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THIN-LAYER CHROMATOGRAPHY OF BIFLAVONYLS ON SILICA GEL

STRUCTURE–CHROMATOGRAPHIC BEHAVIOUR CORRELATIONS

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SUMMARY

The chromatographic behaviour of 28 biflavonyls, including partially and fully methylated ethers, has been examined in five solvent systems. Benzene–pyridine–formic acid (36:9:5) has been found to be the most satisfactory developing system both for identification and separation of biflavonyls and their methyl ethers while benzene–pyridine–ethyl formate–dioxan (5:1:2:2) has been claimed best for fully methylated biflavonyls. Both solvent systems have been used for the first successful separation of biflavonyls and their methyl ethers by preparative thin-layer chromatography. Relative R_F values coupled with variations in the shades of the spots developed by spraying with diazotised sulphanilic acid may be used for ascertaining, approximately, the extent of the methylation in partial methyl ethers of the same series. The characteristic shades of fully methylated biflavonyls in UV light have been found to provide a means for their quick and satisfactory identification.

An attempt has been made to correlate the structure of biflavonyls and their methyl ethers with their chromatographic behaviour. Isomeric pairs of fully methylated biflavonyls involving different interflavonyl linkages, such as hinokiflavone (C4′–O–C8″, C4′–O–C6″), amentoflavone (C3′–C8″, C3′–C6″), cupressuflavone (C8–C8″) and agathisflavone (C6–C8″) have easily been distinguished and oriented.

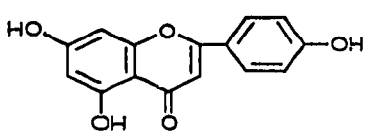
INTRODUCTION

A number of papers and review articles have appeared on the separation and identification of flavonoid pigments, especially by paper chromatography in aqueous and alcoholic solvent systems[1–4]. Correlations between structure and chromatographic behaviour have also been discussed[5–7]. Although biflavonyls have been detected in leaf extracts of gymnosperms by paper chromatography[8–11] and more recently by thin-layer chromatography[12–14], an extensive study has not been undertaken so far probably because a few members belonging to two series of biflavonyls were available. In this communication we wish to present a comprehensive review on thin-layer chromatographic studies using five solvent systems of twenty-eight biflavonyls and their derivatives representing all the series known to date. While most of the solvent

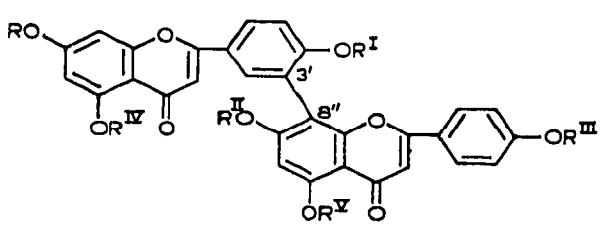
TABLE I

ORIGIN OF BIFLAVONYLS

Biflavonyls	*Source*
Amentoflavone (II), podocarpusflavone A (III), isoginkgetin (VI) and kayaflavone (VIII)	*Podocarpus gracilior* Pilger[18]
Bilobtin (IV) and ginkgetin (VII)	*Ginkgo biloba*[19]
Sotetsuflavone (V)	*Cycas revoluta*[10]
Cupressuflavone (XIII) and isocryptomerin (XXVI)	*Cupressis funebris*[20]
7,7″-Dimethyl ether of cupressuflavone (XIV), 4,4‴,7,7″-tetramethyl ether of cupressuflavone (XV) and 4′,4‴,7,7″-tetramethyl ether of amentoflavone (X)	*Araucaria cookii and A. cunninghamii*[21]
Agathisflavone A (XVIII) and agathisflavone B (XIX)	*Agathis palmerstronii*[22]
Agathisflavone (XVII)	Demethylation of Agathisflavone hexamethyl ether[25]
Cryptomerin B (XXVII) and hinokiflavone (4′–O–6″) (XXIV)	Taxodium macronatum[20]
Cryptomerin A (XXV)	*Cryptomeria japonica*[23]
Fully methylated derivatives of: amentoflavone (3′–8″) (XI), cupressuflavone (XVI), agathisflavone (XX), and hinokiflavone (4′–O–6″) (XXVIII)	By complete methylation of the parent compounds
Pentamethyl ether of hinokiflavone (4′–O–8″) (XXIX)	Synthetic[24]
Hexamethyl ether of amentoflavone (3′–6″) (XII)	Wessely Mosser rearrangement product of amentoflavone (3′–8″) (ref. 20)
Sciadopitysin (IX)	*Scidopitys verticillata* (Courtesey Dr. N. KAWANO)[26]
Morelloflavone (XXI)	*Garcinia morella* (Courtesey Dr. K. VENKATARAMAN)[27]
GB-1 (XXII) and GB-2 (XXIII)	*Garcinia buchnanii* (Courtesey Dr. B. JACKSON)[28]



