CHROMATOGRAPHIC BEHAVIOUR OF SOME STEREOCHEMICALLY INTERRELATED FLAVONOID COMPOUNDS IN AQUEOUS MEDIUM

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INTRODUCTION

The most versatile pair of solvent systems used for studying the complexity of plant extracts by two-dimensional paper chromatography consists of a partitioning mixture for the first direction followed by dilute acetic acid for the second. This combination, due to ROBERTS AND WOOD[1], might not be ideal for all complex mixtures of flavonoid compounds, but offers the advantage of separation based on different principles for each direction.

In the partitioning systems, for example, butyl alcohol–water mixtures often containing acetic acid, separation depends principally on the number of hydroxyl groups substituent on the C_{15} skeleton, and also on the configuration, *i.e. cis-* or *trans-*arrangement, of the main substituent groups at C atoms 2 and 3 in the heterocyclic ring (see BATE-SMITH AND WESTALL[2], BRADFIELD AND BATE-SMITH[3], ROBERTS[4,5], ROUX AND EVELYN[6] and ROUX AND MAIHS[7]).

During "adsorptive" separations in water, the mobility of the C_{15} compound is apparently dependent on the non-planar nature of the compound (see ROUX[8], ROBERTS, CARTWRIGHT AND WOOD[9], ROUX AND EVELYN[6] and ROBERTS[10]), and separations of the optical isomers of catechins and epicatechins have been shown to occur (ROBERTS AND WOOD[1]). ROUX AND EVELYN[6] showed that structural differences are also responsible for variations of R_F in water or dilute acetic acid. These factors may be evaluated more accurately now that the stereochemical interrelationship between many flavonoid compounds has been established as a result of the work of KING, CLARK-LEWIS AND FORBES[11], FREUDENBERG[12], BIRCH, CLARK-LEWIS AND ROBERTSON[13] and WEINGES[14,15].

EXPERIMENTAL AND RESULTS

Origin of substances

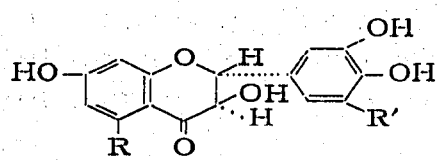
(—)-7,3′,4′-Trihydroxyflavan-3,4-diol from *Schinopsis* spp.[16,17,18] was hydrogenated under conditions established by WEINGES[14] to give (+)-7,3′,4′-trihydroxyflavan-3-ol [(+)-fisetinidol]. (—)-Fustin from *Cotinus coggygria*[15] was a gift from Dr. K. WEINGES. (+)-Trihydroxyflavan-3,4-diol was similarly hydrogenated to (—)-7,3′,4′-trihydroxyflavan-3-ol[19]. (+)-Fustin was isolated from the heartwood of *Acacia mollissima*[20]. (+)-Dihydrorobinetin was obtained from the heartwood of *Robinia pseudacacia*[14]

and hydrogenated under conditions established by FREUDENBERG AND ROUX[21] to (+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol[14]. (—)-Robinetinidol was isolated from the bark of *A. mollissima*[22]. The R_F of (—)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol was determined by using the racemate obtained by the hydrogenation of (±)-dihydrorobinetin[23]. (+)-Catechin, (—)-epicatechin, (—)-epicatechin gallate, (+)-gallocatechin, (—)-epigallocatechin and (—)-epigallocatechin gallate were isolated from air-dried tea leaves and from the bark of *Acacia pycnantha*[24].

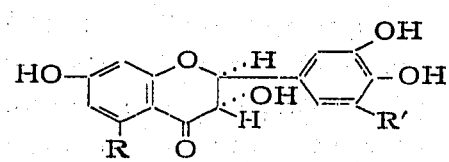
Chromatographic methods

The pure substances were applied within the concentration range 10–20 μg on Whatman No. 1 chromatographic paper and the chromatograms mounted on a stainless steel frame. Chromatograms were developed simultaneously with 2% aqueous acetic acid by upward migration to a point 13–14 inches (33–36 cm) from the starting line over a period of about 6–7 h. After drying the solvent front was located under ultra-violet light and accurately marked. R_F values were calculated and the average of at least three values obtained in the presence on each sheet of the reference compounds (+)-catechin (R_F, 0.35) and (—)-robinetinidol (R_F, 0.42).

