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SULPHATED FLAVONOIDS—AN UPDATE

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IN MEMORY OF TONY SWAIN, 1922–1987

Key Word Index—Flavonoid sulphates; structure; distribution; phytochemistry; spectroscopy; chemical synthesis; enzymology; review.

Abstract—The increasing knowledge of various aspects of flavonoid sulphates calls for an update of previous reviews. This article describes the recent advances in their structural variation and distribution patterns in plants. The methods used in their phytochemical analysis and structural determination are outlined. Furthermore, the recently developed chemical and enzymatic methods for the synthesis of specifically sulphated flavonoids are reviewed, and two hitherto unreported, position-specific flavonol sulphotransferases are described.

INTRODUCTION

Organic sulphur compounds are known for their ubiquitous occurrence in plants [1] as amino acids, coenzymes, aliphatic sulphides, glucosinolates and thiophene derivatives, to mention a few. Although the first flavonoid sulphate has been reported as early as 1937 [2], it was not until 1975 that flavonoid sulphates have been considered to be of common occurrence in a number of plant families [3–6]. Most of these compounds are sulphate esters of common hydroxyflavones and hydroxyflavonols or their methyl ethers, and less commonly of their glycosylated derivatives. In some flavonoid glycosides, however, the sulphate group may be linked to the sugar moiety, mostly at the 3- or 6-position. Recent publications have also reported the natural occurrence of the sulphated derivatives of hydroxycinnamic acids [7–9], coumarins [10], anthraquinones [11] and cyanogenic glycosides [12,13]. On the other hand, sulphate esters of flavonoid classes other than flavones, flavonols and dihydroflavonols have yet to be described. Whereas the physiological role of flavonoid sulphation in plants is not clear [3], in animal tissues sulphate groups may be conjugated with a variety of molecules, such as phenols, steroids, bile acids or xenobiotics (for review see [14]), thus altering their solubility properties and metabolic activity.

Since earlier reviews [3, 4, 6], there has been an increasing number of reports dealing with various phytochemical aspects of sulphated flavonoids, that warrant an update. This review describes the recent advances in their structural variation and natural distribution in higher plants. The phytochemical studies reported here led to the development of new analytical methods for their identification, such as the use of HCl and aryl sulphatase shifts in UV spectral analysis, and of ion pairing coupled with ^{1}H NMR. The use of sulphation shifts in ^{13}C NMR and of FABMS, as applied to their structural determination, are also outlined. The recently developed chemical and enzymatic methods for the synthesis of specifically sulphated flavonoids are also reviewed. Whereas the enzymes involved in the glycosylation [15, 16] and methylation [17, 18] of flavonoids have been extensively studied, little is known of the enzymatic sulphation in plants. This is mainly due to the lack of availability of specifically sulphated compounds to be used as enzyme substrates and reference products, as well as the need for an accurate, rapid enzyme assay for the detection and purification of flavonoid sulphotransferases.

STRUCTURAL VARIATION AMONG FLAVONOID SULPHATES

The 56 known flavonol sulphates are listed in Table 1. This is three times the number described in the previous review [4], which reflects the increasing rate of discovery of these natural conjugates. Eighteen of the 22 flavonol aglycones listed in Table 1 are *O*-methylated, so that there seems to be an association between *O*-methylation and sulphate formation. The flavonol with the largest number of sulphate derivatives, however, is quercetin, which is unmethylated. This apparent contradiction is due to the fact that polysulphation is a feature of the occurrence of quercetin in species of *Flaveria* [31, 32]. If it assumed that the 5-hydroxyl is not normally available for sulphation because of H-bonding to the 4-carbonyl, then most of the possible di- and trisulphates have now been described from this genus. Two typical *Flaveria* conjugates are the 3,7,3′- and 3,7,4′-trisulphates of quercetin (Fig. 1). The fully sulphated 3,7,3′,4′-tetrasulphate is also present.

A quercetagetin methyl ether has also been obtained in sulphated form from *Flaveria*, i.e. the 3,3′-disulphate of patuletin from *F. chloraefolia* [32]. However, a wider range of methylated quercetagetin sulphates are present in another genus of the same plant family, namely *Brickellia* (see Table 1). While quercetagetin derivatives are well represented in these plants, the occurrences of the

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Table 1. The known flavonol sulphates

Compound	First reported source	Reference
Kaempferol		
3-Sulphate	*Acrotema uniflorum*	19
7-Sulphate	*Frankenia pulverulenta*	20
3,7-Disulphate	*Reaumuria mucronata*	21
3,7,4′-Trisulphate	*Acrotema uniflorum*	19
3-β-(3″-Sulphatoglucoside) 3-β-(6″-Sulphatoglucoside)	*Cystopteris fragilis*	22
3-α-(6″-Sulphatoglucoside)	*Asplenium filix-foemina*	23
3-Sulphatorhamnoside	*Davidsonia pruriens*	24
3-Glucuronide-7-sulphate	*Frankenia pulverulenta*	20
3-Sulphatorutinoside	*Adiantum capillus-veneris*	25
3-(6″-Sulphatogentiobioside)	*Asplenium fontanum*	26
Kaempferol 7-methyl ether (Rhamnocitrin)		
3-Sulphate	*Ammi visnaga*	27
Kaempferol 7,4′-dimethyl ether		
3-Sulphate	*Tamarix aphylla*	28
6-Hydroxykaempferol 3-methyl ether		
7-Sulphate	*Neurolaena lobata*	29
6-Hydroxykaempferol 6-methyl ether (eupafolin)		
3-Sulphate	*Flaveria chloraefolia*	30
6-Hydroxykaempferol 6,7-dimethyl ether (eupalitin)		
3-Sulphate	*Flaveria chloraefolia*	30
Quercetin		
3-Sulphate	*Oenanthe crocata*	27
3,7-Disulphate	*Flaveria bidentis*	31
3,3′-Disulphate	*Flaveria chloraefolia*	32
3,4′-Disulphate	*Flaveria bidentis*	33
3,7,3′-Trisulphate	*Flaveria bidentis*	34
3,7,4′-Trisulphate	*Flaveria bidentis*	32
3,7,3′,4′-Tetrasulphate	*Flaveria bidentis*	35
Quercetin		
3-Sulphatorhamnoside	*Davidsonia pruriens*	24
3-(3″-Sulphatoglucoside)	*Asplenium septentrionale*	36
3-Glucuronide-7-sulphate	*Frankenia pulverulenta*	20
3-Sulphatorutinoside	*Arecastrum romanzoffianum*	37
3-Acetyl-7,3′,4′-trisulphate	*Flaveria bidentis*	38
Quercetin 7-methyl ether (Rhamnetin)		
3-Sulphate	*Ammi visnaga*	27
3,5,4′-Trisulphate-3′-glucuronide	*Tamarix aphylla*	39
Quercetin 3′-methyl ether (Isorhamnetin)		
3-Sulphate (persicarin)	*Polygonum hydropiper*	2
7-Sulphate	*Frankenia pulverulenta*	20
3,7-Disulphate	*Flaveria bidentis*	40
3,4′-Disulphate	*Iphiona scabra*	41
3,7,4′-Trisulphate	*Acrotema uniflorum*	19
3-Glucuronide-7-sulphate	*Frankenia pulverulenta*	20
3-Sulphatorutinoside	*Arecastrum romanzoffianum*	37
Quercetin 4′-methyl ether (Tamarixetin)		
3-Sulphate	*Tamarix laxa*	42
Quercetin 7,3′-dimethyl ether (Rhamnazin)		

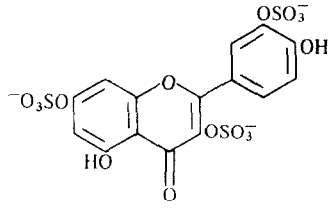
Table 1. *Continued*

Compound	First reported source	Reference
3-Sulphate Quercetin 7,4′-dimethyl ether (Ombuin)	*Polygonum hydropiper*	43
3-Sulphate	*Flaveria chloraefolia*	44
3,3′-Disulphate 6-Hydroxyquercetin 3-methyl ether (quercetagetin 3-methyl ether)	*Acrotema uniflorum*	19
7-Sulphate Quercetagetin 6-methyl ether (Patulctin)	*Neurolaena oaxacana*	45
3-Sulphate	*Brickellia californica*	46
7-Sulphate Quercetagetin 6-methyl ether (Patuletin)	*Lasthenia conjugens*	47
3,3′-Disulphate	*Flaveria chloraefolia*	32
3-Glucoside-7-sulphate Quercetagetin 6,7-dimethyl ether (Eupatolitin)	*Lasthenia conjugens*	47
3-Sulphate Quercetagetin 6,3′-dimethyl ether (Spinacetin)	*Brickellia veronicaefolia*	48
3-Sulphate Quercetagetin 3,6,3′-trimethyl ether (Jaceidin)	*Flaveria chloraefolia*	30
4′-Sulphate Quercetagetin 6,7,3′-trimethyl ether (Veronicafolin)	*Brickellia glutinosa*	49
3-Sulphate Quercetagetin 6,7,4′-trimethyl ether (Eupatin)	*Brickellia veronicaefolia*	48
3-Sulphate Quercetagetin 6,3′,4′-trimethyl ether	*Brickellia californica*	46
3-Sulphate 8-Hydroxyquercetin (Gossypetin)	*Decachaeta haenkeana*	50
3-Sulphate	*Malva sylvestris*	51
8-Glucuronide-3-sulphate	*Malva sylvestris*	52
8-Glucoside-3-sulphate	*Malva sylvestris*	51
Myricetin 3-Sulphatorhamnoside	*Davidsonia pruriens*	24
Dihydromyricetin 3-Gallate-3′-sulphate	*Myrica rubra*	53

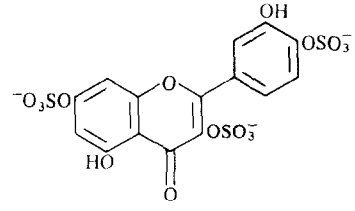
isomeric 8-hydroxyquercetin (gossypetin) sulphates are much rarer. In fact, only the 3-sulphate, the 3-sulphate-8-glucoside and 3-sulphate-8-glucuronide of gossypetin have so far been described, from *Malva sylvestris* (Malvaceae) [52].