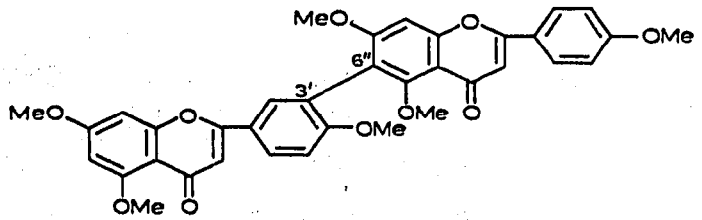
Apigenin (I)



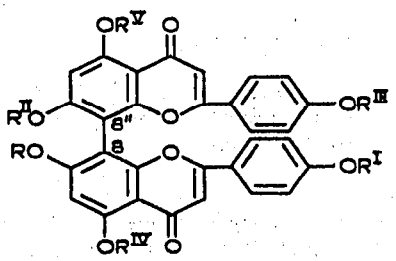
	R	R^{I}	R^{II}	R^{III}	R^{IV}	R^{V}
Amentoflavone (II)	H	H	H	H	H	H
Podocarpusflavone A (III)	H	H	H	Me	H	H
Bilobtin (IV)	H	Me	H	H	H	H
Sotetsuflavone (V)	H	H	Me	H	H	H
Isoginkgetin (VI)	H	Me	H	Me	H	H
Ginkgetin (VII)	Me	Me	H	H	H	H
Kayaflavone (VIII)	H	Me	Me	Me	H	H
Sciadopitysin (IX)	Me	Me	H	Me	H	H
Amentoflavone tetramethyl ether (X)	Me	Me	Me	Me	H	H
Amentoflavone hexamethyl ether (XI)	Me	Me	Me	Me	Me	Me

(*Continued on p. 486*)

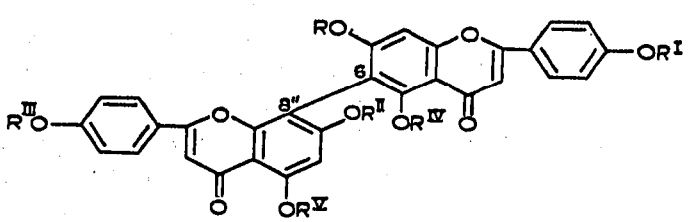
TABLE I (*continued*)



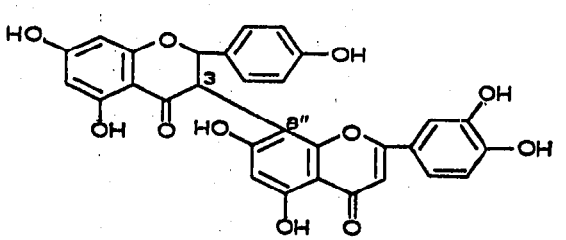
Amentoflavone hexamethyl ether (3′–6″) (XII)



