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 C₆-C₃-C₆ 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 C₃ 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 C₆-C₃-C₆; few of them are C₆-C₁-C₆ (xanthone, for example). A few of them, such as homoisoflavones and rotenoids, possess C₆-C₄-C₆ structure skeleton. In most cases, C₃ part is formed to be a hexatomic or pentagon ring with C₆ 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, methoxy, methyl, isopentenyl, methylenedioxy, benzyl, nitro groups and so on, could be substituted at ring A and/or B.

Figure 1. Basic structure of flavonoids.
| Structure type | Basic structure | Structure type | Basic structure |
|---------------|----------------|---------------|----------------|
| Flavone       | ![Flavone Structure](image1) | Chalcone      | ![Chalcone Structure](image2) |
| Flavonol      | ![Flavonol Structure](image3) | Dihydrochalcone | ![Dihydrochalcone Structure](image4) |
| Flavanone     | ![Flavanone Structure](image5) | Anthocyanidin | ![Anthocyanidin Structure](image6) |
| Flavanonol    | ![Flavanonol Structure](image7) | Flavan        | ![Flavan Structure](image8) |
| Isoflavone    | ![Isoflavone Structure](image9) | Aurone        | ![Aurone Structure](image10) |
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

| Structure type | Basic structure | Structure type | Basic structure |
|----------------|-----------------|----------------|----------------|
| Isoflavanone   | ![Isoflavanone](image) | Homoisoflavanone | ![Homoisoflavanone](image) |
| Rotenoid       | ![Rotenoid](image) | Xanthone       | ![Xanthone](image) |
| Pterocarpin    | ![Pterocarpin](image) | Neoflavonoid   | ![Neoflavonoid](image) |

Table 1. Main structure types of flavonoids.
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 CO₂ 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/(NH₄)₂SO₄ and acetone/K₂HPO₄ 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\textsubscript{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\textsubscript{18}, C\textsubscript{8}, C\textsubscript{2}, amino or phenyl columns could be applied, whereas C\textsubscript{18} and C\textsubscript{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
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\(_6\)D\(_5\)N, (CD\(_3\))\(_2\)CO and CD\(_3\)OD, 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

| 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 flavanone         | 270–295      | 300–330 (shoulder peak) |
| Chalcone                        | 220–270 (weak peak) | 340–390             |
| Aurone                          | 230–270 (weak peak) | 370–430             |
| Anthocyanadin                   | 270–280      | 465–560              |

Table 2. The spectral characteristics of UV-VIS spectrum of flavonoids.
well dissolved in DMSO-$d_6$, and the resonance signals of flavonoids are rarely overlapped by solvent peaks (about $\delta 2.5$). 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. $^1H$-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 $^1H$-NMR spectrum.

4.3.1.1. Protons on ring C

$^1H$-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 $\delta 5.7–6.9$ 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             | 6.3–6.8 (s)                |                            | The signals maybe overlapped by H-6 or H-8.                 |
| Flavonol              | None signal.               |                            |                                                             |
| Isoflavone            | 6.7–7.8 (s)                |                            | The signal is at rather low field because of influence of oxygen atom at position 1 and carbonyl at position 4. |
| Chalcone              | $\alpha$: 6.7–7.4 (d, $J = 17$ Hz) |
|                       | $\beta$: 7.3–7.7 (d, $J = 17$ Hz) |
| Flavanone             | 6.0–5.5 (dd, $J = 11$, 5 Hz) | $\delta 2.3–2.8$ (2H)      | H-2 is coupled by two protons of position 3.                |
|                       |                            | (dd, $J = 17$, 11 Hz)      |                                                             |
|                       |                            | (dd, $J = 17$, 5 Hz)       |                                                             |
| Flavanonol            | 4.8–5.0 (d, $J = 11$ Hz)   | 4.1–4.3 (d, $J = 11$ Hz)   | Configurations of both C-2 and C-3 are R.                   |
| Flavanone-3-O-         | 6.0–5.6                    | 4.3–4.6                    | After glycosidation of 3-OH, resonance signals of both H-2 and H-3 shift to low field. |
| glycoside             |                            |                            |                                                             |
| Aurone                | Exocyclic proton: 6.5–6.7 (s) |                            |                                                             |

