CHAPTER I

THEORETICAL
The chemical investigation of plants has been a favourite area of research in organic chemistry leading to the isolation of a number of new compounds and elucidation of structure of many novel and complex molecules. Flavonoid constitutes one of the most characteristic and largest group of naturally occurring phenols in higher plants. Many flavonoids are easily recognized as flower pigment in most angiosperm family. However, their occurrence is not restricted to flowers only but includes all parts of the plant. The major flavonoids occur almost universally in higher plants, including mosses and ferns. Despite several positive reports, the occurrence of flavonoids in bacteria, algae and fungi is doubtful.

The structure of flavonoids, which form a large group of biosynthetically hybrid molecules, derived from a combination of shikimic acid and acetate precursors by claisen condensation step, are based on a C_{15} skeleton with a chromane ring bearing a second aromatic ring (ring B) in position 2,3 or 4 (I). In few cases, the six membered heterocyclic ring (ring C) occurs in an isomeric open form or is replaced by a five membered ring.
Various subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification. Examples of six major subgroups [Chalcones (II), Flavanones (III), Flavones (IV), Flavonoids (V), Anthocyanidins (VI) and Isoflavones (VII)] are given in Chart-I. All of them bear B ring in the position 2 except in isoflavones in which the ring B occupies position 3. A group of chromane derivatives with ring B in position 4 (4-phenylcoumarins-neoflavones (VIII)) is treated together with other coumarin derivatives. Another small group comprises oligomeric flavonoids biflavonyls (IX), and proanthocyanidins (X).

Altogether there are many hundreds of differently substituted flavonoid aglycones which have been very systematically reviewed by Wollenweber and Dietz\(^2\). Most of these occur as glycosides with different combination of sugars attached hydroxyl groups. The sugars are often further substituted by aryl residue; the details of which are described in the later part of this thesis.

The flavonoids are of commercial interest as antioxidants for fats and oils\(^3,4\). The antioxidant property of flavonoids has been studied. Robenetin and gossypetin were claimed as the most potent and of economic important in the tanning of leather, the fermentation of tea, the manufacture of cocoa, and in the flavour qualities of food stuffs\(^5,6\).
Chart - I

(II)

(III)

(IV)

(V)

(VI)

(VII)
Chart I
Numerous physiological activities have been attributed to flavonoids. The potent uses of flavonoids may be listed as heart stimulants, antiviral effects, antifungal activity, antihemolytic activity, antibacterial activity, contraceptive drugs, anthelmintic activity, Vit P activity, spasmylytic and antihistamine activity, antihypertensive action, treatment of allergic diseases, analgesic and bronchodilator effects etc.

Recently new trends in pharmacological research of flavonoids have been elaborated. Flavonoids have been used as free radical scavengers and as uncouplers of oxidative phosphorylation. In extensive screening programmes of plant products for anticancer drugs, it is not surprising that claims have been made that flavonoids may contribute to, or be effective in combating certain types of cancer. Several flavonoids are moderately active against laboratory cultures of malignant cells. Eupatin, eupatorin, centaureidin and 3,4'-dimethyl quercetin are quite active against Eagles carcinoma of the nasopharynx in cell cultures. The effect of flavonoid on cancer cells in various pharmacokinetics of a new flavanolignan couple has recently been studied.
The term glycoside embraces a large and remarkably varied group of organic compounds having the properties in common of furnishing saccharides or their oxidation products, the glycones and one or more other substances (not in frequently aromatic in nature) the aglycones, when hydrolysed by acid or a specific hydrolytic enzyme. In combination with sugars, representative of nearly all classes of compounds occur in plants, chiefly in fruits, in flowers and barks. These compounds corresponds in structure to the simple synthetic methyl glucosides having hemiacetal linkage and accordingly a glycoside can be represented by a general formula (XI) where R stands for the non-sugar moiety.

\[
\begin{align*}
\text{CH}_2\text{OH} \\
\text{H} & \text{H} \\
\text{H} & \text{OH} \\
\text{H} & \text{OH}
\end{align*}
\]

( XI )

Glycosides are thus regarded as derivatives of sugars in which the reducing or the potential aldehydic group of the sugar is substituted by condensation with an alcohol or a phenol to form a hemiacetal. The oligo- and polysaccharides are also the glycosidic condensation products of monosaccharides, one of the
component sugars behaving as a reducing sugar and the other one acting as an alcohol. Customarily, they are neither included nor dealt with glycosides for they fail to furnish a non-sugar part - the aglycone on hydrolysis.

The classification of glycosides is based upon the nature of aglycone. The aglycones includes representatives of many of the numerous group of hydroxyl compounds occurring in plants, ranging from small molecules such as ethyl alcohol, acetone, cyanohydrin to large ones such as triterpenes, steroids (Cardiac glycosides, saponins etc.), hydroxyanthoquinones, anthocyanins and anthoxanthins.

