SELECTIVE SPRAY REAGENTS FOR THE IDENTIFICATION AND ESTIMATION OF FLAVONOID COMPOUNDS ASSOCIATED WITH CONDENSED TANNINS

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INTRODUCTION

Condensed tannins which are ubiquitously distributed in Nature, are in most instances accompanied by complex mixtures of flavonoid substances. Some of these function as important "precursors" in the biogenesis of the accompanying tannins, and their isolation is an essential prerequisite for satisfactory identification[1,2].

Paper chromatography furnishes a direct method of determining the complexity of the polyphenolic mixtures derived from barks and heartwoods, and usually assists in the prediction of the separation procedures which should be used for the effective resolution of components; for example, partition separations on cellulose or in the Craig machine or adsorption separations on cellulose. Furthermore, the identity of the compounds to be separated may often be established tentatively from a knowledge of the relation between R_F and the degree of hydroxylation and distribution of hydroxyl groups[3-5], coupled with the use of selective spraying reagents. Frequently it is also possible to estimate the concentration of flavonoid compounds in tannin mixtures by means of paper chromatography[6,7].

This paper discusses the accuracy with which chemical structure may be predicted through the use of five selective spray reagents, and describes the detail of a densitometric method of estimating flavonoid compounds based on the use of some of these reagents.

EXPERIMENTAL

Spray reagents

The following spray reagents have been found effective for the identification of flavan-3-ols (catechins), flavan-3,4-diols (leuco-anthocyanidins), flavonols, 2,3-dihydroflavonols, and may also be applied to flavanones, aurones and chalkones.

(*i*) *Ferric reagent.* 3 g AnalaR ammonium ferric sulphate (iron alum) in 100 ml water, used immediately after solution. The reagent gives sharp and distinct blues and greens with pyrogallol and catechol groups respectively. The ferric ion also coordinates weakly with mono- and *meta*-hydroxyphenols to give weak purple colorations observed only if the substances are present on chromatograms in excessively high concentrations.

TABLE I

COLOURS DEVELOPED BY SOME FLAVONOID COMPOUNDS AND THEIR CONSTITUENT PHENOLS WITH BIS-DIAZOTISED BENZIDINE

Compound	*Position of phenolic OH groups*	*Colour*
Phenols		
Phloroglucinol	1,3,5	deep purple
Resorcinol	1,3	claret-maroon
Phenol	1	very pale yellow*
Catechol	1,2	pale yellow**
Pyrogallol	1,2,3	pale reddish yellow**
Catechins or flavan-3-ols		
(+)-Gallocatechin and (—)-epimer	5,7,3′,4′,5′	claret-maroon
(—)-Epigallocatechin gallate	5,7,3′,4′,5′	claret-maroon
(+)-Catechin and (—)-epimer	5,7,3′,4′	claret-maroon
(—)-Epicatechin gallate	5,7,3′,4′	claret-maroon
(—)-Robinetinidol	7,3′,4′,5′	canary-yellow
(±)-Epifisetinidol	7,3′,4′	pale yellow
Leuco-anthocyanidins or flavan-3,4-diols		
Leuco-delphinidin	5,7,3′,4′,5′	claret-maroon
Leuco-cyanidin	5,7,3′,4′	claret-maroon
Leuco-robinetinidin	7,3′,4′,5′	canary-yellow
Leuco-fisetinidin	7,3′,4′	pale yellow
Leuco-guibourtinidin	7,4′	very pale yellow*
2,3-Dihydroflavonols or flavanonols		
Dihydromyricetin (Ampeloptin)***	5,7,3′,4′,5′	golden yellow
Dihydroquercetin (Taxifolin)	5,7,3′,4′	pale reddish yellow
Pinobanksin	5,7	red[11]
Strobobanksin	5,7 (6-methyl)	orange-yellow[11]
Dihydrorobinetin	7,3′,4′,5′	golden yellow
Dihydrofisetin (Fustin)	7,3′,4′	pale yellow
Flavanones		
Cryptostrobin	5,7 (8-methyl)	orange-yellow[10]
Strobopinin	5,7 (6-methyl)	yellow[10]
Pinocembrin	5,7	red[10]
Pinostrobin	5 (7-methoxy)	orange-red[10]
Flavonols		
Myricetin	5,7,3′,4′,5′	golden brown
Quercetin	5,7,3′,4′	reddish yellow
Robinetin	7,3′,4′,5′	golden brown
Fisetin	7,3′,4′	pale yellow
Flavones		
Chrysin	5,7	red
Tectochrysin	5 (7-methoxy)	very pale yellow*

