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Isolation and Structure Characterization of Flavonoids

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Abstract

Flavonoids are one of the most important classes of secondary metabolites from natural products due to their several applications in medicine, foods, diet industries, and so on. Even though a huge number has been reported from natural and synthetic sources, scientists are still interested in flavonoids and derivatives. The biggest challenge for working on secondary metabolites is related to the use of the predicted theoretical method to isolate the expected compound and finally analyse the spectroscopic data to elucidate and fully characterize the structure. This chapter was designed to document useful techniques for isolation and structure characterization of flavonoids. Besides the well-known methods that have been used so far, we would also put together updated information about novel challenge techniques published in recent articles on isolation and characterization of flavonoids. Our data were obtained mainly from academic library and from reported data online by using research links such as Google Scholar, Scopus, SciFinder, Scirus, PubMed, and so on. Our field experience on phytochemistry of isolation and characterization of flavonoids was also used in this chapter.

Keywords: natural products, flavonoids, techniques, isolation, characterization

1. Introduction

Flavonoids are a large group of C-15 ($C_6-C_3-C_6$) secondary metabolites widespread in higher plants and are also detected in some lower plants such as algae. An important number has been reported from natural and synthetic sources due to their several applications in pharmaceutical and diet industries. Flavonoids occur in natural products specially blooming plant species, and colours of flowers could be indicative for the class of compounds. Flavonoids are mostly obtained as yellow pale, white, red, purple, blue, and so on from species of several plant families but are known to be widespread in the Fabaceae family. Flavonoids could

be detected in natural products by using some analytical methods such as the Shinoda [1], sodium hydroxide [1] and *p*-dimethylaminocinnamaldehyde tests [2].

2. Classification and basic skeletons of flavonoids

Flavonoids have a diversity of chemical structures constituted of 15 carbon atoms in their basic skeletons with a C₆-C₃-C₆ framework made by two aromatic rings (A and B) linked by a three-carbon unit that may or may not form a third ring (C). Generally, carbons are referred to by a numbering system, which utilizes ordinary numerals for the A- and C-rings and 'primed' numerals for the B-ring (1–3), but this is not respected when referring to chalcones (3) [3, 4]. The B-ring could be linked to C-ring at position C-2, C-3 or C-4 to form most classes of this secondary metabolite known as the flavonoids (flavone, flavonol, flavonone, flavan), isoflavonoids (isoflavone, isofavonone, isoflavonol, isoflavan, rotenoid, coumestane, pterocarpin, isoflavene) and neoflavonoids (4) (arylcoumarin, neoflavene, etc.), respectively. Some minor flavonoids are also known such as aurone, chalcone, and dihydrochalcone which is the first class of flavonoids obtained by biosynthesis and therefore the precursor of other classes. The chemical structure diversity of flavonoids is particularly obtained from glycosylation, methoxylation, prenylation, hydroxylation that usually took place with some specific positions in these different classes [3, 5–7] (Figure 1).

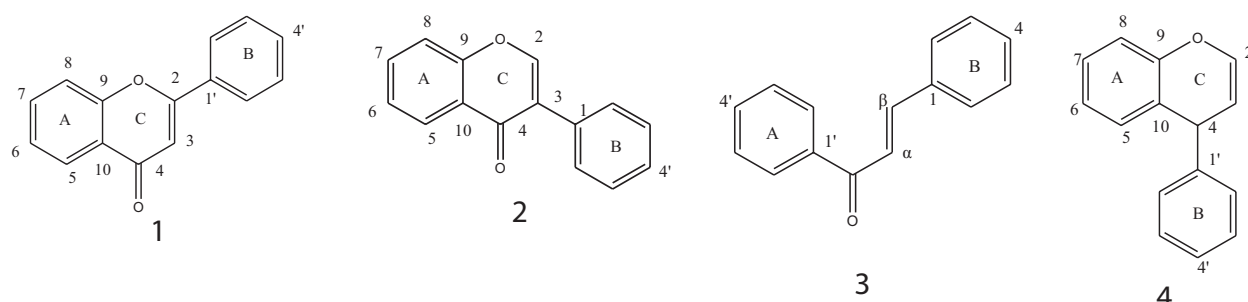


Figure 1. Predominant basic skeletons of flavonoids and carbon atoms numbering pattern.