Besides O-linked glycosides, some of the C-glycosides (a direct C-C link between the sugar and the non-sugar part) have been known for over a hundred years in crystalline form, but it has been only during last two decades that they have been extensively studies.

O-Glycosides

Flavonoid derivatives can have a great diversity of structures. These C\textsubscript{15} compounds commonly have a number of hydroxyl groups on both the A and the B rings. The flavonols, flavanones and anthocyanidins have a hydroxyl group at C-3 too. This polyhydric character together with the fact that a number
of different monosaccharides may form glycosides either as single residue or as oligosaccharide means that many glycosidic forms are possible.

The flavonols constitute the largest and most variable class of flavonoid glycosides. The hydroxyl group at C-3 is usually, but not always, glycosylated. In flavones and flavanones glycosylation occurs mainly at C-7, 4', 6 and 8 hydroxyl groups and occasionally at C-5.

The general nature of phenol and the linkage between carbohydrate and phenol is most important in the study of glycosides. Carbohydrates consists of pentoses, hexoses, 6-deoxyhexoses, hexuronic acids, disaccharides and linear and branched trisaccharides.

Monosaccharides

Ten monosaccharides found to occur in association with flavones and flavonols are listed in Table-1. Amongst these ten monosaccharides, arabinose is definitely known to occur in both pyranose and furanose forms while other sugars (except apiose) are normally in the pyranose form. Galacturonic acid and glucuronic acid are found to occur as the methyl ester.
Table 1: Monosaccharides of flavone and flavonol glycosides

<table>
<thead>
<tr>
<th>Pentose</th>
<th>Hexoses</th>
<th>Uronic acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Apiose</td>
<td>D-Allose</td>
<td>D-Galacturonic acid</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>D-Galactose</td>
<td>D-Glucuronic acid</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>D-Glucose</td>
<td></td>
</tr>
<tr>
<td>L-Rhamnose</td>
<td>D-Mannose</td>
<td></td>
</tr>
</tbody>
</table>

The nature of the linkage of the monosaccharide to the phenolic hydroxyl is the point of significance. This is usually assumed to be the β- for glucosides, galactosides, glucuronides and xylosides and α- for arabinosides and rhamnosides. However, exceptions do exist since a β-linked 3-arabinosides of quercetin is well known and other β-linked arabinoside may be present in plants. On the other hand, α-linked glucosides and galactosides are also possible and need to be distinguished from the much more common β-derivatives.

Disaccharides:

Twenty one disaccharides have been fully characterized to occur as the component of flavone or flavonol glycosides. However, apart from these, there are still many diglycosides which have not been fully characterized.
The first disaccharide based on glucose and galactose have been characterized, i.e. glucose (β 1 → 4) galactose and galactose (β 1 → 4) glucose, as have two disaccharides based on glucose and mannose. The first digalactose, galactose (β 1 → 4) galactose reported in the literature is a myricetin derivative from *Vitex negundo*.

A novel disaccharide, glucose (α 1 → 4) rhamnose, has been isolated from the root bark of *Artocarpus lakoocha*. In this disaccharide the normal order pentose hexose is reversed and the rhamnose is directly linked to the flavonol with the galactose as the terminal sugar. Recently a new disaccharide of this type has been isolated from the leaves of *Mesua ferrea*. This disaccharide occurs linked to the 3′-hydroxyl of 5′C-methyl-eriodictyol.

**Trisaccharide**

The presence of trisaccharides in flavonoids is confined to flavonols as either kaempferol and or quercetin or simple methyl ether of these aglycones. So far 17 trisaccharides have been fully characterized.

**C-Glycosides**

C-Glycosylflavonoids (C-glycoflavonoids) occur widely in
nature and the field of C-glycosylflavonoids has grown rapidly in recent years. The aglycones involved belong to different types, and so far, representatives have been found in the groups of anthrones, anthraquinones, flavones, flavonols, dihydrochalcones, xanthones and isocoumarins. Due to their resistance of acid hydrolysis, the nature of sugar moiety and its linkage with aglycone have remained the most difficult problem in C-glycosyl chemistry. The structure elucidation of C-glycosides has now been simplified by the invention of $^{13}$C-NMR spectroscopy and mass spectrometry.

C-Glycosylflavones often occur in nature as O-glycosides and most of these are monosides. They can be divided into two types (i) $X$ (or $-X'$)-O-glycosyl C-glycosylflavones, in which the O-glycosyl moiety is bound to a phenolic hydroxyl group of the flavone and (ii) $-X''$-O-glycosyl C-glycosylflavones (C-diholosyl flavones) in which the O-glycosyl moiety is bound to an alcoholic hydroxyl group of the C-glycosyl residue.