Eighteen of the 56 flavonol sulphates (Table 1) contain both sulphate and sugar (or other) substitution. The sulphate glycosides fall into two classes: those were the sugar is separately attached to a different hydroxyl from that linked to sulphate (e.g. kaempferol 3-glucuronide-7-sulphate); and those where the sulphate is linked to the flavonol through the sugar substituent. The second class is represented by kaempferol 3-β-(6″-sulphatoglucoside) from *Cystopteris fragilis* [22]. The related α-linked 3-(6″-sulphatoglucoside) has been reported, remarkably enough, from another fern, *Asplenium filix-foemina* [23]. The position of sulphation in these conjugates was established by methylation and hydrolysis to yield 2,3,4-trimethylglucose. In other cases, the mode of linkage of sulphate to the sugar residue is not usually known.

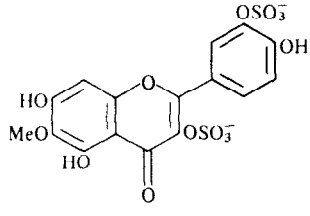
Only one dihydroflavonol sulphate has so far been reported. This is the 3-gallate-3′-sulphate of dihydromyr-



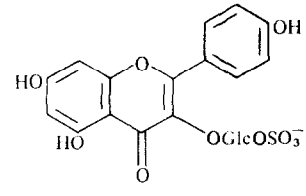
Quercetin 3,7,3′- trisulphate



Quercetin 3,7,4′- trisulphate



Patuletin 3,3′- disulphate



Kaempferol 3 - (6″- sulphatoglucoside)

Structures of some flavonol sulphates

Fig. 1.

Table 2. The known flavone sulphates

Compound	First reported source	Reference
Apigenin		
7-Sulphate	*Bixa orellana*	20
7-Sulphatoglucoside	*Phoenix canariensis*	54
7-Sulphatogalactoside	*Tetracera stuhlmanniana*	19
7-Sulphatoglucuronide	*Fuchsia procumbens*	55
Scutellarein 6-methyl ether (Hispidulin)		
7-Sulphate	*Iphioria scabra*	40
4′-Sulphate	*Lippia nodiflora*	56
7,4′-Disulphate	*Lippia nodiflora*	56
Luteolin		
7-Sulphate	*Bixa orellana*	20
3′-Sulphate	*Lachenalia unifolia*	57
4′-Sulphate	*Daucus carota*	27
7,3′-Disulphate	*Zostera marina*	58
7-Sulphatoglucoside	*Phoenix roebelenii*	37
7-Sulphatoglucuronide	*Fuchsia excorticata*	55
7-Sulphate-3′-glucoside	*Mascarena verschafeltii*	37
7-Sulphatorutinoside	*Phoenix roebelenii*	37
7-Sulphate-3′-rutinoside	*Mascarena verschafeltii*	37
Luteolin 3′-methyl ether (Chrysoeriol)		
7-Sulphate	*Zostera marina*	58
7-Sulphatoglucoside	*Juncus effusus*	59
Luteolin 4′-methyl ether (Diosmetin)		
7-Sulphate	*Zostera marina*	58
3′-Sulphate	*Lachenalia unifolia*	57
7,3′-Disulphate	*Lachenalia unifolia*	57
6-Hydroxyluteolin		
6-Sulphate	*Lippia nodiflora*	56
7-Sulphate	*Lippia nodiflora*	56
6,7-Disulphate	*Lippia nodiflora*	56

Table 2. *Continued*

Compound	First reported source	Reference
6-Methoxyluteolin (Nepetin)		
7-Sulphate	*Lippia nodiflora*	56
3',4'-Disulphate	*Lippia nodiflora*	56
6-Hydroxyluteolin 3'-methyl ether (Nodifloretin)		
7-Sulphate	*Lippia nodiflora*	56
6,7-Disulphate	*Lippia nodiflora*	56
6-Hydroxyluteolin 6,3'-dimethyl ether (Jaceosidin)		
7-Sulphate	*Lippia nodiflora*	56
7,4'-Disulphate	*Lippia nodiflora*	56
8-Hydroxyluteolin (Hypolaetin)		
8-Sulphate	*Bixa orellana*	20
8-Glucoside-3'-sulphate	*Malva sylvestris*	51
8-Hydroxyluteolin 4'-methyl ether		
8-Glucoside-3'-sulphate	*Althaea officinalis*	60
Tricetin		
3'-Sulphate, 7,3'-disulphate	*Lachenalia unifolia*	57
	Lachenalia unifolia	57
Tricetin 3',5'-dimethyl ether (Tricin)		
7-Sulphatoglucoside	*Saccharum officinarum*	61
7-Sulphatoglucuronide	*Cyperus polystachyos*	62
7-Disulphatoglucuronide	*Cyperus polystachyos*	62
Vitexin		
7-Sulphate	*Washingtonia robusta*	37
7-Sulphatoglucoside	*Washingtonia robusta*	37
7-Sulphatorutinoside	*Washingtonia robusta*	37
Isovitexin		
7-Sulphate	*Phoenix roebelenii*	37
Orientin		
7-Sulphate	*Phoenix roebelenii*	37
7-Sulphatoglucoside	*Arecastrum romanzoffianum*	37
Iso-orientin		
7-Sulphate	*Phoenix roebelenii*	37

icetin, which occurs in *Myrica rubra* (see Table 1).

The 45 known flavone sulphates, included in Table 2, are mainly based on apigenin, luteolin or their 6- and 8-hydroxy derivatives. Four derivatives of tricetin and three of tricin are also reported. Of the 20 new flavone sulphates identified since the last review [4], 12 occur in a single source *Lippia nodiflora*. These are various mono- and disulphates of 6-hydroxyflavones, typically such compounds as hispidulin 7-sulphate and nodifloretin 6,7-disulphate [56] (see Table 2). Flavone trisulphates have provisionally been detected in this same plant, but insufficient materials were available for full characterization. Such trisulphates have not been fully identified in any plants as yet, but they can be expected to occur naturally, by analogy with the tri- and tetrasulphates known in the flavonol series.

Some flavones with both sugar and sulphate attachments are known. Typical examples are hypolaetin 8-glucoside-3'-sulphate [51] and the 7-sulphatoglucuronides of apigenin, luteolin and tricin [55, 62]; the linkage between the sulphate and the glucuronic acid moieties in these latter derivatives has yet to be determined. Various sulphates of glycosylflavones are also known (Table 2), such as the 7-sulphates of vitexin and orientin and their 6-isomeric forms.

Flavonoid sulphates are usually isolated as their potassium salts. This is expectable, since potassium is a major cation in the plant cell. Other cations have been reported in a few instances. Sodium salts were obtained of the 12 6-oxygenated flavone sulphates of *Lippia nodiflora* [56]. The presence of sodium rather than potassium in these salts was apparent from the Fast Atom Bombardment-mass spectrometric analyses. A calcium salt was isolated of eupatin 3-sulphate from *Brickellia californica* and *B. laciniata*; other sulphates present in these two plants were obtained as the potassium salts [46]. Recently, Barron and Ibrahim [63] isolated quercetin 3-sulphate and patuletin 3-sulphate from *Flaveria chloraefolia* as an equimolecular mixture, associated with three inorganic ions: sodium, potassium and/or calcium. There is thus apparent natural variation in the way these anionic conjugates occur in the plant cell. However, there is as yet little

information on the precise organellar localisation of flavonoid sulphates within the plants were they occur.

