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# MÉTODOS DE CARACTERIZACIÓN DE FLAVONOIDES EN LA GRANADA (*PUNICA GRANATUM*): UNA REVISIÓN

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**Resumen.-** La granada es un alimento ampliamente consumido en el mundo, debido a sus características nutricionales y a que contiene una elevada cantidad de polifenoles que le confieren una gran actividad antioxidante y debido a su capacidad para transferir electrones a agentes oxidantes aporta efectos beneficiosos en la salud humana entre los que se encuentran sus propiedades anticancerígenas, antidiabéticas, antiinflamatorias, antihipertensión, antifibróticas, antienvejecimiento y cardiovasculares. Estos compuestos se pueden extraer de múltiples formas y estos métodos de extracción se suelen clasificar en dos grupos: convencionales (extracción Soxhlet, maceración e hidrodestilación) y no convencionales (extracciones asistidas por ultrasonido, microondas y presión), sin embargo, el método seleccionado dependerá del uso que se le dará al extracto. Una vez obtenidos los extractos, los flavonoides se pueden caracterizar estructuralmente. Este procedimiento analítico dependerá del interés del análisis que incluye técnicas cromatográficas (cromatografía de gases, cromatografía en capa fina y cromatografía líquida de alta eficiencia) y espectrofotométricas (espectroscopia UV-Vis, espectroscopia de masas, espectroscopia de resonancia magnética nuclear, entre otras).

**Palabras Clave**: Agroindustria, análisis químico, efectos fisiológicos, fisiología vegetal.

# FLAVONOID CHARACTERIZATION METHODS IN POMEGRANATE (*PUNICA GRANATUM*): A REVIEW

***Abstract.-*** Pomegranate is a widely consumed food in the world, due to its nutritional characteristics and because it contains a high amount of polyphenols that give it a high antioxidant activity and due to its ability to transfer electrons to oxidizing agents it provides beneficial effects on human health among which are its anticancer, antidiabetic, anti-inflammatory, antihypertensive, antifibrotic, antiaging and cardiovascular properties. These compounds can be extracted in multiple ways and these extraction methods are usually classified into two groups: conventional (Soxhlet extraction, maceration and hydrodistillation) and unconventional (ultrasound, microwave and pressure assisted extractions), however, the method selected will depend on the intended use of the extract. Once the extracts are obtained, the flavonoids can be structurally characterized. This analytical procedure will depend on the interest of the analysis, which includes chromatographic techniques (gas chromatography, thin layer chromatography and high performance liquid chromatography) and spectrophotometric techniques (UV-Vis spectroscopy, mass spectroscopy, nuclear magnetic resonance spectroscopy, among others).

***Keywords***: Agroindustry, chemical analysis, physiological effects, plant physiology.

## Introduction

Nowadays the consumption of food by the general population is carried out in a more selective way due to the knowledge about nutrition and how this can affect your life (Tenorio & Tenorio, 2021), that is why it is of greater importance to know the chemical composition of the food.

Pomegranate is a well-known and widely consumed fruit in the world, which is rich in polyphenols that give it a high antioxidative activity (Fahmy et al., 2020). It is also a low-calorie fruit with excellent nutritional characteristics (high content of carbohydrates, protein, fiber and minerals) (Magangana et al., 2020), which draws the attention of the population that consumes it. That is the reason why the production of pomegranates currently exceeds 2 billion tons per year (Pienaar & Barends-Jones, 2021). India, the country from which the pomegranate comes from, has been the world leader in the production of pomegranates for 8 years and is one of the main exporters of pomegranates, whether fresh or treated (Bustamante, 2019). Due to its high antioxidant activity, pomegranate possess anti-aging and anti-

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cancer properties; therefore, its detection is of interest to the industry. Most of the natural antioxidants are polyphenolic compounds including polyphenolic acids, flavonoids and anthocyanins (Karak, 2019).

Flavonoids have a high affinity for some proteins and for other biological macromolecules, such as hormones and nucleic acids, and for divalent ions of metals (Vijayakumar et al., 2020). They also have a great capacity to act as free radical receptors, and catalyze the transfer of electrons, which gives it a high antioxidant power. Plants use synthesized flavonoids for a multitude of functions, including signaling, attracting pollinators, protection from ultraviolet light, and protection against attack by different microorganisms (Mathesius, 2018). These compounds are classified into flavones, flavonoids, isoflavones, flavanones, flavonoids, and anthocyanidins (Shen et al., 2022).