3. Isolation techniques of flavonoids

3.1. Sample preparation

Flavonoids especially those in plants could be extracted from several parts such as roots, barks, leaves, fruits, woods and flowers. Samples are more often dried and ground before the extraction process. This initial treatment of samples helps in facilitating the extraction yields as well as preserving constituents. In some cases, the extraction is carried out on fresh plant materials. The dried plant materials have been reported in several investigations to contain most flavonoids than fresh samples [8–10]. Obviously, the ground samples always gave higher yields of extraction, and this could be justified by the fact that the solvent has contact

with surface constituents when the powder has smaller particles. The extraction yields of flavonoids from natural products are also affected by some factors such as temperature, time and ratio of water in case of aqueous mixing solvents [11].

Several methods have been used for extracting flavonoids in plant materials. These include maceration, infusion, decoction, percolation, hot continuous extraction (soxhlet), ultrasound-assisted extraction and microwave-assisted extraction, using solvents as water, ethanol, methanol, n-butanol, acetone, ethyl acetate, chloroform, and so on. Polar solvents are used to obtain flavonoid glycosides, whereas non-polar solvents extracted mostly their aglycones. Most of the investigations conducted in the extraction of flavonoids in plant materials have been done by maceration and infusion [10, 12]. A herbal tea from *Viscum album* L. was prepared using maceration and infusion to yield 31 and 43% flavonoid-like substances, respectively [13]. Ethanol, methanol and acetone are among the best solvents for extracting flavonoids [14, 15]. Acetone was reported to be the best solvent to extract flavonoids from a bitter melon—*Momordica charantia*—and *Tagetes patula* while the ethanol extract from *Trigonella foenum-graecum* had the highest flavonoid contents [11, 12, 16].

Decoction process is presented as a simple, cheap and convenient extraction method that may be useful in poor-equipped laboratories. A Thai medicinal plant called Siamese neem tree (*Azadirachta indica* A. Juss. var. *siamensis* Valetton) is well known to have flavonoids (rutin and quercetin) as main bioactive constituents. The decoction provided an extract with the highest amount of total flavonoids (17.54 mgRE/g extract) when using six different extraction techniques such as maceration, percolation, decoction, soxhlet extraction, ultrasonic extraction and microwave-assisted extraction in dried young flowers [17]. However, it may also depend on the plant material including the part used, number of constituents present and some conditions mentioned above that influence the extraction process. This was the case for the whole plant of *Senecio anteuphorbium* collected from Sidi Ifni, Southern Anti-Atlas of Morocco that was extracted using soxhlet extraction, decoction and maceration, and the methanolic extract from the soxhlet extraction showed the highest total flavonoid content (26.59 ± 0.24 mg QE/gE or 39.47 ± 1.01 mg RE/gE) while the aqueous maceration had the lowest (6.52 ± 0.09 mg QE/gE or 9.68 ± 0.22 mg RE/gE) [18].

The extraction of powered seeds of *Ziziphus mauritiana* using different methods such as maceration, decoction, soxhlet extraction and sonication with 50 and 80% ethanol, and water (decoction) as solvents, was reported and the high total flavonoid contents was obtained from the sonication technique [19].

In the basic mechanism of the extraction techniques, the microwave-assisted extraction follows several steps when comparing to conventional extractions. These include the penetration of the solvent into the solid matrix, the solubilization and/or breakdown of constituents, the transportation of the solute outside of the solid matrix, the migration of the solute from the external surface of the solid into the solution, the movement of the extract with respect to the solid, and the separation and discharge of the extract and solid [20–22]. The main difference between the microwave-assisted extraction and conventional extractions being the directions of heat and mass gradients during the extraction: for the first process, both move from inside to outside while in the second case, the mass transfer goes from inside to outside when heat occurs from outside to inside of the substrate [20–22].

