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Review Article

Chromatographic methods for the identification of flavonoids

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Abstract

A current direction of the development of the pharmaceutical industry is obtaining and using extracts from medicinal plants containing various active principles, including flavonoids. The aim of this work is to provide a relevant characterization of some of the most important groups of compounds with a relevant importance in the food and pharmaceutical field. Also, the chromatographic methods will be analysed in the context of the investigation of flavonoids, determining the specific peculiarities. Phenolic compounds are responsible for the main organoleptic characteristics of most plants and also of their transformation products. They contribute especially to color, bitterness and astringency in drinks. In addition, depending on their nature, they can have a nutritional and pharmacological interest.

Key words: flavonoids; chromatographic methods; plant products; thin layer chromatography

Introduction

Flavonoids are a group of plant-derived compounds called polyphenolic compounds recognized as antioxidants. There are about 6,000 different types of known flavonoids. Some flavonoids are used as food supplements or for other purposes, contributing to maintaining health. Flavonoids are a class of plant secondary metabolites that are part of the broader group of polyphenols. These compounds are found naturally in plants and are responsible for the vibrant colors of fruits, vegetables and flowers. There are six subclasses of flavonoids: anthocyanidins, flavan-3-ols, flavanones, flavones, flavonols, and polymers. Flavonoids have been widely studied and found to have numerous health benefits. They are powerful antioxidants, helping to combat oxidative stress and protect cells against damage caused by free radicals. Flavonoids may also have anti-inflammatory, antiviral, antibacterial and anti-carcinogenic effects [1]. Flavonoids include various classes of natural substances, many of which give flowers their yellow, orange, red, or blue color, respectively, to flowers and fruits in particular. As natural pigments present in plants, in addition to being substances that change color and decorate plants throughout their life, they also constitute a protection against the harmful effects produced by oxidizing agents, such as ultraviolet rays, environmental pollution, *etc*. [2]. Flavonoids are present in plants, especially in the systematic group of *Angiosperms*, and only a few have been detected in fungi and algae, so there are no representatives of the plant kingdom that do not contain constituents from the class of flavonoids [3]. All the organs of the plant, especially the young ones and especially the epidermis, young leaves, buds, buds or barely opened flowers are rich in flavonoids. The heteroside (water-soluble) forms accumulate in vacuoles and concentrate in the leaf epidermis, and in the case of flowers they are stored in the epidermal cells, while the aglycones are distributed in the leaf cuticle and in the wood [4]. Flavonoids are major functional components of many vegetables or animal preparations with medical uses. Flavonoids are also used in the treatment of numerous diseases, inhibiting specific enzymes, stimulating hormones and neurotransmitters and reducing the activity of free radicals. Due to the fact that flavonoids contain a variable number of phenolic groups in their chemical structure and due to their excellent properties to form chelates with iron and other transition metals, they have a high antioxidant capacity. For the human body, the antioxidant activity is highlighted due to the fact that they protect the cells of the body from free radicals that are formed as a result of numerous processes that use oxygen as an energy source and thus play an essential role in the protection of oxidative degradation phenomena. Their antiradical properties are due to hydroxyl and superoxide radicals, reactive groups that are involved in the initiation of lipid peroxidation processes, their ability to modify the synthesis of eicosanoids, to prevent platelet aggregation (antimicrobial effect) and to protect basic lipoproteins from oxidation. Although some studies indicate that some flavonoids possess a pro-oxidative action, this occurs only at high doses, with most investigations confirming the existence of anti-inflammatory, antiviral and anti-allergic effects, as well as the protective role in various pathologies [5].

Metabolism of flavonoids after ingestion, the significance of different varieties of flavonoids depends on the type of flavonoids, geography, culture and season considered. in recent studies, the number of flavones

and flavonols that can be ingested is estimated by some authors to be several tens of mg/day, and by other authors to 115 mg or 1-2 g of total flavonoids daily [6].

In order to obtain more precise results, considerable efforts have been made in different countries in the food field to determine vitamin C and flavonoids, studies being focused on flavones and/or flavonols, flavanones, catechins and anthocyanins.

