Chapter 2

Isolation and Structure Identification of Flavonoids

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Additional information is available at the end of the chapter

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Abstract

Flavonoids, which possess a basic C15 phenyl-benzopyrone skeleton, refer to a series of compounds in which two benzene rings (ring A and B) are connected to each other through three carbon atoms. Based on their core structure, flavonoids can be grouped into different flavonoid classes, such as flavonols, flavones, flavanones, flavanonols, anthocyanidins, isoflavones and chalcones. Flavonoids are often hydroxylated in positions 3, 5, 7, 3′, 4′ and/or 5′. Frequently, one or more of these hydroxyl groups are methylated, acetylated, prenylated or sulfated. In plants, flavonoids are often present as O- or C-glycosides. The O-glycosides have sugar substituents bound to a hydroxyl group of the aglycone, usually located at position 3 or 7, whereas the C-glycosides have sugar groups bound to a carbon of the aglycone, usually 6-C or 8-C. The most common carbohydrates are rhamnose, glucose, galactose and arabinose. This chapter mainly introduces the methods of isolation and structure identification of flavonoids.

Keywords: flavonoids, structures and classification, extraction, isolation, structure identification

1. Introduction

Flavonoids are important natural organic compounds of secondary metabolites that are produced during the long process of natural selection. They widely exist in the roots, stems, leaves, flowers and fruits of higher plants and ferns [1], which are of numerous categories and chemical structures. Because of the special chemical structures, flavonoids possess wide range of physiological and biochemical effects to the cells of mammal and other kinds of animals. Firstly, flavonoids possess strong chemical reactivity. For example, some flavonoids have antioxidant activity via scavenging free radicals in organism [2]. Furthermore, flavonoids possess various pharmacological activities of inhibiting the activity of enzymes, antitumor,

antibiosis, antivirus, anti-inflammatory and so on [3–9]. The potential treatment and prevention effects have been shown in degenerative diseases such as tumors, aging and cardiovascular diseases [10–15]. Additionally, some compounds of flavonoids possess potential application prospects as weak hormones at treating menopausal syndrome of women [14–17].

2. Structures and classification

Flavonoids generally refer to the natural products of C_6-C_3-C_6 basic structure. Most of them are the chromone derivatives with the core structure of 2-phenylchromone and made up of three rings of A/B/C as shown in **Figure 1**.

According to connection mode of ring A with B, the connection position of ring B, the oxidation level of C_3 substructure and degree of polymerization, various type of flavonoids could be classified, as shown in **Table 1**.

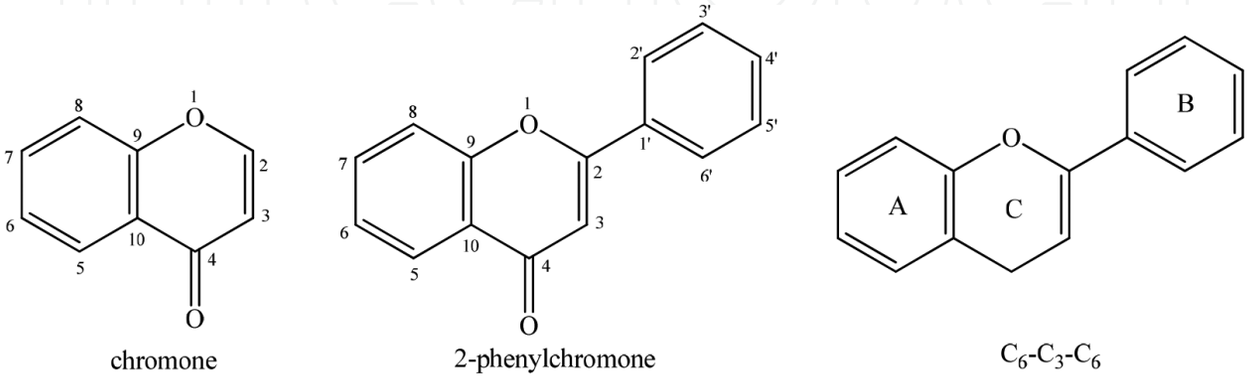
The main factors of the structure diversity of flavonoids are as follows:

2.1. Change of ring system, degree of oxidation and number of core structure

Most of the flavonoids possess the core structure of C_6-C_3-C_6; few of them are C_6-C_1-C_6 (xanthone, for example). A few of them, such as homoisoflavones and rotenoids, possess C_6-C_4-C_6 structure skeleton. In most cases, C_3 part is formed to be a hexatomic or pentagon ring with C_6 part. It could also be aliphatic chain, such as chalcone and dihydrochalcone. Supposing that the double bond of ring C was hydrogenated, dihydro derivative was formed, such as flavanone and flavononol. Commonly, ring B is connected to C-2; it might be connected to C-3 or C-4 in a few cases, such as isoflavone and neoflavonoid. Most of the flavonoids have only one core structure; some of them possess two, however. We called them biflavonoids.

2.2. Various substituents at ring A and B

Generally, hydroxyl, methoxyl, methyl, isopentenyl, methylenedioxyl, benzyl, nitro groups and so on, could be substituted at ring A and/or B.



C_6-C_3-C_6

Figure 1. Basic structure of flavonoids.

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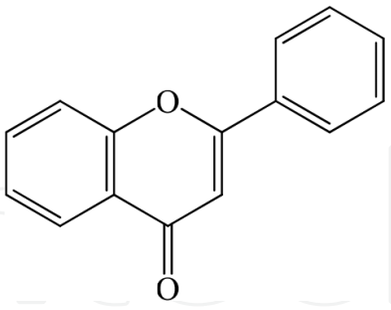
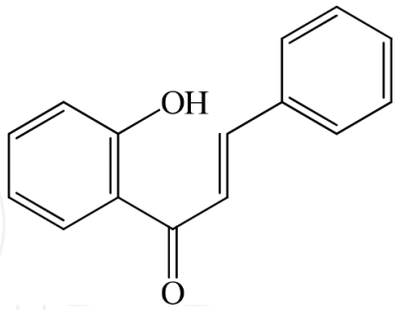
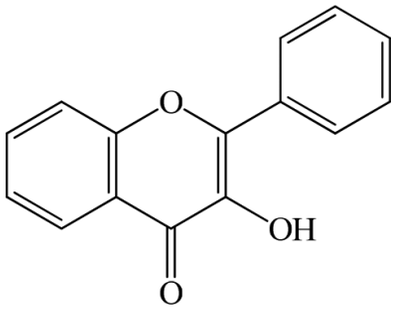
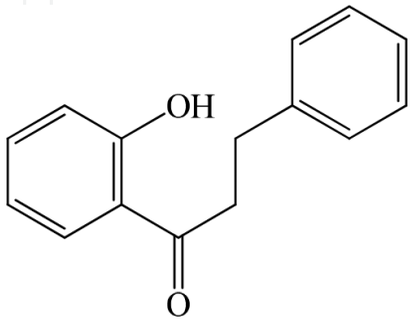
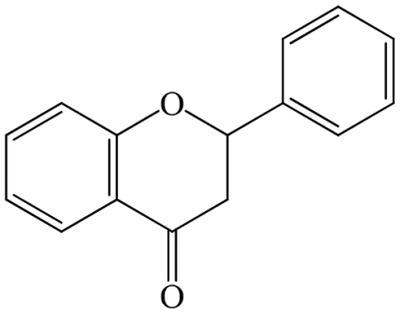
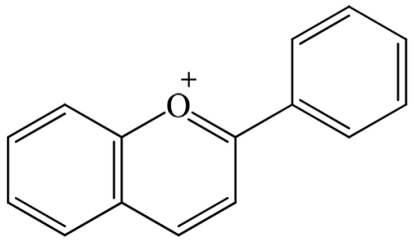
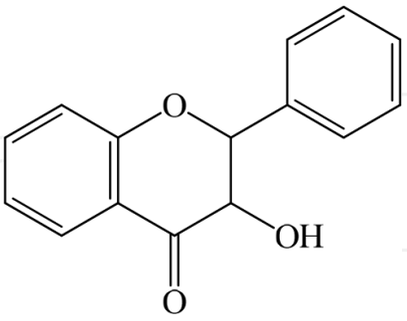
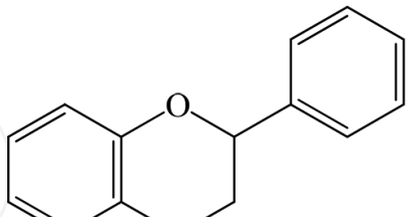
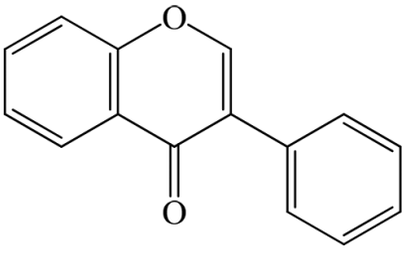
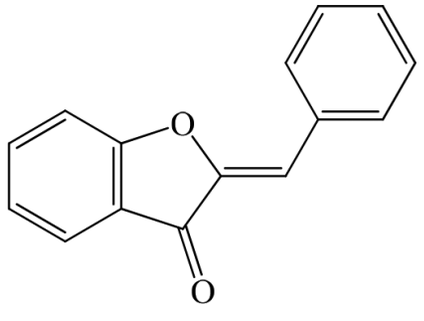
Structure type	Basic structure	Structure type	Basic structure
Flavone		Chalcone	
Flavonol		Dihydrochalcone	
Flavanone		Anthocyanidin	
Flavanonol		Flavan	
Isoflavone		Aurone	