Following the traditional Indian medicinal preparations, Krishnan and Rajan recently reported a suitable extraction of flavonoids from *Terminalia bellerica* Roxb., by the microwave-assisted solid-liquid method, an investigation conducted in view to study the influence of solvent-to-feed ratio and temperature on kinetics and thermodynamics of aqueous extraction [23]. Total flavonoids with good yield (1.13%) obtained under optimum conditions (ultrasonic power 500 W, extraction time 20 min, material solvent ratio 1:20, and ethanol concentration 30%) using ultrasound-assisted extraction were reported from the corn silk (*Zea mays* L.), a Chinese medicinal herb, with a recommendation for this plant to be developed as food natural antioxidant reagents [24]. Ultrasonic extract of flower from *Lythrum salicaria* L. was reported to possess good scavenging of hydrogen peroxide owing to the higher phenolic and flavonoid contents when using three methods of extraction such as percolation, ultrasonic-assisted extraction and polyphenol fraction [25].

All these techniques allow to have flavonoids in the crude extract with good yield before the application of different fractionation and purification procedures for their isolation.

3.2. Chromatography as a main tool for isolation of flavonoids

The isolation of flavonoids from natural sources is conducted by repeated and successive chromatography techniques such as open column chromatography (CC), preparative thin-layer chromatography (prep. TLC), centrifugal preparative thin-layer chromatography (CPTLC), high-speed counter-current chromatography (HSCCC), medium-pressure liquid chromatography (MPLC), high-pressure preparative liquid chromatography (prep. HPLC), and so on.

In column chromatography method, stationary phases could be normal or reverse phase silica gels, Sephadex (LH-20, G-10, G-25 and G-50). In view to have flavonoids-rich fractions, it is recommended to use some preliminary liquid-liquid extraction methods or polymeric resins such as Diaion HP-20, Amberlites (XAD-2, XAD-7) from the crude extract. These polymeric resins are very useful when the absorption of extracts is eluted in the open column chromatography with an increasing gradient of methanol in water.

The open column chromatography (CC) is still the most useful and easy isolation technique for natural products isolation and by means that of purification of flavonoids. The choice of the good solvents system for mobile phase is important and should be made from the check-up TLC on the crude or the flavonoids-rich fraction. Combination and polarity of solvents should be used depending on the class of flavonoids targeted. After the flash column, some major fractions could directly be subjected to Sephadex LH-20 or prep. TLC if they do not contain complex mixture of flavonoids. During the separation process, constituents from the flavonoid-rich fractions could have closer retention factors (R_f) based on their polarities. The change in phase of the adsorbent in some cases is useful to have good separation in either small open CC purification or prep. TLC. Several investigations reported the isolation of new flavonoids using CC. This included two dihydrochalcones, rare natural resources secondary metabolites, from *Eriosema glaumerata* [26], two polyhydroxylated flavones having antioxidant activity from *E. robustum* [3], one isoflavanol from *Kotschyia strigosa* [27], two

glucoside isoflavones from *Iris kashmiriana* [28], four dimeric chalcone derivatives from *Uvaria siamensis* [29], five flavonoids from *Millettia griffithii* [6], one pterocarpan, three isoflavones from the root, stem bark and leaves of *Erythrina schliebenii* [30], four flavonoid C-glycosides with anti-inflammatory properties from the leaves of *Piper aduncum* [31]. The number of recent published articles using CC is indicative for the useful and convenience of this method. Nevertheless, the prep. HPLC technique has been widely used for isolating commonly polyphenols and more specifically flavonoids. The suitability of this method for this class of secondary metabolites is associated with its high absorption in UV that is used as detector during the isolation.

The advantage of this technique is also associated with its analytical version that could help in qualitative characterization of flavonoids in the analysing sample. The diode array detector (DAD) and photodiode array detector (PDA) are commonly used. Further detectors such as mass spectrometry (SM) and nuclear magnetic resonance (NMR) could be combined with UV for more characterization of each flavonoid detected [32–35].