These characteristics of the mentioned compounds bring beneficial effects to human health (Tiwari & Husain, 2017 Sarkar et al., 2022). That is why the interest in the consumption of flavonoids has increased and explain why these types of compounds have an important role within functional foods (Premathilaka et al., 2022).

The pomegranate in the last years has attracted the interest of researchers due to the high content of compounds products of the secondary metabolism of the fruit and that have various applications including medical, food and cosmetic applications (Kori et al., 2020: Arlotta et al., 2020), this review article presents the main methods of extraction, purification and characterization of flavonoids applied to pomegranate extracts.

## Chemical constituents of pomegranate

About the half of the entire fruit weight corresponds to the peel, a vital supply of bioactive compounds like flavonoids, phenolics, proanthocyanidins, and ellagitannins, minerals, primarily metal, phosphorus, sodium, magnesium, nitrogen and calcium, and polysaccharides (Kushwaha et al., 2013). The eating part of the pomegranate which it´s about the fifty percent of the fruit, contains eighty percent arils and twenty percent seeds (Melgarejo et al., 2020). Arils contain eighty-five percent water, ten percent total sugars, mainly fruit sugar like aldohexose, and 1.5 percent organic acids, like vitamin C and malic acid, pectin and bioactive compounds like flavonoids, anthocyanins and other phenolics compounds (Sreekumar et al., 2014).

One of the most teams of compounds to blame for many of the purposeful properties of the fruits, among which is the pomegranate fruit, are polyphenols compounds in any of its different structures (Cervantes et al., 2022).

The term "phenolics" covers an enormous and various cluster of chemical compounds. These molecules will classify in different ways (Truzzi et al., 2021). One among them is to classify them consistent with the quantity of carbons within the molecule as shown in table 1.

Table 1. Classification of the phenolic compounds (taken and modified from Truzzi et al., 2021).

| ***Chemical structure*** | ***Class*** |
|---|---|
| *C6* | simple phenolics |
| *C6-C1* | phenolic acids and related compounds |
| *(C6-C1)n* | hydrolyzable tannins |
| *C6-C2* | acetophenones and phenylacetic acids |
| *C6-C3* | hydroxycinnamic acid and coumarins |
| *(C6-C3)2* | lignans |
| *C6-C1- C6* | benzophenones and xanthones |
| *C6-C2- C6* | stilbenes |
| *C6-C3- C6* | flavonoids |
| *(C6-C3- C6)n* | proanthocyanidins |

*Note*. This table shows the classification of polyphenols according to their chemical structure and the number of carbons present, as well as the name assigned to them.

## Flavonoids

Within the phenols, the group that stands out the most are the flavonoids, these molecules are natural low molecular weight (MW) compounds, which share a common diphenylpyrans skeleton (C6-C3-C6), made up of two aromatic rings (A and B) linked by a three-carbon aliphatic chain, which can combine with one oxygen and two carbons from ring A, to form a third ring, called ring C, of the pyran type, or to a lesser extent of the furan type (Safe et al., 2021). In plants, flavonoids have a multitude of functions including the signaling, attracting pollinators, protecting from UV

light and phytopathogens (Miklavcic, 2019). These compounds can be classified into anthocyanidins, flavonones, isoflavones, flavanols and flavones (Brodowska, 2017).
A significant number of flavonoids are identified to have health benefits to humans (Ballard & Junior, 2019). They include anti-inflammation, anti-cancer, cardiovascular and neurological protective functions (Miklavcic, 2019).

Extraction methods
It is solely attainable to conduct additional separation, identification, associated characterization of polyphenol compounds and then proceed with an applicable extraction method.

There are different extraction strategies which will be accustomed extract bioactive compounds from plants. This may rely on the utilization that may tend to the extract once processed (Alara et al., 2021).
The extraction strategies of bioactive compounds may be classified into two groups: conventional and unconventional ways (Jahromi, 2019).

Conventional strategies area based on the extraction power of various solvents in use and the application of warmth and/or intermixture. These strategies are: Soxhlet Extraction, Maceration and Hydrodistillation (Osorio, 2020).
Unconventional strategies have arisen as ecological alternatives to the standard strategies, like ultrasound, microwave, and pressure aided extractions, applied with different types of solvents or even without the use of them, to scale back the energetic cost and solvent demand (Hidalgo & Almajano, 2017).