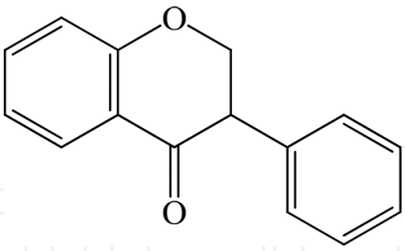
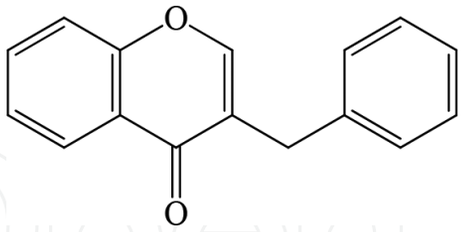
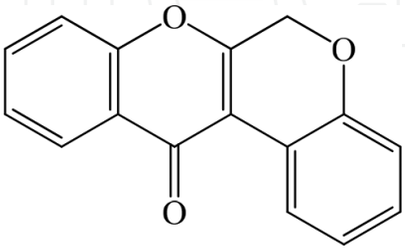
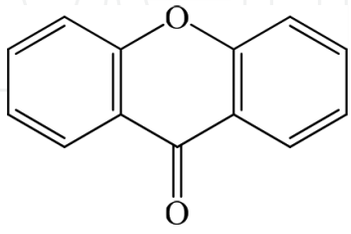
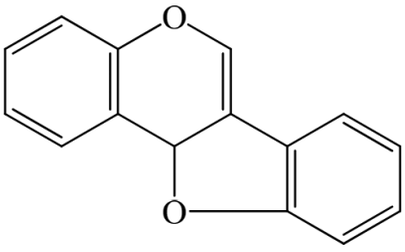
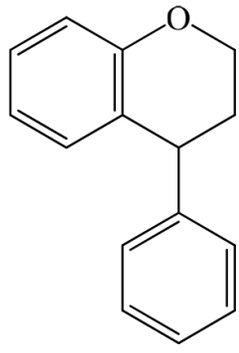
Structure type	Basic structure	Structure type	Basic structure
Isoflavanone		Homoisoflavanone	
Rotenoid		Xanthone	
Pterocarpin		Neoflavonoid	

Table 1. Main structure types of flavonoids.

2.3. Glycosidation

Flavonoids are often glycosided to be O-glycosides or C-glycosides. During the glycosidation of flavonoids, almost every hydroxyl group could be the reaction position. However, the glycosidation reaction mostly occurred at 7-OH of flavone, flavanone and isoflavone, 3- and/or 7-OH of flavonol and flavanonol 3- and/or 5-OH of anthocyanidin. The glycosyl group of C-glycosides is often connected to C-6 and/or C-8.

2.4. Formation of complexes

Complexes of flavonoids could be formed with other types of compounds, such as phenylpropanoids, coumarins and alkaloids.

3. Extraction and isolation

3.1. Extraction

Traditional extraction methods of flavonoids often cause the problems of inefficiency, high energy consumption, more solvent consumption and so on. The new extraction methods and

technologies occurred in recent years promoted the development of flavonoids. Because of the numerous types of flavonoids, the single extraction methods generally cannot meet the requirement. Traditional and modern methods should be applied together according to the extraction purpose.

3.1.1. Traditional extraction methods

Ethanol and methanol are frequently used to extract flavonoids. The common extraction methods include dipping, percolation, reflux, continuous reflux and so on. The alcohol of high concentration (90–95%) is applied to extract free flavonoids, and the alcohol at the concentration of about 60% is applied to extract flavonoid glycosides. For example, reflux method was applied to extract total flavonoids from leaves of *Ginkgo biloba* with 70% ethanol, and the product yield was significantly higher than the water decoction method [18].

Hot water extraction method is applied to flavonoid glycosides. It possesses the advantages of low cost, safety, simple equipment and could be applied in industrial production, but much water-soluble impurities, such as proteins and saccharides might be mixed into the product.

Most of the flavonoids are acidic because of hydroxyl or carboxyl groups, so they could be extracted with alkaline water or alkaline dilute alcohol. The commonly used solvents include dilute sodium hydroxide, lime water, 5% sodium hydroxide dilute ethanol solution and so on. Water-soluble impurities, such as tannins, pectins and mucilages, could be precipitated because of the formation of calcium salts during the extraction with lime water. It has often showed good results if 5% sodium hydroxide dilute ethanol solution was used. However, the product yield might be reduced because some flavonoids obtained after acidification might be dissolved in dilute ethanol solution. It should be noted that the concentration of alkali should not be excessive during the extraction, and the acidity of the solvent should not be excessive during acidification. Additionally, sodium borate should be used if adjacent phenolic hydroxyl groups are existed in the structures. Extraction of rutin from Flos Sophorae Immaturus is one example [19].

3.1.2. New extraction technologies

3.1.2.1. Supercritical fluid extraction (SFE)

The goal of selected extraction, isolation or purification might be achieved via controlling temperature, pressure and regulating the type and consumption of cosolvent during the supercritical fluid extraction. Cosolvent (e.g. ethanol) is usually added to induce product yield. For example, the product yield of supercritical CO_2 extraction of flavonoids from *Licorice* has been raised 2.2 times than the ordinary alcohol extraction [20].

3.1.2.2. Ultrasonic extraction

This method has been used in the quality analysis and small amount extraction of flavonoids. It's still seldom used in industrial production, however. For instance, ultrasonic extraction was used in the extraction of flavone from the bud of *Sophora japonica*, and the product yield was higher than reflux extraction method [21]. Ultrasonic extraction is superior to reflux method from the perspectives of energy saving, time saving and technology.

3.1.2.3. Microwave-assisted extraction

It has obtained good results in the extraction of flavonoids. However, it is confined to laboratories so far. It also can be applied combined with other methods to induce product yield. For example, refluxing extraction was used after treatment with microwave for a short time during the extraction of flavonoids from *Ophiopogon japonicus*. The product yield was induced significantly [22].

3.1.2.4. Enzyme method

The impurities, such as starches, pectins and proteins, could be removed after enzymolysis. Long extraction time is the limitation of this method. However, the mild operational conditions could overcome the shortcomings that some bioactive components may be decomposed under high temperature.

3.1.2.5. Macroporous adsorption resin

It has been used in the separation and enrichment of flavonoids. Suitable types should be chosen according to the nature of target constituents.

3.1.2.6. Ultrafiltration

The molecules of different molecular weight are separated depend on the pressure difference between both sides of ultrafiltration membrane. Proteins, polypeptide, polymeric pigments and starches could be removed largely. It possesses advantages of simple operations such as no need to heat and destroy the molecular structures. It could remove 69.4% pectins and 66% proteins during the preparation of soybean isoflavones [23].

3.1.2.7. Aqueous two phase extraction (ATPE)

Aqueous two phase system (ATPS) is formed when either two polymers and kosmotropic salt, or two salts (one chaotropic salt and the other a kosmotropic salt) are mixed at appropriate concentrations and at a particular temperature. The distribution coefficients are different in specific ATPS of different substances. The separation objective will be achieved via selective distribution between the two phases after adding substances into the system. It possesses advantages of time-saving, simple operation, mild condition, being easy to expand process, large treatment capacity and so on. The commonly used ATPS are high polymer system (e.g. PEG-Dextran system), high polymer-inorganic salt system and PEG-sulfate/phosphate system. The distribution characteristics of puerarin in the two-phase aqueous systems of PEG/$(NH_4)_2SO_4$ and acetone/K_2HPO_4 have been studied [24], and the best system has been determined.