Several works on isolation of flavonoids from natural products using prep. HPLC have been published so far, and some of these compounds, recently reported, are documented in **Table 1** as well as their sources, column characteristics and mobile phases used (**Table 1** and **Figure 2**).

The application of other chromatography techniques, such as circular liquid chromatography (CLC), centrifugal preparative thin layer chromatography (CPTLC), high speed counter current chromatography (HSCCC), medium pressure liquid chromatography (MPLC), and so on, has also led to the isolation of numerous structures of flavonoids [39, 47–49]. Most flavonoids were isolated with combination of these techniques with prep. HPLC: four flavonoids (4',5-dihydroxy-3',7-dimethoxyflavanone, 5-hydroxy-7,3',4'-trimethoxyflavanone, 5,4'-dihydroxy-3,7,3'-trimethoxyflavone, and 5-hydroxy-3,7,4'-tetramethoxyflavone) were isolated from *Pogostemon cablin* (Blanco) Benth. using the HSCCC technique with two phase solvent system made of n-hexane–ethyl acetate–methanol–water (11:5:11:5, v/v/v/v) followed by further purification on prep. HPLC [39]. The combination of HSCCC and semi-prep. HPLC was used to isolate three flavonoid glycosides (orientin, vitexin, quercetin-3-O-neohesperidoside) from *Trollius ledebouri* Reichb. [48]. Two new flavonoids (*rac*-6-formyl-5,7-dihydroxyflavanone and 2',6'-dihydroxy-4'-methoxy-3'-methylchalcone) were recently reported from *Eugenia rigida* using CPTLC and prep. HPLC [50]. Flavoalkaloids and flavonol glucosides were reported from *Astragalus monspessulanus* using the combination of CC, low-pressure liquid chromatography (LPLC) and prep. HPLC [51].

Flavonoids could also be isolated as enantiomers from natural products. Lachnoisoflavones A (**5**) and B (**6**) were isolated from *Crotalaria lachnophora* using prep. HPLC as two enantiomer isoflavones as preliminary indicated by their $[\alpha]_D$ value [0.002 (c 0.1, MeOH)] [36]. The presence of the racemic mixture of **5** was successfully confirmed by a chiral HPLC-MS² separation that exhibited, on the chromatogram, two signals having the same peak area (**Figure 3**) [36]. This indicates the advantage of HPLC techniques for the isolation and structure characterization of flavonoids.

