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SEPARATIONS OF SOME COUMARINS OF HIGHER PLANTS BY LIQUID CHROMATOGRAPHY

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SUMMARY

The behaviour of 67 coumarins occurring in higher plants was examined by high-performance liquid chromatography. Resolutions of neutral coumarins were achieved on a normal-phase silica column (10-μm particles) in hexane–ethyl acetate solvent systems and on a C_{18} reversed-phase column (5 μm) with aqueous acetonitrile or aqueous methanol. Phenolic coumarins were resolved by stepwise elution with increasing concentrations of aqueous methanol containing acetic acid, sometimes with the addition of tetrahydrofuran, on the reversed-phase column. A mixture of ten coumarins simulating the neutral coumarin fraction of *Citrus aurantifolia* was fully resolved by the use of the two columns in sequence.

INTRODUCTION

Coumarins occur in well over 100 plant families, predominantly among the higher plants[1]. It is not uncommon in some of these families, most notably the Guttiferae, Rutaceae, and Umbelliferae, to encounter species which elaborate ten, twenty, or even more coumarins, and four or five from the same species are quite often reported. The advent of various chromatographic procedures has greatly eased the task of resolving such complex mixtures, but has also pointed up an even greater separation problem than had been previously recognized, by revealing numerous minor coumarin constituents, many as yet unidentified.

Until now most applications of high-performance liquid chromatography (HPLC)[2] to coumarins have concerned the resolution of mixtures from specific higher plants[3–8], as well as the separation and analysis of aflatoxins[9–14], complex coumarins formed by moulds of the genus *Aspergillus*, where much interest has arisen from the need to determine traces of these potent hepatotoxins in foodstuffs. The only more extensive study of which we are aware is that of Vande Casteele *et al.*[15], conducted in the course of an examination of the behaviour of a large number of phenolic compounds on reversed-phase columns. These workers used a combination of isocratic and linear gradient elutions with methanol and aqueous formic acid, and in many cases achieved good separations of a total of 43 coumarins, 23 of which are naturally occurring.

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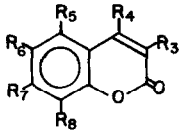
TABLE I

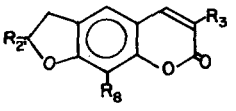
STRUCTURES OF COUMARINS EXAMINED BY HPLC

Note: positions not otherwise designated are unsubstituted.



Structure 1

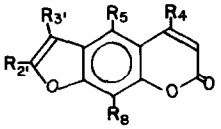
1 (No substituents)
2 $R_7 = OH$
3 $R_7 = OCH_3$
4 $R_7 = OCH_2\text{-}CH=C(CH_3)_2$
5 $R_6 = CH_2\text{-}CH=C(CH_3)_2$, $R_7 = OH$
6 $R_6 = CH_2\text{-}CHOH\text{-}C(OH)(CH_3)_2$, $R_7 = OH$
7 $R_6 = CH_2\text{-}CH=C(CH_3)\text{-}(CH_2)_2\text{-}CH=C(CH_3)_2$, $R_7 = OH$
8 $R_6 = CH_2\text{-}CH=C(CH_3)_2$, $R_7 = OCH_3$
9 $R_7 = OH$, $R_8 = CH_2\text{-}CH=C(CH_3)_2$
10 $R_7 = OCH_3$, $R_8 = CH_2\text{-}CH=C(CH_3)_2$
11 $R_7 = OCH_3$, $R_8 = CH_2\text{-}CH=C(CH_3)\text{-}CH_2\text{-}O\text{-}CO\text{-}C(CH_3)=CHCH_3$
12 $R_7 = OCH_3$, $R_8 = CH(O\text{-}CO\text{-}CH_2\text{-}CH(CH_3)_2)\text{-}CO\text{-}CH(CH_3)_2$
13 $R_5 = R_7 = OH$
14 $R_5 = R_7 = OCH_3$
15 $R_5 = OCH_2\text{-}CH=CH_2$, $R_7 = OH$
16 $R_5 = OCH_2\text{-}CH=C(CH_3)\text{-}(CH_2)_2\text{-}CH=C(CH_3)_2$, $R_7 = OCH_3$
17 $R_5 = R_7 = OCH_3$, $R_8 = CH_2\text{-}CH=C(CH_3)_2$
18 $R_6 = R_7 = OH$
19 $R_6 = OCH_3$, $R_7 = OH$
20 $R_6 = R_7 = OCH_3$
21 $R_3 = C(CH_3)_2\text{-}CH=CH_2$, $R_6 = R_7 = OCH_3$
22 $R_7 = R_8 = OH$
23 $R_7 = OH$, $R_8 = OCH_3$
24 $R_7 = R_8 = OCH_3$
25 $R_5 = R_7 = OCH_3$, $R_8 = OH$
26 $R_5 = R_7 = R_8 = OCH_3$
27 $R_6 = OCH_3$, $R_7 = R_8 = OH$
28 $R_6 = R_8 = OCH_3$, $R_7 = OH$
29 $R_6 = R_7 = R_8 = OCH_3$
30 $R_6 = OCH_3$, $R_7 = OCH_2\text{-}CH=C(CH_3)_2$, $R_8 = OH$
31 $R_6 = R_8 = OCH_3$, $R_7 = OCH_2\text{-}CH=C(CH_3)_2$