3.2. Isolation

3.2.1. General methods

The isolation of flavonoids includes the separation of flavonoids and other kinds of compounds, and the obtaining of monomeric compounds. The choosing of isolation methods is

made primary according to polarity, acidity, molecular weight difference and special structure. Chromatography is still the first choice to isolate flavonoids.

3.2.1.1. Silica gel chromatography

Silica gel chromatography is the main method to isolate or identify flavonoids. It is applied to isolate low or medium polar constituents. Reversed phase silica gel (e.g. reversed phase C_{18} silica gel) is commonly used to isolate flavonoid glycosides.

3.2.1.2. Polyamide chromatography

Polyamide is a good adsorbent to isolate flavonoids. The adsorption strength hinges on hydrogen bonding associated between polyamide and flavonoids, which depends on the number and positions of hydroxyl groups in the molecules of flavonoids.

3.2.1.3. Polydextran gel chromatography

The most commonly used polydextran gel is sephadex LH-20 during the isolation of flavonoids. Adsorption is the main mechanism during the isolation of free flavonoids, and the adsorption strength is mainly based on the phenolic hydroxyl groups. However, molecular sieve effect plays the leading roles during the isolation of flavonoid glycosides.

3.2.2. Application of new isolation technologies

3.2.2.1. High-performance liquid chromatography (HPLC)

This technology has been widely used in the isolation and quality analysis of flavonoids and other kinds of natural products. The determination of chromatographic condition is the key to achieve separation purpose.

3.2.2.1.1. Choice of stationary phases

Silica gel and amino columns are mostly used during the operation of normal phase chromatography. In the reversed phase, HPLC (RP-HPLC), C_{18}, C_8, C_2, amino or phenyl columns could be applied, whereas C_{18} and C_8 columns are mostly used among them.

3.2.2.1.2. Choice of mobile phases

Methanol-water and acetonitrile-water system are commonly applied in RP-HPLC. In order to improve separation performance, minute quantity of acid (e.g. trifluoroacetic acid) could be added into mobile phase.

3.2.2.1.3. Detection

All of the flavonoids are able to absorb ultraviolet rays, so generally they could be detected by UV detectors. It is usually detected at 254–280 nm or 340–360 nm for flavones, flavonols and the corresponding glycosides, 520–540 nm for anthocyanidins and the corresponding glycosides, 250 nm for chromones.

3.2.2.2. High-speed counter current chromatography (HSCCC)

High-speed counter current chromatography (HSCCC) has been applied successfully to the isolation of flavonoids. The method is simple and quick to operate, and could get product with high purity. Furthermore, it is suitable to industrial production. For example, an HSCCC system has been employed to separate seven flavonoids from a methanolic extract of the leaves of *Oroxylum indicum* by a one-step isocratic elution using a chloroform-methanol-water (9.5:10.5) two-phase system [25].

3.2.2.3. Molecular imprinting technology (MIT)

Molecular imprinting technology (MIT) has been applied in recent years to isolation and active screening of flavonoids. As the study [26] of Pakade et al., molecularly imprinted polymers (MIPs) targeting quercetin were prepared from 4-Vinylpyridine and ethylene dimethacrylate (EDMA) under various solvent systems with the aim to form MIPs with high recognition for the quercetin molecule in aqueous systems at high temperature. The slopes for the effect of extraction time revealed that the mass transfer of the analytes was higher at 84°C than at 25°C. Also, the binding capacity for the most promising MIP and its corresponding NIP was higher at 84°C. The binding capacity for the MIP was similar to 30 μmol/g at 25°C and 120 μmol/g at 84°C, while for the corresponding NIP, it was similar to 15 and 90 μmol/g, at 25 and 84°C, respectively.

4. Structure identification of flavonoids

Generally, structure determination of flavonoids can be achieved easily because of the systematic research of their structures and the progress of spectroscopic technologies (Nuclear Magnetic Resonance spectroscopy, especially). Series of spectroscopic technologies, such as IR, UV, NMR, and MS, are often used during structure identification of flavonoids. In rare cases, total synthesis should be applied to verify the elucidated structures.

4.1. Ultraviolet spectrum (UV)

The positions, types and number of substituents in the conjugated systems could be speculated via means of UV spectrum. Most of the flavonoids in methanol possess two main absorption bands. Band I is at 300–400 nm, which is caused by electron transition of cinnamoyl group. Band II is at 240–280 nm, which is caused by electron transition of benzoyl group, as shown in **Figure 2**. The structure types and oxygen-bearing substituent types of flavonoids could be determined by the peak locations, shapes and strengths of band I and II, as shown in **Table 2** [27].

The locations and shapes of Band I and II will be affected by the substituents attached to rings A and B. Normally, red shift of band I increases accordingly when the number of hydroxyl groups located at ring B increases. Similarly, red shift of band II increases accordingly when the number of hydroxyl groups located at ring A increases, but it has trifling impact to band I, with the exception of 5-OH. The corresponding bands will be violet shifted 5–15 nm if the

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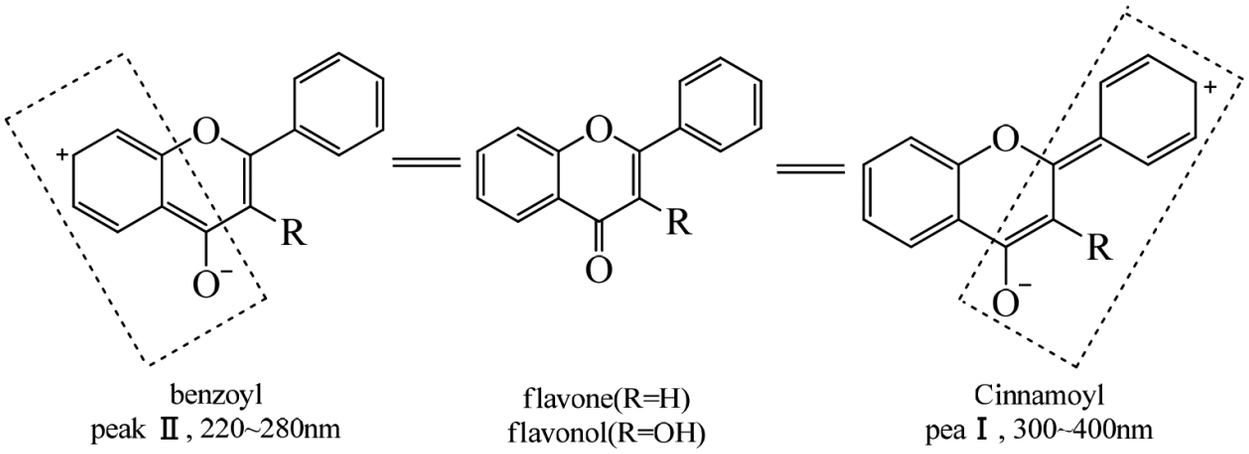

Figure 2. UV spectrum of flavonoids.

Structure type	Band II (nm)	Band I (nm)
Flavone	250–280	304–350
Flavonol (3-OH is substituted)	250–280	328–357
Flavonol (3-OH is free)	250–280	358–385
Isoflavone	245–270	310–330 (shoulder peak)
Flavanone and flavanonol	270–295	300–330 (shoulder peak)
Chalcone	220–270 (weak peak)	340–390
Aurone	230–270 (weak peak)	370–430
Anthocyanidin	270–280	465–560

Table 2. The spectral characteristics of UV-VIS spectrum of flavonoids.

particular hydroxyl is glycosided. Furthermore, the influence of the hydroxyl groups will almost disappear if they are acetylated.

4.2. Infrared spectrum (IR)

It is used mainly to determine the types of functional groups, substitution modes of aromatic rings and so on. The all functional groups, such as carbonyl, phenolic hydroxyl, phenyl and glycosyl, have possessed corresponding IR absorptions. The absorption band of hydroxyl groups are in the 3200–3650 cm^{-1} region, carbonyl groups are in 1660–1680 cm^{-1} region and the vibrations of benzene rings are at about 1500, 1580 and 1600 cm^{-1}.

4.3. Nuclear magnetic resonance spectrum (NMR)

Nuclear magnetic resonance spectrum (NMR) is the most powerful method to elucidate the structures of flavonoids. Kinds of solvents, such as $CDCl_3$, DMSO-d_6, C_5D_5N, $(CD_3)_2CO$ and CD_3OD, could be employed while performing NMR experiments. DMSO-d_6 is the optional solvent among them to perform NMR to flavonoids. Almost all kinds of flavonoids could be

well dissolved in DMSO-d_6, and the resonance signals of flavonoids are rarely overlapped by solvent peaks (about δ2.5). Furthermore, NMR signals of phenolic hydroxyl groups could be displayed clearly with DMSO-d_6 as the solvent. The drawback of this solvent is high boiling point, which leads to difficulty in sample recovery.