Paper chromatography

Due to their solubility and different Rf values, as well as their property to fluoresce in ultraviolet light, flavonoids can be easily identified by chromatographic techniques. Paper chromatography is one of the oldest methods used for flavonoid analysis, since 1958, 228 flavonoids have been analysed using various solvent systems in various proportions. The most frequently used solvent systems that lead to a good separation of the flavonoid fractions (both glycosides and aglycones) are the two Forestal systems:

- Forestal reagent for glycosides: glacial acetic acid: HCl conc.: water (15:3:82, v/v; and
- Forestal reagent for aglycones: glacial acetic acid: HCI conc.: water (30:3:10, v/v).

Although they are the most used, the Forestal systems produce structural changes or destroy some flavonoids on the chromatogram, so they cannot be used when the elution of flavonic spots on the chromatogram is desired, preferring in this sense 15% acetic acid aqueous solutions (for flavonoids) and acetic acid 60% (for aglycones) or simply water, because it protects the benzopyran nucleus [1].

For the identification of flavonoids by paper chromatography, Whatmann, Schieicher and Schull paper and others are most often used. The solvents used are water, respectively aqueous solutions of organic acids, butanol and mixtures of organic acids, neutral mixtures of organic solvents with low polarity, water and methanol. The hydrophilic systems are used for the separation of glycosides, and the lipophilic ones for the separation of aglycones. The most used solvent systems, in various proportions, are: ethyl-methyl-ketone: acetone: formic acid: water; methyl isobutyl ketone: formic acid: water; chloroform: methanol: formic acid: water; benzene: formic acid: water; m-cresol: acetic acid: water; phenol saturated with water; benzene: methanol: water [2].

Aqueous solutions of polyvalent metals can be used for detection (lead acetate, aluminum chloride, *etc*. give color reactions), solutions of sodium carbonate, methanolic sodium hydroxide, ethanolic solution of ferric chloride, Rhodamine B solution, p-Toluenesulfonic acid. Thus, on the basis of the zirconium-citric acid reaction, used to sprinkle the chromatograms, information is obtained about the substituents from C3 or C5, namely, 5-oxy-flavones with the free hydroxyl group can be identified (rhamnazine, kaempferol, morin, quercetin, myricetin), which give color reaction with $ZrOCl_2$ and yellow fluorescence with citric acid. If the OH group in position 3 is missing or glucosidated, the color reaction with $ZrOCl_2$ does not occur and fluorescence does not appear in the presence of citric acid. As a spraying reagent for visualizing flavonoids and some of their glycosides, aqueous NaOH solution is also used for the usual or two-dimensional chromatogram [3].

For flavones, the borax solution is also used as a spraying reagent, which has the same performance as the reagents mentioned above or the iodine/potassium iodide system. The latter does not give colors with fully methylated flavones and with 3-hydroxy-3',4',4,7-tetramethoxyflavones, but the color intensifies in the presence of vicinal methoxy groups. Flavones, such as apigenin, kaempferol, quercetin give blue or gray-blue spots, while all the corresponding glycosides give yellow to yellow-brown spots. The possibility of flavonoids to present fluorescence in UV light is also used in the case of paper chromatography. In case of visualization of the chromatogram with $AICI_3$, flavonoids give yellow fluorescence, unlike coumarins, which do not give fluorescence. The identification of isoflavones is the most difficult, because they do not fluoresce in UV light. With $FeCl_2$, isoflavones that have a free OH group at C3 give color in daylight. Flavones and flavonols, which have an OH group at the carbon atom, are visualized on the chromatogram with the Wiison reagent and green fluorescent spots appear [4].

