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# AN REVIEW ON VARIOUS METHOD OF EXTRACTION AND ISOLATION OF FLAVANOIDS FROM MEDICINAL PLANTS

**P. Venkatesh[1*], T. Durga Snehitha[1], B. Jhansi[1], K. Kishore Babu[1], P. Madhavi[1]**

[1]Department of Pharmaceutical Chemistry, Sir C R Reddy College of Pharmaceutical Sciences, Eluru-534007, Andhra Pradesh, India.

## ABSTRACT

Flavonoids are important group of polyphenols widely distributed among the plant flora. The compounds are derived from parent compounds known as flavans. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants.Flavonoids are mainly present in Citrus fruits as their glycosyl derivatives. This review provide information about various methods of extraction, isolation and determination of flavanoids from medicinal plants.

**Key words:** Flavonoids, Extraction, Isolation, Total flavanoid content

**Author for correspondence:**
**P. Venkatesh**,
Department of Pharmaceutical Chemistry, Sir C R Reddy college of Pharmaceutical Sciences, Eluru-534007, Andhra Pradesh, India.

**Email: venkat7pharma@rediffmail.com**

## INTRODUCTION

Flavonoids are important group of polyphenols widely distributed among the plant flora. Stucturally, they are made of more than one benzene ring in its structure (a range of C15 aromatic compounds) and numerous reports support their use as antioxidants or free radical scavengers (Kar, 2007). The compounds are derived from parent compounds known as flavans. Over four thousand flavonoids are known to exist and some of them are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Other group of flavonoids include flavones, dihydroflavons, flavans, flavonols, anthocyanidins, proanthocyanidins, chalcones and catechin and leucoanthocyanidins.

A flavonoid skeleton is composed of two aromatic rings (commonly designated as A and B), which are connected through a pyrone ring (C) in the case of

flavones, or a dihydropyrone ring in the case of flavanones, as shown in Figure-1.

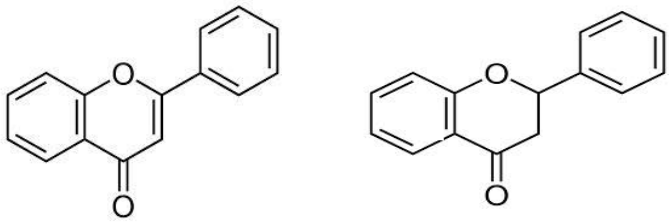

**Figure-1 Structure of flavone and falvanone**

Flavonoids are mainly present in Citrus fruits as their glycosyl derivatives. Aglycones (the forms lacking the sugar moieties) occur less frequently in juices, owing to their lipophilic nature and hence their low solubility in water. The presence of a relatively large number of flavonoids in Citrus juices is a result of the many different combinations that are possible between polyhydroxylated aglycones and a limited number of mono- and disaccharides. The most common sugar moieties include D-glucose and L-rhamnose. The glycosides are usually O-glycosides, with the sugar moiety bound generally to the aglycone hydroxyl group at C-7, or at the C-3 in some cases. In addition to these, C-glycosides have also been detected in various Citrus fruits or juices.

## EXTRACTION METHODS

### Soxhelet extraction

Plants collected were washed in running tap water to remove dust. Aerial part (stems, leaves, flower) of collected plants were separated, shade dried, powdered, weighed and stored separately for extraction. Each of the dried powdered and weighedsample was soxhlet extracted in 80% methanol for 24 hrs and filtered. The filtrate obtained from each sample was subsequently extracted in petroleum ether, diethyl ether and ethyl acetate following the method of Subramanian and Nagarajan (2). Petroleum ether fraction was discarded due to its being rich in fatty substances. Ether fraction was used for free flavonoids whereas ethyl acetate fraction for bound flavonoids. Ethyl acetate fraction of each sample was hydrolysed further with 7% $H_2SO_4$ for 24 hrs and was then re-extracted with ethyl acetate. The fraction obtained was repeatedly washed with distilled water to neutrality, dried and weighed.