In both types, the O-glycosyl group and the C-glycosyl-flavone can be easily identified after acid hydrolysis. In the first type the O-glycosyl position can be deduced from the comparison of UV spectral shifts before and after hydrolysis, as in the case of flavone O-glycosides. In the second type, however, the UV spectral shifts remain unchanged after hydrolysis and location of the O-glycosidic bond is much more difficult than in the case of flavone-O-diholosides, since the C-glycosyl...
moiety can't be detected from the flavone by acid hydrolysis.

**Acylated glycosides:**

The flavonoid glycosides also occur in acylated form with acids, such as p-coumaric, caffeic, sinapic, ferulic, gallic, benzoic, p-hydroxybenzoic, acetic and malonic. The most common acyl groups appear to be p-coumaric, gallic, acetic and ferulic acids. Monoacylation is usual, but substances with two same or different acyl groups have been reported\(^{32,33}\). Several pairs of isomeric acyl derivatives are known. These may occur together in the same plant while acyl migration might possibly occur during isolation and handling\(^{34,35}\).

Acylated glycosides may be recognized by their high chromatographic mobility on paper in solvents such as 15% acetic acid and phenol and low mobility in water, when compared with the corresponding unacylated glycosides. Acylated glycosides have also distinctive spectral properties, those acylated with aromatic acids are readily distinguished by UV spectroscopy, since the aromatic acid absorption is superimposed on the normal flavonoidic spectral bands. The acyl groups can then be removed by mild alkaline hydrolysis and the acid present recovered and identified by standard procedures.

The usual linkage of acyl group is through one of the sugar hydroxyles and any one of the three or four free hydroxyls
may be involved. The direct linkage of acyl group to a phenolic hydroxyl also occasionally occurs\textsuperscript{36-38}.

The most complex acyl derivative reported in the literature is probably an acacetin derivative from \textit{Coptis japonica} leaves, which not only has three acetyl residues attached variously to two different monosaccharide units, but also contains the only known tetrasaccharides, namely 6-rhamnosylsophorotriose\textsuperscript{39}. 
ULTRA-VIOLET SPECTROSCOPY

UV spectroscopy has become a major technique for the structure analysis of flavonoids for two main reasons. The first is that only a small quantity of pure material is required. The second reason is that the structural informations obtained about flavonoids from UV is considerably enhanced by the use of specific reagents which reacts with one or more functional groups on the flavonoid nucleus. The addition of these reagents separately to an alcoholic solution of the flavonoid induces structurally significant shift in the UV spectrum. The commonly used shift reagents are sodium methoxide (NaOMe), sodium acetate (NaOAc), sodium acetate/Boric acid (NaOAc/H₃BO₃), Aluminium chloride (AlCl₃) and Aluminium chloride/Hydrochloric acid (AlCl₃/HCl).

The UV spectra of most flavonoids consists of two major absorption maxima, one of which occurs in the range of 240-285 nm (Band II) associated with A-ring Benzoyl system (XII) and second higher wavelength band (I), occur in the range of 320-380 nm, associated with B-ring cinnamoyl absorption (XIII).
Substitution in B-ring specially at 4' stabilizes the cinnamoyl chromophore resulting in a bathochromic shift of band I whereas substitution in the A-ring has a similar effect on the position of Band II. The presence of a free hydroxyl group at C-5 and C-3 position is established by measuring the spectra in the presence of AlCl$_3$. Compounds having a free 5'-hydroxyl group absorb at higher wavelength and methylation of this hydroxyl group brings about a hypsochromic shift of 10-15 nm of both bands. The hydroxyl groups at C-7 and 4' position are more acidic than others and their occurrence is established by bathochromic shift of Band I and Band II on the addition of fused sodium acetate. The presence of hydroxyl group at 4' position is also confirmed by a large bathochromic shift in Band I without a decrease in intensity on the addition of sodium methoxide. The presence of ortho-dihydroxyl group in ring A and ring B is identified by a bathochromic shift in Band I in the presence of AlCl$_3$/HCl and sodium acetate/Boric acid respectively.

In flavanones and isoflavones, due to the absence of cinnamoyl chromophore the high wavelength band is either totally absent or present only as inflection. Thus it is difficult to distinguish between flavanones and isoflavones with the help of UV spectrum alone.
The ultra-violet spectra of biflavonoids are very similar to that of the monoflavonoid unit with the only difference that the molecular extinction coefficient (Σ) of the biflavone is approximately double as compared to the corresponding monoflavonoid. This demonstrates the presence of two isolated chromophores of flavonoids per molecule of biflavonoids.