4.3.1. ^{1}H-NMR spectrum

It provides information of chemical shifts, coupling constants and proton number. The types of flavonoids, substituted modes, number and configurations of glycosyls and so on, could be determined via ^{1}H-NMR spectrum.

4.3.1.1. Protons on ring C

^{1}H-NMR characteristics of protons on ring are shown in **Table 3** [28].

4.3.1.2. Protons on ring A

The ordinary substitution modes are 5,7-dioxygenation, 7-oxygenation, 5,6,7-trioxygenation and 5,7,8-trioxygenation, See **Figure 3**.

4.3.1.2.1. 5,7-Dihydroxyl substituted

5,7-Dihydroxyl flavonoids are most common. For this type of flavonoids, the signals of H-6 and H-8 are shown at δ5.7–6.9 as doublets, and the signal of H-6 is always at the higher field than H-8. The signals of both H-6 and H-8 shift to lower field after glycosidation of 7-OH.

Type	2-H	3-H	Note
Flavanone		δ6.3– 6.8 (s)	The signals maybe overlapped by H-6 or H-8.
Flavonol	None signal.		
Isoflavone	δ7.6–7.8 (s)		The signal is at rather low field because of influence of oxygen atom at position 1 and carbonyl at position 4.
Chalcone	α-H: δ6.7–7.4 (d, J = 17 Hz) β-H: δ7.3–7.7 (d, J = 17 Hz)		C-2 and C-3 form a *trans* double bond.
Flavanone	δ5.0–5.5 (dd, J = 11, 5 Hz)	δ2.3– 2.8 (2H) (dd, J=17, 11 Hz); (dd, J = 17, 5 Hz)	H-2 is coupled by two protons of position 3.
Flavanonol	4.8–5.0 (d, J = 11 Hz)	δ4.1–4.3 (d, J = 11 Hz)	Configurations of both C-2 and C-3 are R.
Flavanone-3-O-glycoside	δ5.0–5.6	δ4.3–4.6	After glycosidation of 3-OH, resonance signals of both H-2 and H-3 shift to low field.
Aurone	Exocyclic proton: δ6.5–6.7 (s)		

Table 3. Chemical shifts and coupling constants of ring C of common flavonoids.

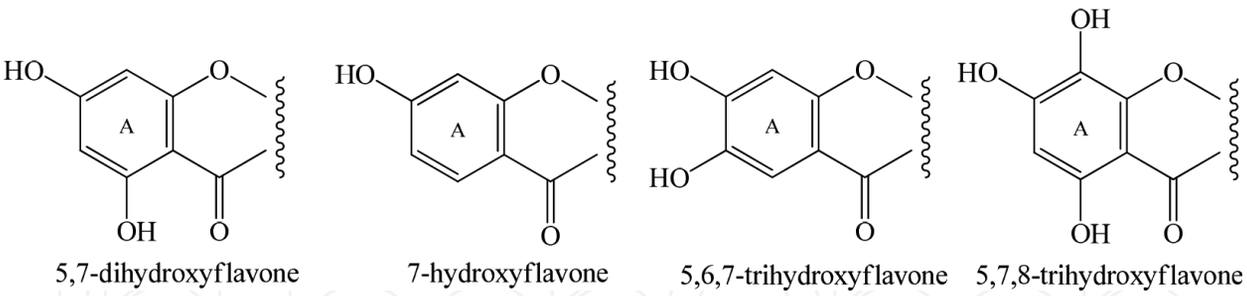

Figure 3. Substitution modes of ring A.

4.3.1.2.2. 7-Hydoxyl substituted

Signal of H-5 is shown to be a doublet since vicinal coupling exists between H-5 and H-6. Additionally, the chemical shift is at rather low field (about δ8.0) because of the shielding effect of carbonyl at position 4. H-6 is affected by H-5 and H-8, so it has showed a double-doublet (dd, J=2.0, 8.0 Hz). H-8 is showed to be a doublet (J=2.0Hz) because of the vicinal relationship with H-6. Signals of both, H-6 and H-8 are at δ6.3–7.1. The chemical shifts of protons on ring A are shown in **Table 4** [28].

4.3.1.3. Protons on ring B

There are a variety of substituted modes of ring B, such as non-substitution, 4′-oxygenation, 2′-oxygenation, 3′,4′-dioxygenation, 2′,4′-oxygenation, 3′,4′,5′-trioxygenation and 2′,4′,5′-trioxygenation, as shown in **Figure 4**. Generally, signals of protons on ring B are showed at slightly lower field, and the chemical shifts are usual at δ6.7-8.1. The substitution modes and structural information could be determined via the chemical shifts and coupling constants of ring B.

4.3.1.3.1. None substituent on ring B

For this mode, there are five protons on ring B. Signals of H-2′ and H-6′ are shown at lower field than H-3′, H-4′ and H-5′ because of the shielding effect of ring C. Furthermore, the peak shapes of all of the protons are complicated because of the coupling effects of the vicinal- and

	Type	H-5	H-6	H-8
5,7-Dihydroxyl	Flavone, flavonol, isoflavone		δ6.0–6.2 d	δ6.3–6.5 d
	7-O-Glucosides of above compounds		δ6.2–6.4 d	δ6.5–6.9 d
	Flavanone, flavanonol		δ5.75–5.95 d	δ5.9–6.1 d
	7-O-Glucosides of above compounds		δ5.9–6.1 d	δ6.1–6.4 d
7-Hydroxyl flavonoids	Flavone, flavonol, isoflavone	δ7.9–8.2 d	δ6.7–7.1 d	δ6.7–7.0 d
	Flavanone, flavanonol	δ7.7–7.9 d	5.7–6.0 d	δ5.9–6.1 d
5,6,7-Trihydroxyl flavonoids				δ6.95 s
5,7,8-Trihydroxyl flavonoids			δ6.3 s	

Table 4. Chemical shifts of protons on ring A.

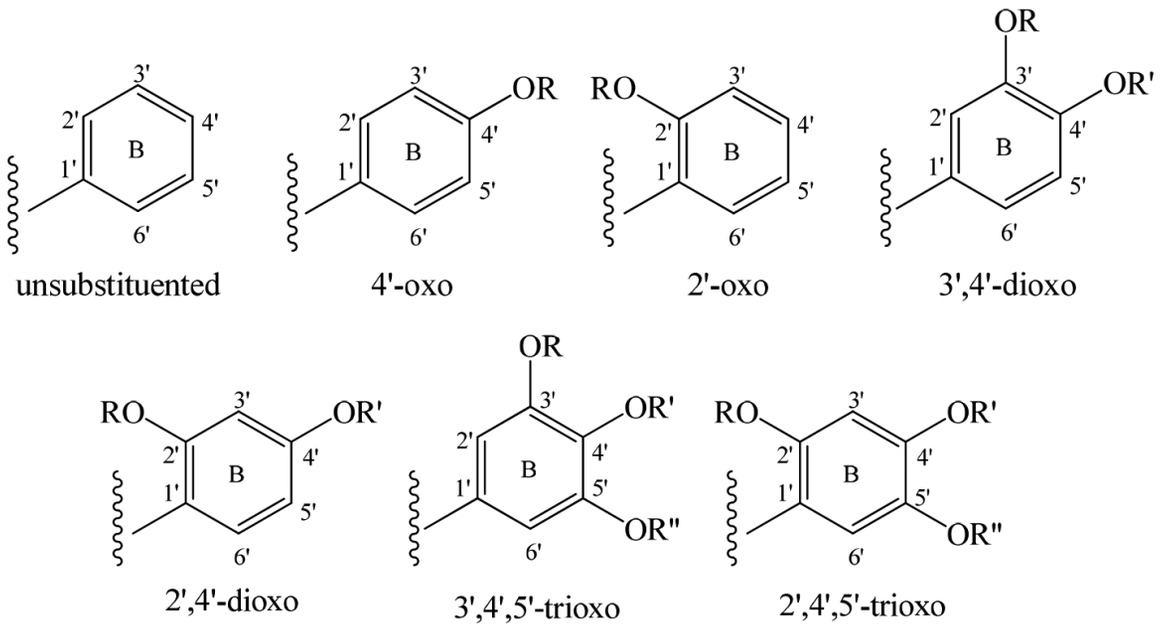

Figure 4. Substitution modes of ring B.

meta-coupling. The signals of H-2′ and H-6′ are usually at *δ*7.1–7.6 and of H-3′, H-4′ and H-5′ are at *δ*7.9–8.2.