* Most colours develop instantaneously or over a few minutes but phenol, leuco-guibourtinidin and tectochrysin take periods of up to 10–15 min to develop.

** On aging catechol often turns pale brown and pyrogallol a brown yellow.

*** The name ampelopsin has been applied to both 3-β-glucosidopetunidin (WILLSTÄTTER AND ZOLLINGER[12]) and 5,7,3′,4′,5′-pentahydroxyflavanonol (KOTAKE AND KUBOTA[12]). The former has historical preference and the name ampeloptin was intended for the latter (personal communication from Dr. T. KUBOTA).

(*ii*) *Ammoniacal silver nitrate reagent.* This reagent due to PARTRIDGE[8] has been described by BATE-SMITH[3,9]. 14 g silver nitrate is dissolved in 100 ml water and 6 *N* ammonium hydroxide added until the silver oxide formed, just dissolves. In the cold the ammoniacal silver nitrate is instantly reduced by vicinal phenolic hydroxy groups, *e.g.*, *ortho*-dihydroxy and *ortho*-trihydroxy groups. Others, for example mono- and *meta*-hydroxyphenols reduce the ammoniacal silver nitrate very slowly. The shade of coloration developed, namely black, grey or brown, depends entirely on the concentration of the hydroxyphenol on the chromatogram and on the nature of the reducing group. Catechol, protocatechuic acid and some other catechol-containing units, if present in sufficiently high concentration give characteristic metallic grey colorations. Pyrogallol-containing units usually give black colorations if present in sufficiently high concentration and brown at low concentration. Catechol groups may give either black, grey or brown colours depending on concentration.

(*iii*) *Bis-diazotised benzidine.* This reagent due to LINDSTEDT[10] consists of two solutions: (a) Benzidine (5 g) or benzidine hydrochloride (6 g) stirred with conc. HCl (14 ml) and the suspension dissolved in water (980 ml). (b) 10% sodium nitrite. Two parts (b) are added to three parts (a) and the reagent used immediately after mixing. This reagent is one of the most selective available; the colours produced with flavonoid compounds and constituent phenols are given in Table I.

(*iv*) *Vanillin–toluene-p-sulphonic acid.* This reagent due to ROBERTS, CARTWRIGHT AND WOOD[4] is used by spraying a solution of vanillin (2 g) and toluene-*p*-sulphonic acid (1 g) in absolute ethanol (100 ml) and heating the chromatogram in an oven at 80–100° for 5–10 min.

"Flavonoid" substances containing phloroglucinol nuclei, *e.g.*, catechins, gallocatechins, leuco-cyanidin, leuco-delphinidin, dihydroquercetin, dihydromyricetin etc. give a strong violet-red colour, while those containing resorcinol nuclei, *e.g.*, robinetinidol, leuco-fisetinidin, fustin, etc., give weak pink colours after more prolonged heating.

(*v*) *Toluene-p-sulphonic acid.* This reagent due to ROUX[13] is a 3 % solution of toluene-*p*-sulphonic acid in absolute ethanol. After being sprayed lightly, chromatograms are heated at 80–100° for 5–10 min. Leuco-anthocyanidins, not hydroxylated in the 5 position, for example melacacidin, leuco-robinetinidin and leuco-fisetinidin give scarlet colorations. Leuco-anthocyanidins hydroxylated in the 5 position, for example leuco-delphinidin and leuco-cyanidin give yellow-red colours on more prolonged heating[5].