**INFRA-RED SPECTRA**

The Infra-red spectrum provides a valuable information of functional groups in molecule. The IR spectrum of flavone shows the carbonyl band at 1660 cm$^{-1}$ (SD Aromatic C=O, 1670 cm$^{-1}$) owing to the conjugation with olefinic double bond. Introduction of a hydroxyl group at 5-position does not alter the band position appreciably. Luteolin and apigenin shows the carbonyl band at 1655 and 1660 cm$^{-1}$ respectively$^{43}$. The IR spectrum of flavanone shows the carbonyl absorption at 1680 cm$^{-1}$, the standard value for aromatic ketones. The shifts of carbonyl band to 1620 cm$^{-1}$ in 5-OH flavonone is largely due to electron donation by the orthohydroxyl group, coupled with chelation. Consequently methylation of the 5-OH produces only a small hypsochromic shift of 10 cm$^{-1}$. A similar shift towards long wavelength of 4′-substituted flavanone is however, attributed to intermolecular hydrogen bonding$^{44}$. The IR spectrum
of isoflavonones are similar to those of flavones. Chelation of the 5-OH in all cases has the effect of broadening the O-H stretching band to a point where it can no longer be made out. The IR spectra of alkylated flavonoids give some indication of the presence of or absence of gem-dimethyl groups and an epoxide linkage but these points can now be better established with the help of NMR spectra.

NUCLEAR MAGNETIC RESONANCE (NMR) SPECTROSCOPY

Since flavonoid compounds contain, in general, very few protons nuclear magnetic Resonance spectroscopy is a useful tool in the structure elucidation of this class of compounds. By the use of NMR studies of Silyl derivatives, Double irradiation techniques, Solvent induced shift studies, Lanthanide induced shift studies (LIS) and one can come to the structure of flavonoids without tedious and time consuming chemical degradation and syntheses.

The valuable contribution in this field have been made by Batterham and Highet, Mabry, Massicot, Clark-Lewis, Kawano and Pelter and Rahman. These studies have been simplified the task of determining the substitution pattern of flavonoids with the help of spectroscopy. The most commonly occurring hydroxylation pattern in natural flavonoid is 4',5,7-trihydroxy system (XIV).
A number of workers\textsuperscript{40} have preferred to use the TMSi ether derivatives of flavonoids with \(\text{CCl}_4\) as solvent. The trimethyl silyl ether derivatives can be conveniently prepared by treatment of the compound with hexamethyl disilazane and trimethyl silyl chloride in pyridine and the spectrum is then measured in carbon tetrachloride with tetramethyl silane as external or internal reference. The most important advantages that this system offers are (i) all hydroxy flavonoids and their glycosides may derivatized and rendered \(\text{CCl}_4\)-Soluble (ii) There are no interfering signals and no deuterated solvent is required, (iii) The sample is readily recovered by treatment with aqueous methanol (iv) Partial trimethyl silylation can yield information about C-3, 6 and 8 substitution and (v) TMSi ethers are readily converted in to acetate or methyl ethers\textsuperscript{40}.

\textbf{Nuclear Magnetic Resonance in trimethyl silylated flavonoids}
normally occurs between 0 and 9 ppm. The chemical shifts of the protons of ring A and B proves to be independent of each other but are affected by the nature of ring C.

**A-Ring Protons**

The peaks arising from ring A protons in most flavonoids occur upfield from other peaks and are readily recognized. There are different types of substitutions in the ring A among the flavonoids.

(i) **H-5, H-6 and H-8 signal in 7-oxygenated flavonoids**

The additional C-5 proton in these compounds is strongly deshielded by the 4-keto group and its signal appears at a very low field (δ 8.0 ppm). It appears as a doublet (J = 9Hz) due to ortho coupling with H-6. The signals for H-6 (a, quarter, q, J = 9Hz and 2.5Hz) and H-8 (a, doublet, d, J = 2.5Hz) occur at lower field then in the 5,7-dihydroxyflavonoids and may even reverse their positions relative to one another.

(ii) **6-H and 8-H protons in 5,7-dioxygenated flavonoids**

The two A ring protons, H-6 and H-8 give rise to two doublets (J = 2.5Hz) in the range δ 5.7 - 6.9 in flavones, flavonols, isoflavones etc. The H-8 doublet occurs consistently downfield than the signal for the H-6. H-8 and H-6 doublets are
also clearly distinguished from each other by their widely
different paramagnetic induced shifts. Depending upon the
nature of the substituents the chemical shift may vary accor-
dingly. For instance when a sugar is attached to the oxygen
at C-7 the signal for both H-6 and H-8 are shifted downfield.

