THE CHROMATOGRAPHY OF FLAVONOID AGLYCONES IN THE SOLVENT SYSTEM BENZENE–ACETIC ACID–WATER

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(Received May 25th, 1962)

INTRODUCTION

Although a large number of solvent systems have been used for the paper chromatography of flavonoid compounds[1–3] few are capable of separating flavone, isoflavone and flavanone aglycones over a wide range of R_F values. The most commonly used solvent system *n*-butanol–acetic acid–water (4:1:5 by vol., or its many single phase variations) clearly separates flavonoid glycosides with good definition of spots, but tends to concentrate most aglycones over a narrow range of high R_F values. Aqueous solvents containing various amounts of acetic acid likewise give narrow spreads of R_F values for non-glycosidic flavonoids (see Tables in refs. [1,3,4]).

In work on ether-soluble polyphenols[5] and hydrolysates of plant extracts we have found the solvent system benzene–acetic acid–water (125:72:3 by vol.) to be most useful for the separation of these compounds. This single phase benzene solvent was devised by SMITH[6], as an improvement on the organic phase of benzene–acetic acid–water (2:2:1 by vol.) commonly used for the chromatography of phenolic acids[6,7]. Excellent separations were obtained with this as the first solvent in two-dimensional chromatography, followed by 2 *N* ammonia[5] or aqueous acetic acid as the second solvent. In the present study the chromatographic behaviour in this solvent system of 43 natural or synthetic flavones, isoflavones, flavanones, flavonols and flavanonols is reported.

EXPERIMENTAL

Solutions of the flavonoid compounds in ethanol (10–20 μg of material) were applied as narrow bands (*ca.* 0.5 × 2 cm) on Whatman No. 1 paper and chromatographed in benzene–acetic acid–water (125:72:3 by vol.) by the descending technique. All compounds were chromatographed under comparable conditions at temperatures in the range 20° ± 2°. One hour or longer was allowed for the preliminary equilibration of the papers. Under these conditions the solvent took 3.5–4 h to descend 40 cm. Because of its volatility, the present solvent system is very sensitive to temperature effects, and phasic separation in the paper has sometimes been observed at higher temperatures, particularly with thicker grades of paper.

Location of compounds was by the usual methods (*viz.* U.V. ± NH_3, U.V. + $AlCl_3$)[1]. In the case of the flavanones and flavanonols the identity of the spots was confirmed by spraying with the specific $NaBH_4$ reagent[8]. Diazotised sulphanilic acid (Pauly's reagent) was used as a general spray reagent. This reagent was prepared as described by SMITH[6] with the following slight modifications. A 0.69 % solution of $NaNO_2$ was used instead of the 5 % solution and the reagent was applied by spraying instead of dipping.

RESULTS

R_F values for the compounds studied are given in Table I. These values were obtained under closely comparable conditions and in most cases represent average values of

TABLE I

R_F VALUES OF FLAVONOID AGLYCONES IN BENZENE–ACETIC ACID–WATER (125:72:3) AND THEIR COLOUR REACTIONS WITH SULPHANILIC ACID

*Compound**	R_F	*Colour with diazotised sulphanilic acid***
Flavanone		
1 5,7,3′,4′-OH (Eriodictyol)	0.29	Br-O
2 7,4′-OH	0.45	R-Br
3 5,7,4′-OH (Naringenin)	0.49	O-Br
4 7,4′-OH; 3′-OMe	0.55	R-Br
5 5,7,3′-OH; 4′-OMe (Hesperetin)	0.60	O
6 5,7,4′-OH; 3′-OMe (Homoeriodictyol)	0.61	O
7 7-OH	0.79	P
8 5,7-OH (Pinocembrin)	0.80	Br-O
9 4′-OH; 8,3′-OMe	0.85	P
10 6,7,3′,4′-OMe	0.87	—
11 5-OH; 7,4′-OMe	0.93	O
Flavanonol		
12 5,7,3′,4′-OH (Taxifolin)	0.09	Br-O
13 5,7-OH (Pinobanksin)	0.66	Y-Br
Isoflavone		
14 5,7,3′,4′-OH (Orobol)	0.20	Pu
15 7,4′-OH (Daidzein)	0.35	R-Br
16 5,7,4′-OH (Genistein)	0.41	Br
17 5,7,3′-OH; 4′-OMe (Pratensein)	0.54	Br-O
18 7-OH; 4′-OMe (Formononetin)	0.72	—
19 5,7-OH; 4′-OMe (Biochanin A)	0.77	Br
20 5,7,2′-OH; 6,5′-OMe (Podospicatin)	0.76	Br-O
21 5,2′-OH; 6,7,5′-OMe	0.86	Br-O
Flavone		
22 5,7,3′,4′-OH (Luteolin)	0.17	Br-O
23 5,7,4′-OH (Apigenin)	0.33	Br-O
24 5,7,3′-OH; 4′-OMe (Diosmetin)	0.47	R-Br
25 5,7,4′-OH; 3′-OMe (Chrysoeriol)	0.47	Br-O
26 5,7,8-OH (Norwogonin)	0.51	—
27 5,6,7-OH (Baicalein)	0.61	—
28 5,7-OH; 4′-OMe (Acacetin)	0.75	Br-O
29 5,7-OH (Chrysin)	0.78	Br-O
30 5-OH; 7-OMe (Tectochrysin)	0.93	Br-O