Estimation of flavonoid compounds by the maximum colour density method

For the estimation of flavonoid compounds in tannins the effective resolution of components on two-dimensional paper chromatograms is necessary. Improved resolution may be obtained by using larger sheets of paper so that the components are as widely separated from each other as possible. This assists the task of measuring the maximum colour densities of individual spots. The method followed is similar to that used by BLOCK[14,15] for the estimation of amino acids on two-dimensional chromatograms.

(*a*) *Apparatus.* Chromatograms are developed in two 20 in. × 20 in. × 8½ in. all-glass tanks (Shandon Scientific Co.) using upward migration in both directions. Micropipettes of 10 μl capacity ("Elphor", Bender and Hobein, Munich) are used for applying the substances, and even spraying is obtained with an all-glass spray-gun (Kopp Laboratory Supplies Inc., New York).

(*b*) *Paper.* Whatman No. 1 chromatographic paper cut into 16 in. squares are used. Spots of ½ in. diameter are applied at a point in one corner, 1 in. from each edge.

(*c*) *Preparation of standard catechin and tannin solutions.* 10 mg flavonoid compound is dissolved in 10 ml pure absolute ethanol. 10 μl quantities are applied on the spot of the above area until the desired concentration of the substance was obtained. Tannin solutions of 1 g per 10 ml are made up in ethanol or methanol and 10 μl applied on a spot of equal size.

(*d*) *Solvents.* For the first direction the upper phase of butan-1-ol–acetic acid–water (4:1:5) is used, and after drying in a strong current of air in a hood, the paper is irrigated with 2% acetic acid in water. Four chromatograms are run simultaneously. Development took place at 21° in a constant temperature room.

(*e*) *Time of development.* In the butanol–acetic acid–water mixture the chromatograms were run for approximately 20 h, and in 2 % acetic acid for 8 h.

(*f*) *Development of colour and choice of spray reagents.* The papers may be sprayed with one of two reagents, the choice of reagent depending on the chemical nature of the flavonoid to be estimated. All chromatograms should be sprayed on both sides of the paper.

(i) Ammoniacal silver nitrate spray. In the best concentration range this reagent gives rather similar brown-black spots with compounds containing either pyrogallol or catechol nuclei. Where the choice of solvents and size of the chromatographic paper enables well-separated spots this reagent may be used to advantage, but it suffers from the disadvantage that it does not differentiate between substances containing differing groups. The use of dilute reagent must be avoided as the full intensity of the spot is not developed merely by heavy spraying. Immediately after spraying the paper sheets are washed three times with chloride-free water in a large (20 in. × 24 in.) stainless steel dish, then treated with 0.1 % sodium thiosulphate, prepared from distilled water, for 3–4 min, and finally washed for 20 min in running tap water. All the washings are carried out in the dish and the chromatograms are allowed to air-dry in the dish to avoid tearing. Work in too high a concentration range should be avoided as the dense spots tend to "bleed" during washing.

(ii) Bis-diazotised benzidine spray. Only the freshly mixed solutions should be used. Flavonoid compounds containing a combination of resorcinol (A nucleus) and catechol (B nucleus) groups give very pale yellow colours usually unsuitable for measurement. Those containing resorcinol and pyrogallol (A and B nuclei) groups give deeper colorations ranging from canary-yellow to golden brown (see Table I) and may be estimated, although the silver nitrate reagent is preferred in both these instances. A combination of phloroglucinol (A nucleus) and catechol or pyrogallol (B nuclei) in catechins, *e.g.* (+)-catechin and (+)-gallocatechin, and flavan-3,4-

diols, *e.g.* leuco-delphinidin and leuco-cyanidin, gives an intense claret-maroon colour suitable for measurement. For estimating degradation-products obtained from flavonoid compounds the intense purple produced with phloroglucinol and the claret-maroon with resorcinol are also suitable.