(iii) **H-6/H-8 Signal in 5,7,8/5,6,7-trisubstituted flavonoids**

NMR provides the requisite information for differenti­
tiating 6 or 8 substituted isomers of 5,7,8/5,6,7-trisubstituted
flavonoids with a high degree of surety. Horowitz and Gentili\(^{58}\) were able to fix up the structure for the two isomers of Vitexin,
viz. vitexin and isovitexin. The H-6 proton signal appears at
about \(\delta 0.2-0.3\) ppm upfield than H-8 signal.

**B-Ring Proton**

All B-ring protons appear around \(\delta 6.7-7.9\) ppm, a region
separate from the usual A-ring protons. The signals from the
aromatic protons of a substituted B-ring in a flavone appears
as a broad peak centered at about \(\delta 7.45\). The presence of
C-ring double bond causes a shift of 2',6'-protons and the
spectrums shows two broad peaks one centred at \(\delta 8.00\) (2',6')
and the other at \(\delta 7.6\) (3',4',5')\(^{50}\). The presence of substi-
tution in one or more position causes a change.
(i) H-2', 6' and H-3', 5' signal in 4'-oxygenated flavonoids

With the introduction of 4'-hydroxy group, the B-ring protons appear effectively as a typical four peak pattern of two doublets called $A_2B_2$ pattern ($J = 8 \text{Hz}$ each). The H-3' and H-5' doublet always occur upfield from the H-2', 6' doublet due to shielding effect of the oxygen substituent and to the deshielding influence of C-ring functions on H-2' and H-6'. The position of H-2' and H-6' signal depends to some extent on the oxidation level of ring C.

(ii) H-2', H-5' and H-6' signals in 3', 4'-dioxygenated flavonoids

The NMR spectrum of 3', 4'-dioxygenated flavonoids are a bit complex and gives the normal ABX pattern. The H-5' proton in flavones and flavonols in such system appears as a doublet centered between $\delta$ 6.7 and 7.1 ppm ($J = 8 \text{Hz}$) and the H-2' and H-6' signals, which often overlap, usually between $\delta$ 7.2 and 7.9 ppm.

(iii) H-2' and H-6' protons in 3', 4', 5'-trioxygenated flavonoids

In 3', 4', 5'-trihydroxylated flavonoids H-2' and H-6' are equivalent and appear as a two proton singlet in the range $\delta$ 6.5-7.5 ppm. Methylation shift of about 1 ppm downfield when the compound is analysed in DMSO-d$_6$.

(iv) H-2 and H-3 signals in flavanones and flavanonols

The spectra of flavones (saturated heterocyclic ring)
contain typical ABX pattern multiplets arising from the C-2 proton and the two C-3 protons. The C-2 proton is split by the C-3 protons into quartet \( J_{\text{cis}} = 5\,\text{Hz}, \ J_{\text{trans}} = 11\,\text{Hz}, \) double doublet) and occurs near \( \delta 5.2 \) ppm, the precise position depending on the substitution of ring B. The two C-3 protons occur as two quartets \( J_{H-3a, H-3b} = 17\,\text{Hz} \) at \( \delta 3.0 \). However, they often occur as two doublets, since two signals of each quartet are of low intensity.

The C-2 proton in dihydroflavonols appears near \( \delta 4.9 \) as a doublet \( J = 11\,\text{Hz} \) coupled to the C-3 proton which comes at about \( \delta 4.2 \) as doublet\(^{59}\).

**Hydroxy Protons:**

The position of hydroxy groups in flavonoids can not be detected by NMR spectra of their trimethylsilylated derivative and thus cannot be used for their detection. The NMR spectra of parent compound in DMSO-\( d_6 \), however, can give good information for the detection of phenolic hydroxyl protons. The hydroxyl protons of 3,5,7-trihydroxyflavone occur at \( \delta 12.40 \) ppm (5-OH), 10.93 ppm (7-OH) and 9.70 ppm (3-OH)\(^{40}\).

**Sugar Protons:**

The sugar protons in the flavone glycosides are denoted as C-1", C-2" and so on while the protons of the terminal sugar in
disaccharides are designated as C-1'”, C-2’” and so on. In
the PMR spectra of TMS derivative of the glycoside, the non-
anomeric protons resonate between δ 2.9-4.3 while the anomeric
protons resonate between δ 4.3-5.8. The axial anomeric protons
are observed between δ 4.3-5.0 and the equatorial anomeric
protons between δ 4.7-5.8. The chemical shift of the C-1’”,
proton of the sugar directly attached to the flavonoids hydroxyl
group depends both on the nature of the flavonoid and on the
position and stereochemistry of the attachment of it. For
instance in flavone glycosides with sugar on either C-5, C-7 or
C-4’ the C-1’” proton signal appear near δ 5.0, while in flavonol
3-O-glycosides the C-1’” proton signal appears much more down-
field i.e. at about δ 5.8. The coupling constant of C-1’” proton
with C-2’” proton in β-linked glycosides is about 7Hz40, due to
diaxial coupling. In the naturally occurring α-linked rhamnosides,
the diequitorial coupling between H-1’” and H-2’” give rise to a
coupling constant of only 2Hz. The rhamnose C-methyl appears
as a doublet (J = 6.5Hz) or multiplet in the region δ 0.8-1.2.