Structure 2

32 $R_{2'} = C(OH)(CH_3)_2$
33 $R_3 = C(CH_3)_2\text{-}CH=CH_2$, $R_{2'} = C(OH)(CH_3)_2$
34 $R_{2'} = C(OH)(CH_3)_2$, $R_8 = OH$

TABLE I (*continued*)



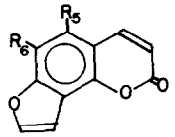
Structure 3

35 (no substituents)
36 $R_{2'} = CH(CH_3)_2$, $R_{3'} = OCH_3$
37 $R_4 = R_8 = R_{2'} = CH_3$
38 $R_5 = OH$
39 $R_5 = OCH_3$
40 $R_5 = OCH_2{-}CH{=}C(CH_3)_2$

41 $R_5 = OCH_2{-}CH(O)CH{-}C(CH_3)_2$

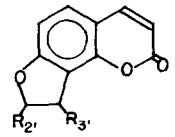
42 $R_5 = OCH_2{-}CH(C(OH)(CH_3)_2){-}O{-}CO{-}C(CH_3){=}CHCH_3$

43 $R_5 = OCH_2{-}CH{=}C(CH_3){-}(CH_2)_2{-}CH{=}C(CH_3)_2$
44 $R_8 = OH$
45 $R_8 = OCH_3$
46 $R_8 = OCH_2{-}CH{=}C(CH_3)_2$
47 $R_8 = OCH_2{-}CH{=}C(CH_3){-}(CH_2)_2{-}CH{=}C(CH_3)_2$
48 $R_5 = OCH_3$, $R_8 = OH$
49 $R_5 = R_8 = OCH_3$
50 $R_5 = OCH_3$, $R_8 = OCH_2{-}CH{=}C(CH_3)_2$
51 $R_5 = OCH_2{-}CH{=}C(CH_3)_2$, $R_8 = OCH_3$



Structure 4

52 (no substituents)
53 $R_5 = OCH_2{-}CH{=}C(CH_3)_2$
54 $R_5 = R_6 = OCH_3$

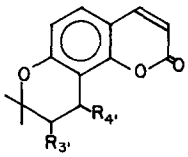


Structure 5

55 $R_{2'} = C(OH)(CH_3)_2$
56 $R_{2'} = C(O{-}CO{-}C(CH_3){=}CHCH_3)(CH_3)_2$
57 $R_{2'} = C(O{-}CO{-}CH_3)(CH_3)_2$, $R_{3'} = O{-}CO{-}C(CH_3){=}CHCH_3$
58 $R_{2'} = C(O{-}CO{-}CH_2{-}CH(CH_3)_2)(CH_3)_2$, $R_{3'} = O{-}CO{-}CH_2CH(CH_3)_2$

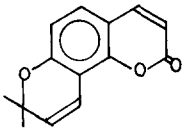
(Continued on p. 326)

TABLE I (*continued*)



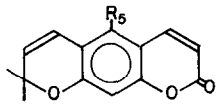
Structure 6

59 $R_{3'}$ = OH
60 $R_{3'}$ = O–CO–C(CH$_3$)=CHCH$_3$
61 $R_{3'}$ = O CO–CH$_3$, $R_{4'}$ = O CO–CH$_2$C(CH$_3$)$_2$
62 $R_{3'}$ = O–CO–CH$_3$, $R_{4'}$ = O–CO–C(CH$_3$)=CHCH$_3$
63 $R_{3'}$ = O–CO–CH(CH$_3$)C$_2$H$_5$OH, $R_{4'}$ = O–OC–CH$_3$
64 $R_{3'}$ = O–CO–C(CH$_3$)=CHCH$_3$, $R_{4'}$ = O–CO–CH$_3$



Structure 7

65 (No substituents)



Structure 8

66 (No substituents)
67 R_5 = OCH$_3$

We have now taken advantage of a relatively large collection of natural coumarins in our laboratory to examine the behaviour of 67 of these compounds (for structures see Table I) on both normal and reversed-phase columns, and have succeeded in resolving virtually all pairs by the use of these columns individually or in sequence. Although this number is still but a small fraction of the total coumarins occurring in nature, it is reasonably representative of the recognized categories.

EXPERIMENTAL

Materials

The coumarins used in this study derive from a wide variety of sources, natural and synthetic. A few were commercial samples; herniarin (3), our reference standard for neutral coumarins, is obtainable from K&K Labs. (Plainview, NY, U.S.A.) or Pfaltz and Bauer (Stamford, CT, U.S.A.). Some were prepared here, but many others were gifts from workers in other institutions, to whom we are most grateful.

Reagent grade solvents were used in mobile phases without further purification.