When different substances are applied to the same spot, a variable amount of mutual interference often occurs during migration resulting in slightly anomalous R_F values[7]. Only pure crystalline materials run singly from individual spots were



(Ia)



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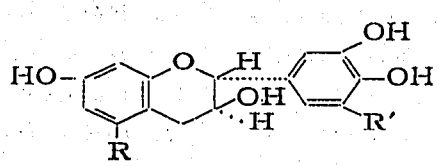
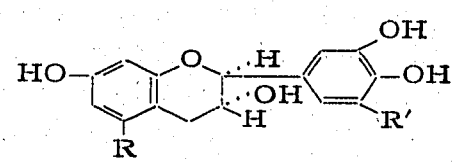


(IIa)



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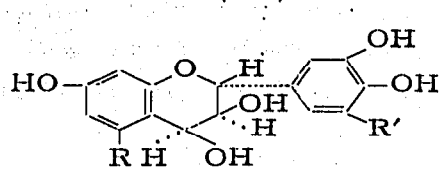
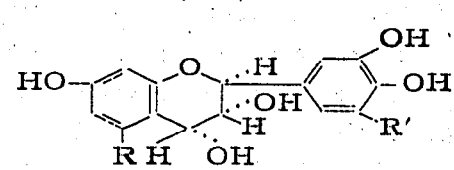


(IIIa)



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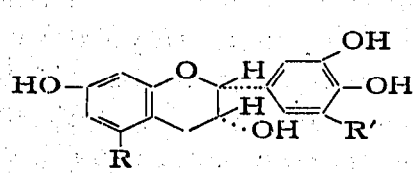
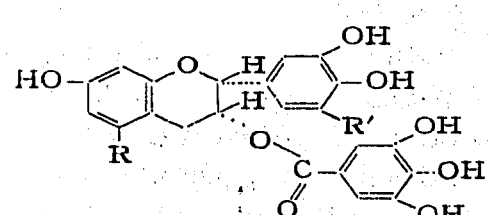


(IV)



(V)

compared, exceptions being the pure racemate (±)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol and the (±)-2,3-dihydroflavonols. The R_F values of compounds having the formulae I–V are given in Table I. Values of the (±)-2,3-dihydroflavonols are the average for the optical isomers, which appear to have close values as shown for the enantiomorphous (+)- and (—)-fustins (R_F, 0.37 and 0.35 respectively).

TABLE I

R_F VALUES IN 2% ACETIC ACID OF FLAVAN-3-OLS, FLAVAN-3,4-DIOLS, 2,3-DIHYDROFLAVONOLS, AND FLAVAN-3-GALLATES

Compound	R_F
(*a*) *Resorcinol series*	
(*1*) $R = R' = H$	
(+)-Fustin (Ia)	0.37
(—)-Fisetinidol (IIa)	0.48
(+)-7,3′,4′-Trihydroxyflavan-3,4-diol (IIIa)	0.52
(—)-Fustin (Ib)	0.35
(+)-Fisetinidol (IIb)	0.43
(—)-7,3′,4′-Trihydroxyflavan-3,4-diol (IIIb)	0.47
(*2*) $R = H$; $R' = OH$	
(+)-Dihydrorobinetin (Ia)	0.34
(—)-Robinetinidol (IIa)	0.42
(+)-7,3′,4′,5′-Tetrahydroxyflavan-3,4-diol (IIIa)	0.46
(—)-7,3′,4′,5′-Tetrahydroxyflavan-3,4-diol (IIIb)	0.40
(*b*) *Phloroglucinol series*	
(*1*) $R = OH$; $R' = H$	
(±)-Dihydroquercetin (I)	0.28
(+)-Catechin (IIa)	0.35
(—)-Epicatechin (IV)	0.30
(—)-Epicatechin gallate (V)	0.23
(*2*) $R = OH$; $R' = OH$	
(±)-Dihydromyricetin (I)	0.24
(+)-Gallocatechin (IIa)	0.32
(—)-Epigallocatechin (IV)	0.24
(—)-Epigallocatechin gallate (V)	0.20