Names and sources	Classes	Mobile phases	Column characteristics
Lachnoisoflavones A (5) and B (6), <i>Crotalaria lachnophora</i> [36]	Isoflavone	H ₂ O (0.1% FA) – MeOH (0.1% FA), gr.	Nucleodur C ₁₈ 5 μm (250 × 16 mm)
Mansoins A (7) and B (8), <i>Mansoa hirsute</i> [37, 38]	Flavanone	H ₂ O – MeCN, gr.	Luna OSD C ₁₈ 5 μm (250 × 21.2 mm) & (250 × 10 mm)
4',5-Dihydroxy-3',7-dimethoxyflavanone (9), 4',5-dihydroxy-3,7,3'-trimethoxyflavone (10), <i>Pogostemon cablin</i> (Blanco) Benth [39]	Flavanone Flavonol	MeOH – AcOH (0.1% aq.), is. (7:3) (75:25)	YMC C ₁₈ 5 μm (250 × 10 mm)
Brutieridin (11), Melitidin (12), <i>Citrus bergamia</i> [40]	Flavanone	H ₂ O (0.1% FA) – MeCN, gr.	ONYX C ₁₈ (100 × 3 mm)
Cyanidin 3-[3''-(O-β-D-glucopyranosyl)-6''-(O-α-L-rhamnopyranosyl)-O-β-D-glucopyranoside] (13), Cyanidin 3-rutinoside (14), <i>Asparagus officinalis</i> [41]	Anthocyanin	[AcOH:MeCN:H ₂ O (1:4:5)] – [AcOH:H ₂ O (1:9)], gr.	Cosmosil 5C ₁₈ AR II (250 × 20 mm)
O3--(6-E-Feruloyl)-β-D-glucopyranosyl-(1→2)-[β-D-xylopyranosyl-(1→2)-]α-L-rhamnopyranosyl-quercetin, Galocatechin, <i>Alphitonia neocaledonica</i> [42]	Flavone Flavanol	MeCN – H ₂ O (3:7 and 6:4), is. MeCN – H ₂ O (0.025% TFA), gr.	Luna C ₁₈ 5 μm (250 × 10 mm)
Pelargonidin 3-(6''-p-coumarylglucoside)-5-(4''-malonylglucoside), Pelargonidin 3-(6''-malonylglucosid, <i>Ficus padana</i> Burm. L. [43]	Anthocyanin	[H ₂ O (2% FA) – MeCN:H ₂ O:FA (49:49:2)], gr.	Shimpack PRC-ODS 5 μm (250 × 20 mm)
Isoschaftoside (15), Orientin (16), Isoorientin (17), <i>Mauritia flexuosa</i> [44]	Flavone	MeCN – H ₂ O, gr. [MeOH (0.1% TFA) – H ₂ O (0.1% TFA)], gr.	Shimpack C ₁₈ 5 μm (250 × 20 mm)
Trilobatin (18), Phloretin (19), 3-Hydroxyphloretin (20), Phlorizin (21), <i>Malus crabapples "Radiant"</i> [45]	Dihydrochalcone	MeOH – 0.01% TFA (3:2)	Agilent Extend C ₁₈ (250 × 9.4 mm)
Diplotrin A (22), Diplotasin (23), <i>Mimosa diplotricha</i> [46]	Flavone	H ₂ O – MeOH (2:3), is.	Cosmosil 5C ₁₈ -AR-II 5 μm (250 × 20 mm)

FA, formic acid; TFA, trifluoroacetic acid; gr, gradient polarity, is, isocratic.

Table 1. Some recent flavonoids isolated from natural products using HPLC as well as columns and mobile phases used.