Instrumentation

Separations were accomplished either on a μPorasil normal phase high resolution column (Waters Assoc., Milford, MA, U.S.A.), a silica column of particle size 10 μm, with surface area of 300–350 m^2/g, 300 × 3.9 mm I.D., column volume *ca.* 3 ml, or on a Waters Nova-Pak C_{18} reversed-phase silica column of particle size 5 μm, with surface area 250 m^2/g, 7% by weight carbon loading, 150 × 3.9 mm I.D., column volume *ca.* 1.5 ml. A Waters Model M-6000A pump was used for solvent

delivery, and injection was by a Rheodyne Model 7125 injection valve (Rheodyne, Cotati, CA, U.S.A.). Effluent was continuously monitored on a Cecil CE 272 linear readout UV spectrophotometer equipped with a flow cell of 8-μl capacity (Cecil, Cambridge, U.K.), and the signal recorded. UV absorption spectra were recorded on a Unicam SP 800A spectrophotometer (Pye Unicam, Cambridge, U.K.).

Chromatographic procedure

Solvent was introduced at a flow-rate of 1.0 ml/min. Samples of 13–25 μg of each coumarin were injected.

Monitoring of the UV absorption was done at 335 or 310 nm. Most coumarins exhibit a broad absorption band in the vicinity of 335 nm and this is the most generally useful wavelength to detect coumarins. However, furanocoumarins and a few others have rather weak absorption at 335 and a stronger peak close to 310. Except when one is measuring near the limits of detection a satisfactory peak is obtained at either wavelength, and in the case of some mixtures one setting must be chosen, normally 335. On the other hand, if the mixture being examined is known to consist mainly of furanocoumarins the preferred choice would be 310. Herniarin (3), which absorbs appreciably at both wavelengths, was used as a reference standard for neutral coumarins, and umbelliferone (2) for phenolic coumarins, with retention volumes (V_R) relative to those of these two standards being expressed to minimize the influence of any minor uncontrolled variables in the system.

Solvent systems employed in this study were, for normal-phase separations of neutral coumarins: ethyl acetate–hexane*, 1:4 (system 1) or 1:3 (system 2), and for reversed-phase separations usually water–acetonitrile, 1:1 (system 3) or water–methanol, 8:2 (system 4). For phenolic coumarins two series of methanol–water–acetic acid mixtures (systems 5 and 6), introduced stepwise, were employed. These were modified from the series used by Rodriguez' group[16] to separate phenolic acids, and are described in detail in the Results and Discussion section. Limited use was also made of an acidified ternary mixture, water–methanol–tetrahydrofuran–acetic acid, 80:15:5:1 (system 7) in the phenolic series. All these aqueous solvent mixtures were degassed before use at aspirator vacuum for 1–2 min.

RESULTS AND DISCUSSION

Neutral coumarins

Data are presented in Table II for normal-phase chromatography of 49 neutral coumarins, embracing 16 simple coumarins (lacking any ring system apart from the benzopyrone nucleus), 8 pyranocoumarins and dihydropyranocoumarins, 19 furanocoumarins and dihydrofuranocoumarins, and 5 coumarins bearing free alcoholic hydroxyl groups which we have arbitrarily segregated from the other categories. Two of these latter in which the hydroxyl groups are esterified with angelic acid were not thus segregated.

The two solvent systems employed, with differing proportions of hexane and ethyl acetate, evidently complement each other to some degree. In solvent system 1

* Because of questions of hexane toxicity recently raised, solvent mixtures in which isooctane was substituted were tested. Limited experience suggests that retention volumes are not appreciably altered.

TABLE II

NORMAL-PHASE LIQUID CHROMATOGRAPHY OF NEUTRAL COUMARINS

$R_H = \frac{V_R \text{ (sample)}}{V_R \text{ (herniarin)}}$; these values were calculated from herniarin retention volumes recorded in each series of analyses, which approximated 8.8 ml in system 1 and 7.2 ml in system 2.

	R_H in solvent system 1	*R_H in solvent system 2*
Simple coumarins		
5-Geranyloxy-7-methoxycoumarin (16)	0.63	0.73
Suberosin (8)	0.71	0.70
O-Prenylumbelliferone (4)	0.77	0.73
Coumarin (1)	0.81	0.84
Rutacultin (21)	0.90	1.04
Osthol (10)	0.91	0.96
Citropten (14)	0.96	1.00
Coumurrayin (17)	1.00	1.04
Herniarin (3)	1.00	1.00
Macrocarpin (11)	1.22	1.19
7,8-Dimethoxycoumarin (24)	1.84	1.65
Puberulin (31)	2.00	1.81
5,7,8-Trimethoxycoumarin (26)	–	2.08
Scoparone (20)	2.49	–
Paniculatin (12)	2.65	1.89
6,7,8-Trimethoxycoumarin (29)	2.68	2.46
Pyranocoumarins and dihydropyranocoumarins		
Seselin (65)	0.74	0.77
Lomatin angelate (60)	0.84	0.81
Xanthoxyletin (67)	0.87	0.92
Xanthyletin (66)	0.94	0.96
Suksdorfin (61)	1.09	1.08
Pteryxin (62)	1.19	1.11
Visnadin (63)	1.26	1.15
Isopteryxin	1.35	1.23
Furanocoumarins and dihydrofuranocoumarins		
Bergamottin (43)	0.53	0.62
Lanatin (53)	0.53	0.62
8-Geranyloxypsoralen (47)	0.59	0.65
Isoimperatorin (40)	0.63	0.69
Cnidilin (51)	0.72	0.73
Columbianetin angelate (56)	0.78	0.81
Angelicin (52)	0.81	0.81
Pimpinellin (54)	0.84	0.85
Trioxsalen (37)	0.84	0.85
Athamantin (58)	0.91	0.85
Imperatorin (46)	1.06	0.96
Phellopterin (50)	1.09	1.08
Psoralen (35)	1.09	1.12
Bergapten (39)	1.12	1.12
Xanthotoxin (45)	1.28	1.23
Isopimpinellin (49)	1.31	1.31
Oxypeucedanin (41)	1.43	1.31
Edultin (62)	3.84	2.92