RELATED PHENOLIC SULPHATES

Other phenolic compounds besides flavonoids occur in plants bound to sulphate. Perhaps the most common are hydroxycinnamic acid esters, particularly of p-coumaric and caffeic acids. Most of those that have been investigated are sulphates of either caffeoylquinic acid (as various isomers) or 1-caffeoylglucose and they are often found in similar plants to those which contain flavonoid sulphates [4]. Of the few of these conjugates which have been fully characterised, one may note the 2″-, 3″- and 4″-sulphates of 1-caffeoylglucose found in different ferns [8, 9]. Caffeic acid 3-sulphate has also been detected in several members of the Polygonaceae [4]. Two sulphated anthraquinones have been found in *Rumex pulcher* and provisionally detected in other *Rumex* and in *Rheum* species [11]. They are sulphates of emodin monoglucoside and of emodin dianthrone diglucoside, in which the location of the sulphate link to the glucose is still uncertain. The betalain pigments of the Centrospermae also occur occasionally with sulphate linked through a sugar moiety. Both the 6′- and 3′-sulphates of betanin have been reported from the roots of *Beta vulgaris* and the fruits of *Rivinia humlis* respectively [4]. Three coumarin sulphates have been found in the roots of a maritime plant, the umbellifer *Seseli libanotis* [10]. These, however, have the sulphate attached through an alcoholic group of an isoprenoid substituent, rather than through a phenolic group.

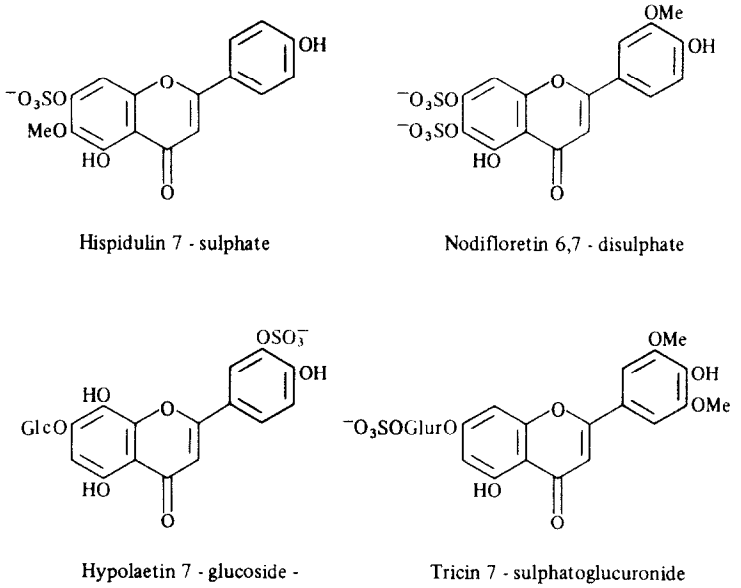
From our electrophoretic surveys, it is apparent that a large number of other as yet unidentified phenolic conjugates with sulphate attachment remain to be characterised in land plants. Marine organisms also contain a significant number of phenolic metabolites which occur in sulphated form. Two typical examples are 3,4-dibromo-5-hydroxymethyl catechol 1,2-disulphate from the red alga *Polysiphonia* and comantherin sulphate from the crinoid *Comantheria perplexa* [64] (see Fig. 2).

Natural distribution

General. Flavonoid sulphates have now been found in at least 250 species belonging to 17 dicotyledonous and 15 monocotyledonous families (Tables 3 and 5). Since many of these families are widely separated from each other in any accepted taxonomic arrangement of the angiosperms, the presence/absence of sulphates has no overall systematic significance. The only general point is that they have so far only been found in families which are herbaceous and/or advanced morphologically.

Flavonoid sulphates have also been recorded in ferns, but relatively rarely. They occur in *Adiantum capillaris-veneris* (Adiantaceae), *Asplenium filix-foemina*, *A. fontanum* and *A. septentrionale* (Aspleniaceae) and *Cystopteris fragilis* (Athyriaceae) [23, 25, 26, 36, 65]. They appear to be uncommon in these families. In this laboratory, we have surveyed 14 species of Athyriaceae and 11 species of Aspleniaceae of Malaysian origin and have not so far detected flavonoid sulphates in these plants, although sulphate esters of hydroxycinnamic acids are clearly present. There are no records so far of flavonoid sulphates in the Bryophyta or in gymnosperms.

In the dicotyledons. There are six new families, in addition to the 11 families previously known in 1976 to have sulphates present [4]. They are the Davidsoniaceae, Dilleniaceae, Malvaceae, Myricaceae, Onagraceae and Verbenaceae (Table 2). In addition, there have been several new generic or species records in families already known to synthesize these conjugates. This is especially



Hispidulin 7 - sulphate

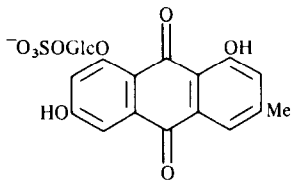
Nodifloretin 6,7 - disulphate

Hypolaetin 7 - glucoside - 3′- sulphate

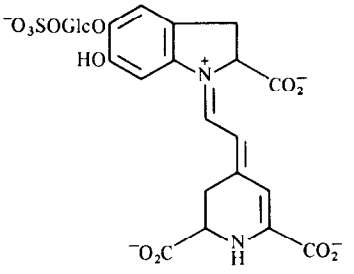
Tricin 7 - sulphatoglucuronide

Some typical flavone sulphates

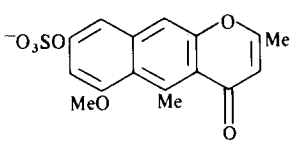
Fig. 2.



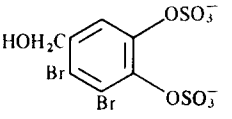
Emodin glucoside sulphate



Betanin 6' - sulphate



Comantherin sulphate



Dibromocatechol sulphate

Some miscellaneous phenolic sulphates

Fig. 3.

true of the Compositae, where 24 genera have been found to produce mainly flavonol sulphates. Complex mixtures of sulphates are often present in individual species of the Compositae. This is true of species in the genus *Flaveria* (Senecioneae), as illustrated in Table 4 for *F. bidentis* and *F. chloraefolia*. So far, the reported Composite genera fall into four tribes: the Inuleae (8 genera), Eupatorieae (2), Senecioneae (3) and Heliantheae (1). Further surveys are, however, needed to determine whether only members of these tribes in such a large plant family are capable of producing sulphates.

The new finding of sulphates in Onagraceae refers to the presence of the 7-sulphatoglucuronides of apigenin and luteolin in leaves of the ornamental genus *Fuchsia* [55]. These sulphates occur specifically in *F. procumbens*, *F. perscandens* and *F. excorticata* but are not present in any of the other species that have been used to breed new garden forms of this plant. Hence, the presence/absence of sulphates in plants of hybrid origin can usefully indicate, together with other flavonoid characters, which species are likely to have been involved as parents.

The first finding of flavonoid sulphates in the Verbenaceae was of 12 6-oxygenated flavones in aerial parts of the maritime plant, *Lippia nodiflora* [56]. This is a cosmopolitan species, so that different geographical populations could be surveyed; they all proved to contain the same constituents, although an earlier study of the flavonoids in this plant in India failed to yield any of these conjugates. A related species *L. canescens* was also found to have the same compounds, but a third taxon, the lemon-scented *L. tryphylla* completely lacked sulphates.

In the monocotyledons. The records of flavonoid sulphates in families of the monocotyledons (Table 5) are taxonomically meaningful, since negative data are available for all these families. Previously, flavonoid sulphates were reported in only nine of the 15 families listed (Table 5). The new family records are in the Araceae, Commelinaceae, Cyperaceae, Iridaceae, Marantaceae and Orchidaceae.

In Araceae, these sulphates are quite uncommon, being recorded in less than 10% of species surveyed, while in Commelinaceae, they are distinctly rare: there is a single report in *Tradescantia hirta* after an extensive survey [75]. In the Cyperaceae, sulphates were originally thought to be absent [4], following a survey of 65 European species. However, they have subsequently been detected in Australian species, where they are fairly frequent, and in a few African and South American species [62, 76]. In the remaining three new families—Iridaceae, Marantaceae and Orchidaceae—they are again of uncommon occurrence (see Table 5).

Overall then, the family with the most frequent occurrence of sulphates remains the Palmae, where they are present in over half the species. They are also common (in 10–25% of species) in three families which have always been placed near each other in any plant classification: the Gramineae, Cyperaceae and Juncaceae. Whether the sulphate character links these three families to the Palmae, usually regarded as being very different morphologically from the grasses, is a moot point, but there are other chemical features in the flavonoid pattern which are shared between the palms and the grasses [37].