	R	R^{I}	R^{II}	R^{III}	R^{IV}	R^{V}
Cupressuflavone (XIII)	H	H	H	H	H	H
Cupressuflavone dimethyl ether (XIV)	Me	H	Me	H	H	H
Cupressuflavone tetramethyl ether (XV)	Me	Me	Me	Me	H	H
Cupressuflavone hexamethyl ether (XVI)	Me	Me	Me	Me	Me	Me

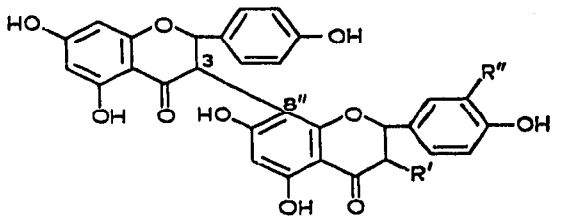


	R	R^{I}	R^{II}	R^{III}	R^{IV}	R^{V}
Agathisflavone (XVII)	H	H	H	H	H	H
Agathisflavone A (XVIII)	Me	H	H	H	H	H
Agathisflavone B (XIX)	Me	H	H	Me	H	H
Agathisflavone hexamethyl ether (XX)	Me	Me	Me	Me	Me	Me

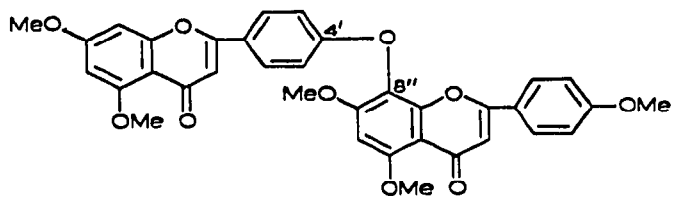


Morelloflavone (XXI)

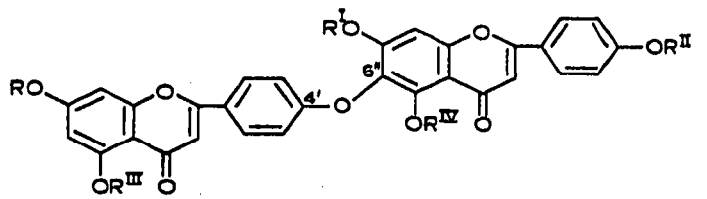
TABLE I (*continued*)



	R^I	R^{II}
GB-1 (XXII)	OH	H
GB-2 (XXIII)	OH	OH



Hinokiflavone pentamethyl ether (4'–O–8") (XXIX)



	R	R^I	R^{II}	R^{III}	R^{IV}
Hinokiflavone (XXIV)	H	H	H	H	H
Cryptomerin A (XXV)	H	H	Me	H	H
Isocryptomerin (XXVI)	H	Me	H	H	H
Cryptomerin B (XXVII)	H	Me	Me	H	H
Hinokiflavone pentamethyl ether (XXVIII)	Me	Me	Me	Me	Me

systems are of qualitative interest only, two of them have worked so well that they have been used successfully in these laboratories for the separation for the first time of parent biflavonyls and their partially and fully methylated derivatives by preparative thin-layer chromatography. An attempt was also made to correlate the structure and chromatographic behaviour of these compounds.

EXPERIMENTAL

All reagents used were of BDH "Analar" grade excepting formic acid (E. Merck) and ethyl formate (Bush & Co.). Using a thin-layer applicator (Desaga, Heidelberg), glass plates (20 × 20 cm) were coated with a well-stirred suspension of Silica

Gel G (E. Merck, 50 g–95 ml water) to give a layer approximately 0.5 mm in thickness. After drying for 2 h at room temperature, the plates were heated at 110–120° for 1 h and preserved in a desiccator until required. Pure samples of fully methylated biflavonyls (1 mg/ml in $CHCl_3$) and parent and partial methyl ethers (1 mg/ml in pyridine) were applied with suitable microliter pipettes at the starting line (2 cm from the lower edge of the plate and 2 cm apart from each other). The plates were mounted on a stainless steel frame and placed in Desaga glass chamber 10 × 22 × 21 cm containing 200 ml of solvent. When the solvent front had travelled 15 cm from the starting line the development was interrupted and the plates were dried at room temperature. Both $FeCl_3$–EtOH and diazotised sulphanilic acid (prepared according to SMITH[29]) were used as spray reagents.