TABLE II (*continued*)

	R_H *in solvent system 1*	R_H *in solvent system 2*
Alcoholic coumarins		
Heliettin (33)	–	1.77
Lomatin (59)	–	2.03
Columbianetin (55)	–	2.31
Ostruthol (42)	–	2.77
Marmesin (32)	–	5.35

V_R values ranged from 4.7 ml for the furanocoumarins bergamottin (43) and lanatin (53) to 33.8 ml for edultin (62), whereas in system 2 they were between 4.4 ml for bergamottin and lanatin and 38.2 ml for marmesin (32), one of the slow-running alcoholic coumarins not examined with system 1. In each group where they were compared, system 1 gave a wider range of retention volumes, and in some cases significantly better resolutions, as with O-prenylumbelliferone (4) and 5-geranyloxy-7-methoxycoumarin (16), coumurrayin (17) and rutacultin (21), and isopimpinellin (49) and oxypeucedanin (41). Instances were nevertheless noted where system 2 separated more efficiently: *e.g.* imperatorin (46) and phellopterin (50), and xanthotoxin (45) and isopimpinellin (49), but resolutions were sometimes inadequate or non-existent in both systems, the commonly co-occurring bergapten (39) and psoralen (35) being a notable example.

Relationships between structures and V_R values appear in general to conform to accepted theory[2]. Coumarins bearing the non-polar isoprenoid substituents, on either carbon or oxygen, such as suberosin (8), bergamottin (43), and 8-geranyloxypsoralen (47), tend to be eluted early, with R_H values usually below 0.8, but this effect is modified by the presence of certain methoxyl substituents on the benzene ring, which can markedly increase retention. Thus the introduction of a 7-methoxyl group into coumarin (1) (to yield herniarin, 3) produces a rise from 0.84 to 1, and a second methoxyl at position 8 (7,8-dimethoxycoumarin, 24) from 1 to 1.72. Both trimethoxycoumarins examined had R_H values above 2. Interestingly, introduction of a 5-methoxyl group into herniarin (3) (citropten, 14) or into psoralen (35) (bergapten, 39) has no appreciable effect on V_R, indicating that position isomerism has a role to play in this context. Prenylation of citropten (14) at position 8 (coumurrayin, 17) has little effect on retention; hence it would appear that the retarding effect of the methoxyl groups is dominant. In the psoralen series the successive introduction of first an 8- and then a 5-methoxyl group (xanthotoxin, 45, isopimpinellin, 49) does result in measurable increases of V_R.

The polar alcoholic coumarins, as expected, all have high retentions, with the R_H values varying in the five compounds tested from 1.77 to 5.35 in system 2. Esterification lowers these values to the normal range, although one of the diesters, edultin (62), again has a high value.

As normal phase systems had failed to resolve certain combinations of our neutral coumarins, recourse was had to reversed-phase liquid chromatography. Solvent systems composed of 50% aqueous acetonitrile (system 3) were most widely useful here, and some of the results are presented in Table III. Adjacent coumarins

TABLE III

COMPARISON OF RETENTIONS OF SELECTED NEUTRAL COUMARINS ON A REVERSED-PHASE COLUMN ELUTED BY SOLVENT SYSTEM 3 WITH THOSE ON A NORMAL-PHASE COLUMN

The R_H values were calculated from herniarin retention volumes recorded in each series of analyses. For system 3 this approximated 2.5 ml. Values in parentheses are R_H values by normal-phase HPLC, solvent system 1, arranged in order of increasing magnitude.