4.3.1.3.2. 4′-Oxygenation

In this circumstance, ring B is a symmetrical substructure. One AA′BB′ coupling system is formed by four aromatic protons. The spectral characteristics are show in **Table 5** [28].

4.3.1.3.3. 3′,4′-Dioxygenation

In this circumstance, one ABX coupling system is formed by three aromatic protons, and three groups of signals are displayed as H-2′(1H, d, $J \approx 2.0$Hz), H-5′(1H, d, $J \approx 8.0$Hz) and H-6′(1H, dd, $J \approx 2.0$, 8.0Hz). The chemical shifts of protons on ring B are shown in **Table 6 [**28].

Type	**H-2′, H-6′**	**H-3′, H-5′**
Flavanone	*δ*7.1–7.3	
Flavanonol	*δ*7.2–7.4	
Isoflavone	*δ*7.2–7.5	
Chalcone	*δ*7.4–7.6	*δ*6.5–7.1
Aurone	*δ*7.6–7.8	
Flavone	*δ*7.7–7.9	
Flavonol	*δ*7.9–8.1	

Table 5. Chemical shifts of protons on ring B of 4′-oxygenated flavonoids.

Type	Substituent mode	H-2′	H-5′	H-6′
Flavone	3′,4′-OH and 3′-OH, 4′-OCH_3	*δ*7.2–7.3	*δ*6.7–7.1	*δ*7.3–7.5
Flavonol	3′,4′-OH and 3′-OH, 4′-OCH_3	*δ*7.5–7.7		*δ*7.6–7.9
	3′-OCH_3, 4′-OH	*δ*7.6–7.8		*δ*7.4–7.6
	3′,4′-OH, 3-O-glc	*δ*7.2–7.5		*δ*7.3–7.7
Isoflavone, flavanone, flavanonol	3′,4′-OH	*δ*6.7–7.1		*δ*6.7–7.1

Table 6. Chemical shifts of protons on ring B of 3′,4′-dioxygenated flavonoids.

4.3.1.3.4. 2′-Oxygenation

ABCD coupling system is formed by the rest protons of ring B. The peak shapes are rather complicated. Signals of H-3′ and H-5′ are usually displayed at *δ*6.8–6.9, H-4′ at about *δ*7.2 and H-6′ at *δ*7.4–7.5.

4.3.1.3.5. 3′,4′,5′-Trioxygenation

If identical substituents are attached to C-3′ and C-5′, which allows the formation of a symmetrical substructure of ring B, H-2′ and H-6′ will display to be a singlet at *δ*6.5–7.5.

4.3.1.3.6. 2′,4′,5′-Trioxygenation

In the cases of this substituent mode, either of the two protons on ring B displays to be a singlet. Generally, signals of H-6′ in flavones and flavonols are showed at *δ*7.2–7.5, H-3′ at *δ*6.4–6.6. Signals of H-6′ are shown at slight higher field.

4.3.1.3.7. 2′,4′-Dioxygenation

In the cases of this mode, H-3′ will be showed at *δ*6.00–6.6 (d, $J \approx 2.0$ Hz), H-5′ at *δ* 6.6–6.5 (dd, $J \approx 2.0$, 8.0 Hz) and H-6′ at *δ*7.0–7.4 (d, $J \approx 8.0$ Hz). See **Table 7**.

4.3.1.4. Common substituents

The proton chemical shifts of common substituents of flavonoids are shown in **Table 8** [28].

4.3.2. ^{13}C-NMR spectrum

Strong regularities are also shown in ^{13}C-NMR spectra of flavonoids. The types of flavonoids, number and connection positions of glycosyls could be elucidated from ^{13}C-NMR spectra.

4.3.2.1. Identification of skeleton structures of flavonoids

The core structures are difficult to be elucidated by resonance signals of aromatic protons. However, the characteristic signals of carbons in ring C allowed the identification of different types of flavonoids, see **Table 9**.

Substituent mode	H-2′	H-3′	H-4′	H-5′	H-6′
None substituent on ring B	δ7.1–7.6 (m)	δ7.9–8.2 (m)	δ7.9–8.2 (m)	δ7.9–8.2 (m)	7.1-7.6 (m)
2′-Oxygenated		δ6.8–6.9 (m or dd)	δ7.2 (m or dd)	δ6.8–6.9 (m or dd)	7.4–7.5 (m or dd)
4′-Oxygenated	δ7.1–8.1 (2H, d, *J*≈8.0Hz)	δ6.5–7.1 (2H, d, *J*≈8.0Hz)		δ6.5–7.1 (2H, d, *J*≈8.0Hz)	7.1–8.1 (2H, d, *J*≈8.0Hz)
2′,4′-Dioxygenation		δ6.00–6.6(d, *J*≈2.0Hz)		δ6.30–6.50 (1H, dd, *J*≈8.0,2.0Hz)	7.0–7.4(d, *J*≈8.0Hz)
3′,4′-Dioxygenation	δ7.2–7.8(1H, d, *J*≈2.0Hz)			δ6.7–7.1(1H, d, *J*≈8.0Hz)	6.7–7.9(1H, dd, *J*≈2.0, 8.0Hz)
3′,4′,5′-Trioxygenation	δ6.5–7.5 (2H, s, H-2′,6′) as identical oxygen-bearing substituents are connected to position 3′,4′ and 5′.				
2′,4′,5′-Trioxygenation		δ6.4–6.6(s)			δ7.2–7.5(s)

Table 7. Chemical shifts of protons on ring B of various substituent modes [28].

Proton type	Chemical shift
Phenolic hydroxyl	5-OH (δ12.0–14.0),7-OH (δ10.8–11.0), 4′-OH (δ9.8–10.6), 3′-OH (δ9.2–10.4)
Methyl	C-6,8 (δ2.0–2.5); rha-CH_3 [δ9.2–10.4 (d, *J*≈6.5Hz)] CH_3CO [glc: δ1.65–2.10 (3H,s); aromatic -CH_3:δ2.3–2.5 (3H,s)
Methoxyl	δ3.5–4.1 (3H,s)
O-CH_2-O	δ6.0
Isopentenyl	δCH_2 (3–3.4); CH (5.2);CH_3 (δ1.7–1.8)
Terminal protons of glycosyl	δ4.5–5.5

Table 8. Chemical shifts of the protons on common substituents.

4.3.2.2. Determination of substituent modes of flavonoids

The substituent modes of core structures of flavonoids could be determined by the signals of aromatic carbons. The chemical shifts of carbons in ring A and B, if they are not substituent, are shown in **Table 10** [28].

4.3.2.2.1. Signal characteristics of ring A

Usually, the substituents, such as hydroxyl, methoxyl and isopentenyl groups, are attached at position 5 or/and 7 of ring A, which leads to the changes of chemical shifts of other carbons in ring A. It is shown in **Table 11** [28].

4.3.2.2.2. Signal characteristics of carbons on ring B

The signal characteristics of carbons on ring B are shown in **Table 12** [28].

Type	C-2	C-3	C-4
Flavone	δ160.0–165.5	δ104.0–112.0	δ175.0–184.0
Flavonol (3-OH is free)	δ145.0–149.5	δ135.6–139.0	δ172.0–178.0
Flavonol (3-OH is free)	δ155.7–157.0	δ133.2–134.3	δ172.0–178.0
Isoflavone	δ149.8–156.5	δ120.3–126.0	δ174.5–182.0
Flavanone	δ75.0–80.5	δ42.0–44.6	δ189.5–197.2
Flavanonol	δ82.7–83.5	δ71.2–73.0	δ188.0–197.0
Chalcone	δ136.9–145.4	δ116.8–128.1	δ188.0–194.6
Dihydrochalcone	δ39.2–45.5	δ28.5–31.2	δ199.6–205.5
Aurone	δ146.1–147.7	δ111.0–113.3	δ180.5–182.7
Flavan-3-ol	δ77.5–82.7	δ65.1–68.2	δ27.5–28.6
Flavan-3,4-diols	δ80.0–82.5	δ72.5–74.4	δ69.8–72.0

Table 9. Chemical shifts of carbons in ring C of flavonoids [28].