In flavonoids diglycosides, the C-1, proton of the terminal
sugar (H-1’”), being relatively remote from the flavonoid nucleus,
resonates upfield from H-1’”. The extent, however, can vary
depending upon the position of attachment of terminal sugar61.
Methylated41 and acetylated41,61-62 derivatives have also been used
for disaccharide linkage determination.
**Acetyl and Methoxyl Protons:**

In the NMR spectra of acetylated flavonoids (CDCl₃) the position of methyl signals of acetyl group can also give useful informations about the position of acetyl group by which the position of the hydroxyl group can be confirmed. The methyl signals of 4'- and 7-O-acetyl group appear in the range δ 2.30–2.35 ppm. While the methyl signal of a 5-O-acetyl group appears at about δ 2.45 ppm. The aliphatic acetoxyl signals of sugars generally appears in the range of δ 1.65-2.10 ppm. The position of the aliphatic acetoxyl group of sugars also help in the location of sugar moiety in C-glycosylflavonoids. Within the aliphatic acetoxyl group signals, the 2''-O-acetyl signal appears at δ 1.70-1.75 ppm in 8-C-glycosylflavonoids and δ 1.80-1.83 ppm in 6-C-glycosylflavonoids and 6''-O-acetyl in 8-C-glycosylflavonoid appears at δ 1.90-1.95 ppm and in 6-C-glycosylflavonoids it appears between δ 1.98-2.04 ppm.

Methoxyl proton signals with few exceptions appear in the range of δ 3.5–4.1 ppm.
The mass spectra of a wide variety of organic natural products have been studied only during last few years. The introduction of inlet system suitable for volatilization of high molecular weight ($M^+$, 300-1200) organic materials has greatly increased the utility of mass spectrometry. Generally the fragmentation is related to the structure of the intact molecule. Electron impact mass spectrometry of both flavonoid aglycone and glycosides serves as a valuable aid in determining their structures, especially when only very small quantities (i.e. less than 1 mg) of the compounds are available. It has been applied successively to all classes of flavonoid aglycones and recently to a number of different types of glycosides. The flavonoid aglycones and glycosides have been subjected to GLC-Mass spectrometry in the form of their permethyl ethers, perdeuteriopropyl ethers and trimethylsilyl ethers.

**Flavones**

The most flavonoids yield intense peaks for the molecular ion ($M^+$) and indeed this is often the base peak. In addition to the molecular ion, flavonoids usually afford major peaks for $[M-H]^+$ and, when methoxylated ($M-\text{CH}_3$)$^+$. Perhaps the most useful fragmentation in terms of flavonoid identification are those which involves the cleavage of intact A- and B-ring fragments. Kingston has recently discussed in detail the mass spectra...
of a large number of flavones, flavonols and their ether derivatives. He has summarized the number in which mono-flavones fragment as follows:

(a) Flavones with fewer than four hydroxy groups do not readily fragments, a consequence of the stability of their molecular ion.

(b) Flavones with fewer than four hydroxy groups tend to undergo decomposition predominantly by way of the retro-Diels-Alder (RDA) process\(^68,69\). This and other common fragmentation processes are shown in (Scheme-1) using apigenin (XV)\(^68\) as a typical example.

(c) An \((M-1)^+\) ion is often found in the mass spectra of flavones, its origin is, however, obscure.

(d) The presence of ion (C) (Scheme-1), frequently more intense when a 3-hydroxy group is present, is attributed to the alternative mode of retro-Diels-Alder fragmentation as depicted in Scheme-1.

(e) Doubly charged ions are frequently present.

(f) When heavily substituted with hydroxyls and methoxyls, the flavones tend to fragment in a less predictable manner, retro-Diels-Alder process becomes insignificant and the spectrum is dominated by the molecular ion and ions at \(M-15, M-28\) and \(M-43\)\(^69,75\).
**Flavanone**

Flavanones (XVI) typically fragment by the RDA reaction (path-A and path-B) and yield same ions from path-A as observed for flavanones. However, the most important B-ring ion from path-A contain an ethylene group\(^6\)\(^8\). The fragmentation pattern are given in Scheme-2.