The main objective of those new strategies is to scale back the extraction time and thus increase its efficiency in terms of milligrams extracted per hour of extraction (Caldas et al., 2018), as shown in table 2.

Table 2. Flavonoid extraction in Saussurea medusa Maxim, using conventional and unconventional methods (taken and modified from Gao & Liu, 2005).

| ***Technique*** | ***Flavonoids % (w/w)*** | ***Time (hours)*** | ***Efficiency (mg/h)*** |
|---|---|---|---|
| *Solvent a room temperature* | 3.0% | 24 | .02 |
| *Heat reflux* | 3.9% | 6 | .13 |
| *Soxhlet* | 4.1% | 20 | .04 |
| *Ultrasound* | 3.5% | .5 | 1.4 |
| *Microwave* | 4.1% | .1 | 8.2 |

*Note*. The table shows the comparison in the efficiency of the extraction strategies, wherever it's determined that unconventional strategies (microwave and ultrasound) are favored during this section (Gao & Liu, 2005).

There is a statistical method which has the name of Response Surface Methodology (RSM), supported on a second-order polynomial model is sometimes used to see the most effective union of process specifications to confirm largest extraction effectiveness (Hasani et al., 2020). Consistent with the second order polynomial model, a response surface plot is created to analyze the optimum treatments and largest extraction efficiency. Once the experimental tests have been carried out, we can obtain data to observe the interaction between the process variables (Hidalgo & Almajano, 2017), once the structural characterization of the flovonoids present has been performed, this type of statistical techniques can help to correlate the type of compounds present with their beneficial properties.

Structural characterization of flavonoids
Many procedures applied to the analysis of flavonoids have been developed, which reflects the complexity of the analysis matrices and the diversity of scenarios that are presented in the analytical challenge (Jug et al., 2018). Therefore, the design of the analytical procedure will depend on the interest of the analysis. For example, in flavonoid profiling and quantification studies, the most useful approach has been chromatography, preferably chromatography efficiency (HPLC) due to the complexity of the matrix that normally is analyzed, and the structural diversity of the compounds present. On the other hand, when identification is required, the use of spectrometric techniques is necessary (Lysiuk & Hudz, 2017).

NMR spectroscopy is undoubtedly one of the tools most powerful available to scientists for characterization structural of organic compounds and of those whose structure is unknown (Silva, 2017). However, Mass spectroscopy and UV-Vis spectroscopy are useful tools in the confirmation of structures or in the identification of certain structural characteristics. Although UV-Vis spectroscopy has been displaced by other spectroscopic methods, it may be useful in determining some structural characteristics of shape simple and with the use of more economic and affordable resources to the most labs (de Villiers et al., 2016).

Infrared and Raman spectroscopy, among others, have also been used for structural characterization purposes.

Techniques of Chromatography

Chromatography is predicated on the principle that when the molecules of a mixture are applied to a surface or enter a solid, the liquid stationary phase (stable phase) is separated from each other by moving with the help of a mobile phase. Factors effective for this separation method include molecular properties related to surface assimilation (liquid-solid), partitioning (liquid-solid), and changes in affinity or molecular weight (Coskun, 2016).

TLC (Thin layer chromatography)

Around the 1950´s the use of paper chromatography was used on several occasions for the determination of phenolic compounds, mostly when flavonoids are involved, but it never came to replace thin layer chromatography (TLC). The above because it is a very simple and low-cost technique, in addition to offering great versatility in qualitative analysis of phenolic compounds from different samples with the use of suitable adsorbents and reagents (Poblocka et al., 2016). Thin-layer chromatography is a "solid-liquid adsorption" chromatography. A phase coated plate is used stationary (polyamide, silica gel, modified silica) of small thickness constant throughout the plate, supported by a plate that can be made of glass or some other support. The mobile phase is liquid and moves up the plate, dragging the components throughout it, causing the separation of the different compounds (Choma & Jesionek, 2015).

The most used mobile phase or eluents in TLC are petroleum ether, methylene chloride, ethyl acetate, cyclohexane, acetone, toluene, diethyl ether, among others; and the degree of elution of substances depends on both their polarity and the polarity of the eluent used (Córdova, 2017).

GC (Gas Chromatography)

At the beginning, the gas chromatography technique was intended for facilitate the determination of different polyphenols. However, as the sample must be gasified, it is necessary to resort to the derivation phase, so that once carried out for experimentation, it was extremely complicated to characterize these compounds (Viñas & Campillo, 2019).