Table 3. Chemical shifts and coupling constants of ring C of common flavonoids.
4.3.1.2.2. 7-Hydroxyl 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 $\delta 8.0$) 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 ($J=2.0$Hz) because of the vicinal relationship with H-6. Signals of both, H-6 and H-8 are at $\delta 6.3–7.1$. 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’,A’-dioxxygenation, 2’,A’-oxygenation, 3’,A’,5’-trioxygenation and 2’,A’,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 $\delta 6.7-8.1$. 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 | $\delta 6.0–6.2$ d | $\delta 6.3–6.5$ d |
| 7-O-Glucosides of above compounds | $\delta 6.2–6.4$ d | $\delta 6.5–6.9$ d | |
| Flavanone, flavanonol       | $\delta 5.75–5.95$ d | $\delta 5.9–6.1$ d | |
| 7-O-Glucosides of above compounds | $\delta 5.9–6.1$ d | $\delta 6.1–6.4$ d | |
| 7-Hydroxyl flavonoids       | Flavone, flavonol, isoflavone | $\delta 7.9–8.2$ d | $\delta 6.7–7.1$ d | $\delta 6.7–7.0$ d |
| Flavanone, flavanonol       | $\delta 7.7–7.9$ d | $5.7–6.0$ d | $\delta 5.9–6.1$ d |
| 5,6,7-Trihydroxyl flavonoids|         |         | $\delta 6.3$ s |
| 5,7,8-Trihydroxyl flavonoids|         |         | $\delta 6.95$ s |

Table 4. Chemical shifts of protons on ring A.
meta-coupling. The signals of H-2' and H-6' are usually at δ7.1–7.6 and of H-3', H-4' and H-5' are at δ7.9–8.2.

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'-Dioxgenation

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 ≈ 2.0Hz), H-5'(1H, d, J ≈ 8.0Hz) and H-6'(1H, dd, J ≈ 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     | 87.1–7.3   |            |
| Flavanonol    | 87.2–7.4   |            |
| Isoflavone    | 87.2–7.5   |            |
| Chalcone      | 87.4–7.6   | 86.5–7.1   |
| Aurone        | 87.6–7.8   |            |
| Flavone       | 87.7–7.9   |            |
| Flavonol      | 87.9–8.1   |            |

Table 5. Chemical shifts of protons on ring B of 4'-oxygenated 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 δ6.8–6.9, H-4' at about δ7.2 and H-6' at δ7.4–7.5.

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 δ6.5–7.5.

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 δ7.2–7.5, H-3' at δ6.4–6.6. 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 δ6.00–6.6 (d, J ≈ 2.0 Hz), H-5' at δ 6.6–6.5 (dd, J ≈ 2.0, 8.0 Hz) and H-6' at δ7.0–7.4 (d, J ≈ 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].

| Type                  | Substituent mode                        | H-2'          | H-5'          | H-6'          |
|-----------------------|-----------------------------------------|---------------|---------------|---------------|
| Flavone               | 3',4'-OH and 3'-OH, 4'-OCH₃             | 87.2–7.3      | 86.7–7.1      | 87.3–7.5      |
| Flavonol              | 3',4'-OH and 3'-OH, 4'-OCH₃             | 87.5–7.7      | 87.6–7.9      |               |
|                       | 3'-OCH₃, 4'-OH                          | 87.6–7.8      | 87.4–7.6      |               |
|                       | 3',4'-OH, 3-O-glc                       | 87.2–7.5      | 87.3–7.7      |               |
| Isoflavone, flavanone, flavanonol | 3',4'-OH                                  | 86.7–7.1      | 86.7–7.1      |               |

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

4.3.2. 13C-NMR spectrum

Strong regularities are also shown in 13C-NMR spectra of flavonoids. The types of flavonoids, number and connection positions of glycosyls could be elucidated from 13C-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.
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].
4.3.2.2.3. Signal characteristics of common substituents

The carbon chemical shifts of common substituents are shown in Table 13 [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 |
| Flavanol              | δ82.7–83.5  | δ71.2–73.0 | δ188.0–197.0 |
| Chalcone              | δ146.1–147.7 | δ111.0–113.3 | δ180.5–182.7 |
| Dihydrochalcone       | δ39.2–45.5  | δ28.5–31.2 | δ199.6–205.5 |
| Aurone                | δ136.9–145.4 | δ116.8–128.1 | δ188.0–194.6 |
| Flavan-3-ol           | δ77.5–82.7  | δ65.1–68.2 | δ275.5–28.6 |
| 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'   | δ28.5–132     |
| 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    | Flavone, flavonol     | δ160     | δ109–111 | δ135–137 | δ107–108 | δ157–160 | δ111     |
|                 | Flavanone, flavanonol | δ128     | δ109.5–110 | δ165–166 | δ100     | δ162–164 | δ114–116 |
|                 | Isoflavone            | δ127     | δ115     | δ161–163 | δ102–104 | δ156–158 | δ117–119 |
| 7,7-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].
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 ¹H-NMR. The terminal carbons of O-glycosides are at δ95–105 and at δ71–80 for C-glycosides. Furthermore, the number of glycosyls could be determined by combined analysis of ¹H and ¹³C-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 ¹H-NMR spectra. The absolute configurations, however, should be determined by chemical methods and gas chromatography.