Thin layer chromatography

In thin layer chromatography, work variants can be obtained by combining a series of mobile phases and a single stationary phase. Since the information on thin-layer chromatography of flavonoids is numerous, some researchers have tried to centralize them through a statistical processing. Graphical representations, called dendrograms, were thus obtained, based on which the effective chromatographic analysis scheme can be found, as well as the probable Rf values for the flavonoid compounds separated in a concrete situation. Through thin-layer chromatographic analysis combined with UV-VIS spectrophotometric analysis, structural differentiations between 5,6-dihydroxy and 5,8-dihydroxy-flavones could be made. High-performance chromatography systems on modified silica gel were researched by binding some organic compounds (isopropanol, ethyl acetate, methyl-ethyl-ketone, dipropyl-ether, tetrahydrofuran) and elution with n-hexane [5].

Column chromatography (liquid-solid chromatography)

The following adsorbents are used: magnesol, silica gel, cellulose or polyamide powder, calcium sulfate, Sephadex LH-20, Sephadex G-10, G-25 and G 50. Sephadex LH-20 is recommended for the separation of proanthocyanidins, and as a solvent for organic solvents are used for elution; methanol and ethanol are used for the elution of proanthocyanidins, and acetone for the separation of high molecular weight polyphenols. Al_2O_3 is not a good adsorbent for the separation of flavonoids, because it forms stable compounds with them. It is used for the separation of some flavonols by elution with dilute acid or acidic methanol. Polyamide is preferred, because it allows the separation of phenols and their derivatives, polyhydroxyphenols, flavones, flavanones, quinones and tannins. The amido groups in the polyamide structure are responsible for the retention power for the formation of hydrogen bridges with phenolic hydroxyl groups, so that phenols are retained depending on the number of hydroxyl groups, o-diphenols being more easily retained than monophenols. The adsorbed substance is eluted by replacing it with a solvent. The most used solvents for elution are water, methanol, ethanol, acetone, dilute alkaline solutions, formamide and dimethylformamide [6].

The separation of flavonoid aglycones from a methanolic extract of *Crataegus fruits* was carried out using as stationary phase: Sephadex LH-20 and mobile phase: methanol: ethyl acetate mixture = 1:2. The same Sephadex LH-20 adsorbent was also used to isolate quercetin, myricetin and their dihydro compounds, from the methanolic extract of *Pinus contorta* bark, using the methanol: ethanol = 1:1 mixture as elution solvents. Myricetin, kaempferol, quercetin and their glycosides are separated very well on adsorbents such as: silica gel, nitrocellulose and polyacrylamide, using water: methanol = 1: 1 as elution solvent. Barley and hop extracts were analyzed with good results using chromatography liquids, method proposed for standardization in beer analysis. Flavonoid aglycones and glycosidic flavonols were separated in an isocratic regime, under the following working conditions: Sephadex LH-20 column, elution with ethanol for flavonols, and for glycosides an acetic acid-water mixture to which a third component, tetrahydrofuran, is added, acetonitrile or methanol. The detection was carried out in UV at 350 nm, and the identification of the components was done with the help of two-dimensional thin layer chromatography [7].

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography (HPLC) is a technique that offers accuracy, speed and high sensitivity. Compared to gas chromatography, derivatization is not necessary, and detection can be easily done spectrophotometrically. Using a solvent system (methanol-acetic acid-water) and a Ci8 column, 34 flavonoid aglycones were initially analysed, for which correlations were found between retention times and some structural details. Methylation of the hydroxyl groups in the A ring reduces the polarity of the compound and produces an increase in the retention time, while methylation in the B ring reduces the retention time of the aglycone. The identification of flavonoids from pharmaceutical and plant extracts was carried out using various mobile and stationary phases.