Ring A	Chemical shift	Ring B	Chemical shift
C-5	δ124–126	C-2′,6′	δ126.0–130.0
C-6	δ124–126	C-3′,5′	δ128–129
C-7	δ133–135	C-4′	δ128.5–132
C-8	δ116.5–119	C-1′ (flavone, flavonol, isoflavone)	δ130–133
C-9	δ154–156	C-1′ (flavanone, flavonol)	δ138–139
C-10	δ121–124		

Table 10. Chemical shifts of carbons in ring A and B if they are not substituent.

Substituent mode	Type	C-5	C-6	C-7	C-8	C-9	C-10
5-Oxygenated		≈δ160	δ109–111	δ135–137	δ107–108	δ157–160	δ111
7-Oxygenated	Flavone, flavonol	δ126	δ114	δ162–164	δ100–102	δ157	δ114–116
	Flavanone, flavanonol	δ128	δ109.5–110	δ165–166	δ100	δ162–164	δ114–116
	Isoflavone	δ127	δ115	δ161–163	δ102–104	δ156–158	δ117–119
5,7-Dioxygenation	Flavone, flavonol, isoflavone	δ160–162	δ97–100	δ162–165	δ93–95	δ155–158	δ103–109
	Flavanone, flavanonol	δ162–163	δ93–97	δ165–167	δ93–97	δ161–163	δ100–104

Table 11. Chemical shifts of carbons in ring A of flavonoids.

4.3.2.2.3. Signal characteristics of common substituents

The carbon chemical shifts of common substituents are shown in **Table 13** [28].

Substituent mode	Type	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′
4′-Oxygenated	Flavone, flavonol, isoflavone	δ121–123	δ130	δ115	δ157–161	δ115	δ130
	Flavanone, flavanonol	δ128–130					
3′-OH,4′-OCH_3 (or 3′,4′-dihydroxyl)	Flavone, flavonol, isoflavone	δ121–125	δ113–114	δ145–147	δ149–151	δ112–116	δ118–122
3′-OCH3,4′-OH		δ121–125	δ110–111	δ150–152	δ148	δ115–117	δ118–122
3′-OH,4′-OCH_3 (or 3′,4′-dihydroxyl)	Flavanone, flavanonol	δ128–129	δ111–115	δ144–147	δ146–148	δ112–116	δ118–120
3′-OCH_3,4′-OH		δ131–132	δ111–115	δ147–149	δ146–147	δ112–116	δ118–120
2′,4′-Dioxygenation	Flavonoids	δ108–113	δ156–158	δ102–104	δ157–162	δ104–108	δ131–132
3′,4′,5′-Trioxygenation		δ120–126	δ106–109	δ146–153	δ93–97	δ136–142	δ106–109

Table 12. Chemicals shifts of carbons on ring B of flavonoids.

4.3.3. Glycosides of flavonoids

In plants, flavonoids are often present as O- or C-glycosides. The O-glycosides have sugar substituents bound to a hydroxyl group of the aglycone, usually located at position 3 or 7, whereas the C-glycosides have sugar groups bound to a carbon of the aglycone, usually 6-C or 8-C. The most common carbohydrates are rhamnose, glucose, galactose and arabinose.

Generally, the chemical shifts of terminal protons of glycosyls are at δ4.5–5.5 in ^{1}H-NMR. The terminal carbons of O-glycosides are at δ95–105 and at δ71–78 for C-glycosides. Furthermore, the number of glycosyls could be determined by combined analysis of ^{1}H and ^{13}C-NMR spectra. It is an effective method to determine the connection positions of glycosyls by glycosylation shifts, as shown in **Table 14** [28].

The configurations of glycosyls should be determined. The relative configurations of some glycosyl groups could be determined sometimes by coupling constants of terminal protons in ^{1}H-NMR spectra. The absolute configurations, however, should be determined by chemical methods and gas chromatography.

Substituent		Chemical shift
CH_3		6-CH_3(δ6–10);8-CH_3(δ20–30);$COCH_3$(δ17–22)
OCH_3		δ55–57
Isopentenyl [-$CH_2CH{=}CHCH_3$]		CH_2(δ21–22); CH(δ122–124; CH(δ129–131); CH_3(δ17–27)
O-CH_2-O		δ100–101
Terminal carbon of glycosyls	O-glycosides	δ95–105
	C-glycosides	δ71–80

Table 13. Chemical shifts of carbons of common substituents on flavonoids.

Glycosylation position	2	3	4	5	6	7	8	9	10	1′	2′	3′	4′	5′	6′
7-O-glu					+0.8	−1.4	+1.1		+1.7						
7-O-rha					+0.8	−2.4	+1.0		+1.7						
3-O-glu	+9.2	−2.1	+1.5	+0.4					+1.0	−0.8	+1.1	−0.3	+0.7		+1.5
3-O-rha	+10.3	−1.1	+2.0	+0.6					+1.1						
5-O-glu	−2.8	+2.2	−6.0	−2.7	+4.4	−3.0	+3.2	+1.4	+4.3	−1.3	−1.2	−0.4	−0.8	−1.0	−1.2
3′-O-glu	−0.5	+0.4									+1.6		+1.4	+0.4	+3.2
4′-O-glu	+0.1		+1.0							+3.7	+0.4	+2.0	−1.2	+1.4	

Table 14. Glycosylation shifts (average values) of flavonoids in ^{13}C-NMR spectrum.

As for the spectral method, the types and configurations could be speculated by the chemical shifts of glycosyl carbons in ^{13}C-NMR spectra, as shown in **Table 15** [28].

4.4. Mass spectral characteristics of flavonoids

ESI-MS and FAB-MS are widely applied in the studies of flavonoids. While the positive ion mode is employed, quasi-molecular ion peaks such as $[M+H]^+$, $[M+Na]^+$, $[M+K]^+$ and $[M+NH_4]^+$ will be displayed. $[2M+H]^+$, $[2M+Na]^+$ and so on will also be shown if the sample is concentrated. The MS fragmentation pathways of flavone and flavanone are shown in **Figures 5** and **6**.

4.5. Determination of absolute configuration

The absolute configuration should be determined if chiral atoms are existed in the structures. The main methods to elucidate absolute configuration include circular dichroism (CD), optical rotatory dispersion (ORD) and X-ray single crystal diffraction. Circular dichroism and ORD are mainly introduced here.

Type of glycosyl	C-1″	C-2″	C-3″	C-4″	C-5″	C-6″
β-D-glu	δ104.0	δ74.1	δ76.8	δ70.6	δ76.8	δ61.8
α-D-glu	δ100.0	δ72.2	δ74.1	δ70.6	δ72.5	δ61.6
β-D-gal	δ104.5	δ71.7	δ73.8	δ69.7	δ76.0	δ62.0
α- D- gal	δ100.1	δ69.2	δ70.5	δ70.2	δ71.6	δ62.2
β-D-man	δ102.3	δ71.7	δ74.5	δ68.4	δ77.6	δ62.6
α-D-man	δ102.2	δ71.4	δ72.1	δ68.3	δ73.9	δ62.5
β-D-rha	δ102.4	δ71.8	δ74.1	δ73.4	δ73.4	δ17.9
α-D-rha	δ102.1	δ71.2	δ71.5	δ73.3	δ69.5	δ17.9

Table 15. Carbon chemical shifts of common glycosyls.