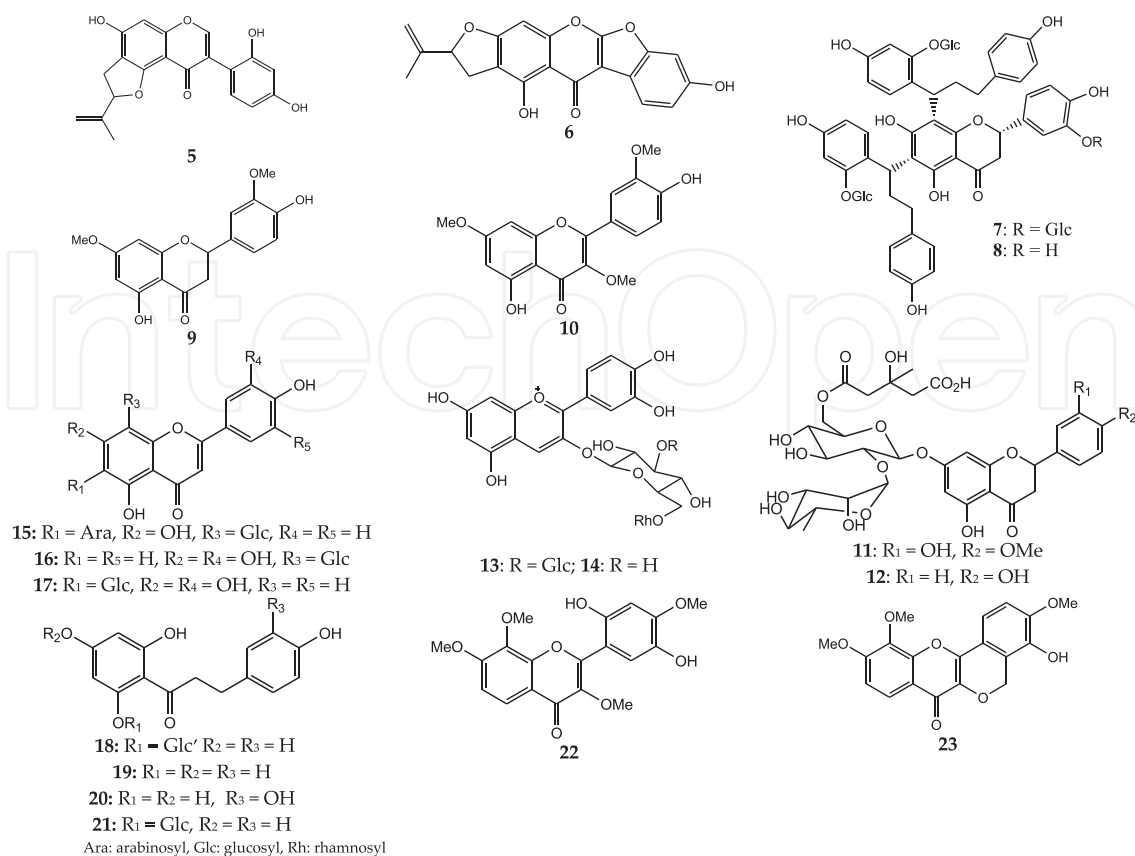


Figure 2. Some flavonoids recently isolated by Prep. HPLC from natural resources.

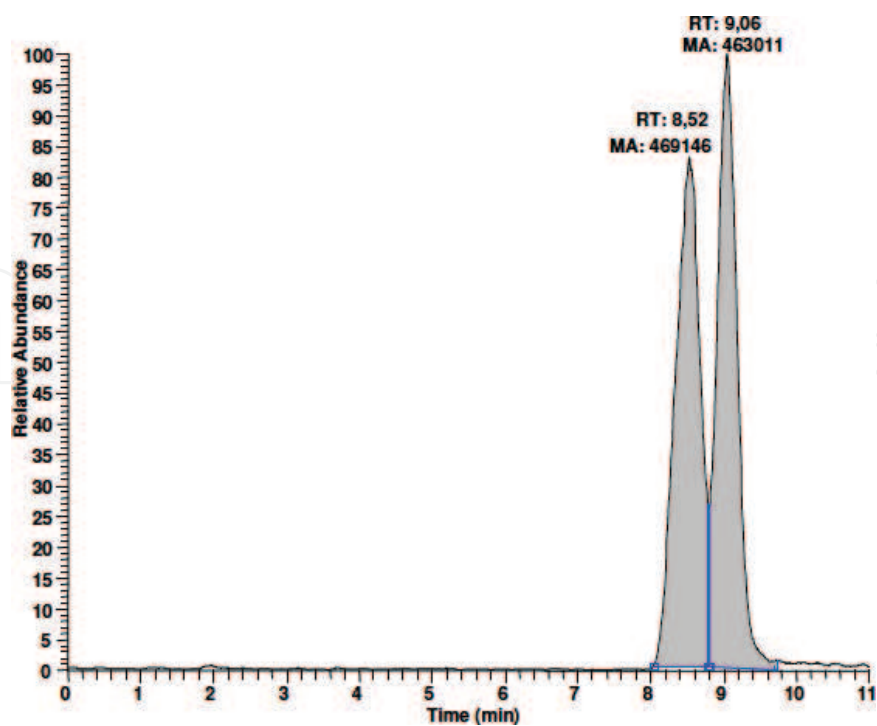


Figure 3. Chromatogram of chiral separation by LC-MS² of 5.