Solvent systems. The following five mixtures were studied as developing solvent systems:

Benzene–pyridine–formic acid (36:9:5)[15]	BPF
Toluene–ethyl formate–formic acid (5:4:1)[16]	TEFF
Toluene–pyridine–acetic acid (10:1:1)[17]	TPA
Benzene–ethyl acetate–acetic acid (8:5:2)	BEAA
Benzene–pyridine–ethyl formate–dioxan (5:1:2:2)	BPEFD

RESULTS

All the spots were located in UV light but the spots of parent biflavonyls and their partial methyl ethers were also revealed by using $FeCl_3$–EtOH and diazotised sulphanilic acid as chromogenic reagents. R_F values of biflavonyls, their partial and fully methylated derivatives (Table II) were obtained under closely comparable conditions and were calculated to an average of three values.

Both the chromogenic reagents, $FeCl_3$–EtOH and diazotised sulphanilic acid were found useful in revealing the spots of biflavonyls and their partial methyl ethers. Diazotised sulphanilic acid, however, had the additional advantage of giving an approximate idea about the extent of methylation as the colour changes from dark brown to yellow with increasing methylation. Examination of the fully methylated biflavones (BPF) in UV light provided a fairly good diagnostic test for the identification of different series of biflavones (Table II). The spots were compact and the differences in the R_F values of all the members of a series in the BPF system were so marked (Fig. 1, chromatogram 1) that it became the developing system of choice, not only for satisfactory identification but also for quantitative purposes. The solvent system was found to work equally well for biflavonyls with reduced heterocyclic rings where most of the systems proved unsuccessful. In BPEFD, although the spots were compact, the R_F value differences between the parent compound and its partial methyl ethers in a series were small (Fig. 1, chromatogram 4). The system, however, proved to be the most satisfactory in the quantitative separation of mixtures of fully methylated biflavonyls. BEAA, TPA and TEFF (most widely used by KAWANO *et al.*[12,13,19]) were found to be of some value for qualitative work (Fig. 1, chromatograms 2, 3, 5) but of no value at all in quantitative separations. The spots were either too elongated or too closely spaced, and in some cases travelled with the solvent front. BEAA and TPA were found unsatisfactory for morelloflavone, the GB series and fully methylated biflavonyls (Table II).