### Solvent Extraction

Polymethoxyflavones (PMF's) are low polar compounds and can be extracted using non polar solvents such as hexane and polar solvents including water (3), ethanol and methanol (4). Moreover, these compounds were extracted from various parts of citrus such as peel, leaves and cold pressed oil. Raman *et al*. (5), reported extraction of *C.reticulata* peels using non polar hexane solvent followed by treating with 10% sodium hydroxide solution. The mixture was later extracted with diethyl ether, washed with water and subjected to adsorptive separation using cation exchange resin Dowex 50WX2 to yield nobiletin and tangeretin. Chaliha et al. (6), reported extraction of *C. jambhiri* peels using petroleum ether solvent in a Soxhlet apparatus for separation of PMF's. Jayaprakasha et al. (7) , reported extraction of *C. reticulate* (Blanco Coorg Mandarin) using hexane and chloroform successively in a Soxhlet apparatus. The extracts were subjected to further separation using silica gel column chromatography for Isolation of desmethylnobiletin, nobiletin and tangeretin.

### Supercritical Fluid Extraction

Apart from citrus peel and leaves, cold pressed oil is a rich source of PMF's. Extraction of these compounds from the precipitate of winterized (storing the oil at -20° C for long duration of time) citrus peel oil was commonly reported (8-10). These compounds were also extracted from peel oil extract using super critical fluid extraction. Recently, the optimum conditions for extraction of nobiletin and tangeretin from *C. depressa* Hayata by supercritical CO2 was developed by comparing various combinations of pressure and percentage of modifier ethanol solvent. Optimum extraction was achieved by ethanol (85%) as a modifier in supercritical CO2 maintained at 80 °C and 30 MPa ofpressure. Also, the %yield of PMF's by SFE was 107% as compared to conventional solvent extraction yielding 100%.

**Preparative Thin Layer Chromatography (prep-TLC)**

Among the reported separation methods of PMF's, prep-TLC is the most economical. It is relatively low in cost and does not require sophisticated instrumentation. However, this method is limited by the low amount of sample loaded and yield. Successive separations may be required for obtaining pure PMF's. Lee*et al*., (11) reported separation of these compounds from peel oil of various Citrus fruits. Citrus oil was mixed with 2-propanol and distilled water in a decantation funnel and extracted with hexane. The 2-proponol/water phase was concentrated, mixed with water and liquid-liquid extraction was conducted using benzene. The organic phase was separated, concentrated and dehydrated by adding anhydrous sodium sulfate. The extract was placed on a TLC plate containing silica and eluted with benzene:acetone (3:1, v/v). The separated compounds were visualized by their fluorescence and the individual bands were collected and analyzed by HPLC and mass spectrometry.

**Preparative-HPLC**

Increased interest in investigating the biological activity of PMF's and advancement in chromatographic techniques led to exploring isolation of PMFs using prep-HPLC. Chen at al., (11) reported the separation of these compounds from cold pressed Dancy tangerine peel oil solids using prep-HPLC. The procedure involved a combination of normal phase chromatography and C18 prep-HPLC. The dried tangerine oil solids were loaded to a open silica gel column and eluted with increasing polarity gradient of benzene/ethyl acetate, ethyl acetate, ethyl acetate/2-propanol and 2-propanol. The fractions with similar PMF's were pooled and purified using C18 prep-HPLC with a gradient mobile phase of methanol/water and ethanol/water. The procedure was applied for separation of PMF's from Dancy tangerine leaves leading to the isolation of pure compounds. However, use of solvents such as benzene for isolation studies should be avoided due to their carcinogenic and mutagenic properties. Li et al., (13) reported a gram-scale isolation method of nobiletin using a combination of normal phase flash chromatography and prep-HPLC. The procedure involved initial separation of orange peel extract using silica gel flash column eluted with a gradient solvent system of ethyl acetate and hexanes. The collected fractions containing nobiletin and 5,6,7,4'-tetramethoxyflavone were concentrated and further separated on a Regis chiral column connected to a prep-HPLC. The solvent system consisted of 35% ethanol and 65% hexanes with a flow rate set at 85 mL/min resulting in isolation of gram amounts of nobiletin and 5,6,7,4'-tetramethoxyflavone. Similar procedure was further applied for isolation of other PMF's from cold-pressed orange peel oil.