	R_H		R_H
Bergamottin	−* (0.53)	Osthol	3.5 (0.91)
Lanatin	14.0 (0.53)	Xanthyletin	2.0 (0.94)
5-Geranyloxy-7-methoxycoumarin	1.7 (0.63)	Citropten	1.2 (0.96)
Imperatorin	4.1 (0.63)	Herniarin	1 (1)
Suberosin	4.4 (0.71)	Coumurrayin	6.3 (1.0)
Cnidilin	3.7 (0.72)	Imperatorin	2.8 (1.06)
Seselin	3.9 (0.74)	Phellopterin	3.2 (1.09)
O-Prenylumbelliferone	0.6 (0.77)	Suksdorfin	5.2 (1.09)
Columbianetin angelate	10.8 (0.78)	Psoralen	1.0 (1.09)
Angelicin	1.5 (0.81)	Bergapten	1.2 (1.12)
Coumarin	1.1 (0.81)	Pteryxin	3.9 (1.19)
Lomatin angelate	9.6 (0.84)	Xanthotoxin	1.1 (1.28)
Pimpinellin	2.6 (0.84)	Isopimpinellin	1.3 (1.28)
Trioxsalen	4.8 (0.84)	Isopteryxin	4.4 (1.35)
Rutacultin	3.7 (0.90)	Oxypeucedanin	1.6 (1.43)

* Very high V_R; not recovered.

in this table, which have closely similar R_H values on the normal phase column in solvent system 1, show better separations in the reversed-phase system, sometimes spectacularly so, as with the three compounds of R_H (0.84). This column retained bergamottin (43) too strongly to permit practical recovery, but another solvent system, 80% aqueous methanol (system 4), yielded an entirely satisfactory resolution of bergamottin and lanatin (53), with respective R_H values of 2.0 and 2.8.

Despite these data, reversed-phase liquid chromatography is by no means clearly superior to normal-phase, in which many other coumarins were found to be better resolved.

Phenolic coumarins

Coumarins bearing free phenolic hydroxyl groups are readily separable from neutral coumarins by extraction out of ethereal or ethyl acetate solutions into dilute alkali. Although the need for accomplishing this separation chromatographically therefore does not arise, the phenolic coumarins are accompanied by other acidic substances, so that their further purification by physical means may pose potentially the greater problem. Conversely, owing to the pattern of their natural occurrence, the separation of phenolic coumarins from one another is inherently less difficult than that of the neutral coumarins. Among the natural coumarins now known those with a free phenolic hydroxyl group are decidedly in the minority[1], and with rare exceptions —notably the genus *Mammea* (Guttiferae)— more than two or three have not been reported to occur in a single species. It is arguable, however, that their

TABLE IV

RETENTIONS OF PHENOLIC COUMARINS ON A REVERSED-PHASE COLUMN ELUTED STEPWISE WITH SOLVENT SYSTEM 5

$R_U = \frac{V_R \text{ (sample)}}{V_R \text{ (umbelliferone)}}$. These values were calculated from umbelliferone retention volumes recorded in each series of analyses, which approximated 12 ml.

	R_U		R_U
Aesculetin (18)	0.48	Rutaretin (34)	1.68
Daphnetin (22)	0.65	Xanthotoxol (44)	1.68
5,7-Dihydroxycoumarin (13)	0.83	Peucedanol (6)	1.71
Fraxetin (27)	0.87	8-Hydroxybergapten (48)	1.77
Umbelliferone (2)	1.00	Bergaptol (38)	1.84
Scopoletin (19)	1.12	Capensin (30)	2.11
Hydrangetin (23)	1.24	Lacoumarin (15)	2.15
Isofraxidin (28)	1.37	Osthenol (9)	2.60
Leptodactylone (25)	1.66		

relative paucity may be illusory, and due in part to the greater difficulty of examining the acidic fractions of plant extracts which, as mentioned above, often contain many compounds other than coumarins. Application of liquid chromatography to the study of these acidic fractions may well reveal that phenolic coumarins are more commonly occurring than is now recognized.

Separations of the phenolic coumarins were done by reversed-phase liquid chromatography on the Nova-Pak C_{18} column, by stepwise elution in which varying proportions of water, methanol, and acetic acid were employed. The most extensively used system (System 5) involved a protocol in which this mixture in the ratio of

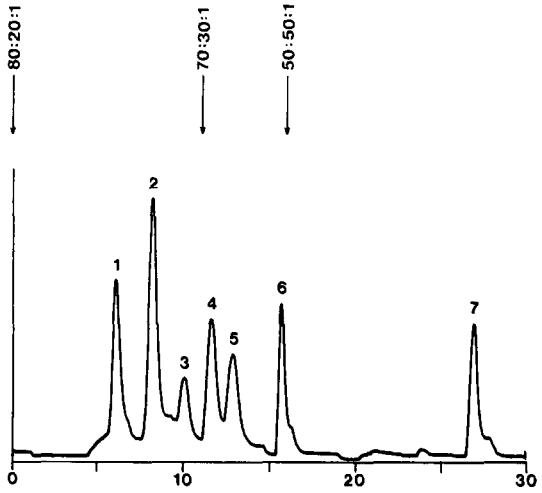


Fig. 1. Separation of a mixture of phenolic coumarins by reversed-phase liquid chromatography with solvent system 5. Abscissa is elution volume (ml). Ratios are water–methanol–acetic acid. Band 1 is aesculetin (18), 2 is daphnetin (22), 3 is an unidentified impurity, 4 is 5,7-dihydroxycoumarin (13) + fraxetin (27), 5 is umbelliferone (2), 6 is isofraxidin (28), 7 is capensin (30).