(continued on p. 451)

TABLE I *(continued)*

*Compound**	R_F	*Colour with diazotised sulphanilic acid***
Flavonol		
31 5,6,7,3′,4′-OH (Quercetagetin)	0.00	—
32 7,8,3′,4′-OH	0.02	—
33 6,7,3′,4′-OH	0.02	—
34 7,3′,4′-OH (Fisetin)	0.09	Br-O
35 5,7,5′,6′-OH	0.09	Br-O
36 5,7,3′,4′-OH (Quercetin)	0.10	Br-O
37 5,7,4′-OH (Kaempferol)	0.30	Br-O
38 7,3′,4′-OMe	0.89	—
39 Flavonol	0.91	—
Flavonol 3-methyl ether		
40 5,7-OH; 3′,4′-OMe	0.77	Br-O
41 5,7-OH; 4′-OMe	0.79	Br-O
42 5,7,3′,4′-OMe	0.86	—
43 5-OH; 7,3′,4′-OMe	0.93	O-Y

* Sources of compounds: compounds 3, 5, 23, 31, 34, 36, 37 were from commercial sources; 17, 28 were isolated from plant materials[9,10]; 15, 16, 18, 19 were previously synthesised[11]; the rest were donated by other workers (see ACKNOWLEDGEMENTS).

** Br = brown; O = orange; Pu = purple; R = red; P = pink; Y = yellow; — = no colour.

5 or 6 individual determinations. They are reproducible to within ± 0.02. Good discreet spots were obtained from all compounds studied. The colours given with the diazotised sulphanilic acid reagent are also listed in Table I. Glycosides and anthocyanidins move very slowly in this solvent and were not included in the study.

DISCUSSION

It can be seen from the R_F values given that the benzene–acetic acid–water solvent system is capable of separating aglycones of all the classes of flavonoid compounds tested giving a good range of R_F values for a large variety of structural patterns. This solvent system, being much less polar, complements the polar aliphatic solvents typified by butanol–acetic acid–water. Corresponding R_F values in benzene–acetic acid–water are in general much lower than in the butanol solvent, with the result that substances such as the flavonoid aglycones which are very mobile in the latter are well distributed in the former. Glycosides and the more highly hydroxylated aglycones are not very mobile in the present solvent system however, and are best separated in butanol–acetic acid–water.

With the exception of bisdiazotised benzidine[12], diazotised aromatic amines do not appear to have been used extensively as spray reagents for flavonoid compounds although they are commonly used for other phenolic substances[6,13]. We have found diazotised sulphanilic acid to be an excellent reagent, usually giving shades of brown but often more distinctive colours which are useful as further aids to identification. It gives less intense backgrounds than other diazonium salts and the colours of the spots keep for long periods without appreciable change.

The well known general principles governing the behaviour of phenolic compounds

in partition chromatography[3,14,15] are well borne out in the present study. In Fig. 1 the R_M values [14][$R_M = \log (1/R_F - 1)$] of some selected structures are presented. It is readily apparent that the order of R_F values is the same in each class indicating that the effects of structural variations upon R_F values are similar in all classes of flavonoids studied. Comparison of compounds having the same substitution patterns (graphs A, B, C, etc. in Fig. 1) show that in all cases R_F values vary regularly in the order: flavanone > isoflavone > flavone. ROUX and co-workers[15–17] have noted the greater mobility of flavan derivatives in both adsorption and partition systems, and attribute this to the non-planar ring structure in these compounds.

