TIMES (t_R) VALUES OF FLAVONES AND FLAVONOLS



Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyl and glycosyloxy*		
(a) Flavones					
1 Tricetin-7-glucoside	5,3′,4′,5′	–	7-β-D-Glucopyranosyloxy	13.99	20
2 Vitexin	5,7,4′	–	8-β-D-Glucopyranosyl	14.44	C. Roth, Karlsruhe, G.F.R.
3 Luteolin-3′,7-diglucoside	5,4′	–	7,3′-Di-β-D-glucopyranosyloxy	14.60	20
4 Luteolin-5-glucoside	7,3′,4′	–	5-β-D-Glucopyranosyloxy	15.15	20
5 Luteolin-7-glucoside	5,3′,4′	–	7-β-D-Glucopyranosyloxy	15.52	20
6 Tricetin-3′-methyl ether-7-glucoside	5,4′,5′	3′	7-Glucosyloxy	15.76	21
7 Apigenin-7-neohesperidoside	5,4′	–	7-β-D-(2-O-α-L-Rhamnopyranosido)glucopyranosyloxy	16.77	Synthetic
8 Apigenin-7-glucoside	5,4′	–	7-β-D-Glucosyloxy	16.78	21
9 Diosmin	5,3′	4′	7-β-D-(6-O-α-L-Rhamnopyranosido)glucopyranosyloxy	17.10	G. Hrazdina
10 Isoëtin	5,7,2′,4′,5′	–	–	17.19	Synthetic
11 Luteolin-5-methyl ether	7,3′,4′	5	–	17.43	22
12 Tricetin	5,7,3′,4′,5′	–	–	17.59	23
13 Luteolin-3′-glucoside	5,7,4′	–	3′-β-D-Glucopyranosyloxy	17.95	20
14 7,4′-Dihydroxyflavone	7,4′	–	–	18.74	22
15 Chrysoeriol-5-methyl ether	7,4′	5,3′	–	19.16	22
16 Tricetin-3′-methyl ether	5,7,4′,5′	3′	–	19.38	21
17 Luteolin	5,7,3′,4′	–	–	19.34	23
18 3′,4′-Dihydroxyflavone	3′,4′	–	–	19.80	Synthetic
19 Genkwanin-4′-glucoside	5	7	4′-β-D-Glucosyloxy	20.48	24

20 Tricin	5,7,4′	3′,5′	–	20.87	Synthetic
21 Tricetin-7-methyl ether	5,3′,4′,5′	7	–	20.92	Synthetic
22 Apigenin	5,7,4′	–	–	20.93	23
23 Diosmetin	5,7,3′	4′	–	20.96	Synthetic
24 Chrysoeriol	5,7,4′	3′	–	21.09	22
25 4′-Hydroxyflavone	4′	–	–	21.27	Synthetic
26 3′,4′-Dimethoxy-7-hydroxyflavone	7	3′,4′	–	21.32	Synthetic
27 Primetin	5,8	–	–	21.65	J. Chopin
28 7-Hydroxyflavone	7	–	–	21.68	Synthetic
29 Luteolin-7-methyl ether	5,3′,4′	7	–	22.53	Synthetic
30 2′-Hydroxyflavone	2′	–	–	22.86	Synthetic
31 Tricetin-3′,4′,5′-trimethyl ether	5,7	3′,4′,5′	–	23.24	Synthetic
32 Chrysin	5,7	–	–	23.83	Synthetic
33 Tricetin pentamethyl ether	–	5,7,3′, 4′,5′	–	23.88	Synthetic
34 5-Methoxyflavone	–	5	–	23.95	Synthetic
35 Luteolin-7,4′-dimethyl ether	5,3′	7,4′	–	24.21	Synthetic
36 Flavone	–	–	–	24.29	Synthetic
37 Acacetin	5,7	4′	–	24.29	Synthetic
38 Genkwanin	5,4′	7	–	24.31	24
39 2′-Methoxyflavone	–	2′	–	24.65	Synthetic
40 7-Methoxyflavone	–	7	–	24.77	Synthetic
41 4′-Methoxyflavone	–	4′	–	24.88	Synthetic
42 5-Hydroxyflavone	5	–	–	26.24	Synthetic
43 Tectochrysin	5	7	–	27.39	Synthetic
(b) Flavonoles					
44 Kaempferol-3-sophoroside-7-glucoside	5,4′	–	3-β-D-(6-O-β-D-Glucopyranosido)glucopyranosyloxy-7-β-D-glucopyranosyloxy	10.05	20
45 Quercetine-3,7-diglucoside	5,3′,4′	–	3-β-D-Glucopyranosyloxy,7-β-D-glucopyranosyloxy	11.28	25
46 Kaempferol-3-sinapoylsophoroside-7-glucoside	5,4′	–	3-β-D-2-(2-O-Sinapoyl-β-D-glucopyranosyl)glucopyranosyloxy, 7-β-D-glucopyranosyloxy	11.84	20
47 Kaempferol-3,7-diglucoside	5,4′	–	3-β-D-Glucopyranosyloxy, 7-β-D-glucopyranosyloxy	12.64	20
48 Kaempferol-3-rutinoside-7-glucoside	5,4′	–	3-β-D-(6-O-α-L-Rhamnopyranosyl) glucopyranosyloxy, 7-β-D-glucopyranosyloxy	12.98	20