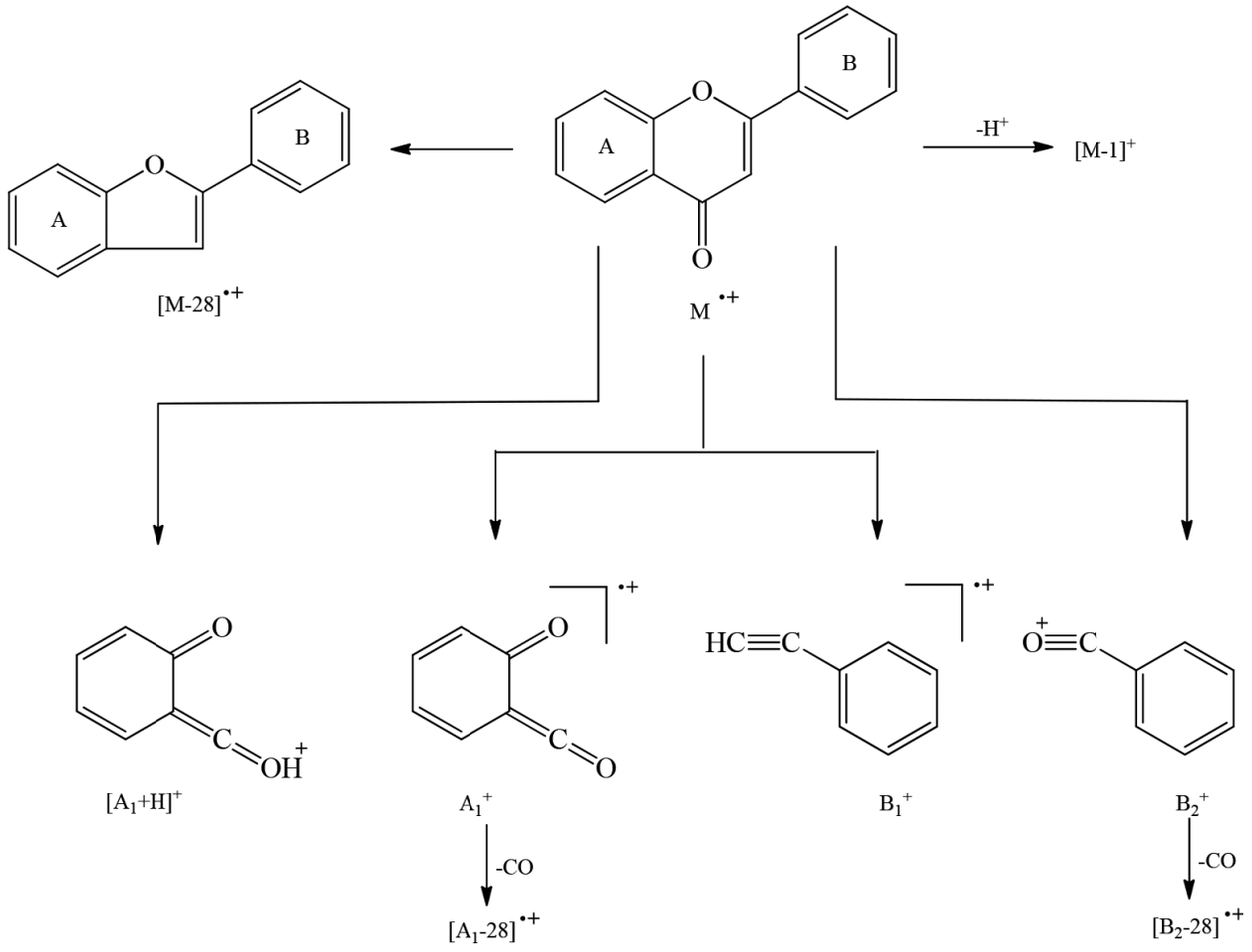

Figure 5. MS fragmentation pathway of flavone.

4.5.1. Optical rotatory dispersion (ORD)

For the flavonoids possess chiral centers, their optical activities (589.0 nm, Na-D light source) are correlative with spatial configurations, as shown in **Table 16** [27].

4.5.2. Circular dichroism (CD)

It is the most used method to elucidate the absolute configurations of flavonoids via cotton effect (CE) of CD spectra.

4.5.2.1. Flavanone

Most of the protons of flavanones at position 2 are axial ($J \approx 11.0$Hz). The characteristics of CE are shown in **Table 17**.

As reported in literature [29], the absolute configurations of the enantiomeric flavanone pair (2S)-6-formyl-5,7-dihydroxyflavanone (1a) and (2R)-6-formyl-5,7-dihydroxyflavanone (1b) were assessed via their chiroptical data. The ECD curves of compound (1a) showed sequential positive and negative cotton effects near 310 and 280 nm for the $n \rightarrow \pi^*$ and $\pi \rightarrow \pi^*$ electronic transitions, respectively. These cotton effects are reminiscent of flavanones exhibiting

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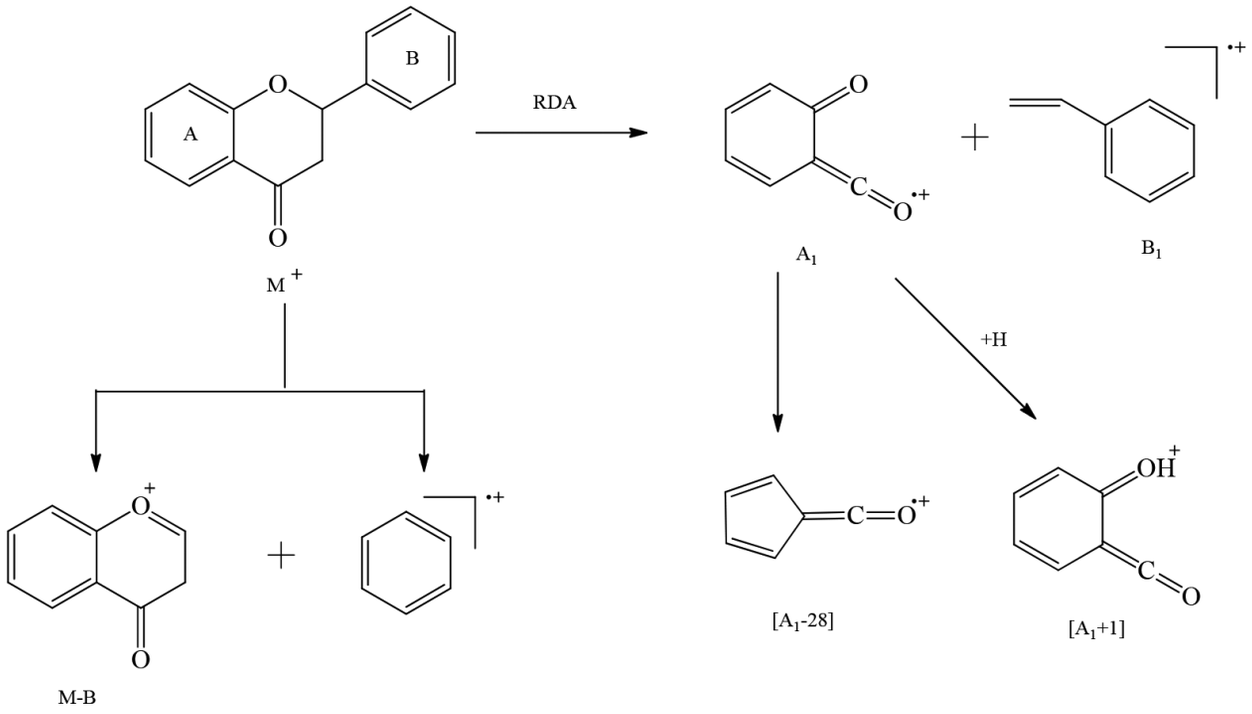

Figure 6. MS fragmentation pathway of flavanone.

Type	Optical activity	Spatial structure
Flavan	–	2S
Flavanonol (*trans*-form)	+	2R,3R
Epicatechin (*cis*-form)	-	2R,3R
Epicatechin (*cis*-form)	+	2S,3S
Catechin (*trans*-form)	+	2R,3S
Pterocarpin (*cis*-form)	–	6aR, 11aR
6a-Hydroxyl pterocarpin (*cis*-form)	–	6aS, 11aS
6a-Hydroxyl pterocarpin (*trans*-form)	+	6aR, 11aR

Table 16. Optical activities of flavonoids.

CE		Absolute configuration of C-2
270–290 nm	**320–330 nm**	
–	+	S
+	–	R

Table 17. Relationship between CE and absolute configurations of flavanones.

P-helicity of the conformational flexible heterocycle with a C-2 equatorial B ring and, hence, (2S) absolute configuration. The mirror image related to ECD spectrum of **1b** accordingly confirmed its (2R) absolute configuration. It is shown in **Figure 7**.

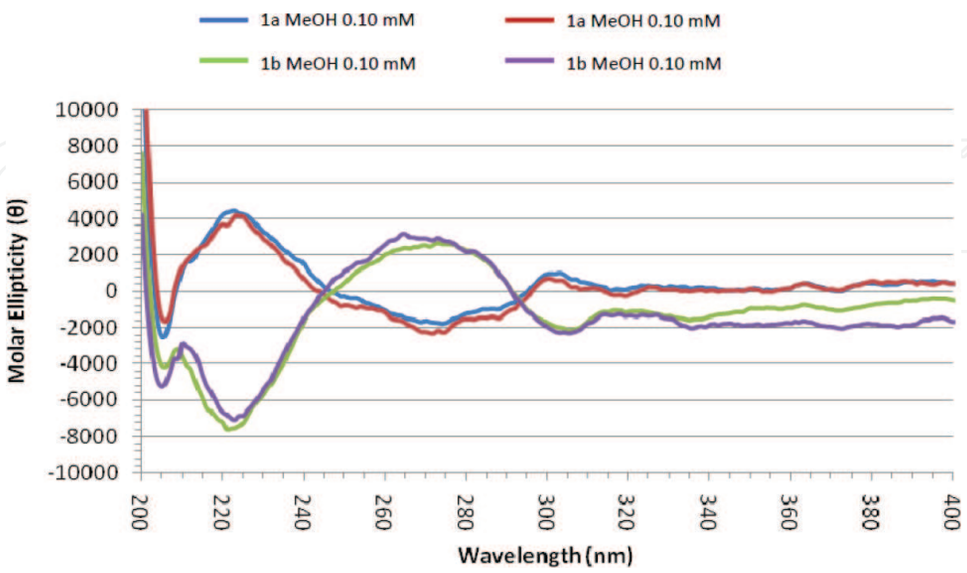

Figure 7. ECD spectra for compounds **1a** and **1b**.