The other significant occurrence of flavonoid sulphates in monocot families is in the members of the Fluviales, notably the Alismataceae, Hydrocharitaceae, Zannichelliaceae and Zosteraceae. These are all water plants and the widespread occurrence of sulphates of either flavonoids or hydroxycinnamic acid esters in these plants supports the view that sulphate conjugation represents an ecological adaptation to an aquatic habitat. The frequency of sulphates in tropical and subtropical grasses and their concomitant infrequency in temperate grasses

Table 3. Natural distribution of flavonoid sulphates in dicotyledons

Family	Genera and species	References
Bixaceae	*Bixa orellana*	20
Chenopodiaceae	*Atriplex* (3), *Chenopodium* (2), *Halochemum strobilaceum*, *Petrosimonia oppostifolia*, *Suaeda* (3).	20; Harborne, J.B., unpublished results
Cistaceae	*Helianthemum squamatum*	20
Compositae	*Brickellia glutinosa*, *B. california*, *B. veronicaefolia*, *B. laciniata*, *B. baccharidea*, *B. chlorolepis*	46, 48, 49, 66–68
	Callilepis laureola	68
	Epaltis gariepina	47
	Decachaeta haenkeana	50
	Flaveria chloraefolia	30,32,44
	F. bidentis	31, 33–35, 38,40
	Helichrysum angustifolium	
	Inula erithmoides, *Lasthenia* (2), *Phagnalon graecum*, *Pulicaria* (2), *Sphaeranthus incisus*	47,69
	Iphiona scabra	41
	Neurolaena (3)	29,45
	Senecio (5)	70
Davidsoniaceae	*Davidsonia pruriens*	24
Dilleniaceae	*Acrotema* (1), *Davilla* (2), *Dillenia* (3), *Schumacheria* *Tetracera* (14)	19,71
Frankeniaceae	*Frankenia corymbosa*, *F. ericifolia*, *F. laevis*, *F. pulverulenta*, *F. thymifolia*	20
Guttiferae	*Hypericum eradnatum*, *H. elodes*, *H. grandiflorum*	20
Malvaceae	*Althaea officinalis*	60
	Malva sylvestris	51,52
Myricaceae	*Myrica rubra*	53
Onagraceae	*Fuchsia excorticata*, *F. procumbens*, *F. perscandens*	55,72
Polygonaceae	*Polygonum hydropiper* var. *vulgare*	2, 43
	P. perfoliatum, *P. thunbergii*	Harborne, J. B., unpublished results
Rosaceae	*Acaena* (5), *Dendriopterium*, *menendezii*, *Potentilla* (3), *Sanguisorba minor*, *S. officinalis*	Harborne, J. B., unpublished results
Tamaricaceae	*Tamarix africana*, *T. aphylla*, *T. cananensis*, *T. gallica*, *T. hispida*, *T. pentandra*, *T. laxa*, *T. smyrnensis*, *Myricaria germanica*.	20, 28, 39, 42
	Reaumuria mucronata	21
Umbelliferae	*Ammi visnaga*, *Daucus carota** *Oenanthe* (6)	27, 73
Verbenaceae	*Lippia nodiflora* & *L. canescens*	56
Plumbaginaceae	*Armeria maritima*, *Limonium* (10), *Plumbago europaea*	Harborne, J. B., unpublished results

The figure in parentheses indicates the number of positive species in any given genus.

*This indicates that sulphates are variably present in these plants.

Table 4. Flavonol sulphates identified in *Flaveria bidentis* and *F. chloraefolia*

Flaveria bidentis	*Flaveria chloraefolia*
Quercetin	Quercetin
3-sulphate	3-sulphate
3,4′-disulphate	3,3′-disulphate
3,7-disulphate	3,4′-disulphate
3,7,3′-trisulphate	Patuletin
3,7,4′-trisulphate	3-sulphate
7,3′,4′-trisulphate-3-acetate	3,3′-disulphate
3,7,3′,4′-tetrasulphate	6-Methoxykaempferol
Isorhamnetin	3-sulphate
3-sulphate	Spinacetin
3,7-disulphate	3-Sulphate
	Eupalitin
	3-sulphate
	Eupatolitin
	3-sulphate
	Eupatin
	3-sulphate
	Ombuin
	3-sulphate

also indicates a correlation between sulphate synthesis and plant habitat.

Sulphates are also of taxonomic interest at lower levels of classification in these plants. There are several examples of this in flavonoid studies of the Australian Cyperaceae [76] and of the Palmae [37]. In the Zosteraceae, too, there are correlations between sulphate occurrences and subgeneric classification in *Zostera* and with specific classification in *Halophila* [83]. Populational variation linked to leaf size has also been detected in the *Halophila ovalis–H. minor* complex of the Indo-Pacific Ocean [84].

ISOLATION AND PURIFICATION

Because of the instability of the sulphate ester bonds, flavonoid sulphates may be hydrolysed during extraction and purification, thus resulting in their corresponding aglycones. Furthermore, their co-occurrence with flavonoid glycosides renders their separation somewhat difficult, since both types of compounds are polar in nature.

Isolation

The general methods used for the isolation and identification of sulphated flavonoids have been described in earlier reviews [3–5]. These methods consist of extrac-

Table 5. Natural distribution of flavonoid sulphates in monocotyledons

Order or family	Genera and species	References
Araceae	*Anthurium hookeri**, *Gonatropus boivinii**, *Scindapsus* sp.*, *Anchomanes difformis**, *Philodendron ornatum**	74
Commelinaceae	*Tradescantia hirta*	75
Cyperaceae	*Cyperus polystachyos, C. bulbosus, C. laevigatus, C. sanguinolentus, C. flavescens, C. subulatus, C. flavus, C. brevifolius Schoenus ericetorum, S. fluitans, S. pleiostemoneus, S. scabripes, Cyperus enervis, Isolepis habra, I. hookeriana, I. inundata, I. prolifera, Eleocharis equisetina, E. pusilla, Fimbristylis ferruginea, Scleria mackaviensis, Carex longebrachiata*	62, 76
Fluviales		
Alismataceae	*Damasonium stellatum* *Sagittaria falcata*	58, 77
Hydrocharitaceae	*Halophila engelmanii* *Thalassia tertudinum* *Halophila baillonis* *H. ovalis, H. stipulacea* *Thalassia hemprichii, Enhalus acoroides*	
Zannichelliaceae	*Zannichellia palustris*	58

Table 5. *Continued*

Order or family	Genera and species	References
Zosteraceae	*Zostera angustifolia*, *Z. marina*, *Z. nana* *Z. americana*, *Z. asiatica*, *Z. capensis*, *Z. capricorni*, *Z. caulescens*, *Z. japonica*, *Z. muellerii*, *Z. noltii*, *Z. novazelandica*, *Phyllospadix iwatensis*, *P. japonicus*, *P. scouleri*, *P. serrulatus*, *P. torreyi*	58, 77, 78
Gramineae	*Ichnanthus pallens*, *Panicum maximum*, *P. convexum*, *P. humboldtianum*, *Bothriochloa bladhii*, *B. caucasica*, *B. edwardsiana*, *B. insculpta*, *B. ischaemum*, *B. radicans*, *Cymbopogon procerus*, *Dimeria ballardii*, *Erianthus maximus*, *Saccharum barberi*, *S. eduli*, *S. officinarum*, *S. robustum*, *S. sinense*, *S. spontaneum*, *Tripsacum dactyloides*, *Zea mays*, *Chloris roxburghiana*, *Cynodon dactylon*, *C. lemfuensis*, *Spartina townsendii*, *Eragrostis horizontalis*, *Cortaderia bifida*, *C. pilosa*, *C. richardii*, *C. selloana*, *Gynerium sagittatum*, *Chionochloa conspicua*, *C. rigida*, *Notodanthonia racemosa*, *Stipagrostis garubensis*, *Microlaena stipoides*	61
Iridaceae	*Iris sanguinea*, *Calydorea speciosa*, *Cipura paludosa*, *Cypella lauthalii*, *Eleutherine bulbosa*, *Gelasine azurea*, *Tigridia pavonia*, *Trimezia steyermarkii*, *Pillansia templemannii*, *Crocus tommasinianus*, *Melasphaerula graminea*, *Synnotia villosa*	79
Juncaceae	*Juncus maritimus*, *J. acutus*, *J. jacquini*, *J. balticus*, *J. inflexus*, *J. effusus*, *J. conglomeratus*, *J. squarrosus*, *J. gerardi*, *J. acutiflorus*, *J. articulatus*, *Marsippospermum grandiflora*, *Rostkovia magellanica*, *Prionium serratum*	59
Liliaceae	*Bellevalia flexuosa*, *Lachenalia uniflora* *Lachenalia aloides*, *Ipheion uniflorum*	57; Williams, C. A., unpublished results
Marantaceae	*Maranta bicolor*, *M. leuconeura* var. *kerchoviana*, *Stromanthe sanguinea*	80