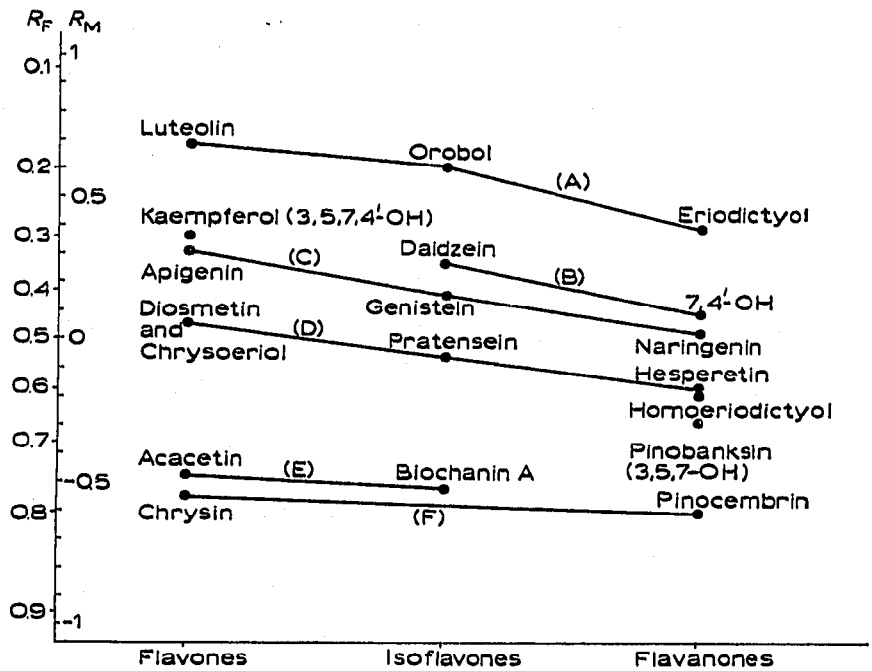

Fig. 1. Relation of R_M to structure in different classes of flavonoids when chromatographed in benzene–acetic acid–water. Graph (A) 5,7,3',4'-OH; (B) 7,4'-OH; (C) 5,7,4'-OH; (D) 5,7,3'-OH; 4'-OMe and 5,7,4'-OH; 3'-OMe; (E) 5,7-OH; 4'-OMe; (F) 5,7-OH.

The principle that hydroxyl groups decrease and methoxyl groups increase the R_F value is also well demonstrated. The positive ΔR_M effect of a hydroxyl group (other than a 5-OH, to be discussed below) is clearly illustrated in Fig. 1 (*cf.* graphs A and C, C and F, and D and E). In contrast to the observation of BATE-SMITH AND WESTALL[14] that in butanol–acetic acid–water the rise in R_F for the methylation of a hydroxyl group is much less than the rise in R_F caused by the complete removal of a hydroxyl group, the lyophilic effect of a methoxyl group is much greater in benzene–acetic acid–water. Methylation almost completely reverses the effect of hydroxylation (*cf.* graphs C, E, and F, Fig. 1) or actually causes a greater R_F rise than dehydroxylation (*cf.* graphs A, C, and D, Fig. 1). The greater lyophilic effect of the methoxyl group in benzene solvents is also apparent in the previous results of SIMPSON AND GARDEN[18].

The abnormal effect of 5-OH and 3-OH groups in partition chromatography, due to their formation of hydrophobic chelate ring systems with the 4-carbonyl group has been clearly demonstrated by SIMPSON and co-workers[18,19]. In the present work the

increase in R_F due to the introduction of a 5-OH group is again illustrated in Fig. 1 (*cf.* graphs B and C, and B and F). Numerous other examples can be found in results listed in Table I. The suppression of the normal positive ΔR_M contribution of a hydroxyl, due to hydrogen bonding of the 3-OH group, can be demonstrated by comparisons of the R_M values for kaempferol, luteolin and apigenin, and for those of pinobanksin, naringenin, and pinocembrin (Fig. 1). The second example suggests that simultaneous hydrogen bonding of both 5- and 3-OH groups[18] is also possible in flavanonols.

The benzene–acetic acid–water solvent system, because of excellent separations it gives with many classes of phenolic compounds, is recommended as the solvent of choice for the first direction in the two-dimensional paper chromatography of less water-soluble polyphenols in plant extracts. Also because of the regular manner in which mobility varies with structure in the classes of flavonoids studied, comparisons of R_M values in this solvent would be useful for the prediction of structures of unknown substances belonging to these classes of natural products.

ACKNOWLEDGEMENTS

The authors wish to thank Professors L. H. BRIGGS, T. A. GEISSMAN, T. R. SESHADRI and W. B. WHALLEY, and Doctors R. M. HOROWITZ, J. B. HARBORNE and H. R. ARTHUR, for their generous gift of samples. One of us (A.O.T.) was the recipient of an ICI (NZ) Research Scholarship.

SUMMARY

The R_F values of 43 natural and synthetic flavonoid aglycones in the solvent system benzene–acetic acid–water (125:72:3 by vol.) are recorded. Diazotised sulphanilic acid has been found to be a useful general spray reagent for these compounds.

Excellent separations are obtained for all the classes of compounds studied. The effect of structural variation upon R_F is similar for all classes. All the known effects of hydroxylation, methylation and chelation on the chromatographic behaviour of flavonoids were observed in this solvent system. R_F values for compounds having the same substitution pattern vary in the order flavanone > isoflavone > flavone.

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