4.5.2.2. Flavanonol

Four possible structures are existed in nature because of the existence of two chiral centers (C-2 and C-3), while 2R, 3R configurations are commonest. The relative configuration could be determined by coupling constant between H-2 and H-3 and then CD spectrum is employed to elucidate the absolute configuration, as shown in **Table 18** [30].

4.5.2.3. 3-Hydroxyl flavans

Similarly, C-2 and C-3 are also the chiral centers of 3-hydroxyl flavans. The characteristics are shown in **Table 19** [31].

4.5.2.4. 4-Hydroxyl flavans

The relative configuration could be determined by coupling constant of H-2 and H-4 combined with NOE spectra and then CD spectrum could be employed to elucidate the absolute configuration, as shown in **Table 20** [32].

Relative configuration	Cotton effect (300–340)	Absolute configuration
trans-	+	2R, 3R
	−	2S, 3S
cis-	+	2R, 3S
	−	2S, 3R

Table 18. Relationship between absolute configurations of flavanonol with CE.

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Relative configuration	CE		Absolute configuration
	240 nm	280 nm	
Trans-	+	−	2R, 3S
	−	+	2R, 3R
Cis-	−	+	2S, 3R
	+	+	2S, 3S

Table 19. Relationship between absolute configurations of 3-hydroxyl flavans with CE.

Relative configuration	CE		Absolute configuration
	240 nm	280 nm	
Trans-	−	−	2S, 4S
	+	+	2S, 4R
Cis-	−	−	2S, 4S
	+	+	2R, 4R

Table 20. Relationship between absolute configurations of 4-hydroxyl flavans with CE.

Relative configuration between C-2 and C-3	Relative configuration between C-3 and C-4	Cotton effect (280 nm)	Absolute configuration
Cis-	*Cis-*	+	2S,3S,4S
	Trans-	+	2S,3S,4R
Trans-	*Cis-*	+	2S,3R,4R
	trans-	+	2S,3R,4S
Cis-	*Cis-*	−	2R,3R,4R
	Trans-	−	2R,3R,4S
Trans-	*Cis-*	−	2R,3S,4S
	Trans-	−	2R,3S,4R

Table 21. Relationship of absolute configurations of 3,4-dihydroxyl flavans with CE.

4.5.2.5. 3,4-Dihydroxyl flavans

More absolute configurations are existed because of three chiral centers, as shown in **Table 21** [33].

4.5.2.6. Flavans

The cotton effects of flavans are show in **Table 22** [34].

4.5.2.7. Isoflavans

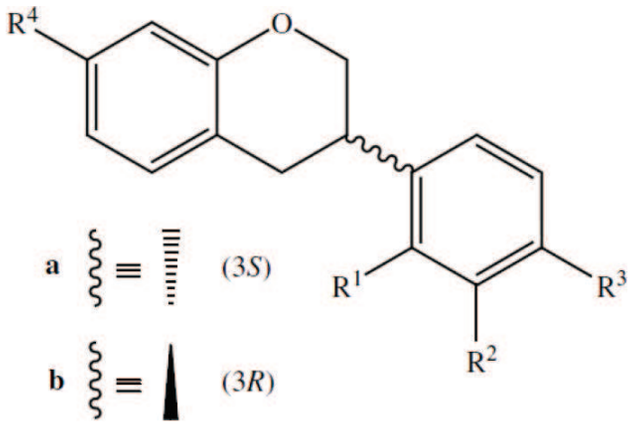
The CE characteristics are shown in **Table 23**.

Cotton effect (280 nm)	Absolute configuration
+	2R
−	2S

Table 22. Cotton effects of flavans.

CE		Absolute configuration
260–320 nm	**220–260 nm**	
+	−	3R
−	+	3S

Table 23. Cotton effects of isoflavans.



Compound	R^1	R^2	R^3	R^4
30a/b	OH	H	OMe	OH
31a/b	H	H	OMe	H
32a/b	H	OMe	OMe	H
33a/b	OMe	H	OMe	H
34a/b	H	H	OMe	OMe
35a/b	OMe	H	H	OMe
36a/b	OMe	H	OMe	OMe

Figure 8. Synthetic isoflavans (31a/b–36a/b).

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Versteeg et al. [35] synthesized six isoflavans and their enantiomers (31a/b-36a/b), and used authentic 3S- and 3R-vestitol (30a and 30b) derivatives to establish the absolute configuration at C3 of the synthetic isoflavans (**Figure 8**). (3S)-Isoflavans with oxygenation at both the A- and B-rings (34a, 35a and 36a) display positive and negative CEs in the 240 (1La) and 270–280 nm (1Lb) regions, respectively, and conversely for the 3R-enantiomers (34b, 35b and 36b) (**Figure 9**).

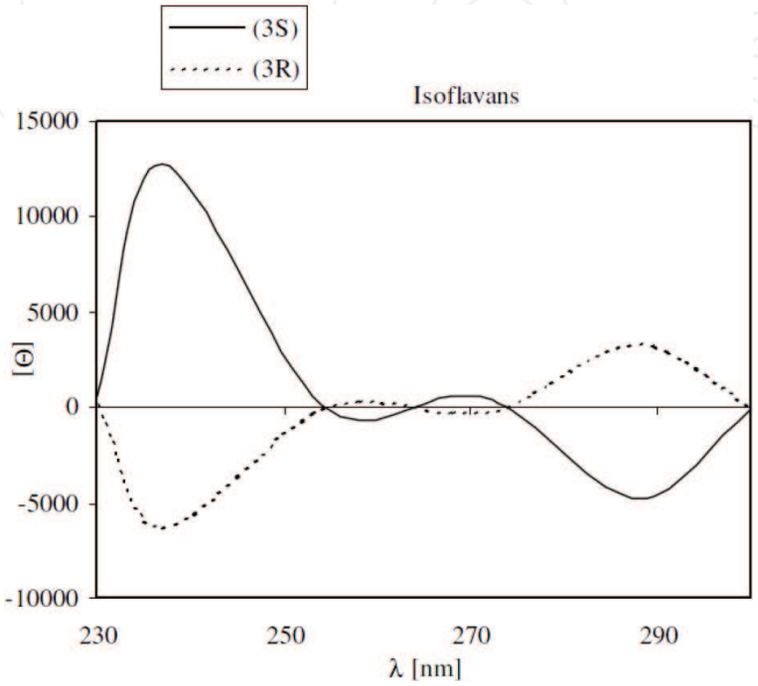
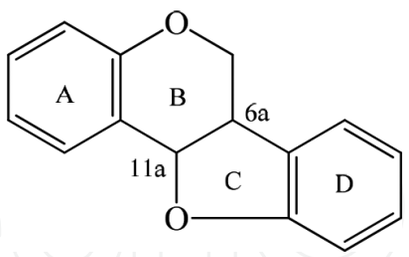


Figure 9. CD spectra of isoflavans with oxygenation at both the A and B rings.



Relative configuration of between C-6a and C-11a	CE		Absolute configuration
	260–310 nm	220–250 nm	
Trans-	+		6aS, 11aR
	−		6aR, 11aS
Cis-	+	−	6aR, 11aR
	−	+	6aS, 11aS

Table 24. Relationship of absolute configurations of pterocarpins with CE.

4.5.2.8. Pterocarpins

The spectral characteristics are shown in **Table 24** [36].

The relationships between the CE and absolute configurations will change after a hydroxyl group is attached to position 6a, as shown in **Table 25** [37].

Relative configuration of between C-6a and C-11a	CE		Absolute configuration
	260–310 nm	220–250 nm	
Trans-	−	−	6aS, 11aR
	+	+	6aR, 11aS
Cis-	+	−	6aS, 11aS
	−	+	6aR, 11aR

Table 25. Relationship of absolute configurations of 6a-hydroxyl pterocarpins with CE.

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