TABLE II

R_F VALUES OF BIFLAVONYLS, AND THEIR PARTIAL AND FULLY METHYLATED DERIVATIVES

Compound	*Solvent system*					*Colour (solvent BPF)*
	BPF	*TEFF*	*TPA*	*BPEFD*	*BEAA*	
Apigenin (I)	0.52	0.62	0.40	0.74	0.67	Brown[a]
Amentoflavone (II)	0.17	0.33	0.07	0.43	0.27	Dark brown[a]
Podocarpusflavone A (III)	0.37	0.53	0.16	0.52	0.48	Light brown[a]
Bilobtin (IV)	0.37	0.53	0.16	0.52	0.48	Light brown[a]
Sotetsuflavone (V)	0.37	0.53	0.16	0.52	0.48	Light brown[a]
Isoginkgetin (VI)	0.54	0.57	0.34	0.71	0.70	Orange-brown[a]
Ginkgetin (VII)	0.54	0.57	0.34	0.71	0.70	Orange-brown[a]
Kayaflavone (VIII)	0.61	0.61	0.55	0.82	0.79	Orange[a]
Sciadopitysin (IX)	0.61	0.61	0.55	0.82	0.79	Orange[a]
Amentoflavone tetramethyl ether (X)	0.76	0.63	0.66	0.91	0.88	Yellow[a]
Cupressuflavone (XIII)	0.16	0.30	0.07	0.36	0.29	Dark brown[a]
Cupressuflavone dimethyl ether (XIV)	0.50	0.57	0.34	0.61	0.65	Orange-brown[a]
Cupressuflavone tetramethyl ether (XV)	0.76	0.63	0.62	0.90	0.85	Yellow[a]
Agathisflavone (XVII)	0.16	0.23	0.04	0.35	0.27	Dark brown[a]
Agathisflavone A (XVIII)	0.27	0.44	0.10	0.41	0.35	Brown[a]
Agathisflavone B (XIX)	0.43	0.57	0.23	0.61	0.59	Light brown[a]
Morelloflavone (XXI)	0.02	0.19	0.02	0.25	0.27	Dark brown[a]
GB-1 (XXII)	0.14	0.37	0.04	0.27	0.38	Reddish brown[a]
GB-2 (XXIII)	0.06	0.19	0.02	0.23	0.27	Reddish brown[a]
Hinokiflavone (XXIV)	0.32	0.37	0.13	0.47	0.35	Brown[a]
Cryptomerin A (XXV)	0.57	0.49	0.33	0.61	0.73	Light brown[a]
Isocryptomerin (XXVI)	0.57	0.49	0.33	0.61	0.73	Light brown[a]
Cryptomerin B (XXVII)	0.67	0.55	0.49	0.69	0.80	Orange-brown[a]
Amentoflavone (3′–8″) hexamethyl ether (XI)	0.40	0.35	0.09	0.09	0.06	Bright yellow[b]
Amentoflavone (3′–6″) hexamethyl ether (XII)	0.50	0.52	0.27	0.41	0.27	Blue[b]
Cupressuflavone hexamethyl ether (8–8″) (XVI)	0.43	0.47	0.17	0.17	0.13	Orange[b]
Agathisflavone hexamethyl ether (6–8″) (XX)	0.45	0.48	0.20	0.32	0.20	Bright yellow with greenish tinge[b]
Hinokiflavone pentamethyl ether (4′–O–6″) (XXVIII)	0.52	0.53	0.28	0.55	0.33	Yellowish blue[b]
Hinokiflavone pentamethyl ether (4′–O–8″) (XXIX)	0.44	0.45	0.11	0.13	0.08	Dull yellow[b]

[a] Diazotised sulphanilic acid as spray reagent.
[b] In UV light.

DISCUSSION

All biflavonyls except morelloflavone and those of the GB series are derived from apigenin units with a C–C or C–O–C interflavonyl linkage. It is well known that if there are several substituents in the same molecule, the effect of each substituent on the adsorption affinity is very approximately additive[30]. A comparison of the R_F values of the biflavonyls (II, XIII, XVII) consisting of two apigenin units with the R_F values of apigenin (I) itself proves that the aforesaid generalisation is far from