For the identification of flavonoids from alcoholic extracts of *Betula pendula* Roth; UV detection 210-400 nm. under these conditions, the compounds: myricetin-3-glycoside, quercetin-3-arabinopyranoside, quercetin-3-arabinofuranoside, quercitrin and apigenin derivatives were separated very well [8]. Rutin, quercetin, eupafolin and hispidulin were identified using a Ci8 column with UV detection at 339 nm [9]. The methanolic extract of *Glycyrrhiza glabra* leaves, purified on silica gel CC (Wako-gel C-200), was analyzed by HPLC with a UV detector at 254 nm. Flavonoids were separated: genistein, pinocembrin, prunetin, 6-prenylnaringenin [10]. The flavonoids, quercetin, kaempferol and myricetin from tomato (*Solanum lycopersicum* L.) were dosed by HPLC, with UV 266 nm detection [11]. The aglycones of the flavonoid's quercetin, kaemferol and isorhamnetin, from *Ginkgo biloba* extracts and their products were identified under different conditions [12]. The identification of quercetin and flavanones glycosides from orange juice was performed by HPLC. UV detection was performed at 280 nm for flavanones, and for flavones and flavonols at 265 nm [13]. The identification and quantification of phenolic compounds from *Phaseolus vulgaris* was carried out on a Nucleosil 120 Cia column. The phenolic structures, proanthocyanidin, (+) catechin, epicatechin, quercetin glycoside, rutin, kaempferol glycoside, as well as daidzein derivatives were identified. This method tried to correlate the linear regression coefficient with the concentration of phenols and the area of the chromatographic peak [14].

The simultaneous analysis of flavonoids and phenolic compounds of different polarities from the leaves of *Betula pendula* and *Betula pubescens* was carried out with the following chromatographic system: HP Hypersil column, and the analysis was monitored at 220, 280, 320, 360 nm [15].

Neutral phenolic compounds {(-) catechin, (-) epicatechin}, flavan-3-ol polymers and flavonol glycosides (isoquercetin, hyperin, quercitrin, avicularin and rutin) from apple juice were separated and quantified by reverse-phase HPLC (RP-HPLC) with Cis column. The UV detection range was between 260-350 nm for flavonols. The chemical composition of *Visnea mocanera* fruits was analysed by TLC and HPLC to establish their composition in phenolic compounds. The analysis of low molecular weight phenolic compounds and 3-flavanols was carried out by ODS; UV detection 266 nm [16].

The determination of flavonoid 5-aglycones was carried out with the chromatographic system: Cis column with reverse phase and UV-VIS detection [17]. The analysis of bioflavonoids from fruits by HPLC chromatography allowed the identification of flavones and their glycosides from grapefruit, apple, and tomato fruits. Flavonoids (quercetin, kaempferol, myricetin), flavones (apigenin, luteolin) from apple, tomato, grapefruit, lemon and orange juices were dosed with a Nova-Pack Cis column, elution was carried out in isocratic mode, UV detection at 280 nm [18].

The different retention times can be interpreted as a result of two opposite tendencies: hydrophobic binding to the column and the formation of bonds with the eluate. Thus, it can be concluded that: in flavones and isoflavones - the carbonyl group is a strong proton acceptor, so if there is a hydroxyl group in its vicinity at C5, a strong hydrogen bond is formed and as a result the interaction with the elution solvent is weak, and the retention time increases. This is valid for aglycones, because in glycosides there are multiple possibilities for the formation of hydrogen bonds. The hydrogen bond with the hydroxyl at C3 is much weaker and as a result the retention time increases less. If there is an OH group at C5, the introduction of another hydroxyl group at C3 usually reduces the retention time and because of this, flavones and flavonols with identical substituents form hard-to-separate pairs. The presence of hydroxyl groups in positions other than 3 and 5 considerably reduces the retention time. These flavonoids can be easily separated. By methylating a hydroxyl group, the retention time changes, and the separation of a flavonoid from its methyl ether is easy, but when a new methoxy group is introduced, the separation is no longer possible. in the case of glycosides, the sugar part introduced has a hydrophilic character, and the contribution to hydrophilic interactions decreases from hexoses to pentoses and methyl-pentoses. Pyranosic and furanosic forms can be clearly separated from arabinose, but with glucose and rhamnose the separation is difficult. The RP-HPLC method with UV spectrophotometric detection was used to determine the majority of components in *Schisandra chinensis* Baill leaves [19].