Table 5. *Continued*

Order or family	Genera and species	References
Orchidaceae	*Gymnadenia conopsea*, *Restrepia elegans*	81
Restionaceae	*Hypolaena fastigiata*, *Elegia parviflora*	Harborne, J.B., unpublished results
Palmae	*Areca alicae*, *Arecastrum romanzoffianum*, *A. romanzoffianum* var. *australe* *Bactris guineensis*, *Butia bonnetii*, *B. capitata*, *B. capitata* var. *pulposa*, *B. eriospatha*, *B. yatay*, *Calamus polystachys*, *Clinostigma samoense*, *Cocothrinax dussiana*, *Copernicia macroglossa*, *Cyrtostachys* sp., *Daemonorops jenkinsiana*, *Gaussia attenuata*, *Gigliolia insignis*, *Iguanura geonomaeformis*, *Jubaea chilensis*, *Kothalsia echinanetra*, *Licuala muelleri*, *Linospadix monostachya*, *Livistona altissima*, *L. chinensis*, *L. mariae*, *Mascarena lagenicaulis*, *M. revaughanii*, *M. verschaffeltii*, *Mauritia flexuosa*, *Microcoelum weddelianum*, *Nannorhops ritchiana*, *Neodypsis decaryi*, *Nypa fruticans*, *Opsiandra maya*, *Phoenix canariensis*, *P. dactylifera*, *P. farinifera*, *P. loureiri*, *P. pusilla*, *P. reclinata*, *P. roebelenii*, *P. sylvestris*, *P. tomentosa*, *Pritchardia affinis*, *P. martii*, *Ptychosperma macarthurii*, *P. propinquum*, *Rhapis excelsa*, *R. flabelliformis*, *Rhopalostylis baueri*, *Rhopaloblaste singaporensis*, *Sabal beccariana*, *S. causiarum*, *Thrinax parviflora*, *Trithrinax acanthocoma*, *Veitchia mervillii*, *Washingtonia filifera* and *W. robusta*	37, 54, 82

tion of the dried material with aqueous alcohol and concentration of of the extract before its partition against organic solvents of increasing polarity, such as hexane, chloroform and ethyl acetate. The latter solvents remove the aglycones and some glycosides, whereas flavonoid sulphate esters remain the aqueous layer.

Detection

Sulphated flavonoids are easily detected, in crude extracts, on cellulose TLC where they display characteristic arrow-shaped spots, and high mobility in water as solvent [3,5]; but with little distinction among the polysulphate esters. In butanol solvents (e.g. BAW, 3:1:1), on the other hand, their mobility is reversed with a separation pattern inversely proportional to the number of sulphate groups (Table 6). However, the fact that flavonoid sulphates are more polar than their corresponding glycosides, and the latter more so than their aglycones, allows the separation of the three flavonoid types using the appropriate solvent systems [3, 5]. Flavonoid sulphate esters can also be resolved from glycosides by HPLC,

Table 6. R_f values and UV fluorescence of various sulphated flavonoids

Sulphated flavonoid	$R_f \times 100$		Fluorescence in UV light†	
	H_2O*	BAW (3:1:1)	NH_4OH	Diphenyl borinate
Apigenin 7-sulphate	36	50	OL	Y
4'-sulphate	39	53	Ap	Y-G
7,4'-disulphate	—	31	Ap	O
Luteolin 7-sulphate	22	39	Y-G	Y
3'-sulphate	24	49	Ap	dG
7,4'-disulphate	—	29	Ap	dO
7,3',4'-trisulphate	—	18	Ap	Ab
Kaempferol 3-sulphate	35	46	Ap	Y-G
7-sulphate	28	45	Y	B
7,4'-disulphate	—	28	Y-O	G
3,7,4'-trisulphate	—	9	Ap	Ap
Quercetin 3-sulphate	39	36	dOL	Y
7-sulphate	24	34	Y	Y-O
3'-sulphate	29	43	G	G
3,3'-disulphate	—	26	dOL	G
3,7-disulphate	—	22	dY	O
7,4'-disulphate	—	27	Y-O	G
3,7,4'-trisulphate	—	9	Ap	Ap
3,7,3',4'-tetrasulphate	—	4	Ap	Ap
3,5,7,3',4'-pentasulphate	—	0	B	B
Rhamnetin 3-sulphate	45	41	Y	O
3,3'-disulphate	—	25	G	dy
3,4'disulphate	—	24	Ap	Ap
3,3',4'-trisulphate	—	16	OL	dY
Isorhamnetin 3-sulphate	45	43	dOL	Y
Tamarixetin 3-sulphate	46	44	Ap	Ab
3,7-disulphate	—	25	Ap	Ab
3,3'-disulphate	—	27	Ap	Y-O
3,7,3'-trisulphate	—	10	Ap	Ab
Eupatolitin 3,4'-disulphate	—	25	Ap	Ap
Eupatin 3-sulphate	74	47	Ap	Ap

*All highly sulphated compounds move close to the solvent front.

†A, absorb; B, blue; G, green; O, orange; OL, olive; Y, yellow; b, brown; d, dull; p, purple.

on reverse-phase C_{18} column, after ion pairing with tetrabutylammonium phosphate [31, 56, 85]. Due to the high affinity of the paired ions to the non-polar reverse phase support, the polarity of flavonoid sulphates is decreased relative to the number of sulphate groups, whereas that of glycosides is not affected. Therefore, with methanol–acetic acid–water, as mobile phase, the flavonoid sulphate esters elute, in order of increasing sulphation, after their corresponding glycosides [31]. However, it was recently shown that, after ion pairing, the flavone disulphates of 6-hydroxyluteolin, nodifloretin, hispidulin and jaceosidin eluted earlier on HPLC than their respective monosulphate esters [56]. Finally, the presence of flavonoid sulphates can be confirmed by electrophoresis on Whatman No. 3 paper using acetic acid–formic acid buffer, pH 2.2 [37], where they move towards the anode with mobilities relative to the number of sulphate groups; by comparison, glycosides and uronides which move towards the anode at pH 4.4, remain immobile at pH 2.2 [56]. On electrophoresis flavonol sulphates move farther than flavone sulphates, and substitution in the 3-position increases mobility over that in the 7- or 3'-positions [4]. Furthermore, the presence of sugar residues on flavonoid sulphates results in increased electrophoretic mobility. However, we found that flavonoid glycoside sulphates can migrate on electrophoresis at the level of monosulphate esters, but that their mobility increases with the number of sugar moieties present on the flavonoid aglycone (Barron, D., unpublished results). Therefore, the use of electrophoretic mobilities in screening for sulphated conjugates should be interpreted with caution.

Purification

Flavonoid sulphates can be purified by precipitation from concentrated aqueous extracts [48, 66, 68], which can also be induced by the addition of ethanol [33, 38, 40]. This is usually followed by column chromatography

on Polyclar using water, aqueous methanol, water–methanol–methyl ethyl ketone–acetone (13:3:3:1) [33], or gradients of aqueous methanol [41, 48, 66]; on cellulose columns using water [51]; Sephadex LH-20 using 80% aqueous methanol [37, 48, 51] or Sephadex G-10 using water and gradients of aqueous methanol [33, 38, 45, 48]. Alternatively, when the initial concentrated aqueous extract is rich in flavonoid glycosides, it is useful to selectively separate the sulphated flavonoid fraction using the ion pairing reagent, tetrabutylammonium hydrogen sulphate (TBAHS), where the latter separate as TBA-salts leaving the highly glycosylated flavonoids in the aqueous layer [30]. However, no information is yet available on the effect of glycosylation on the recovery of sulphated flavonoids after ion pairing. The usefulness of Polyamide in the chromatography of sulphated conjugates may be questionable, since it has been reported to strongly adsorb sulphated flavonoids [10, 24]. This problem can be overcome by using aqueous TBAHS–pyridine, or aqueous methanol containing a base, as solvent systems, which makes it possible to distinguish the isomeric 3-, 7- and 3′-/4′-sulphate esters [30, 32].

IDENTIFICATION

Proper identification and structure elucidation of flavonoid sulphates involve determination of the nature of the parent aglycone and estimation of the number and position of sulphate groups on the flavonoid ring system. This is carried out both at the level of the intact molecule, as well as the intermediates recovered after acid or enzymatic hydrolysis.

Acid hydrolysis

Total acid hydrolysis (*ca* 2 N HCl, 15 min, 95°) yields the flavonoid aglycone and sulphate, as well as the sugar in case of sulphated flavonoid glycosides. After extraction with the appropriate organic solvent, the aglycone can be identified by the classical chromatographic [86–90], UV [90–94] and mass spectroscopic methods [90, 95]. The sulphate ion can be precipitated with barium chloride [96] or detected on cellulose TLC developed with 20% of 0.1 N hydrochloric acid in ethanol, which on spraying with cobaltous hexanitrite the HSO_4^- appears as a white spot on a pale yellow background [37]. The cation associated with the sulphate group can further be determined by atomic absorption [3, 4], as well as FABMS (see later). Characterization of the sugar is carried out in the aqueous layer by paper chromatography or TLC on cellulose [87] or silica gel [97] plates. Partial hydrolysis with organic acids, e.g. 10% acetic acid [22], followed by chromatography allows the detection of intermediate products of the parent molecule, which can be co-chromatographed with the appropriate reference compounds. For example, partial hydrolysis of a sulphated kaempferol glucoside gives rise to kaempferol 3-glucoside, as well as kaempferol, glucose and sulphate [22]. Similarly, this procedure allows the identification of the sulphate esters of glucose and gentiobiose in a sulphated kaempferol gentiobioside [26]. Furthermore, controlled hydrolysis of polysulphated flavonoids affords a number of intermediates relative to the level of sulphation of the flavonoid aglycone; e.g. isorhamnetin 3,7-disulphate gave the 3- and 7-sulphated derivatives [40], whereas quercetin 3,7,4′-trisulphate released the 3-, 4′- and 3,4′-sulphate esters as intermediates [33].