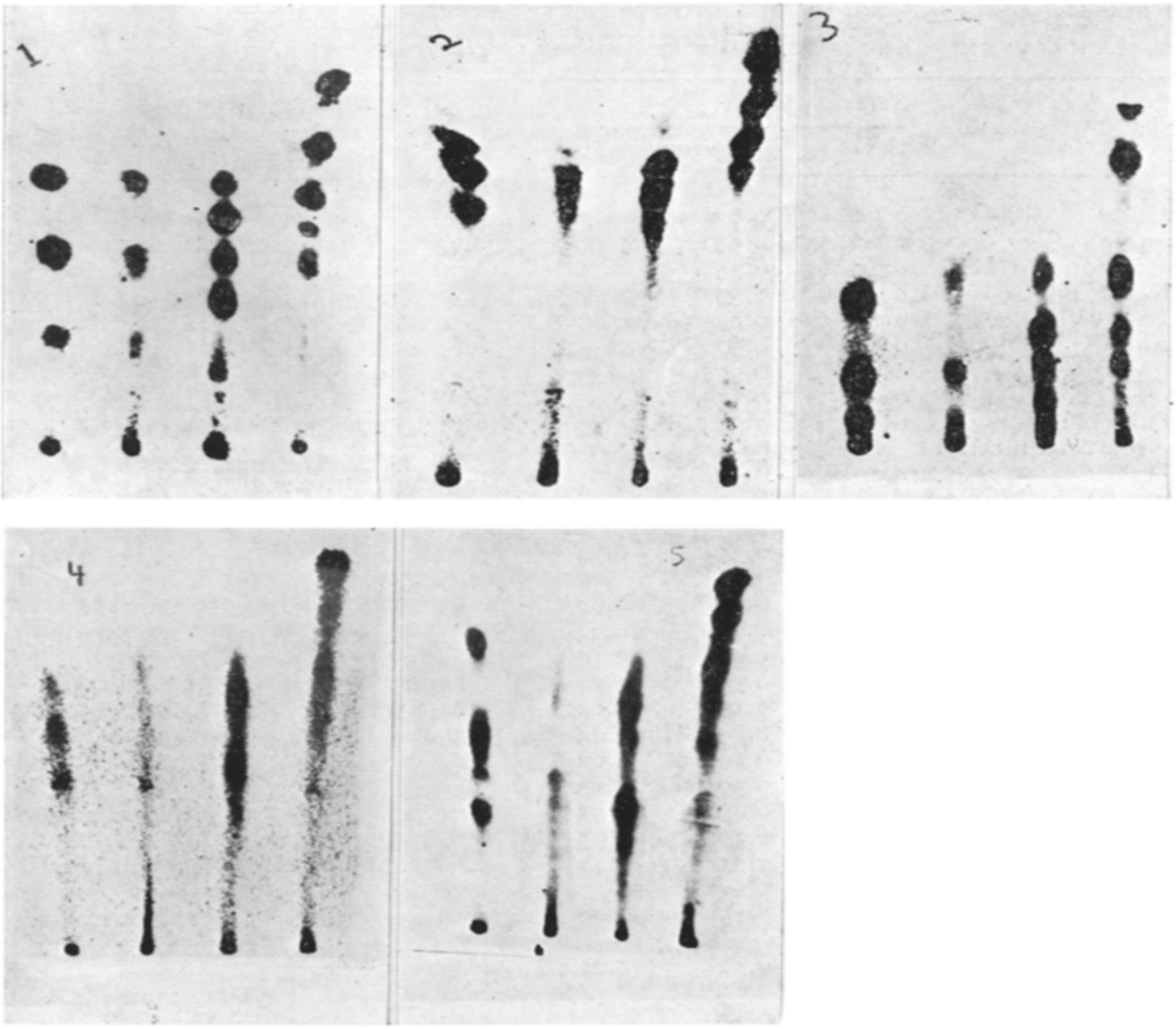


Fig. 1. Chromatograms of biflavonyls from leaf extracts of (left to right) *Podocarpus gracilior* Pilger, *Cryptomeria japonica*, *Agathis palmerstronii* and *Araucaria cookii* in solvent systems: 1, benzene–pyridine–formic acid (36:9:5); 2, toluene–ethyl formate–formic acid (5:4:1); 3, toluene–pyridine–acetic acid (10:1:1); 4, benzene–pyridine–ethyl formate–dioxan (5:1:2:2); 5, benzene–ethyl acetate–acetic acid (8:5:2).

being even very approximate. Amentoflavone (II), cupressuflavone (XIII) and agathisflavone (XVII) having an equal number of phenolic hydroxyls would be expected to show the same R_F values. It has actually been found that their R_F values are so close that they defy identification and separation in cases where they occur together in the same plant. The small differences in the R_F values in BPF of amentoflavone (0.17), cupressuflavone (0.16) and agathisflavone (0.16) may, however, be explained by their relative departure from planarity with subsequent variations in the magnitude of the conjugative effects.