Another mode of cleavage, that helps to characterize the flavanone is the loss of either a hydrogen atom \((M-H)^+\), an aryl radical at C\(_2\) \([M-(B\text{-ring})]^+\) from the molecular ion to give an even electron fragment.
The presence of a hydroxyl or methoxyl group at 4'-position of ring B facilitates. By enhance resonance stabilization of the resulting fragment ion, the formation of p-hydroxy benzyl or p-methoxy benzyl ion \(^{(XVII)}\) or their equivalent tropylium ion. The p-hydroxy/p-methoxybenzyl ion appears as a base peak of significant intensity in the mass spectrum of naringenin/its methyl ether \(^{(XIX)}\).
In the case of 4'-methoxyflavanone (XX) (Scheme-2), $B_3^+$ ion is the base peak while $A_1^+$ and $(A_1+H)^+$ fragment have relative intensities of only 3.5 and 30% respectively. Other ions from 4'-methoxyflavanone are derived by the loss of a hydrogen radical to give the $(M-H)^+$ ion and loss of an aryl radical to produce the diagnostic $[M-(B\text{-ring})]^+$ fragment. Fission of B-ring from the remainder of the molecular ion accompanied by a hydrogen transfer leads to a moderately intense B-ring ion at m/z 108.

The mass fragmentation of 3,5,7-trihydroxy-4'-methoxyflavanone (XXI) is shown in Scheme-3, shows the $[A_1+H]^+$ ion as base peak rather than $B_3^+$ as base peak. Although the intensity of $B_3^+$ is 89%. A most important feature in the mass spectrum is the presence of $A_2^+$ ion (XXII) of 100% intensity formed by cleavage of B-ring accompanied by a hydrogen transfer.

**Flavonoid glycosides**

Mass spectrometry has been extensively used in the structure elucidation of flavonoid O-glycosides as well as flavonoid C-glycosides. Mass spectrometric sequencing of oligosaccharide derivatives has been much refined to furnish not only the sequence of sugars involved but also in many cases the position of their interglycosidic linkage and even information about the stereochemistry at the anomeric centre.
The mass spectrometric study of glycosides can be done in two groups:

(i) Mass spectrometry of Flavonoid O-glycosides.

(ii) Mass spectrometry of Flavonoid C-glycosides.

Flavonoid O-glycosides

The position of a sugar residue in a flavonoid aglycone can be easily recognized from the mass spectrum of the permethylated glycoside. The sugar attached to the position 5 and 3 splits more readily than those at position 7 and as a result the molecular ion peak is of very low intensity or totally absent.

On the other hand, 7-O-glycosides usually show an intense molecular ion peak amounting to 50% or higher of the base peak. The 4' and 3-O-glycosides represent an intermediate case, having small but distinct molecular ion peak.

The sequencing of sugars in flavonoid oligosaccharide derivatives can be determined by mass spectrometry. The principal fragmentation pattern of perdeuteriomethylated flavonoid disaccharide is illustrated in Scheme-4.

Fission of the glycosidic carbon-oxygen bond of the terminal sugar leads to the ion $T_1$ which successively losses $\text{CD}_3$-methanol to give $T_2$ and $T_3$. 
Rupture of the ethereal carbon-oxygen bond between the terminal and the second sugar gives rise to the sequence ion $S$. Fission of the carbon-oxygen bond between the sugar and the aglycone is indicated by the fragment $A$, invariably formed by transfer of hydrogen and followed by loss of CO.

Retention of charge on the disaccharide residue after this type of rupture leads to the oligosaccharide ion $OS$. Peaks due to RDA-cleavage of the flavonoid aglycone are small or absent as it is often observed in highly substituted compounds.\textsuperscript{74}
Sequence of sugars and position of Interglycosidic linkage:

Information about the terminal sugar is obtained from the difference \((M^+ - S)\) and from peaks due to ions of the T-series. The T-series is more reliable induction since the \((M^+ - S)\) value can be charged by H-transfer or, in the case of apiosyl containing compounds, even more complicated reaction. In compounds containing glucose as the second sugar moiety, the OS-T\(_1\) rather than the \((S-A)\) difference seems to be the more useful for mass identification.