80:20:1 was introduced at the outset, with subsequent changes to 70:30:1 at 11 ml and to 50:50:1 at 16 ml (*cf.* Proksch *et al.*[16]). Table IV presents the data from 17 compounds, whose retention volumes ranged from 5.7 ml for the very polar diphenol, aesculetin (18) to 31.7 ml for osthenol (9), a monophenol bearing a prenyl side-chain. Fig. 1 shows the chromatogram of a mixture of seven arbitrarily selected compounds from this group, eluted by system 5. Except for 5,7-dihydroxycoumarin (13) and fraxetin (27), good separations were observed for all these relatively polar coumarins.

In an effort to achieve more rapid elution of less polar phenolic coumarins from this column, a second protocol was adopted in which elution was initiated with water–methanol–acetic acid, 70:30:1, changed after 5 ml to 50:50:1, and after 10 ml to 30:70:1 (system 6). In this system osthenol (9) was eluted with an R_U value of 3.4 (retention volume 14.6 ml), 7-demethylsuberosin (5) 3.7 (16.1 ml), and ostruthin (7) 7.8 (33.7 ml); thus only ostruthin, with its geranyl side-chain, required much over 16 ml for emergence.

A chromatogram of a mixture of ten of the coumarins of Table IV developed with system 6 is reproduced in Fig. 2. Two pairs of positional isomers, bergaptol (38) and xanthotoxol (44), and osthenol (9) and 7-demethylsuberosin (5), were both well resolved.

Four compounds in Table IV have R_U values in the 1.66–1.71 range, too close for resolution in system 5. Much better resolution of these was achieved by the partial substitution of methanol with tetrahydrofuran. Fig. 3 shows the result of isocratic elution with water–methanol–tetrahydrofuran–acetic acid, 80:15:5:1 (system 7). Of

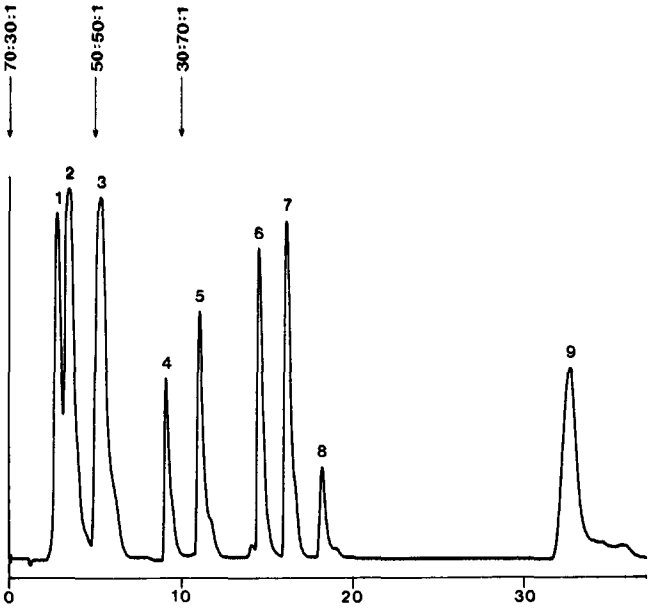


Fig. 2. Separation of a mixture of phenolic coumarins by reversed-phase liquid chromatography with solvent system 6. Abscissa is elution volume (ml). Ratios are water–methanol–acetic acid. Band 1 is aesculetin (18), 2 is daphnetin (22), 3 is umbelliferone (2) + hydrangetin (23) + isofraxidin (28), 4 is xanthotoxol (44), 5 is bergaptol (38), 6 is osthenol (9), 7 is 7-demethylsuberosin (5), 8 is an unidentified impurity, 9 is capensin (30).

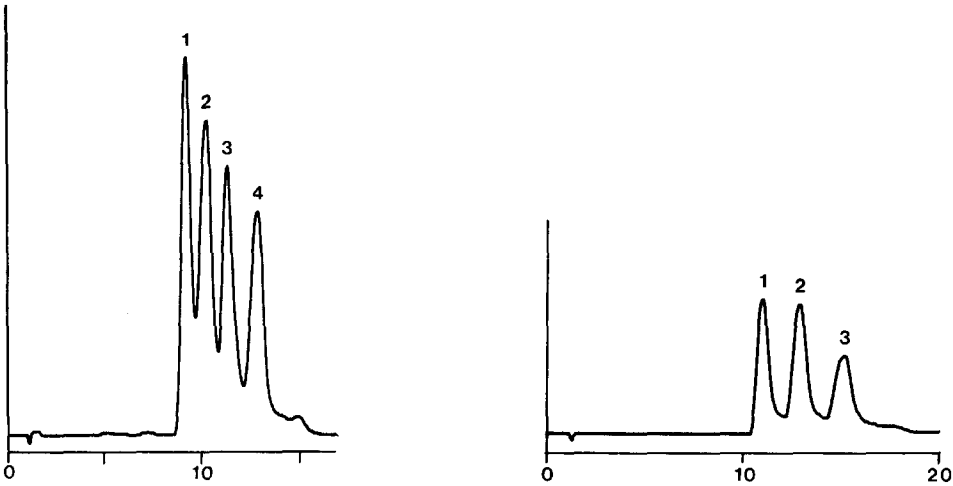


Fig. 3. Resolution of a mixture of four phenolic coumarins by reversed-phase liquid chromatography with solvent system 7. Abscissa is elution volume (ml). Band 1 is rutaretin (34), 2 is leptodactylone (25), 3 is peucedanol (6), and 4 is xanthotoxol (44).