4. Structure characterization of flavonoids

The structure characterization of flavonoids is related to the elucidation of their spectroscopic spectra obtained by techniques such as nuclear magnetic resonance (NMR), mass spectrometry (MS), spectrophotometric ultra-violet (UV) and infrared (IR). Physical properties of the flavonoids as melting point (mp), circular dichroism (CD), optical rotatory power ($[\alpha]_D$) are also useful for full characterization of the isolated flavonoid specially when it contains stereocenter for CD and $[\alpha]_D$. Some chapters in this book would provide more details about the use of spectroscopic analysis to characterize flavonoids. Nevertheless, the NMR spectroscopy is mainly divided into 1D and 2D analyses. The 1D NMR analysis includes the proton (^1H), carbon-13 (^{13}C) and distortionless enhancement by polarization transfer experiment (DEPT) that provide information about the signals of protons, carbons and type of carbons (C, CH, CH_2 or CH_3) in the structure of flavonoid under elucidation. The ^1H NMR spectrum is very useful as it provides the number (integration value) and the type of proton involved. The chemical shift (δ_{H}) values are usually exhibited within 0 (reference standard value for TMS) and 14 ppm in ^1H NMR of flavonoids while in ^{13}C NMR, they (δ_{C}) appeared between 0 and 220 ppm. Characteristic proton and carbon chemical shift values for some flavonoid classes were summarized [52, 53] (Table 2).

The 2D NMR is composed mainly with the proton-proton correlated spectroscopy (^1H ^1H COSY), the heteronuclear multiple quantum coherence (HMQC)/heteronuclear single quantum coherence (HSQC), the heteronuclear multiple bond connectivity (HMBC), the nuclear overhauser spectroscopy (NOESY), the rotative-frame overhauser spectroscopy (ROESY) and the

Chemical shifts (ppm)	^1H
2–3	H-3 (Flavanone), CH_3 aromatic
4–6	H-2 (Flavanone, dihydroflavonol)
6–8	A- and B-ring protons
8–8.5	H-2 isoflavone
12–14	5-OH when C=O at C-4 (usually observed in $\text{DMSO}-d_6$)
Chemical shifts (ppm)	^{13}C
210–170	C=O
165–155 (no ortho/para oxygenation)	Oxygenated aromatic carbons
150–130 (with ortho/para oxygenation)	Oxygenated aromatic carbons
135–125 (para substitution)	Non-oxygenated aromatic carbons
125–90 (with ortho/para oxygenation)	Non-oxygenated aromatic carbons
80–40	Non-oxygenated (C-2, C-3 flavanone/flavanol)
28–35	C-4, flavanol

Table 2. Characteristic proton and carbon chemical shifts for some flavonoids.

total correlated spectroscopy (TOCSY) experiments. The ^{13}C data of flavonoids in several cases could also be assigned from HMQC and HMBC spectra.

The infrared spectroscopy compared to other spectroscopic techniques exhibits little but useful information in the structure characterization of flavonoids. Most of hydroxylated flavones, isoflavones and chalcones or dihydrochalcones showed maxima large band absorptions around $3300\text{--}3600\text{ cm}^{-1}$ due to hydroxyl groups. Additionally, intense band absorption characteristic for flavonoid carbonyl groups ($\text{C}=\text{O}$) is observed around 1680 cm^{-1} and is shifted approximately to 1620 cm^{-1} when the hydroxyl is chelated with a $\text{C}=\text{O}$. From the IR spectrum of flavonoids, a sharp and intense absorption band is also observed between 1600 and 1500 cm^{-1} due to aromatic double bonds (aromatic rings).

Ultra-violet (UV) absorption spectroscopy of flavonoids has two maxima absorptions around $300\text{--}350$ and $240\text{--}285\text{ nm}$ corresponding to bands I and II from A- and B-rings, respectively. This technique is used for identification of the flavonoid type and its oxygenation pattern. UV-shift reagents (AlCl_3 , NaOMe , NaOAc , $\text{NaOAc} + \text{H}_3\text{BO}_3$, $\text{AlCl}_3 + \text{HCl}$) are mostly used in the sample solution to confirm the presence and the substitution pattern of hydroxyl groups in flavonoids. The presence of ortho-dihydroxylated groups could be detected by the bathochromic shift of band I after addition of $\text{NaOAc}/\text{H}_3\text{BO}_3$ while the addition of AlCl_3 led to the bathochromic effect of band I when the flavonoid with a carbonyl at C-4 had hydroxyl group at positions C-3 or C-5. The bathochromic shift of band II occurs especially when NaOAc is added to a solution of flavonoids having a free hydroxyl group at C-7 [3, 54, 55]. Characteristic UV absorption bands I and II due to different classes of flavonoids have been reported elsewhere (Table 3) [54].