Enzymatic hydrolysis

In contrast with mild acid hydrolysis, which may fail to release intermediates [36] or may yield undetectable amounts of products, enzymatic hydrolysis allows for better control of the process and higher yield of products. β-Glucosidase [51] and β-glucuronidase [52] selectively cleave the glucosides and glucuronides which are sulphated on the flavonoid ring, respectively. The position of attachment of the sugar moiety can be determined by comparison of the UV absorption spectra of the intact flavonoid and its hydrolysis product(s). However, when the sulphate group is attached to the uronic acid moiety, enzymatic cleavage releases the aglycone and the sulphated uronic acid [62]. Such specific elimination of the sulphated sugar moiety would be impossible to perform by acid hydrolysis, since the sulphate ester bond is cleaved faster than the glycosidic bond. Sulphate groups can be hydrolysed by a diverse group of sulphatases (sulphohydrolases, EC 3.1.5.-) where the sulphate is in the form of C–O–S, and with apparent specificity for the parent molecule [98,99]. Aryl sulphatase [3–5] acts primarily on phenol sulphate esters, although some reports [23, 26, 62] claim that alcoholic sulphate esters, such as sugar sulphates, can be cleaved as well. In fact, contrary to earlier reports [23, 46, 48], none of the 3-sulphate groups of substituted flavonol 3-sulphates was hydrolysed with aryl sulphatase [30, 32, 63, 100]. This characteristic property has been utilized in the synthesis of flavonol 3-sulphates [100]. However, commercially available aryl sulphatase is usually contaminated with glucosidase and uronidase activities, which poses a problem in the specific hydrolysis of sulphate groups in sulphated flavonoid glycosides/uronides [55], and therefore should be purified before use.

IR and UV spectroscopy

The presence of sulphate groups in flavonoids can be confirmed by the appearance, in their IR spectra, of two strong bands at *ca* 1200 (S=O) and 1040 (C–O–S) cm^{-1} [33, 38, 40]. On the other hand, the position of attachment of the sulphate ester bonds can be deduced from determining their UV spectra in aqueous methanol and after the addition of spectral shift reagents [90–94]. In general, flavonoid sulphates exhibit the same UV spectral characteristics as those of the corresponding methyl ethers [3, 4]. However, recent UV spectral studies [138] of several naturally occurring [30–32, 63] and synthetic [100, 103, 104] sulphated compounds have indicated that sulphation at positions 3 and/or 4′ induce an appreciably more important hypsochromic shift in Band I than those reported for methylation or glycosylation (Table 7), due to the electron withdrawing effect of the sulphate group. Furthermore, unlike methyl groups, the ease of hydrolysis of the sulphate groups with hydrochloric acid [4, 41, 48] and/or aryl sulphatase [4, 41, 48, 104] except the 3-sulphate [100], causes a bathochromic shift in Band I due to formation of the corresponding aglycone or its 3-sulphate ester. The hydrochloric acid reagent is of diagnostic value for the detection of 4′-/3′-sulphated flavones and 3-sulphated flavonols [44]. In addition, the 4′-sulphate shift in Band I is greatly enhanced when position 3

Table 7. Comparison of UV absorption maxima of some naturally occurring and synthetic flavonoid aglycones with their glycosides, methyl ethers and sulphate esters

Substitution	Band I λ_{max}^{MeOH} (nm)										
	Kae*	Que	Rha	isoR	Tam	Pat	Eupl	Jac	Eup	Apig	Lut
Aglycone	367	372	370	370	370	373	363	351	360	336	350
3-OMe	352	358				354	354		347		
3′-OMe		369									349
4′-OMe	362	370	356							332	345
3-glucoside	345	364				353	353				
3-galactoside				357			358				
7-glucoside										335	348
3-sulphate	337	350	345	350	340	348	345		335		
7-sulphate	360	365								330	348
3′-sulphate											330
4′-sulphate								343		315	325
3,7-disulphate	345	350		350	345						
3,3′-disulphate		340	340		340	340					
3,4′-disulphate		345	330	333			320				
7,4′-disulphate	358	360								300	320
3,7,3′-tri-		335							340		
3,7,4′-tri-	305	335		337							
3,3′,4′-tri-		315	315								
7,3′,4′-tri-											305
3,7,3′,4′-tetra-		310									

*Key: Kae, kaempferol; Que, quercetin; Rha, rhamnetin; isoR, isorhamnetin; Tam, tamarixetin; Pat, patuletin; Eupl, eupatolitin; Jac, jaceodin; Eup, eupatin; Api, apigenin; Lut, luteolin.

Table 8. Interpretation of the UV spectral shifts of flavone and flavonol sulphates caused by HCl and aryl sulphatase

Reagent	Shift observed for Band I (nm)		Interpretation
	Flavones	Flavonols	
HCl	+8		3′-sulphate
	+15 to 25		4′-sulphate
	+35		3′,4′-sulphate
		+15 to 20	3-sulphate
		+25	3,3′-disulphate
		+30 to 40	3,4′-disulphate
		+48 to 53	3,3′,4′-trisulphate
Aryl sulphatase		HCl shift = 15 to 20; No aryl sulphatase shift	3-monosulphate
		HCl shift >25; No aryl sulphatase shift	4′-MeO-3,3′-disulphate
		+7 to 10	4′-OH-3,3′-disulphate
		+17 to 22	3,4′-disulphate, with or without free 3′-OH
		+30 to 35	3,3′,4′-trisulphate

was substituted. Thus, 4′-sulphation in flavonols is better detected using aryl sulphatase. The effects of HCl and aryl sulphatase reagents on the UV-spectral shifts of flavones and flavonols are summarized in Table 8.

Methylation. Total methylation of the intact sulphated flavonoid, followed by hydrolysis and UV spectral and/or EIMS analysis reveals the hydroxyl groups that were originally sulphated. The most convenient methylating agent is diazomethane [35, 46], since methylation can be performed in a wide range of solvents, including dimethyl sulphoxide, in which flavonoid sulphates are quite soluble. Methyl iodide in dimethyl

formamide/silver oxide [22] and dimethyl sulphate in acetone/potassium carbonate [23, 26] have also been used as methylating agents. When the sulphate group is attached to the sugar moiety of a sulphated flavonoid glycoside, the positions of sulphation can also be determined by the methylation procedure [22, 23, 26, 36], after which the methylated sugars formed may be identified by paper chromatography [22, 23] or TLC on silica [23, 36], using reference compounds [101].

^{13}C *NMR spectroscopy*

It was not until 1981 that this method was used in the identification of gossypetin 8-glucuronide-3-sulphate [52]. Since then, the spectra of two additional compounds, quercetin 3,7,3′-trisulphate [34] and 7,4′-dimethylkaempferol 3-sulphate [102], as well as a number of naturally occurring [30, 32, 63] and synthetic [100, 103, 104] flavonoid sulphate esters have recently been reported. In ^{13}C NMR analysis, the introduction of *O*-sulphate, an electron withdrawing group, results in a decreased electron density of the carbons *ortho* and *para*, and an increased electron density of the carbon carrying the sulphate group, thus resulting in a downfield shift for the former carbons and an upfield shift for the latter. Calculation of the sulphation shifts of a number of naturally occurring [32, 63] and synthetic [103] compounds demonstrates that sulphation of positions 7, 4′ and/or 3′ induced shifts that were similar to those reported for simple phenol sulphate esters [105], although their magnitudes differed with the position of sulphation and type of compound. Flavonoids with 3′,4′-dihydroxy groups exhibited downfield shifts induced by 3′-/4′-sulphation, which were more pronounced for the *ortho* carbon lacking the hydroxyl than that carrying the phenolic group (Table 9). It is interesting to note that flavonoid sulphation at position 3 did not follow the usual pattern of phenol sulphate esters, since the downfield shift observed for *ortho* C-2 was especially pronounced (Table 9). A similar unexpected shift has been reported for 3-glycosylation [106] and 3-methylation [107], which is characteristic of an olefinic, rather than aromatic, system and reflects the pseudo-olefinic character of the 2,3 double bond in flavonols. On the other hand, the individual effects of 3′- and 4′- sulphation were not cumulative in the case of 3′,4′-*ortho* disulphated compounds such as quercetin 3,7,3′4′-tetrasulphate, since the downfield shifts for the carbons *ortho* and *para* to the sulphate groups were lower than expected, while on the contrary, the upfield shifts for carbons carrying the sulphates tend to be higher [104].