Fig. 4. Resolution of three highly polar coumarins by isocratic elution with water–methanol–acetic acid (80:20:1) from a reversed-phase column. Abscissa is elution volume (ml). Band 1 is umbelliferone (2), 2 is hydrangetin (23), 3 is isofraxidin (28).

particular interest here is the good separation of xanthotoxol (44) and rutaretin (34), two structurally similar compounds. Although system 7 was not further examined, its potential usefulness for the resolution of yet untested mixtures of phenolic coumarins is stressed.

In addition to these four coumarins, three of a more polar structure —umbelliferone (2), hydrangetin (23), and isofraxidin (28)— were eluted as a single peak by system 6, and the observed retention volumes suggest that system 5 would also not fully resolve the latter two. Isocratic elution with the 80:20:1 mixture of system 5 did succeed, as shown in Fig. 4, in separating these three compounds almost completely within 16 ml.

Lack of additional samples has precluded a more extensive study of the behaviour of phenolic coumarins of relatively low polarity, but the single case of ostruthin (7) suggests that, in the presence of such compounds, benefit could be expected from modification of system 6 to provide a third solvent change to an even more concentrated methanol solution after about 20 ml. The protocols adopted here were, of course, arbitrary, and investigators faced with specific separation problems may well find other modifications desirable to achieve optimal resolutions. It should also be mentioned that others[8,15] have used formic acid as a component of eluent mixtures for coumarins, and higher plate numbers have been claimed[15]. As we found resolutions on the Nova-Pak column to be generally quite adequate with acetic acid we did not explore this alternative solvent system, but the possibility of substitution of formic acid for acetic acid in our systems should be borne in mind for possible enhancement of resolutions in more difficult cases.

Resolution of mixtures

We have addressed the question of the practical value of the above procedures for the resolution of the often complex mixtures of coumarins occurring in plant extracts. *Citrus aurantifolia* (Christm.) Swingle, for example, has been reported[1] to elaborate thirteen neutral coumarins, as well as bergaptol (38). As we possessed ten of these thirteen, we prepared a mixture of them as a qualitative simulation of a neutral coumarin fraction from this species. Fig. 5 represents a chromatogram of this mixture obtained with solvent system 1 on the μPorasil normal-phase column. The mixture was resolved into six bands, each of which was collected, and examined by thin-layer chromatography (TLC). Four of these were pure compounds, and the remaining two each consisted of an unresolved mixture of three coumarins. Band 2, containing the less polar mixture, was rechromatographed on the Nova-Pak reversed-phase column with solvent system 4. The more polar band 5 was rechromatographed on this column with solvent system 3. Figs. 6 and 7 show that in both cases clean

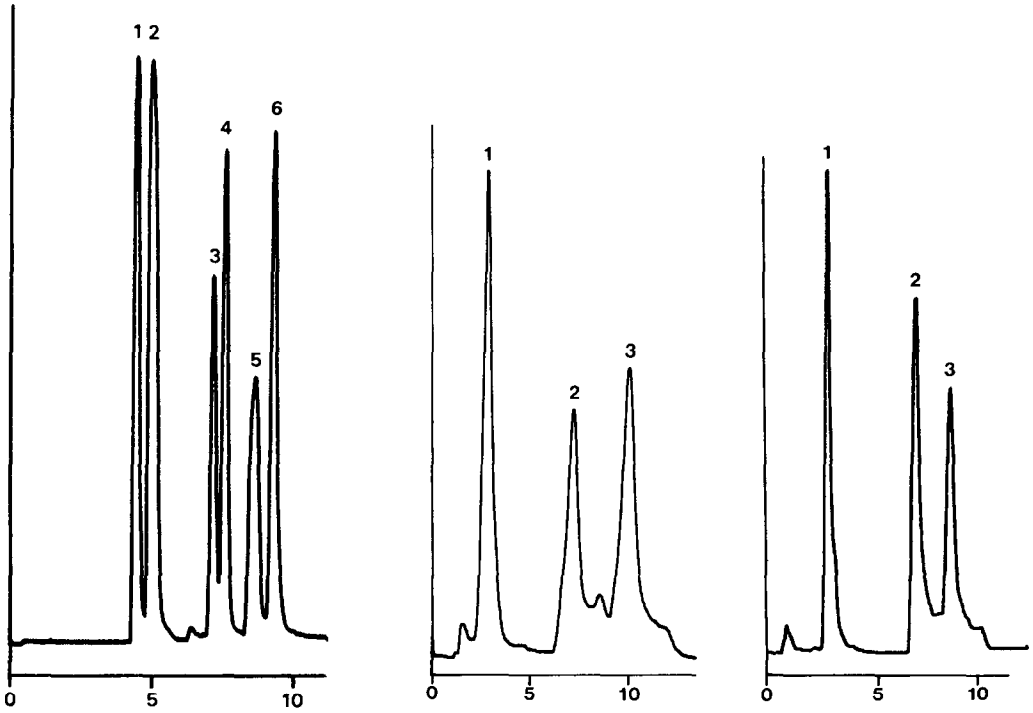


Fig. 5. Separation of coumarins found in *Citrus aurantifolia* by normal-phase liquid chromatography with solvent system 1. Abscissa is elution volume (ml). Band 1 is bergamottin (43), 2 is 5-geranyloxy-7-methoxycoumarin (16) + 8-geranyloxypsoralen (47) + isoimperatorin (40), 3 is xanthyletin (66), 4 is citropten (14), 5 is phellopterin (50) + bergapten (39) + imperatorin (46), 6 is isopimpinellin (49). (Detector wavelength 335 nm.)