Chromatograms sprayed with the reagent are allowed 1–3 min for full development of colours, and immediately washed for 20 min in running tap water to avoid a yellow back-ground due to excess benzidine reagent. The chromatograms are air-dried in a tray. The colours developed appear to be stable. Working in a high concentration range must be avoided due to the "bleeding" of intense spots during washing.

(*g*) *Measurement of colour density*. The maximum colour density of each spot is measured with a densitometer (Fig. 1) in which the intensity of the light source is controlled by a rheostat for coarse adjustment, and by an iris diaphragm for fine adjustment. The light intensity is measured with a selenium barrier layer photocell ("Eel" 37 × 50 mm) coupled directly with a sensitive galvanometer (Pye Scalamp Galvanometer 7891/S). The colour density of each spot is determined by using the blank paper of a particular chromatogram as 100% transmission, and the average of

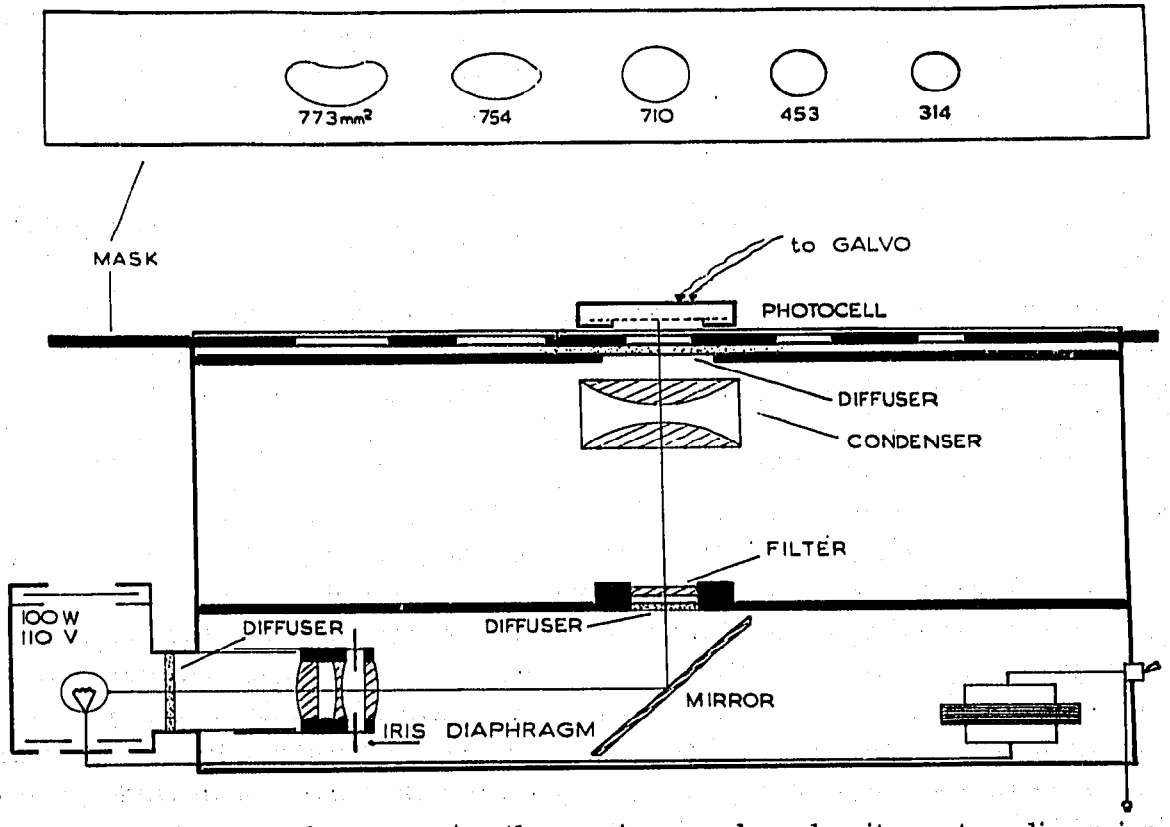

Fig. 1. Simple densitometer for measuring the maximum colour density on two-dimensional paper chromatograms. The sliding "mask" permits the use of different size apertures. One aperture only is selected for the preparation of a standard curve and for subsequent estimation.

replicate analyses at each concentration of flavonoid compound was plotted on semi-logarithmic paper. A straight-line relationship between log concentration and galvanometer reading was obtained for phloroglucinol (benzidine reagent), and for (—)-

robinetinidol, (+)-catechin and (+)-gallocatechin (benzidine and silver nitrate reagents) as in Fig. 2. Where compounds giving a pale yellow colour with the benzidine reagent, *e.g.* fisetinidol, fustin, leuco-fisetinidin and robinetinidol (at low concentra-

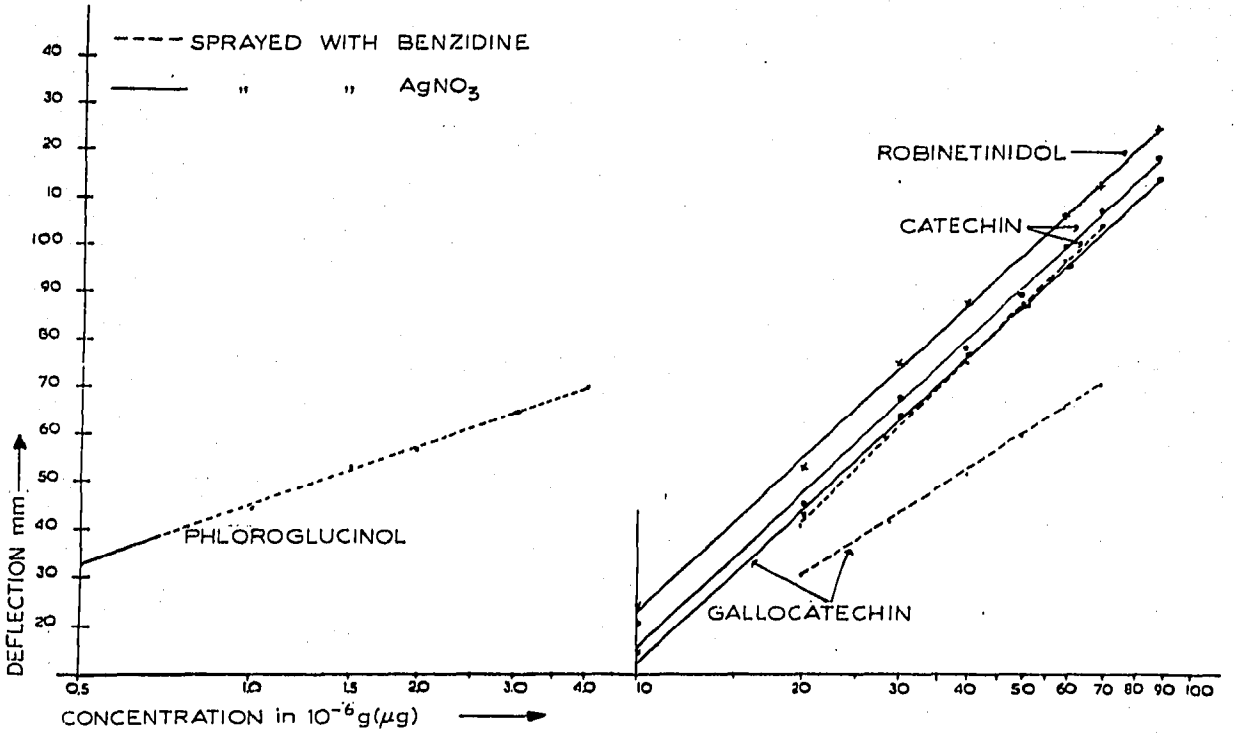

Fig. 2. Calibration curves for the estimation of robinetinidol, catechin, gallocatechin and phloroglucinol by the maximum density method.