Fast atom bombardment mass spectroscopy (FABMS)

Unlike electron impact mass spectrometry (EIMS) which causes the loss of the sulphate ion [3, 4], FABMS is a mild ionization technique that is commonly used in the structural determination of flavonoid glycosides [108, 109] and most recently, flavonoid sulphates [56]. It only requires microgram quantities of the compound and can be carried out in the positive [41, 50] or negative [30, 32, 56, 63, 100, 102–108] mode. It is now well established that flavonoid monosulphate esters give two major, [M] and [M–SO_3], peaks in their negative FABMS spectra. When potassium or sodium are the counter ions, the respective [M + K − H] and [M + Na − H] fragments are observed as well. However, when a mixture of cations, such as K^+, Na^+ and Ca^{2+} are present in the sample, only the former can be visible in the spectrum because of exchange phenomena [63]. Therefore, the recent detection of Na^+ in the flavonoid sulphates of *Lippia* [56] is considered of interest. Very recently, negative FABMS spectra of several natural [32, 56] and synthetic [100, 103, 104] disulphated conjugates revealed the presence of [M + cation], [M − SO_3 + H] and [M − 2SO_3 + H] peaks. Those of trisulphated flavonoids gave ions at [M + 2 cations], [M − SO_3 + 2 cations] and [M − SO_3 + H + cation] [100, 103]. A similar [M + 2Na] fragment was also reported for quercetin 3,7,4′-trisulphate sodium salt [41]. The fragmentation pattern characteristic of flavonoid mono-, di-, and trisulphates is summarized in Table 10.

SYNTHESIS

Synthesis of flavonoid sulphates is an indispensable method for the preparation of specificially sulphated compounds, not only for comparison with the natural products, but also for their use as substrates and reference reaction products in enzymatic studies.

Chemical synthesis

Among the methods used for sulphation of phenolic compounds [111, 112] the sulphamic acid method has

Table 9. ^{13}C NMR sulphation shifts in quercetin sulphates (DMSO-d_6).

	Sulphation shifts* induced by sulphation of quercetin at positions			
Carbon	3†	7‡	3§	4′‖
Ipso	+3.3 (C-3)	+5.0 (C-7)	+4.2 (C-3′)	+4.3 (C-4′)
Ortho	−9.7 (C-2)	−2.7 (C-6)	−6.9 (C-2′)	−3.2 (C-3′)
	−2.0 (C-4)	−4.0 (C-8)	−3.6 (C-4′)	−6.0 (C-5′)
Meta	−1.1 (C-10)	+0.8 (C-5)	−0.6 (C-1′)	−2.0 (C-2′)
		+1.2 (C-9)	−1.6 (C-5′)	−1.6 (C-6′)
Para	—	−2.4 (C-10)	−4.9 (C-6′)	−4.5 (C-1′)

*Refers to δ quercetin-δ sulphate ester; values were calculated for quercetin 3-monosulphate†; −7,4′-disulphate‡; -3′-monosulphate§; -3,4′-disulphate‖ [32, 103].

Table 10. Major peaks observed in negative FABMS spectra of flavonoid mono-, di- and trisulphates

	Flavonoid sulphate conjugate		
Fragments*	Mono-	Di-	Tri-
M + 2 ci			+
M + ci − H	+		
M + ci		+	
M	+		
$M-SO_3+2ci$			+
$M-SO_3+H+ci$			+
$M-SO_3+H$		+	
$M-SO_3$	+		
$M-2SO_3+H$		+	

*M represents the negatively charged sulphate ester, in absence of counter-ion (ci).

been applied to flavonoid compounds [113–116]. According to Yamaguchi [117], sulphamic acid performs sulphation in pyridine through a pyridinium sulphamate intermediate and introduces a sulphate group at the 3′-position of 3′,4′-dihydroxy flavonoids. However, re-examination of this method [118] demonstrated that although quercetin gives the 3′-sulphate in crystalline form, a complex mixture of mono- and disulphate esters are produced. In fact, sulphation of luteolin gives at least five products (Table 11) namely, the 3′-sulphate (75%), 7-sulphate (5%), 7,3′-disulphate (10%) and 7,4′-disulphate (10%) ester derivatives [118]. The fact that this method yields only small amounts of the 3- and 7- sulphate esters, makes it of little use in the synthesis of naturally occurring flavonoid sulphates.

Another method of synthesis involves the use of sulphur trioxide adducts [111, 112, 120]. However, attempts to sulphate quercetin using sulphur trioxide trimethylamine complex and potassium carbonate, resulted in a mixture of mono- to trisulphate ester derivatives, while most of the quercetin remained unreacted (Barron, D., unpublished). Furthermore, the use of *N,N′*-dicyclohexylcarbodiimide (DCC) and sulphuric acid in dimethylformamide (DMF) has been reported in the sulphation of phenols [121, 122], carbohydrates [123], steroids [124], hydroxyamino acids and peptides [125]. However, attempts to use this method for the sulphation of flavonoids resulted in the destruction of DCC before any sulphate ester was formed (Barron, D., unpublished). On the other hand, the use of TBAHS, instead of sulphuric acid, as sulphating agent allowed the synthesis of various specifically sulphated flavonoids in good yield [103]. In this method, TBAHS reacted with flavonoids in pyridine under controlled conditions to give sulphate esters as their TBA-salts, which were easily separated from minor byproducts by gel filtration. Conversion of the latter to the potassium salt was performed with potassium acetate. Using this method with the calculated equivalents of the flavonoid compound, TBAHS and DCC, under different reaction conditions, resulted in stepwise sulphation of positions 3, 7 and 4′ of the flavonoid skeleton, and followed the order 7 > 4′ > 3 [103]. It should be noted, however, that the 3-position of flavonols was less susceptible to sulphation due to its chelation with the adjacent carbonyl group. It could only be sulphated in the presence of a large excess of TBAHS and DCC, over an extended reaction time, to give the flavonol 3,7,4′-trisulphate ester [103]. Moreover, the 3′-hydroxyl group exhibited low reactivity towards the TBAHS + DCC mediated sulphation, possibly due its weak acidic nature. However, it could be sulphated if the 4′-hydroxyl was substituted with a methyl, but not sulphate, group possibly because of steric hindrance of the bulky 4′-sulphate. On the other hand, 4′-sulphation in the presence of 3′-sulphate group could be achieved by using sulphur trioxide-trimethylamine complex in aqueous potassium carbonate [104].

Enzymatic synthesis.

The discovery that highly purified aryl sulphatase mediates the stepwise hydrolysis of sulphate groups esterified to the flavonoid ring, except at position 3 [30, 32, 63], has made it possible to prepare the 3-sulphated flavonol intermediates as well as the flavonoid monosulphates. The stepwise desulphation of polysulphated flavonols followed the order 7/4′ > 3′ > > > 3 [100, 104]. The resistance to enzyme hydrolysis of the 3′-sulphate group was also observed in such compounds as tamarixetin 3,3′- and ombuin 3,3′-disulphates (Barron, D., unpublished results). The naturally occurring flavonoid sulphate esters whose structure has been confirmed by synthesis are listed in Table 11.

BIOSYNTHESIS

Accumulation and turnover

Despite the increasing number of reports on the natural occurrence of flavonoid sulphate esters, very little is known of their accumulation or turnover in plant tissues. The co-occurrence, in *Flaveria bidentis* (Table 4), of flavonol glucosides (3-*O*-glucosides of kaempferol, 6-methoxykaempferol and patuletin) and sulphate esters of quercetin and isorhamnetin [31] allowed the study of their biosynthesis in this tissue. Both individual and total flavonoids (nmol/g fr. wt) were highest in the buds, lowest

Table 11. The synthesized naturally occurring flavonoid sulphates

Compound	Method*	Reference
Apigenin		
7-sulphate	DCC; SA	20, 103
Luteolin		
7-sulphate	DCC; SA	20, 103
3′-sulphate	DCC + ST + AS; SA	20, 103, 104
4′-sulphate	DCC + AS; SA	20, 100, 103
7,3′-disulphate	SA	20
Diosmetin		
7-sulphate	SA	57
7,3′-disulphate	SA	57
Kaempferol		
3-sulphate	DCC + AS; SA	27, 100, 103
7-sulphate	DCC	103
3,7,4′-trisulphate	DCC	103
Quercetin		
3-sulphate	DCC + AS; SA	27, 100, 103
7-sulphate	DCC	103
3,7-disulphate	DCC + AS	100, 103
3,4′-disulphate	DCC + AS	100, 103
3,3′-disulphate	DCC + ST + AS	103, 104
3,7,4′-trisulphate	DCC	103
3,7,3′,4′-tetrasulphate	DCC + ST	103, 105
Rhamnetin		
3-sulphate	DCC + AS	100
Isorhamnetin		
3-sulphate	DCC + AS	100
Tamarixetin		
3-sulphate	DCC + AS	100
Eupalitin		
3-sulphate	DCC + AS	100
Eupatolitin		
3-sulphate	DCC + AS	100
Veronicafolin		
3-sulphate	DCC + AS	100

* AS, aryl sulphatase; DCC, dicyclohexylcarbodiimide and tetrabutylammonium hydrogen sulphate; SA, sulphamic acid; ST, sulphur trioxide-trimethylamine complex.