(Continued on p. 86)

TABLE I *(continued)*

Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyl and glycosyloxy*		
49 Quercetagitrin	3,5,6, 3′,4′	–	7-β-D-Glucopyranosyloxy	13.45	From flowers of *Tagetes erecta*[26]
50 Quercetin-3-sophoroside	5,7,3′,4′	–	3-β-D-(2-O-β-D-Glucopyranosido) glucopyranosyloxy	13.61	25
51 Myricetin-3-galactoside	5,7,3′, 4′,5′	–	3-β-D-Galactopyranosyloxy	13.95	20
52 Gossypitrin	3,5,8,3′,4′	–	7-β-D-Glucopyranosyloxy	14.09	Flowers of *Chrysanthenum segetum*[27]
53 Quercetin-3-(6-O-galloyl) galactoside	5,7,3′,4′	–	3-β-D-(6-O-Galloyl(galactopyran-osyloxy	14.61	20
54 Quercimeritrin	3,5,3′,4′	–	7-β-D-Glucopyranosyloxy	14.61	20
55 Quercetin-3-glucoside-7-rhamnoside	5,3′,4′	–	3-Glucosyloxy, 7-rhamnosyloxy	14.61	28
56 Azaleatin-3-galactoside	7,3′,4′	5	3-Galactosyloxy	14.81	22
57 Kaempferol-3-sophoroside	5,7,4′	–	3-β-D-(2-O-β-D-Glucopyranosido) glucopyranosyloxy	14.91	20
58 Myricitrin	5,7,3′,4′,5′	–	3-α-L-Rhamnopyranosyloxy	14.93	20
59 Robinetin	3,7,3′,4′,5′	–	–	15.01	C. Roth, Karlsruhe
60 Quercetin-7-neohesperidoside	3,5,3′,4′	–	7-β-D-(2-O-α-L-Rhamnopyranosido) glucopyranosyloxy	15.07	G. Hrazdina
61 Patulitrin	3,5,3′,4′	6	7-Glucosyloxy	15.28	Flowers of *Tagetes patula*[29]
62 Hyperosid	5,7,3′,4′	–	3-β-D-Galactopyranosyloxy	15.49	20
63 Azalein	7,3′,4′	5	3-Rhamnosyloxy	15.57	Azalea flowers[30]
64 Gossypetin	3,5,7,8, 3′,4′	–	–	15.64	Leaf of *Ledum palustre*[31]
65 Isoquercitrin	5,7,3′,4′	–	3-β-D-Glucopyranosyloxy	15.69	20
66 Quercetagetin	3,5,6,7, 3′,4′	–	–	15.73	Flowers of *Tagetes erecta*[26]
67 Rutin	5,7,3′,4′	–	3-β-D-(6-O-Rhamnosyl)glucosyloxy	15.76	32