**Supercritical Fluid Chromatography**

This method is one of the ideal methods for separation of PMF's. This method involves use of pressure and temperature combinations maintained at critical point of the mobile phase used. Moreover, the absence of permanent adsorptive loss of sample on to the stationary phase which is commonly noticed in column chromatography makes this method advantageous. Among the various mobile phases used for SFC, CO2 along with methanol seems to be ideal for separation of PMF's. This method was initially used for analyzing the authenticity of citrus oils by quantification of PMF's. The separation of PMF's was conducted using CO2 as mobile phase and methanol as a polar modifier. In another report hydroxy- and methoxy-flavones were separated by supercritical CO2 chromatography on capillary columns using flame ionization and Fourier transform infrared (FT-IR) spectroscopy detection. Recently, a large scale isolation method of four PMF's such as nobiletin, tangeretin, 3,5,6,7,8,3 ',4'-heptamethoxyflavone and 5,6,7 ,4'-tetramethoxyflavone was reported using a combination of normal phase flash column separation and SFC separation (14). The raw material used for the separation was crude sweet orange peel extract. The extract was separated on a silica gel flash column using a gradient mobile phase. Individual fractions were analyzed by LC—ESI-MS and TLC and grouped into 6 groups. The groups that had high concentration of PMF's were subjected to SFC separation using mobile phase of CO2 and methanol. The separated peaks were collected as individual fractions to obtain pure PMF's.

**High-Speed Counter Current Chromatography**

This is a chromatography technique in which liquid—liquid partition is used as a strategy for separations and

unlike other chromatographic techniques does not use any solid support matrix. Due to the characteristic absence of solid support matrix there is no loss of samples by adsorption. This method was first reported as efficient method for the preparative isolation and purification of polymethoxylated flavones from Tangereine peel extracts (15). Tangerine peels were extracted by light petroleum, concentrated and frozen. The sediment was dried and injected to the HSCCC in 15 mL sample injections. The separations were conducted using a two-phase solvent system composed of n-hexane, ethyl acetate, methanol and water (1:0.8:1:1) (vlv). The effluent was monitored with a UV detector at 254 nm and peak fractions were collected according to the elution profile. Similar peaks were pooled and four PMF's nobiletin, 3,5,6,7,8,3',4'-heptamethoxyflavone, tangeretin and 5-hydroxy-6,7,8,3',4'-pentamethoxyflavone were isolated in milligram quantity.

**Flash Chromatography**

FC also called as medium pressure liquid chromatography which is a faster technique of column chromatography. The regulated application of medium pressure enables separation of compounds using large sample volumes, thereby yielding high quantity of pure compounds. Recent technological advances have also enabled conducting separations with online detection and robotic fraction collectors. These advancements have enabled in development of large scale separation of PMF's. Dried peels of Cleopatra mandarin and Marrs sweet orange (*C. sinensis L. Osbeck*) fruits were powdered and extracted using a non polar solvent in a Soxhlet. The extract was concentrated, impregnated with silica gel and subjected to separation using flash chromatography. A gradient solvent system was used for separation and the eluent was monitored at wavelengths of 254 nm & 340 nm. Individual peaks were collected in fractions and pooled after analyzing by HPLC. The isolated compounds were identified as sinensitin, tetramethoxyflavone, nobiletin, and tangeretin using NMR. and mass spectrometry (16).

**ISOLATION METHODS**

A number and variety of methods for the detection and quantification of flavonoid compounds in fruit have already been developed. Several analytical procedures allow the simultaneous determination of the various kinds of flavonoid glycosides as flavanone-O-glycosides, flavone-O-glycosides, flavone-C-glucosides and polymethoxyflavones. Among the various techniques, we will focus on liquid chromatography (LC) and more recent coupled methods as LC-MS, and LC-MS-MS, with brief mention of less widely employed techniques (GC-MS, CE)

**Gas chromatography (GC)**

GC techniques are of limited application in flavonoids analysis as a consequence of the very low volatility of flavonoids, especially glycosylated ones. Usually flavonoids are transformed to more volatile derivatives by suitable derivatization (17).

**High Performance Liquid Chromatography (HPLC)**

HPLC methodologies represent, to date, the most widely used approach to the analysis of phenolics. In most cases, HPLC techniques allow flavonoid profiles in juices to be obtained without the need for preliminary derivatization and sample preparation. Reversed-phase chromatography has been extensively employed for the separation of flavonoids on C8 or C18 columns with polar mobile phases, such as methanol, acetonitrile, tetrahydrofuran or acid solutions. Gradient elution has often been used to obtain the profile of separated flavonoids. Under normal reversed-phase conditions, the more polar compounds are generally eluted first. Thus, diglycosides precede monoglycosides, which in turn precede aglycones. The classes of flavonoids that characterize Citrus species (flavanones, flavones, and, to a lesser extent flavonols/flavanols) have their maximum absorption at specific wavelength ranges: flavanones (280-290 nm), flavones (304-350 nm) and flavonols (352-385 nm). Simultaneously recorded DAD chromatograms of a red orange juice at two different wavelengths, for example, at 284 and 326 nm (Figure-2), highlights the difference in behaviour of flavanones compared to flavones under UV detection (18).