Another chromatographic method for the separation of flavonoids is RP-HPLC coupled isocratically with the NP-TLC system, when the fractions of flavonoids separated on a 100 RP18 column, UV detection 254 nm, are collected, evaporated and applied to silica plates, and the development is carried out in three steps using gradient elution, methanol-ethyl acetate, as mobile phase. HSCCC (High Speed counter current chromatography) has preparative applications for the separation of isorhamnetin, kaempferol and quercetin from fresh extracts of *Ginkgo biloba* and *Hippophae rhamnoides* L, when the analysis is performed using a Microsorb-MV ODS column and monitoring at A = 254 nm [20].

MPLC (Medium pressure liquid chromatography) is a new method for the isolation of natural compounds. Six phenolic compounds (gallic acid, catechin, rutin, naringenin, quercetin and isoramnetin) were separated by this method.

Gas chromatography

The first attempts to separate simple phenols by gas-liquid chromatography date back to 1968, but results regarding phenols with a more complex structure were published in 1962, when results were reported regarding the analysis of 36 flavonoids using as a phase liquid silicone polymer SE-40. This method allowed the separation, isolation and identification of milligram quantities of these compounds, but the question of the influence of methylation, acetylation and hydrogen bonds arose. As a result, no linear correlation was found between the number of substituents and the retention time, which indicates that the substituents placed in different positions of the flavonoid nucleus influence the retention time differently especially for 3- and 5-substituted flavones. However, there are compounds whose retention times are sufficiently different to allow easy identification. Using SE-30 liquid phase, He gas phase, it was found that: as the number of substituents increases, so does the retention time. Methoxylation decreases the retention time while acetylation increases the time, but only for compounds in which there are no hydrogen bonds. If there are hydrogen bonds, the retention time is much lower than the expected value. Compounds with more than two hydroxyl groups and three methoxy groups do not elute in a timely manner on the column. Increasing the temperature brings some advantages, but simultaneously increases the danger of decomposition of the injected compounds and decreases the "Column Life", which is required at high temperatures [21].

The identification of flavonoids, especially trimethylsilyl flavonoids and their aglycones, was carried out using the OV series of silicone polymers

as the liquid phase. Thus, to identify the ethers of trimethyl flavonoids, a column of silicone polymers SE-30 was used, for the identification of anthocyanidins. The flavonoids quercetin, hesperetin, kaempferol, naringenin, Wagonin and Baicalein (5,6,7-trihydroxyflavone) were identified on several types of columns [22].

The gas chromatograms were obtained at 210°C, where the flavonoids show a slightly weaker band and can be detected very well. Gas-liquid chromatography was also used to elucidate the structure of flavonoids by identifying the compounds resulting from their alkaline degradation. Hydroxy- and methoxy-flavonoids were analysed by combining liquid gas chromatography with FTIR spectroscopy. By means of this technique, correlations were made between IR spectral data and retention times, based on which it was possible to gather information on the nature of the substituents, or s - were able to make predictions on the retention time [23].

The association of gas chromatography with mass spectroscopy allowed the detection of 5-methoxyflavones in blood plasma (the sensitivity of the method allowing the detection of 1 ng flavonoid/mL blood plasma), in plant extracts, as well as for the quantification of the antiradical activity of some plant extracts and the analysis of phenolic compounds from solid matrices.

Conclusion

Flavonoids give intense color to plants and perform many other important functions. More precisely, they are responsible for the color tons of food (fruits and vegetables) and plants in general (leaves, flowers and roots), be they blue, red, orange, purple or emerald green. This study revealed that Chromatographic methods are suitable for determining the total content of flavonoids in different plant products. Even if the benefits of liquid chromatography are well known, thin-layer chromatography can be considered a competitive technique in this field, especially due to its characteristic simplicity and at the same time the speed due mainly to the fact that several can be analysed simultaneously on a single plate compound. The repeatability it shows is due to some parameters that define the chromatographic process and, respectively, the retention mechanism.

Conflict of interests

The authors declare no conflicts of financial interest in any product or service mentioned in the manuscript, including grants, equipment, medications, employments, gifts, and honoraria.

This article is a review and vegetal products have not been administered to men or animals.

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