68 Robinin	5,4′	–	3-β-D-(6-O-α-L-Rhamnopyranosido) galactopyranosyloxy, 7-α-L-rhamnopyranosyloxy	15.80	C. Roth, Karlsruhe, G.F.R.
69 Kaempferol-3-glucoside-7-rhamnoside	5,4′	–	3-β-D-Glucopyranosyloxy, 7-α-L-rhamnopyranosyloxy	15.92	20
70 Kaempferol-3-rutinoside-7-glucoside	5,4′	–	3-β-D-(6-O-α-L-Rhamnopyranosido) glucopyranosyloxy, 7-β-D-glucopyranosyloxy	15.92	20
71 Datiscin	5,7,2′	–	3-Rhamnosidoglucosyloxy	15.94	Leaf and stem of *Datisca cannabina*[33]
72 Morin	3,5,7,2′,4′	–	–	16.07	Chromatography of commercial product
73 Guajaverin	5,7,3′,4′	–	3-α-L-Arabopyranosyloxy	16.16	20
74 3′-O-Methylmyricetin-3-rhamnoglucoside	5,7,4′,5′	3′	3-Rhamnosidoglucosyloxy	16.18	34
75 Kaempferol-7-glucoside	3,5,4′	–	7-β-D-Glucopyranosyloxy	16.22	20
76 Myricetin	3,5,7,3′, 4′,5′	–	–	16.54	23
77 Spiraeosid	3,5,7,3′	–	4′-β-D-Glucopyranosyloxy	16.55	20
78 Quercitrin	5,7,3′,4′	–	3-α-L-Rhamnopyranosyloxy	16.55	20
79 Avicularin	5,7,3′,4′	–	3-α-L-Arabofuranosyloxy	16.55	20
80 Quercetin-5,3′-dimethyl ether-3-glucoside	7,4′	5,3′	3-Glucosyloxy	16.63	22
81 Tamarixetin-7-glucoside	3,5,3′	4′	7-Glucosyloxy	16.86	Synthetic
82 Tamarixetin-7-rutinoside	3,5,3′	4′	7-β-D-(6-O-α-L-Rhamnopyranosido) glucopyranosyloxy	16.89	20
83 Fisetin	3,7,3′,4′	–	–	16.99	Chromatography of commercial product
84 Astragalin	5,7,4′	–	3-β-D-Glucopyranosyloxy	17.09	20
85 Isorhamnetin-3-glucoside	5,7,4′	3′	3-Glucosyloxy	17.14	G. Hrazdina
86 Herbacetin	3,5,7,8,4′	–	–	17.21	35
87 Cacticin	5,7,4′	3′	3-β-D-Galactopyranosyloxy	17.23	20
88 Isorhamnetin-3-rutinoside	5,7,4′	3′	3-β-D-(6-O-α-L-Rhamnopyranosido) glucopyranosyloxy	17.40	20
89 Distichin	5,7,4′	3′	3-α-L-Arabopyranosyloxy	17.84	21
90 Kaempferol-3-arabinoside	5,7,4′	–	3-L-Arabinosyloxy	17.94	21
91 Isorhamnetin-3-arabofuranoside	5,7,4′	3′	3-α-L-Arabinofuranosyloxy	18.44	21

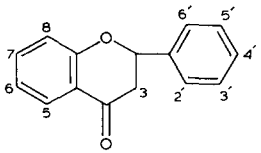
(Continued on p. 88)