in mature leaves, and conspicuously absent in roots. The flavonol sulphate esters predominated (>90% of total flavonoids) in young seedlings, with quercetin 3,7-disulphate as the major constituent (*ca* 50–70%). On the other hand, in mature shoots and flowers, the flavonol glucosides and flavonol sulphate esters were found to occur in almost equal amounts, but with different pattern for the sulphated compounds. Those in the shoots consist mainly of quercetin 3-sulphate, 3,7-disulphate, 3,7,3′,4′-tetrasulphate; with small amounts of isorhamnetin 3-sulphate, 3,7-disulphate and quercetin 3-acetyl-7,3′,4′-trisulphate [31]. In flowers, on the other hand, quercetin 3-sulphate is the predominent compound (>90%) with trace amounts of the di- to tetrasulphated derivatives. These variations in the glucoside-sulphate composition of the different organs seem to indicate a fairly rapid turnover of conjugated flavonoids in *F. bidentis*. Furthermore, this tissue represents an interesting example of the segregation of flavonol aglycones and the transferase reactions involved, i.e. glucosylation of kaempferol and sulphation of quercetin; although quercetin 3-glucoside has previously been reported to occur in the flowers of this species [139]. However, other examples seem to represent a competition between the glucosylation and sulphation reactions for the same aglycones, as indicated by the presence of patuletin 3-glucoside and 3-sulphate in *Brickellia* spp. [46] and *F. chloraefolia* [44, 63] and eupatolitin 3-glucoside and its sulphated analogue in different *Brickellia* spp. [48, 68]. Furthermore, it is interesting to note that whereas the large-leaved forms of *Halophila* (an Australian seagrass) accumulates predominantly sulphated flavonoids, small-leaved plants contain non-sulphated, glycosylated compounds [83, 84]. Although there was no mention in these studies of the aglycones, sugars or the number or position of the sulphate groups involved, both reports demonstrate the systematic and ecological implications on the nature of conjugated flavonoids in this complex genus. Further studies of other species will be required to demonstrate the competition among the different transferase reactions (glucosylation, methylation, sulphation) for similar aglycones.

Biosynthesis from labelled precursors

In contrast with the extensive studies on sulphated phenolic conjugates in animal tissues [14, 126], little is known of their formation in plants, except for two preliminary reports [78, 127] on the incorporation of [$^{35}SO_4^{2-}$] into sulphated flavonoids. The biosynthetic studies of *Flaveria* flavonoids from [^{3}H]cinnamate and [^{35}S]sulphate showed that the terminal bud and adjacent pair of young leaves were the most active organs in flavonoid synthesis [31]. Whereas the cinnamate label was incorporated almost predominantly into flavonol glucosides, that of sulphate was found exclusively in the flavonol sulphate esters, and was proportional to the number of sulphate ester linkages of individual flavonoids. The lack of cinnamate incorporation into sulphated flavonols may be due to a low endogenous level of the sulphate donor, 3′-phosphoadenosine-5′-phosphosulphate (PAPS). The fact that double labelling experiments resulted in constant incorporation ratios of both [^{3}H] amd [^{35}S] labels into flavonoid sulphates, seems to indicate that sulphation is a later step in their biosynthesis.

ENZYMOLOGY

In contrast with the extensive studies on the enzymatic sulphation of endogenous metabolites and xenobiotics in animal tissues [128, 129], almost nothing is known of the sulphation of flavonoids in plants. This apparent lack of interest in the enzymology of sulphated flavonoids might be explained by the inherent difficulties in the preparation of specifically substituted flavonoid substrates and reaction products, as well as the need for a simple, reliable enzyme assay for sulphotransferase activity.

Enzyme assay

The assay for phenol/flavonoid sulphotransferase (EC. 2.8.2.-) is based on the incubation of the enzyme protein with [^{35}S]PAPS, as the sulphate donor, and the appropriate phenolic acceptor.The fact that both the sulphate donor and the sulphated reaction products share similar solubility properties, renders their separation difficult to achieve, since the methods commonly used are inaccurate and time consuming. These methods (for review see ref. [128]) are based on the precipitation of PAPS with a mixture of barium hydroxide, and sulphuric acid or zinc sulphate. Whereas this procedure may be used with various sulphate acceptors, it is of limited use with phenolic carboxylic acids, and can not be used with flavonoid sulphate esters which precipitate as barium salts [130]. Other methods consist of the chromatography of the enzyme reaction products on paper, cellulose thin layers, ECTOLA-cellulose columns, or HPLC. Such techniques are cumbersome and time consuming, especially for routine use in protein purification on different columns, or in conducting enzyme kinetic studies. Very recently, a simple, rapid and accurate enzyme assay was developed for flavonoid sulphotransferases [130]. It is based on the formation of an ion pair between tetrabutylammonium dihydrogen phosphate (TBADP) and the flavonoid suphate ester formed. The latter can then be extracted in an organic solvent, such as ethyl acetate, whereas the sulphate donor remains in the aqueous reaction medium. The reproducibility and accuracy of this assay allowed us to demonstrate, for the first time, the existence of flavonoid sulphotransferase activity in cell free extracts of *F. bidentis* and *F. chloraefolia* [131]. Using *F. bidentis* as the enzyme source and quercetin as substrate, resulted in the formation of mono- to tetrasulphated derivatives as reaction products. These results suggest the existence of a family of sulphotransferase activities that seem to be involved in the sulphation of specific hydroxyl groups of the flavonol molecule, as has been shown with the glucosylation [16] and methylation [18] reactions.

Enzyme purification

The problems encountered in the purification of plant sulphotransferases are quite similar to those already reported from studies with animal tissues [128], and fall in the following categories. First, flavonoid sulphotransferases have a high tendency to form aggregates, which seem to be related to the oxidation state of the proteins. On gel filtration, two peaks of enzyme activity are usually eluted with M_r of 30000 and 60000. The activity ratios of the two peaks is dependent on the presence or absence of SH-group reducing agents. Their addition to the eluting buffers favors aggregation. This unusual behaviour has often been reported from various sulphotransferases in animal tissues [128, 132–134] and was ascribed to intermolecular rearrangements after the reduction of intramolecular disulphide bridges. The second major problem is related to the instability of sulphotransferases during purification, which can be partially prevented by the addition of bovine serum albumin, glycerol and SH-group protectors.

Whereas our work on flavonol sulphotransferases is still in progress, we have accumulated sufficient data to allow an overview of their purification and properties from plant tissues. Previous work with *Flaveria* spp. [131] led to the purification of a flavonol sulphotransferase which showed expressed specificity towards the 3 position of quercetin.

The 3-sulphotransferase was purified to near homogeneity (700-fold) from *F. chlorefolia* (Varin L., unpublished results). The choice of the latter as the enzyme source was dictated by the difficulties encountered in obtaining the 3-sulphotransferase free from other contaminating activities if *F. bidentis* was used as the plant material. The purification of this enzyme involved successive chromatography of the protein extract by gel filtration, affinity chromatography on PAP-agarose and ion-exchange chromatography on Mono-Q. The purified enzyme showed two pH optima at 6.0 and 7.5, an apparent pI of 5.1, and a molecular weight of 32000. The enzyme exhibited expressed specificity for position 3 of flavonols with rhamnetin > isorhamnetin > quercetin > kaempferol > patuletin, as sulphate acceptors. However, it did not accept flavonols with free hydroxyl groups at position 6, 8 or 5′. The K_m of the 3-sulphotransferase for both the sulphate donor (PAPS) and the flavonol acceptor was 0.2 μM and the enzyme was strongly inhibited by PAP with a K_i of 0.1 μM. Other flavonol sulphotransferase activities were detected during purification and have not yet been characterized.

Another flavonol sulphotransferase was partially purified from *F. bidentis* and exhibited specificity towards position 7 of partially sulphated flavonoids (Varin L., unpublished). It accepted both quercetin 3,3′- and 3,4′-disulphates equally well and gave rise to their 7-sulphate

ester derivatives, respectively; both of which are natural constituents of *F. bidentis* (Table 4) [31]. The chromatographic properties of the 7- sulphotransferase were different from those of the 3-sulphotransferase as well as other flavonol sulphotransferase activities found in *Flaveria* spp. It had a pI of 6.5 and bound weakly on both ion exchange and affinity columns.

In view of the strict position specificity of these two enzymes, it seems plausible that sulphation at position 3′ and 4′ of flavonols are catalysed by two distinct enzymes. However attempts to resolve the latter activities has, so far, been impeded by the similarity in their elution properties and instability during purification. In contrast with the sequence of flavonoid methylation (3→3,7→3,7,4′→3,7,4′,5′), previously studied in *Citrus mitis* [135] and *Chrysosplenium americanum* [136], that of polysulphation in flavonoids seems to be different and its elucidation promises to be a challenging task.

The co-occurrence of the desulphation and sulphate conjugation reactions in different organisms is manifested by the ubiquitous occurrence of sulphohydrolases (EC.3.1.5.-) in microorganisms [98], animal [99] and plant ([137] and refs therein) tissues. It is conceivable, therefore, that flavonoid-specific aryl sulphatases exist in plant tissues, especially those which actively synthesize flavonoid sulphate esters. The role of these enzymes in the metabolic regulation of sulphated flavonoids should not be overlooked.

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