In the same series, a monomethyl ether shows an R_F value higher than that of the parent compound, a dimethyl higher than a monomethyl ether, a trimethyl higher than a dimethyl ether etc. For example: see the R_F's of II, III, VI and VIII. The trend is in line with the observation that the increase in R_F values parallels the increase in

methylation[3–6]. Isomeric methyl ethers of the same series (amentoflavone) such as sotetsuflavone (V) bilobtin (IV) and podocarpusflavone A (III) (monomethyl ethers); ginkgetin (VII), isoginkgetin (VI) and podocarpusflavone B (dimethyl ethers); kayaflavone (VIII) and sciadopitysin (IX) (trimethyl ethers) show the same R_F values (Table II) in accordance with the observation that the position of the substituent is of secondary importance[31].

Hinokiflavone (XXIV), a biflavone of a biphenyl ether pattern, shows an R_F of 0.32 (BPF). This is much higher than those of the three biphenyl type biflavones discussed earlier. Hinokiflavone with five free phenolic hydroxyls and the sixth involved in an ether type interflavonyl linkage may be expected to behave, with respect to adsorption affinity, like a monomethyl ether of the biphenyl type biflavones. Similarly the monomethyl ether of hinokiflavone compares with the dimethyl ethers of amentoflavone and cupressuflavone. The validity of this argument is supported by the observation that such mixtures, when actually encountered in these laboratories, could not be separated by TLC (unpublished results).

The adsorption affinity differences widen with the increasing methylation so much that fully methylated biflavones involving various modes of interflavonyl linkages were found to show sizable differences in R_F values. This observation has been exploited extensively and successfully in these laboratories to bring about the quantitative separation, in BPF and BPEFD, of pure samples from fully methylated mixtures of amentoflavone (XI) (R_F 0.40), cupressuflavone (XVI) (R_F 0.43), agathisflavone (XX) (R_F 0.45) and hinokiflavone (XXVIII) (R_F 0.52), although it was not possible to separate them at the partial methyl ether stage. The different shades of the spots of these derivatives in UV light (BPF) were also found to be of some help in their identification. BPF and BPEFD have thus been established as excellent developing systems for the quantitative separation of fully methylated biflavones.

The tendency of the R_F values of the hexamethyl ethers of amentoflavone (XI), cupressuflavone (XVI) and agathisflavone (XX), respectively, to increase may be interpreted as the magnitude of their departure from planarity as a result of steric interactions. Examination of space models reveals that such interactions are greater in the case of agathisflavone hexamethyl ether than in that of amentoflavone hexamethyl ether resulting in subsequent deviations from planarity. This is translated as a decrease in the adsorption affinity and a corresponding increase in the R_F value of agathisflavone hexamethyl ether. Similar arguments may be used to explain why fully methylated biflavonyls involving C-6 in the interflavonyl linkage show higher R_F values than those involving C-8 (solvent BPF): This is shown in Table III.

The low R_F values of morelloflavone (XXI), GB-1 (XXII) and GB-2 (XXIII) as compared to those of (II), (III) and (XVII) may be interpreted as resulting from the increase in the number of phenolic hydroxyls. The increase in R_F value of GB-1 (0.14, BPF) from morelloflavone (0.02, BPF), both having the same number of hy-

TABLE III

Biflavone methyl ethers	*C-8*	*C-6*
Hinokiflavone	0.44	0.52
Amentoflavone	0.40	0.50
Cupressuflavone	0.43	0.45 Agathisflavone

droxyls is attributable to greater mobility as a result of the more non-planar character of the former[3-6]. The lower R_F value of GB-2 (XXIII) (0.06, BPF) as compared to GB-1 (XXII) (0.14, BPF) both with an identical planar configuration may be interpreted as due to the greater phenolic content (8 phenolic hydroxyls).

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