tion), are in close proximity or overlap the ochre colorations of (+)-catechin, (—)-epicatechin, (+)-gallocatechin, or epicatechin and epigallocatechin gallates, the maximum colour density of the latter may be determined by using a suitable green filter.

Adsorption and partition chromatography of flavonoid compounds

(—)-Robinetinidol ((—)-7,3′,4′,5′-tetrahydroxyflavan-3-ol) has the same absolute configuration as (+)-catechin[16], and therefore also as (+)-gallocatechin[17]. The R_F values of (—)-robinetinidol in water-saturated *sec.*-butanol (0.67) and in water (0.42) were determined and compared with those of (+)-gallocatechin (0.54, 0.32)[5]. Similarly the R_F values of (±)-epifisetinidol ((±)-epi-7,3′,4′-trihydroxyflavan-3-ol) (0.79, 0.42–0.47)[5] may be compared with that of (—)-epicatechin (0.60, 0.29). The naturally occurring enantiomorphous[24] (+)- and (—)-leuco-fisetinidins have the same R_F values in *n*-butanol–acetic acid–water (0.76) and in water-saturated *sec.*-butanol (0.68) but differ in their R_F values in water (0.54 and 0.48 respectively). The racemic mixture of these substances does not separate into the optical antipodes in water and runs to the lower R_F value (0.48). Conclusions drawn from these comparisons and from similar comparisons of the chromatographic behaviour of other flavonoid compounds of the resorcinol and phloroglucinol series[5], are described in the discussion section of this paper.

DISCUSSION

Selective spray reagents often furnish conclusive evidence regarding the A and B phenolic nuclei and the heterocyclic ring in flavonoid compounds.

The improved ferric reagent and the ammoniacal silver nitrate reagent afford information regarding the B nucleus when pyrogallol or catechol groups are present. When the B nucleus is monohydroxylated, for example the 4'-hydroxy groups in epiafzelichin, dihydrokaempferol and leuco-guibourtinidin, or unhydroxylated as in the flavones, flavanones and flavanonols typical of pine heartwood components[18], the state of hydroxylation of the nucleus may often be deduced from a knowledge of the hydroxylation of the A and heterocyclic rings coupled with the R_F of the substance in different solvent systems[5]. Other substances which present difficulties are dihydromorin (4',6'-dihydroxy B nucleus), and melacacidin where both A and B nuclei have free catechol groups. Both compounds, however, readily undergo conversions into a flavonol and anthocyanidin respectively which may be identified.

The bis-diazotised benzidine and vanillin reagents afford information regarding the A nucleus when it consists of either phloroglucinol or resorcinol nuclei. The vanillin reagent is particularly sensitive towards the phloroglucinol nucleus giving a mauve-red colour after a short period of heating (often even in the cold) and almost without exception, while with resorcinol nuclei a weak pink is obtained with more prolonged heating. The bis-diazotised benzidine reagent is useful for confirming evidence of the vanillin reagent, giving claret-maroon colours with phloroglucinol-containing catechins and flavan-3,4-diols but not necessarily with phloroglucinol-containing 2,3-dihydroflavonols (see below). The benzidine reagent is of great diagnostic value and especially sensitive in its colour reactions, giving colours which are the sum-total of the colorations of the A and B phenolic moieties. It also reflects the presence of strong hydrogen bondage between 5-hydroxyl and 4-carbonyl groups when these are present. For example (*cf.* Table I), the resorcinol-containing flavonoids, fisetin, fustin, leuco-fisetinidin and fisetinidol give pale yellow colours derived mainly from the catechol B nucleus (yellow) and the monohydroxyphenol (very pale yellow) A nucleus. Robinetin, dihydrorobinetin, leuco-robinetinidin and robinetinidol give canary to deeper yellow colours (depending on concentration) derived mainly from the pyrogallol B nucleus which contributes a reddish yellow colour, and the monohydroxyphenol of the A nucleus contributing a very pale yellow. Where the A nucleus contains *meta*-hydroxy substituents, for example 5,7-dihydroxy groups, the deep claret-maroon colour developed is identical to that of the *meta*-dihydroxyphenol, resorcinol. This intense coloration presumably overshadows the pale yellow or deeper yellow of catechol and pyrogallol B nuclei. Typical examples are the catechins, gallocatechins and their gallates, leuco-cyanidins and leuco-delphindins.