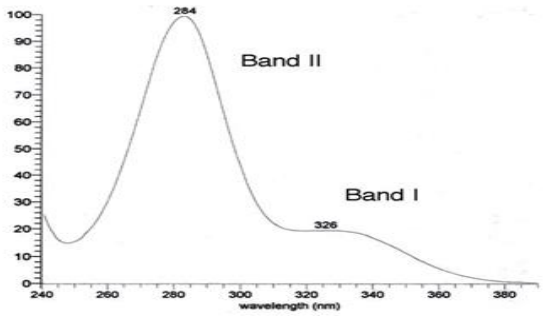


**Figure-2 UV spectrum of flavanones and flavones**

**Other chromatographic and miscellaneous methods.**

Other techniques have been investigated for application to flavonoid analysis, although of less importance than the extensively applied HPLC. Thus, capillary electrochromatography method has been used to detect 7-O-glycosides in Citrus juices . The same technique has been examined in the chiral separation of six diastereoisomeric flavanone-7-O-glycosides in lemon juice. Flavonoid separation in orange juice has also been achieved by using Micellar Electrokinetic Chromatography.

A different technique has been employed for the direct determination of naringin, making use of the formation of naringin-mercury complexes on the surface of a hanging mercury drop electrode. Cathodic stripping voltammetry makes it possible to determine of naringin between 0.1 and 40 mg/L (19).

**Coupled methods**

HPLC coupled with mass spectrometry (tandem HPLC-MS) has often been used for the structural characterization of phenolics. The identification of phenolics collected after HPLC separation has also been carried out using fast atom bombardment mass spectrometry (FAB-MS), electrospray ionization mass spectrometry (ESI-MS) and atmospheric pressure chemical ionization (APCI-MS). In some cases, MS detection may provide enough data for a complete flavonoid structure analysis. However it is more generally used to determine molecular mass and to establish the nature of substituents between the A and the B rings. The ESI or APCI sources are very soft in the ionization and are commonly utilized to obtain TIC chromatograms and mass spectra in correspondence with each peak. Mass spectra, generated in positive mode, show pseudomolecular [M+H]+ ions together with other fragments depending on the voltage applied to the source. An acid (acetic or formic) is often added to mobile phases as a source of protons to assist ionization. Many flavonoids show low sensitivity in positive mode MS analysis and are therefore detected in negative mode. In this case, fragmentations start from pseudomolecular peak [M–H] (20).

HPLC-diode array detection-electrospray mass spectrometry has recently been used for a qualitative and quantitative determination of the flavonoid content of Citrus juices . Often, one-course chromatographic analysis has been performed on crude juice. Two chromatograms at different wavelengths, and in correspondence with each peak UV spectrum along with full mass and MS-MS mass spectra can be recorded simultaneously. This approach has been successfuly applied to problems involving trace analysis of Citrus flavanones, and can be expected to significantly improve the future development of LC-MS applications. Figure 10 provides an example of the HPLC-DAD-ESI-MS-MS analysis of a lemon juice, focusing on the peak corresponding to diosmin . Other interesting coupled methods are those based on HPLC separation coupled with electrochemical detection, being it either colometric or amperometric (21, 22).

**DETERMINATION OF TOTAL FLAVONOIDS**

The aluminum chloride method was used for the determination of the total flavonoid content of the sample extracts 5. Aliquots of extract solutions were taken and made up the volume 3ml with methanol. Then 0.1ml AlCl 3 (10%), 0.1ml Na-K tartarate and 2.8 ml distilled water were added sequentially. The test solution was vigorously shaken. Absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample (23).

**CONCLUSION**

This review detailed explained the various cold, hot and continuous extraction methods of flavanoids. Various methods of chromatographic isolation and HPLC-coupled (HPLC-DAD-ESI-MS-MS) methods were detailed discussed. This review provide good

information for researchers involved in extraction and isolation of flavanoids.

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