TABLE I *(continued)*

Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyl and glycosyloxy*		
92 Galangin-3-rhamnoglucoside	5,7	–	3-Rhamnosidoglucosyloxy	18.51	Leaf and stem of *Datisca cannabina*[33]
93 Quercetin	3,5,7,3′,4′	–	–	18.67	23
94 Patuletin	3,5,7,3′,4′	6	–	18.79	Flowers of *Tagetes patula*[36]
95 3′-O-Methylmyricetin	3,5,7,4′,5′	3′	–	19.04	34
96 Datiscetin	3,5,7,2′	–	–	19.85	Leaf and stem of *Datisca cannabina*[33]
97 Kaempferol-7-rhamnoside	3,5,4′	–	7-α-L-Rhamnosyloxy	20.09	28
98 Kaempferol	3,5,7,4′	–	–	20.48	23
99 Tamarixetin	3,5,7,3′	4′	–	20.86	Synthetic
100 Isorhamnetin	3,5,7,4′	3′	–	21.03	21
101 Rhamnetin	3,5,3′,4′	7	–	22.37	Chromatography of commercial mixture
102 Galangin	3,5,7	–	–	23.97	Rhizoma of *Alpinia officinarum*[37]
103 Rhamnocitrin	3,5,4′	7	–	24.23	Synthetic
104 Galangin-3-methyl ether	5,7	3	–	24.29	Rhizoma of *Alpinia officinarum*[38]
105 Kaempferid	3,5,7	4′	–	24.39	Rhizoma of *Alpinia officinarum*[37]
106 3-Methoxyflavone	–	3	–	24.51	Synthetic
107 Rhamnazin	3,5,4′	7,3′	–	24.53	Chromatography of a commercial mixture with rhamnetine
108 3-Hydroxyflavone	3	–	–	24.88	G. Hrazdina

TABLE II

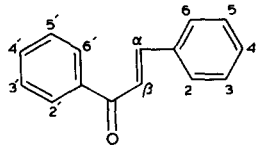
FLAVANONES AND DIHYDROFLAVONOLS



Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyloxy*		
109 Dihydrofisetin	3,7,3′,4′	–	–	11.50	G. Hrazdina
110 Taxifolin	3,5,7,3′,4′	–	–	12.56	W. Steck
111 Eriodictyol-7-glucoside	5,3′,4′	–	7-Glucosyloxy	13.37	Sarsyntex
112 Eriodictyol-7-neohesperidoside	5,3′,4′	–	7-β-D-(2-O-α-L-Rhamnopyranosido)glucopyranosyloxy	14.05	G. Hrazdina
113 Astilbin	5,7,3′,4′	–	3-Rhamnosyloxy	14.72	E. von Rudloff
114 Naringenin-7-glucoside	5,4′	–	7-Glucosyloxy	15.71	Synthetic
115 Naringin	5,4′	–	7-(2-O-Rhamnosido(glucosyloxy	15.71	Sigma
116 Hesperidin	5,3′	4′	7-(6-O-Rhamnosido)glucosyloxy	16.30	Sigma
117 Engelitin	5,7,4′	–	3-Rhamnosyloxy	16.32	E. von Rudloff
118 Neohesperidin	5,3′	4′	7-(2-O-Rhamnosido)glucosyloxy	16.62	W. Steck
119 Eriodictyol	5,7,3′,4′	–	–	16.65	Sarsyntex
120 Naringenin	5,7,4′	–	–	18.71	Sigma
121 Homoeriodictyol	5,7,4′	3′	–	18.72	Leaf of *Eriodictyon californicum*[39]
122 Hesperitin	5,7,3′	4′	–	19.57	Sigma
123 Sakuranitin	5,4′	7	–	21.93	G. Hrazdina
124 Pinocembrin	5,7	–	–	22.41	E. von Rudloff
125 Eriodictyol-7,3′,4′-trimethyl ether	5	7,3′,4′	–	24.30	W. Steck
126 Flavanone	–	–	–	24.53	G. Hrazdina

TABLE III

CHALCONES AND DIHYDROCHALCONES



Substance	*Structure*			*Saturation of α–β double bond*	t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glycosyloxy*			
(a) Dihydrochalcones						
127 Phloridzin	4,4′,6′	–	2′-Glucosyloxy	Yes	16.07	BDH
128 Asebotin	4,6′	4′	2′-Glucosyloxy	Yes	18.23	E. von Rudloff
129 Phloretin	4,2′,4′,6′	–	–	Yes	19.28	G. Hrazdina
(b) Chalcones						
130 2′,4′,6′,3,4-Pentahydroxychalcone	3,4,2′,4′,6′	–	–	No	17.76	Sarsyntex
131 Poncirin chalcone	2′,6′	4	4′-β-D-(2-O-α-L-Rhamnopyranosido)glucopyranosyloxy	No	20.47	G. Hrazdina
132 2-Hydroxy-4,6,2′-trimethoxychalcone	2	4,6,2′	–	No	27.39	W. Steck

cases it may be necessary to separate critical pairs of compounds by use of another solvent system or by another suitable method.