The 2,3-dihydroflavonols belonging to the phloroglucinol series, for example dihydromyricetin (ampeloptin), dihydroquercetin (taxifolin) and dihydrokaempferol (aromadendrin) provide exceptions in that the colour produced with the bis-diazotised

benzidine tends towards yellow and is either pale reddish yellow (dihydrokaempferol, dihydroquercetin) light golden brown (dihydromyricetin), orange-yellow (strobobanksin) or red (pinobanksin). Such behaviour probably reflects strong hydrogen bondage between the 5-hydroxyl and 4-carbonyl. This suggestion finds support in the finding that dihydrorobinetin and dihydromyricetin give completely identical light golden brown colorations with the benzidine reagent. The 2,3-dihydroflavonols of the phloroglucinol series nevertheless show the unmistakable presence of a phloroglucinol nucleus with the vanillin reagent.

Where the pattern of distribution of hydroxyl groups is "unusual", as for example in melacacidin (7,8,3',4'-tetrahydroxyflavan-3,4-diol) the benzidine reagent behaves consistently in giving pale yellow colours, contributed presumably by "free" catechol groups of both A and B nuclei.

In flavonoid compounds some information regarding the heterocyclic ring is obtained by means of the toluene-*p*-sulphonic acid reagent. This sensitive reagent indicates the presence of 3,4-diol groups, by giving pink deep red colorations with flavan-3,4-diols unhydroxylated in the 5 position, for example, leuco-robinetinidin, leuco-fisetinidin and melacacidin[13]. Those flavan-3,4-diols which are hydroxylated in the 5 position, namely leuco-delphinidin and leuco-cyanidin give less characteristic orange colours[5]. The toluene-*p*-sulphonic acid reagent also converts 2,3-dihydroflavonols into flavonols during heating, and the emergence of fluorescence is usually detectable under ultra-violet light[13]. Other information regarding the oxidation state of the heterocyclic ring may be obtained under ultra-violet light where yellow fluorescence indicates either flavones or flavonols, the 5-deoxyflavonols giving exceptionally brilliant greenish-yellow fluorescence[3]. Anthocyanidins and anthocyanins show orange, pink, red and red-blue colorations with absorption-maxima lying in the range 500–555 mμ. Information supplied by the various spray reagents must in all instances be correlated with the R_F of the flavonoid compound in partitioning and adsorption solvent systems.

SWAIN AND HILLIS[19] reviewed methods of estimating total phenols, leucoanthocyanidins, flavonols in plant extracts and ROUX[20] used methods for estimating tannins (total polyphenols) in plant extracts. These methods are applied to heterogeneous mixtures of compounds, and they are of necessity empirical. KING AND WHITE[21] have studied a number of methods for estimating individual groupings in tannins or phenolic compounds. The estimation of individual "flavonoid" compounds in mixtures derived from natural sources has not apparently been attempted.