Differences in hydrogen transfer to some peaks allow prediction of the position of interglycosidic linkage. The sequence peak S is formed without hydrogen transfer (flavonols) or with single hydrogen transfer (flavone, flavanone) in the case of 1 \(\rightarrow\) 6 linked flavonoid disaccharide derivatives, while transfer of two hydrogens takes place in the case of 1 \(\rightarrow\) 2 linked compounds. The aglycone and the oligosaccharide fragments A and OS behave similarly. For 1 \(\rightarrow\) 6 linked derivatives the A+H and OS peaks are prominent, while A+2H and OS+H peaks are observed for 1 \(\rightarrow\) 2 linked compounds. Further \((OS-CD_3-Methanol)\) differentiation as possible by a strong \((OS-CD_3-Methanol)\) peaks present only in the 1 \(\rightarrow\) 2 linked glycosides, and by the S+62 (flavone, flavanone) or S+63 (flavanonols) peak observed only in 1 \(\rightarrow\) 6 linked compounds in Scheme-5.
Flavonoid C-glycosides

The direct mass spectral analysis of a C-glycosylflavone rarely produces an observable molecular ion, the base peak is usually the aglycone fragment with only a CH$_2^+$ group remaining of the original C-glycosyl moiety. This ion is established by rearrangement to a tropylium ion. The additional hydrogen is derived from a sugar hydroxyl group. The other fragments come from pathway-I and II process from the base peak ion giving the B$_2^+$, B$_1^{++}$, A$_1^+$ ions. Besides these fragments, other fragments corres-
ponding to ion (M-18)$^+$, (M-36)$^+$, (M-54)$^+$ are formed by the successive loss of water molecules from the molecular ion.

Mass spectrometry also help to distinguish 6- and 8-C-glycosylflavones as in the case of 6-C glycosylflavone (M-148)$^+$ peak (equivalent to aglycone + $\text{CH}_2^+ + \text{H}$) has 50% to 80% of intensity compare to (M-149)$^+$ peak whereas with 8-C-glycosylflavones (M-148)$^+$ ion is usually only about 25% of the intensity of the (M-149)$^+$ peak.

In the case of di-C-glycosides, the base peak corresponds to $[M-C_5H_{10}O_4 - C_5H_9O_5]^+$ in (XXIII) accord with the presence of a C-linked rhamnose and glucose in this compound is formed. A characteristic of the spectra of di C-glycosides is the series of peaks related to the sequential loss of six molecules of water from the molecular ion, compared with only three from mono-C-glycosides. The mass fragmentation of 8-C-glycosyl-4'-methoxy apigenin (XXIV) is given in Scheme-6.

![XXIV](image)
**Biflavonoids**

A more specific study of the mass spectral fragmentation of the permethyl ether derivatives of amentoflavone, cupressuflavone and hinokiflavone have reported by Seshadri and co-workers. The mass spectra of hexamethyl ether of amentoflavone (XXV), cupressuflavone (XXVI) are similar, molecular ion being the base peak in each case. Together with RDA reaction these compounds also undergo (a) fussion of the C–C or C–O–C linkage between aromatic residues (b) elimination of CO and CHO from the biphenyl ethers (c) rearrangement involving condensation between the phenyl rings.

**C–C linked biflavonoids :**

**Amentoflavone hexamethyl ether (XXV)**

The mode of fragmentation of amentoflavone hexamethyl ether is shown in Scheme-7.

![Scheme-7](image-url)
Amentoflavone hexamethyl ether

\[ \text{Scheme-7} \]
Main peaks (m/z : intensity) : 622 (100), 621 (31), 607 (33), 592 (8), 576 (10), 312 (2), 245 (5), 181 (2), 135 (16) and 132 (3).

Cupressuflavone hexamethyl ether (XXVI)

The mode of fragmentation of cupressuflavone hexamethyl ether (XXVI) is given in Scheme-8.

![Cupressuflavone hexamethyl ether (XXVI)](image)

Main peaks : 622 (100), 621 (38), 607 (8), 592 (18), 576 (4), 312 (7), 311 (14), 245 (11), 135 (26), and 132 (14).

C-O-C-Linked biflavonoids :

Hinokiflavone pentamethyl ether (XXVII)

The mode of fragmentation of hinokiflavone pentamethyl ether (XXVII) which contain a biphenyl ether system, is considerably different from those of amentoflavone, cupressuflavone and agathisflavone hexamethylethers Scheme-9.
Cupressuflavone hexamethyl ether

\[ \text{M}^+, \text{m/e} \ 622(100) \]

\[ \text{m/e} \ 576(4) \]

\[ \text{m/e} \ 621(33) \]

\[ \text{m/e} \ 607(8) \]

\[ \text{m/e} \ 592(18) \]

\[ \text{m/e} \ 135(26) \]

\[ \text{m/e} \ 132(14) \]

(490\(^{++}\) appears at m/e 245)

Scheme 8
Main peaks: 608 (39), 607 (12), 593 (36), 560 (4), 579 (11), 578 (11), 576 (6), 431 (7), 327 (23), 313 (100), 312 (32), 311 (22), 304 (2), 297 (29), 296 (75), 281 (22), 181 (11), 180 (3), 135 (19) and 132 (18).
Scheme-9
REFERENCES