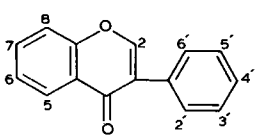
The elution sequence of the individual compounds can best be interpreted by assuming that the compounds are first adsorbed on the hydrophobic stationary phase by "hydrophobic interaction", and that they are subsequently eluted with the mobile phase according to the extent of hydrogen bond formation. Therefore the hydrogen bond donating and/or accepting ability of a given substituent as well as its contribution to the hydrophobic interaction have to be considered. In a methoxyl group, for example, the oxygen is a hydrogen bond acceptor, whereas the methyl group contributes to the hydrophobic interaction. In our system, and with the compounds studied, these two effects balance with the net result that the retention times of tricetin pentamethyl ether (33) and the completely unsubstituted flavone (36) are nearly the same.

The strongest hydrogen bond acceptor in a flavone or isoflavone is the carbonyl group at C-4 which, due to resonance, bears a partial negative charge. If an OH group is present at position 5 a strong internal hydrogen bond is formed between this group and the carbonyl groups, and therefore the latter can no longer interact strongly with the solvent. As a result, the t_R values of the 5-hydroxy-flavones, -isoflavones and -flavonols in Tables I and IV are 1.76–2.29 higher than those of their counterparts not possessing a free 5-OH group. This range applies only to aglycones because for glycosides, which have the ability to form various hydrogen bonds, such generalizations are more difficult (see below).

Hydrogen bonding between the carbonyl group and an OH group in position 3 is much weaker; therefore the t_R value of flavonol (108) is only 0.59 higher than that of flavone (36). If an OH group is present at position 5, introduction of another such group at position 3 usually lowers the t_R values by only 0–1.31. This means that flavones and flavonols, with otherwise identical substitution patterns, are often "critical pairs", which are only poorly separated if at all. On the contrary, introduction

TABLE IV

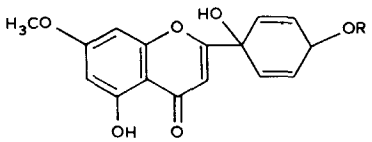
ISOFLAVONES



Substance	*Structure*			t_R *(min)*	*Source ref.*
	OH	OCH_3	*Glucosyloxy*		
133 Iridin	5,3′	6,4′,5′	7	16.51	Rhizoma of *Iris germanica*[40]
134 Daidzein	7,4′	–	–	18.15	J. Sachse
135 Genistein	5,7,4′	–	–	19.60	J. Sachse
136 Pratensein	5,7,3′	4′	–	20.31	J. Sachse
137 Irigenin	5,7,3′	6,4′,5′	–	20.43	Rhizoma of *Iris germanica*[40]
138 Formononetin	7	4′	–	21.85	J. Sachse
139 Biochanin A	5,7	4′	–	23.55	J. Sachse

TABLE V

t_R VALUES OF PROTOGENKWANIN AND ITS 4′-GLUCOSIDE



Substance	*Structure*	t_R *(min)*	*Ref.*
140 Protogenkwanin-4′-glucoside	R = β-D-Glucopyranosyl	15.11	24
141 Protogenkwanin	R = H	17.38	24

into a flavanone of an OH group at position 3 (formation of a dihydroflavonol, *e.g.*, 119 and 110) lowers the t_R value considerably (4.09 in the given example).

Hydroxyl groups at positions other than 3 and 5 reduce the t_R values by 1.43–4.59, provided that no OH group is already present at the *ortho* position to the position considered. If an *o*-OH group is already present the decrease in t_R is only 0.86 to 3.27. This means that all flavonoids, which differ in the number of OH groups, at positions other than 3, can easily be separated.