DISCUSSION

Those mobile flavonoid compounds which have been studied in Table I have the absolute configurations illustrated in formulae I to V. Of these, the members of the group (+)-fustin, (—)-fisetinidol and (+)-7,3′,4′-trihydroxyflavan-3,4-diol, and of the group (+)-dihydrorobinetin, (—)-robinetinidol and (+)-7,3′,4′,5-tetrahydroxyflavan-3,4-diol are interconvertible, and have been shown by WEINGES[14,15] to have the same absolute configuration at C atoms 2 and 3 as (+)-catechin, whereas (—)-fustin, (+)-fisetinidol and (—)-7,3′,4′-trihydroxyflavan-3,4-diol correspond to (—)-catechin. The (+)- and (—)-fustins[19], (+)- and (—)-fisetinidols[20], (+)- and (—)-7,3′,4′-trihydroxyflavan-3,4-diols[25,26] and presumably also the (+)- and (—)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diols are enantiomorphous pairs of compounds. These compounds

as well as (+)-catechin[11,12], (+)-gallocatechin[27], (±)-dihydroquercetin[28] and presumably (±)-dihydromyricetin have a 2,3-*trans* configuration of substituent groups, whereas (—)-epicatechin, (—)-epigallocatechin and their gallates have a 2,3-*cis* configuration[11,12]. Comparison of compounds with the same or with different configurations, under the following headings, is of interest:

Separation of enantiomorphs

ROBERTS AND WOOD[1] first showed that the optical antipodes of catechin and gallocatechin and their epimers could be resolved using water as irrigant. The optical antipodes of 7,3′,4′-trihydroxyflavan-3-ol (fisetinidol) (ΔR_F, 0.05), and of the flavan-3,4-diols, leuco-fisetinidin (ΔR_F, 0.05) and leuco-robinetinidin (ΔR_F, 0.06) may similarly be separated in water or 2 % acetic acid (Table I). Also the 2,3-dihydroflavonols, (+)- and (—)-fustin show differences in R_F (0.37 and 0.35) but the difference between the enantiomeric fustins is small (ΔR_F, 0.02) compared with enantiomeric flavan-3-ols and flavan-3,4-diols (ΔR_F, 0.05–0.06), and effective resolution occurs only after prolonged irrigation. For catechin, gallocatechin, fustin, 7,3′,4′-trihydroxyflavan-3,4-diol and 7,3′,4′,5′-tetrahydroxyflavan-3,4-diol the (+)-form has a higher R_F in water or 2 % acetic acid than the (—)-form. This sequence is reversed for epicatechin and epigallocatechin (see ROBERTS AND WOOD[1]) and also for the fisetinidols (Table I) and will evidently also apply to the robinetinidols of which only the (—)-form is known. This reversed sequence for the fisetinidols confirms the stereochemical interrelationship between (+)-fisetinidol, (—)-fustin and (—)-7,3′,4′-trihydroxyflavan-3,4-diol shown by WEINGES[14] and between those of their mirror-images[19,20]. The same must apply to (—)-robinetinidol in relation to (+)-dihydrorobinetin and (+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol.

Cis–trans relationship

(+)-Catechin and (—)-epicatechin have the same configuration (2 R) at C atom 2 but differ at C atom 3 with (3 S) and (3 R) configurations respectively[13,29]. The effect of R_F on the *trans*-configuration of the 2-phenyl and 3-hydroxyl groups as in (+)-catechin as opposed to their *cis*-configuration as in (—)-epicatechin is shown in the pairs (+)-catechin (0.35) and (—)-epicatechin (0.30) (ΔR_F, 0.05), (+)-gallocatechin (0.32) and (—)-epigallocatechin (0.24) (ΔR_F, 0.08). Similar relative behaviour in the water direction is shown by the pairs (—)-catechin and (+)-epicatechin, (—)-gallocatechin and (+)-epigallocatechin on the two-dimensional chromatograms illustrated by ROBERTS AND WOOD[1]. Catechins (flavan-3-ols) of the "phloroglucinol series" therefore have higher R_F values in aqueous medium compared with the corresponding epicatechins in which the 3-hydroxyl group is inverted. (+)- and (—)-Catechins also have higher R_F values than (+)- and (—)-epicatechins in partitioning mixtures (see BRADFIELD AND BATE-SMITH[3], and ROBERTS[4,5]) and these effects may be due to disparity in their molecular shape[5,30] and possibly to the nearly planar nature of the epicatechins, with their 2-aryl group presumably in equatorial position[5,30].

Galloyl groups in 3-position

Comparison of the R_F values of the pairs (—)-epicatechin (0.30) and (—)-epicatechin gallate (0.25), (—)-epigallocatechin (0.24) and (—)epigallocatechin gallate (0.22) shows that in both instances (ΔR_F, 0.05 and 0.02 respectively) galloylation in the 3-position reduces the R_F slightly. Similar deductions may be made from the R_F values in water for these substances by ROBERTS, CARTWRIGHT AND WOOD[9].