The application of paper chromatography and the elution procedures commonly used for sugars, presents difficulties as the recovery of flavonoid substances from the cellulose is never quantitative. Substantial losses occur apparently due to the residual affinity of flavonoid compounds for cellulose, the losses varying according to the chemical nature of the compound. These difficulties are overcome by the use of two-dimensional paper chromatography coupled with the measurement of maximum colour density similar to that used by BLOCK[14,15] for the estimation of amino acids and amines. Various catechins and phloroglucinol give a straight line relationship

between galvanometer deflection and log concentration using ammoniacal silver nitrate and bis-diazotised benzidine reagents. The choice of spray reagent for estimation depends on the ability of the compound either to reduce strongly or to couple strongly with the reagent. The benzidine reagent is more versatile in that phloroglucinol-containing flavan-3-ols and flavan-3,4-diols, affording a claret-maroon colour, may be estimated in the presence of resorcinol-containing flavonoid compounds, which give various shades of yellow, with the aid of a suitable green filter. By means of these methods catechins have been estimated in tannin mixtures[6], and catechins and their degradation products in the presence of tannins formed by the autoxidation of catechin[7]. This densitometric method is of obvious value in the micro-degradation of flavonoid compounds and tannins.

In previous work[5] dealing with the correlation between structure and chromatographic behaviour of flavonoid compounds comparison of flavan-3-ols (catechins) and 2,3-dihydroflavonols (flavanonols) of the resorcinol series with those of the phloroglucinol series was overlooked. The flavan-3-ols, (—)-robinetinidol and (±)-epifisetinidol have higher R_F values in water (0.42, 0.42–0.47) than their corresponding 5-hydroxy derivatives, (+)-gallocatechin and (—)-epicatechin (0.32, 0.29), of identical absolute configuration[16,17]. A similar effect is evident (see Table I of ref. [5]) in the comparison of the R_F values in water of the naturally-occurring dihydroflavonols, dihydrorobinetin (0.35) and ampeloptin (0.24), fustin (0.36) and taxifolin (0.28), all of which are likely to have the 2,3-*trans* configuration of substituent groups in the heterocyclic ring[22]. The presence of a hydroxyl group in the 5 position, therefore, causes a marked decrease of the R_F in water or in 2–6 % aqueous acetic acid, even in the 2,3-dihydroflavonols of the phloroglucinol series (ampeloptin and taxifolin) where hydrogen bondage between 5-hydroxyl and 4-carbonyl occurs[23].

In partitioning solvents the naturally-occurring enantiomorphous (+)- and (—)-leuco-fisetinidins run to the same R_F (0.68 in *sec.*-butanol) but their R_F values in water differ (0.54, 0.48 respectively) as is the case for catechin, gallocatechin and their epimers[25]. Unlike these catechins, however, the racemic mixture of (+)- and (—)-leuco-fisetinidins does not separate in water due probably to molecular interaction, although their R_F values differ markedly when run individually.

Hydrogenation of the 2,3 double bond in the heterocyclic ring in both the pairs flavone → flavanone[10] and flavonol → flavanonol[5] increases the R_F in partitioning mixtures[10,5].

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SUMMARY

Five selective spray reagents provide information regarding the A and B phenolic nuclei and the heterocyclic ring of flavonoid compounds. This information coupled

with R_F values in two solvent systems, enables the tentative identification of a wide variety of flavonoid substances. LINDSTEDT's bis-diazotised benzidine reagent gives colour reactions which are highly indicative of variations in the distribution of phenolic hydroxyl groups. The benzidine and ammoniacal silver nitrate reagents may be used with two-dimensional paper chromatography and a simple densitometer for estimating flavonoid compounds present in tannins and also the yield of phenols resulting from the degradation of flavonoid compounds and tannins. The benzidine reagent allows for the estimation of flavan-3-ols and flavan-3,4-diols of the phloroglucinol series in the presence of their counterparts of the resorcinol series.

Additional factors in the correlation between structure and chromatographic behaviour of flavonoid compounds are described.

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