Methylation of OH groups, as already mentioned above, more or less prevents the effect of these groups. This means that, on the one hand, with the exception of flavonol-3-methyl ethers, flavonoids and their partial methyl ethers are easily separated, whereas on the other hand, introduction of additional methoxyl groups has little or no effect on the t_R values. Flavonoids differing only in a methoxyl group are therefore often "critical pairs".

Glycosylation of an OH group means not only introduction of a hydrophilic moiety, but also shielding (be it by hydrogen bonding or just by steric hindrance) of some hydrophilic substituents already present. The latter effect accounts for the striking fact that rutinosides and neohesperidosides show the same t_R values as the corresponding glucosides, although rhamnosylation of a phenolic OH, with no *ortho*-OH, always decrease the t_R value. The same shielding effect also plays a rôle if an OH group located *ortho* to another OH group is glycosylated. For example, if one considers the β-D-glucopyranosides 4, 5, 19, 49, 52, 54, 65, 75 and 84, it can be seen that by comparison with the aglycone, glucosylation of a 7- or 4′-OH, without an adjacent *ortho*-OH, decreases the t_R values by 4.26–3.83, whereas in the presence of an *ortho*-OH the decrease is only 2.28–1.55. Finally, the fact that the t_R value of luteolin-5-β-D-glucopyranoside (4) is only 0.37 smaller than that of the corresponding 7-β-D-glucopyranoside* can also be explained in terms of the shielding effect of the sugar on the carbonyl group. The contributions of various types of sugars to the hydrophilic interaction decrease, as expected, from hexoses through pentoses to methylpentoses. Interestingly enough, arabopyranosides and arabofuranosides are clearly separated. However, glucopyranosides and galactopyranosides, as well as arabofuranosides and rhamnopyranosides, are usually "critical pairs", which are not separated.

* This is rather surprising if one considers the large difference in t_R values of the corresponding methyl ethers.

Saturation of the C-ring, *i.e.*, transformation of a flavone to the corresponding flavanone or of a flavonol to a dihydroflavonol, affects the t_R values in a very complex way. The saturation of the C-ring itself produces only a small effect [*e.g.*, transformation of flavone (36) to flavanone (126) increases the t_R value by only 0.24] but when OH groups are present the t_R values are always decreased. This is because the interruption of the conjugation in the system more or less affects the acidity, and therefore the hydrogen bond accepting and donating abilities of all OH groups. This effect is most pronounced with the 3-OH, which is phenolic in the former (36) and alcoholic in the latter (126) (in our examples dihydroflavonols move 4.41–6.11 and flavanones only 1.06–2.78 min faster than their fully unsaturated counterparts).

In the above discussion attention has been paid not only to the separations which can be achieved by our system, but also to the "critical pairs" of related compounds, which are usually not separated. It is hoped that such a discussion provides a guideline in the search for a suitable complementary system. There remains another type of "critical pairs", *i.e.*, pairs of totally unrelated compounds, and in this case the above discussion cannot be used as a guideline in the search for chromatographic conditions which in a most general way would complement the system described in this paper.

ACKNOWLEDGEMENTS

We are greatly indebted to all those who generously furnished us with flavonoid samples; their names are given in Tables I–IV. H.G. thanks the Fonds der Chemischen Industrie for financial support and C.v.S. is grateful for a grant from the Belgian Instituut tot Aanmoediging van het Wetenschappelijk Onderzoek in Nijverheid en Landbouw.