Carbonyl group in the 4-position

Comparison of the 2,3-dihydroflavonols with their stereochemically related flavan-3-ols (catechins), for example, the pairs (+)-fustin (R_F, 0.37) and (—)-fisetinidol (R_F, 0.48) (ΔR_F, 0.11), and (—)-fustin and (+)-fisetinidol (0.35 and 0.43) (ΔR_F, 0.08), shows that introduction of a carbonyl group in the 4-position reduces the R_F considerably. Similar conclusions may be drawn from the comparison of (±)-dihydrorobinetin (0.35) and (—)-robinetinidol (0.42) (ΔR_F, 0.07), (±)-dihydromyricetin (0.24) and (+)-gallocatechin (0.32) (ΔR_F, 0.08), and (±)-dihydroquercetin (0.28) and (+)-catechin (0.35) (ΔR_F, 0.07). The considerable reduction in R_F is evident for both phloroglucinol and resorcinol series of compounds, although in the former group hydrogen bonds between the 5-hydroxyl and 4-carbonyl almost certainly exist[7]. The lower R_F of the dihydroflavonols compared with the catechins may be due to the equatorial arrangement of the bulky 2-phenyl group and also of the 3-hydroxyl group as suggested by MAHESH AND SESHARDI[31]. These equatorial arrangements will confer a nearly planar structure to 2,3-dihydroflavonols, resulting, as in the (—)-epicatechins, in a reduced R_F in water, compared with catechins where the 2-phenyl group is likely to have an axial arrangement[30].

Hydroxylation in the 4-position

The suggestion made by ROUX AND EVELYN[6] that hydroxylation in the 4-position increases the R_F, is confirmed by the comparison of stereochemically related flavan-3-ols and flavan-3,4-diols. Examination of the pairs (—)-fisetinidol (0.48) and (+)-7,3′,4′-trihydroxyflavan-3,4-diol (0.52) (ΔR_F, 0.04), (+)-fisetinidol (0.43) and (—)-7,3′,4′-trihydroxyflavan-3,4-diol (0.47) (ΔR_F, 0.04), (—)-robinetinidol (0.42) and (+)-7,3′,4′,5′-tetrahydroxyflavan-3,4-diol (0.46) (ΔR_F, 0.04) shows that the increase in R_F is smaller than originally anticipated[6]. This effect may be expected as introduction of an aliphatic hydroxyl on the heterocyclic ring should contribute to the solubility of the C_{15} unit as a whole.

Hydroxylation in the 5-position

Comparison of the stereochemically related pairs (+)-catechin (0.35) and (—)-fisetinidol (0.48) (ΔR_F, 0.13), and (+)-gallocatechin (0.32) and (—)-robinetinidol (0.48) (ΔR_F, 0.16) shows that for the flavan-3-ols introduction of a (phenolic) hydroxyl in the 5-position causes a large reduction in R_F. This also applies to the 2,3-dihydroflavonols *e.g.* (±)-dihydromyricetin (0.24) and (±)-dihydrorobinetin (0.35) (ΔR_F,

0.11), and (±)-dihydroquercetin (0.28) and (±)-fusin (0.36) (ΔR_F, 0.08) although the 5-hydroxyl is probably hydrogen-bonded with the 4-carbonyl in this group of compounds[7].

Hydroxylation in the 5'-position

ROBERTS, CARTWRIGHT AND WOOD[9] have shown that with water as irrigant, introduction of a hydroxyl group in the 5'-position causes a slight reduction in the flavan-3-ols of the phloroglucinol series. Similar behaviour is shown in 2 % acetic acid by these compounds, for example (+)-catechin (0.35) and (+)-gallocatechin (0.32) (ΔR_F, 0.03), (—)-epicatechin (0.30) and (—)-epigallocatechin (0.24) (ΔR_F, 0.06), (—)-epicatechin gallate (0.25) and (—)-epigallocatechin gallate (0.22) (ΔR_F, 0.03), and also by flavan-3-ols of the resorcinol series (—)-fisetinidol (0.48) and (—)-robinetinidol (0.42) (ΔR_F, 0.06). 2,3-Dihydroflavonols show the same behaviour, for example (±)-fustin (0.36) and (±)-dihydrorobinetin (0.33) (ΔR_F, 0.03), (±)-dihydroquercetin (0.28) and (±)-dihydromyricetin (0.24) (ΔR_F, 0.04). The above data show that catechins (flavan-3-ols) of the "phloroglucinol" and "resorcinol" series with 2,3-*trans* configuration of substituent groups, those of the "phloroglucinol" series with 2,3-*cis* configuration and their gallates, and 2,3-dihydroflavonols of both series (all 2,3-*trans* configuration[31]) show similar small reductions of R_F (0.03–0.06) with the introduction of a hydroxyl group in the 5'-position, using 2 % acetic acid as chromatographic irrigant.