The mass spectrometry technique is very helpful in the structure elucidation of flavonoids. It is used in the determination of the molecular weight for establishing the distribution of substituents between the A- and B-rings and in the determination of the nature and site of attachment of the sugar(s) in flavonoid C- and O-glucosides. The molecular weight of the basic

Band II (nm)	Band I (nm)	Flavonoid class
250–280	310–350	Flavone
250–280	330–360	Flavonols (3-OH substituted)
250–280	350–385	Flavonols (3-OH free)
245–275	310–330 shoulder	Isoflavone
	C. 320 peak	Isoflavones (5-deoxy-6,7-dioxygenated)
275–295	300–330 shoulder	Flavonones and dihydroflavonols
230–270	340–390	Chalcones
230–270	380–430	Aurones
270–280	465–560	Anthocyanidins and anthocyanins

Table 3. Ultra-violet absorption ranges for flavonoids.

flavonoid nucleus is 222 *a.m.u.* for flavones, isoflavone and aurone; 224 *a.m.u.* for flavanones and chalcones; 238 *a.m.u.* for flavonols; and 240 *a.m.u.* for the dihydroflavonols. The molecular weight of the unknown flavonoid could be deduced by addition of atomic mass units of all its substituents [16 *a.m.u.* (-OH), 30 *a.m.u.* (-OCH₃), and so on] to one of the basic molecular weights above. The loss of some ion-fragments from the molecular or pseudo-molecular ion is very characteristic in the mass spectra of flavonoids. Peaks obtained during this fragmentation process represent accurately the corresponding ion-fragments that are expressed as mass-to-charge ratio (*m/z*). The exact molecular weight for each fragment may be measured to the nearest 0.0001 mass unit if the mass spectrometer is operating in high resolution. This information enables calculation of precise molecular formula from the molecular ion peak and ion-fragments [54]. A prerequisite for successful mass spectrometry is that the flavonoid should be sufficiently volatile in the high vacuum within the mass spectrometer. Most aglycones are sufficiently volatile at probe temperature of 100–230°C, higher temperatures being required for the more polar polyhydroxyflavones and flavonols. Glycosides, anthocyanidins and biflavonoids, however, are not sufficiently volatile and should therefore be derivatized to improve their volatility. Some standard methods used for derivatization of compounds are permethylation or perdeuteromethylation and trimethylsilylation [54].

Natural products in general or flavonoids in particular remain an important source for drug discovery. Determination of their absolute configurations is one of the most challenging tasks in the structure elucidation of chiral flavonoids. It has been proven that the change in absolute configuration of secondary metabolites consequently affected the difference in pharmacological activity of both stereo-compounds. Methods such as chiroptical approaches, chemical synthesis, analytical chemistry, chiral derivatization and X-ray crystallography could be used to determine the absolute configuration of flavonoids. An important investigation was reported on the determination of absolute configuration of natural products and some flavonoids using experimental and calculated electronic circular dichroism (ECD) data [56].

5. Conclusion

The extraction, isolation and characterization of flavonoids from natural products have been carried out successfully by natural product chemists and phytochemists using relevant techniques and new methods. Some of these techniques and methods have been documented in this chapter with illustrations owing to some flavonoids recently reported. It is clear that the HPLC and its combination with other available techniques of isolation are being often used to obtain flavonoids from natural sources especially from plant species. The characterization of flavonoids remains basically focused on the analysis of their spectroscopic, mass and UV data and some chemical investigations depending on the nature of the structure under elucidation. The need of flavonoids in agriculture, food and drug industries still one of the worldwide up-to-date research interests. Natural resources and especially medicinal plants are still available to discover novel or efficient antioxidant flavonoids that could be used as drugs to fight against degenerative diseases one of the issues the global health is facing today.

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