| 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'-OCH₃   | Flavone, flavonol, isoflavone | δ121–125 | δ113–114 | δ145–147 | δ149–151 | δ112–116 | δ118–122 |
| (or 3', 4'-dihydroxyl) |     |       |        |       |       |      |      |
| 3'-OCH₃, 4'-OH   | Flavanone, flavanonol | δ121–125 | δ110–111 | δ150–152 | δ148 | δ115–117 | δ118–122 |
| 3'-OH, 4'-OCH₃   | Flavanone, flavanonol | δ128–129 | δ111–115 | δ144–147 | δ146–148 | δ112–116 | δ118–120 |
| (or 3', 4'-dihydroxyl) |     |       |        |       |       |      |      |
| 3'-OCH₃, 4'-OH   | Flavonoids | δ131–132 | δ111–115 | δ147–149 | δ146–147 | δ112–116 | δ118–120 |
| 2', 4'-Dioxygenation |     |       |        |       |       |      |      |
| 3', 4', 5'-Trioxgenation |     |       |        |       |       |      |      |

**Table 12.** Chemical 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 ¹H-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 ¹H and ¹³C-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 ¹H-NMR spectra. The absolute configurations, however, should be determined by chemical methods and gas chromatography.

| Substituent | Chemical shift |
|-------------|---------------|
| CH₃         | 6-CH₃(δ6–10); 8-CH₃(δ20–30); COCH₃(δ17–22) |
| OCH₃        | δ55–57       |
| Isopentenyl [-CH₂CH=CHCH₃] | CH₂(δ21–22); CH(δ122–124); CH(δ129–131); CH₃(δ17–27) |
| O-CH₂-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.
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.

| 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 |    | +1.0 |    |    |    |    |    |    |    |    |    |    |
| 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].

### Table 15. Carbon chemical shifts of common glycosyls.

| Type of glycosyl | C-1’' | C-2’’ | C-3’’ | C-4’’ | C-5’’ | C-6’’ |
|-----------------|-------|-------|-------|-------|-------|-------|
| β-D-glu         | 8104.0 | 874.1 | 876.8 | 670.6 | 876.8 | 861.8 |
| α-D-glu         | 8100.0 | 872.2 | 874.1 | 670.6 | 872.5 | 861.6 |
| β-D-gal         | 8104.5 | 871.7 | 873.8 | 669.7 | 876.0 | 862.0 |
| α-D-gal         | 8100.1 | 869.2 | 870.5 | 670.2 | 871.6 | 862.2 |
| β-D-man         | 8102.3 | 871.7 | 874.5 | 668.4 | 877.6 | 862.6 |
| α-D-man         | 8102.2 | 871.4 | 872.1 | 668.3 | 873.9 | 862.5 |
| β-D-rha         | 8102.4 | 871.8 | 874.1 | 673.4 | 873.4 | 817.9 |
| α-D-rha         | 8102.1 | 871.2 | 871.5 | 673.3 | 869.5 | 817.9 |

Table 15. Carbon chemical shifts of common glycosyls.
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...
Table 16. Optical activities of flavonoids.

| 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-Hydroxylation pterocarpin (cis-form) | –          | 6aS, 11aS         |
| 6a-Hydroxylation pterocarpin (trans-form) | +      | 6aR, 11aR         |

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

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

Figure 6. MS fragmentation pathway of flavanone.
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.

![ECD spectra for compounds 1a and 1b.](image)

**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.
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.

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

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-                                           | –        | –                      |
|                                                  | +        | +                      |
|                                                  | +        | –                      |
|                                                  | –        | +                      |
| Cis-                                             | –        | 6aR, 11aS              |
|                                                  | +        | 6aS, 11aR              |
|                                                  | +        | 6aR, 11aS              |
|                                                  | –        | 6aR, 11aR              |

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

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