Correlation between mobility, planarity and solubility

Flavonoid compounds which are completely planar, for example anthocyanidins, flavonols, flavones, aurones and chalcones, do not migrate in water[6,8,9,10]. Although this apparent affinity for the cellulose has been ascribed to planarity[8–10] and to special adsorption effects associated with the planar structure[6], it appears more likely that planarity and low solubility are also associated properties in the C_{15} group. The above groups of compounds all have low solubilities in cold water, and flavonols, although not mobile in cold water, migrate on cellulose columns developed with hot water. Furthermore the addition of formic or acetic acid to the aqueous irrigant, allows for the migration of all these substances on a cellulose substrate in a regular manner[32]. Low solubility associated with a planar structure is therefore almost certainly responsible for the zero R_F in water or 2 % acetic acid. Solubility might also be a predominant single factor affecting the migration of those flavonoids which are mobile in predominantly aqueous systems.

Summarised conclusions

Comparisons of some stereochemically related flavonoid compounds as detailed above, has shown that the enantiomers of flavan-3-ols, flavan-3,4-diols and 2,3-dihydroflavonols may be separated in water, sometimes with a reversal in the sequence of migration of the (+)- and (—)-forms. Amongst the flavan-3-ols, those of 2,3-*trans* configuration of substituent groups always have an appreciably higher R_F than the related epimer of 2,3-*cis* configuration in which the 2-hydroxyl group is inverted.

Regarding the functional groups, it was shown that a galloyl group in the 3-position slightly reduces the R_F of the corresponding 2,3-*cis*-flavan-3-ol (ΔR_F, —0.02 to —0.05). The presence of a carbonyl group in the 4-position (2,3-dihydroflavonols) introduces a pronounced reduction in R_F compared with the corresponding flavan-3-ol (ΔR_F, —0.07 to —0.11). Hydroxylation in the 4-position (aliphatic hydroxyl) produces a small increase of R_F, (R_F, +0.04) whereas the introduction of hydroxyl groups in positions 5 and 5′ (both phenolic hydroxyls) produces large (ΔR_F, —0.08 to —0.16) and very small (ΔR_F, —0.03 to —0.06) reductions in R_F values respectively. The solubility of flavonoid compounds in water appears to be a predominant factor in determining their R_F on cellulose substrates.

ACKNOWLEDGEMENTS

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SUMMARY

Factors affecting the chromatographic behaviour of some flavonoid compounds in aqueous medium may be evaluated more accurately now that their stereochemical interrelationships have been established. The effect of the following factors on R_F have been examined: (a) the separation of enantiomorphous flavan-3-ols, flavan-3,4-diols and 2,3-dihydroflavonols; (b) the *cis-trans* relationship of substituents in the heterocyclic ring of flavan-3-ols, (c) the introduction of a carbonyl group in the 4-position, hydroxyl groups in the 4, 5 and 5′-positions, and a galloyl group in the 3-position. The zero R_F of flavonoid compounds of planar structure is likely to be due to their low solubility in water. Solubility appears to be one of the predominant factors affecting the R_F of flavonoid compounds in aqueous medium.

NOTE ADDED IN PROOF

Hydroxylation in the 3-position

(±)-Butin (3-deoxyfustin) (0.22) prepared from butein by alkali isomerization [33] has a lower R_F in 2 % acetic acid (ΔR_F, 0.14) than (±)-fustin (0.36). Similarly (±)-7,3′,4′-trihydroxyflavan-4-ol (0.33, 0.28) obtained from the reduction of (±)-butin [34], has a lower R_F (ΔR_F, 0.19) than either (±)- or (—)-7,3′,4′-trihydroxyflavan-3,4-diols (0.52, 0.47). Substitution of hydroxyls (aliphatic) in 3-position in both flavanones and flavans produces large R_F increases in 2 % acetic acid (ΔR_F, 0.14–0.19) contrasting with the small increase (ΔR_F, 0.04) accompanying hydroxylation (aliphatic) in the 4-position.

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