Fig. 6. Resolution of band 2 of Fig. 5 by reversed-phase liquid chromatography with solvent system 4. Abscissa is elution volume (ml). Band 1 is isoimperatorin (40), 2 is 8-geranyloxypsoralen (47), 3 is 5-geranyloxy-7-methoxy-coumarin (16). (Detector wavelength 310 nm.)

Fig. 7. Resolution of band 5 of Fig. 5 by reversed-phase liquid chromatography with solvent system 3. Abscissa is elution volume (ml). Band 1 is bergapten (39), 2 is phellopterin (50), 3 imperatorin (46). (Detector wavelength 310 nm.)

resolutions of the mixtures from the normal-phase column were achieved.

A phenomenon observed in the normal phase, unexpected in view of the small sample size, was a shift in retention volumes by comparison to those of individually injected coumarins, with earlier elution than anticipated. Such behaviour has been observed previously with other planar molecules, but its theoretical basis appears not to be well understood[17]. The extent to which other systems are subject to this phenomenon is at present uncertain, although recent experience with acidic fractions recovered from a plant[18] has not pointed up so great a problem with phenolic coumarins chromatographed on the reversed-phase column. It would appear prudent when one is approaching the investigation of unknown coumarin mixtures to monitor the individual bands by TLC until their identities have been established.

Finally, we examined by liquid chromatography a neutral lactone fraction recovered by a standard procedure[19] from *Ruta graveolens* L. leaves, which have been reported[1] to contain psoralen (35), bergapten (39) and xanthotoxin (45), as well as minor amounts of one or two other neutral coumarins. Chromatography on the reversed-phase column developed with 30% acetonitrile yielded three well separated major bands which were confirmed by TLC to be the above three furanocoumarins. Minor components were also noted, but were not identified.

It is clear from the results reported here that the normal and reversed-phase columns in this study, with appropriate solvent systems, complement each other very nicely, and, employed individually or in sequence, are of practical value for the resolution of complex mixtures of plant coumarins.

ACKNOWLEDGEMENTS

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REFERENCES

1 R. D. H. Murray, J. Méndez and S. A. Brown, *The Natural Coumarins*, Wiley, Chichester, 1982.
2 H. Engelhardt, *High-Performance Liquid Chromatography*, Springer, Berlin, 1979.
3 F. D. Stermitz and R. D. Thomas, *J. Chromatogr.*, 77 (1973) 431.
4 F. D. Stermitz, R. D. Thomas and M. C. Williams, *Phytochemistry*, 14 (1975) 1681.
5 J. F. Fisher and L. A. Trama, *J. Agr. Food Chem.*, 27 (1979) 1334.
6 S. Shibata and M. Noguchi, *Phytochemistry*, 16 (1977) 291.
7 E. B. Thompson, G. H. Aynilian, R. H. Dobberstein, G. A. Cordell, H. H. S. Fong and N. R. Farnsworth, *J. Nat. Prod.*, 42 (1979) 120.
8 R. C. Beier, G. W. Ivie, E. H. Oertli and D. L. Holt, *Food Chem. Toxicol.*, 21 (1983) 163.
9 R. M. Beebe, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 1347.
10 M. Manabe, T. Goto and S. Matsuura, *Agr. Biol. Chem.*, 42 (1978) 2003.

11 S. Nesheim, *U.S. Nat. Bur. Stand. Spec. Publ.*, 519 (1979) 355; *Chem. Abstr.*, 91 (1979) 73194.
12 W. A. Pons and A. O. Franz, *J. Ass. Offic. Anal. Chem.*, 61 (1978) 793.
13 D. P. H. Hsieh, D. L. Fitzell, J. L. Miller and J. N. Seiber, *J. Chromatogr.*, 117 (1976) 474.
14 G. E. Neal and P. J. Colley, *Biochem. J.*, 174 (1978) 839.
15 K. Vande Casteele, H. Geiger and C. F. Van Sumere, *J. Chromatogr.*, 258 (1983) 111.
16 P. Proksch, C. Wisdom and E. Rodriguez, *Z. Naturforsch., C: Biosci.*, 36C (1981) 357.
17 D. Neale, Waters Scientific, Ltd., private communication.
18 S. A. Brown, D. E. A. Rivett and H. J. Thompson, unpublished results.
19 D. J. Austin and S. A. Brown, *Phytochemistry*, 12 (1973) 1657.