REFERENCES

1 R. S. Ward and A. Pelter, *J. Chromatogr. Sci.*, 12 (1974) 570.
2 J. Adamovics and F. R. Stermitz, *J. Chromatogr.*, 129 (1976) 464.
3 J. F. Fisher and T. A. Wheaton, *J. Agr. Food Chem.*, 24 (1976) 898.
4 L. W. Wulf and C. W. Nagel, *J. Chromatogr.*, 116 (1976) 271.
5 H. Becker, G. Wilking and K. Hostettmann, *J. Chromatogr.*, 136 (1977) 174.
6 W. A. Court, *J. Chromatogr.*, 130 (1977) 287.
7 J. Jerumanis, *European Brewery Convention, Proceeding of the 17th Congress, Berlin, 1979*, p. 309.
8 R. L. Rouseff and S. V. Ting, *J. Chromatogr.*, 176 (1979) 75.
9 D. Strack, K. Fuisting and G. Popovici, *J. Chromatogr.*, 176 (1979) 270.
10 S. Asen, *Hort. Science*, 12 (1977) 447.
11 J. P. Bianchini and E. M. Gaydou, *J. Chromatogr.*, 190 (1980) 233.
12 R. E. Carlson and D. Dolphin, *J. Chromatogr.*, 198 (1980) 193.
13 J. M. Hardin and C. A. Stutte, *Anal. Biochem.*, 102 (1980) 171.
14 R. N. Stewart, S. Asen, D. R. Massie and K. H. Norris, *Biochem. Syst. Ecol.*, 8 (1980) 119.
15 P. Labosky, Jr. and J. A. Sellers, *Wood Sci.*, 13 (1980) 32.
16 G. J. Niemann, *Z. Naturforsch., C.*, 35 (1980) 514.
17 M. Vanhaelen and R. Vanhaelen-Fastré, *J. Chromatogr.*, 187 (1980) 255.
18 G. Hrazdina and A. H. Moskowitz, in G. Charalambous and G. Inglett (Editors), *Quality of Foods and Beverages*, Vol. 1, Academic Press, New York, 1981, p. 341.
19 C. F. Van Sumere, K. Vande Casteele, R. Hanselaer, M. Martens, H. Geiger and L. Van Rompaey, *J. Chromatogr.*, 234 (1982) 141.
20 K. R. Markham, B. Ternai, R. Stanley, H. Geiger and T. J. Mabry, *Tetrahedron*, 34 (1978) 1389.

21 H. Geiger and W. de Groot-Pfleiderer, *Phytochemistry*, 18 (1979) 1709.
22 E. Gehring and H. Geiger, *Z. Naturforsch., C.*, 35 (1980) 380.
23 S. Beckmann and H. Geiger, *Phytochemistry*, 7 (1968) 1667.
24 M. Hauteville, J. Chopin, H. Geiger and L. Schuler, *Tetrahedron Lett.*, (1980) 1227.
25 H. Geiger and K. R. Markham, unpublished results.
26 P. S. Rao and T. R. Seshadri, *Proc. Indian Acad. Sci., Sect. A*, 14 (1941) 265.
27 T. A. Geissman and C. Steelink, *J. Org. Chem.*, 22 (1957) 946.
28 H. F. Aly, H. Geiger, U. Schücker, H. Waldrum, G. Vander Velde and T. J. Mabry, *Phytochemistry*, 14 (1975) 1613.
29 N. R. Bannerjee and T. R. Seshadri, *Proc. Indian Acad. Sci.*, 44A (1956) 284.
30 E. Wada, *J. Amer. Chem. Soc.*, 78 (1956) 4725.
31 J. B. Harborne and C. A. Williams, *Bot. J. Linn. Soc.*, 66 (1973) 37.
32 H. Geiger and S. Beckmann, *Z. Naturforsch., B.*, 20 (1965) 1139.
33 H. Griesebach and H. J. Grambow, *Phytochemistry*, 7 (1968) 51.
34 H. Geiger, *Z. Naturforsch., C.*, 34 (1979) 878.
35 H. Geiger and K. R. Markham, unpublished results.
36 P. S. Rao and T. R. Seshadri, *Proc. Indian Acad. Sci., Sect. A*, 14 (1941) 643.
37 E. Jahns, *Ber. Deut. Chem. Ges.*, 14 (1881) 2385.
38 G. Testoni, *Gazz. Chim. Ital.*, 30II (1900) 327.
39 T. A. Geissman, *J. Amer. Chem. Soc.*, 62 (1940) 3258.
40 W. Baker, *J. Chem. Soc., London*, (1928) 1022.