The sample vaporizes and passes to the mobile phase. The different components of the sample to be analyzed are dispersed between the mobile phase and the stationary phase on the solid support (Coskun, 2016).

This technique is the one with the greatest capacity and sensitivity when analyzing volatile components. It usually works with components of lower molecular weight at 1000 g/mol and a maximum temperature of 400°C (Navarro et al., 2015).

According to the nature of the phase stationary we have two types of GC:

- Gas-solid chromatography.
- Gas-liquid chromatography

HPLC (High-performance liquid chromatography)

High performance liquid chromatography (HPLC) is one of the most common methods for characterizing polyphenolic compounds. With this chromatography technique it is feasible to carry out a structural and specific analysis, in addition to purifying numerous molecules of a sample in a short time. In this technique, the mobile phase passes through columns with a pressure below 400 atmospheres and with a high flow. The use of small particles (generally 5 micrometer particles are used) and the application of high pressure in the solvent flow rate will increase the power in the separation (Coskun, 2016).

The main components of an HPLC equipment are the following: a solvent tank, a high-pressure pump, the column, a detector, and a recorder. A device will help to detect some property of the eluent that will depend on its chemical composition, the detection is carried out continuously (Patil, 2017).

It´s a sensitive and selective technique; it helps quantify isolated substances and is wide used for all categories of flavonoids (Mizzi et al., 2020).

Spectrophotometric techniques

UV-Vis spectroscopy (Ultraviolet-visible spectroscopy)

Molecular UV-Vis spectroscopy, like other spectroscopic techniques, is based on the study of the interaction of electromagnetic radiation with molecules. Energy of the electromagnetic radiation provinces of the UV-Vis region produces electronic transitions (electronic jumps) between a ground state and an excited state (Perkampus, 2013).

UV-Vis spectroscopy is frequently used in quantitative analysis since the Lambert-Beer law establishes a relationship directly proportional between absorbance and concentration of dilute solutions. However, in the case of flavonoids, have established simple experimental procedures, based on the use of bathochromic displacement reagents, which allow inferring structural characteristics (Madaci et al., 2022).

MS (Mass spectroscopy)

Mass spectrometry is an analytical technique that allows the identification of different molecules within a substance by ionizing the sample (Urban, 2016). The identification of the molecules is obtained after bombarding the sample with electrons, which results in the electronic loss of the molecules and their ionization. This results in a characteristic mass distribution (fingerprint) for each molecule (Sephton et al., 2018).

The need to convert neutral molecules into ions to perform the spectrum of masses has conditioned the development of various ionization methods (Awad et al., 2015). Electronic ionization and ionization by electrospray are two of the most common ionization methods used in the structural analysis of flavonoids (Sen et al., 2019). Electron ionization is a "hard" ionization method where a beam of electrons interacts with the molecule and favors the formation of a radical ion called molecular ion M+., caused by the loss of an electron and the electrospray ionization method is frequently used in the analysis of non-volatile or heat-sensitive molecules, this method of ionization causes ionization of the molecule and produces little or no fragmentation, favoring the identification of the molecular mass (Rajawat & Jhingan, 2019).

NMR (Nuclear magnetic resonance)

NMR spectroscopy is a physico-chemical method that is based on the magnetic properties possessed by the atomic cores of the molecules to be analyzed (De Graaf, 2019). This technique is based on the characteristic absorption of energy by the cores that rotate within a strong magnetic field, after being irradiated with another field weaker secondary and perpendicular to the first, thus allowing to identify the atomic configuration in molecules. The cores of some isotopes have a intrinsic movement of rotation around its axis. Their own spin of these particles generates a magnetic moment along the axis of the spin. If such a core is placed in an external magnetic field, its movements could be aligned in favor of or against of that field (Fariña, 2016).

An NMR spectrometer basically consists of an emitter and a detector of radio frequency and a magnet. The sample under study is placed between the two poles of that magnet and is subjected to a radio frequency field by the emitter. At this time, the sample will absorb energy, both magnetic and electrical. When the relationship between the magnetic and electric fields is adequate, a pulse will be emitted, and the receiver will register a signal (De Graaf, 2019).

This technique can only be used to study atomic cores with an odd number of protons or neutrons (or both), which occurs in the next atoms: 1H, 13C, 19F and 31P (Lambert et al., 2019).

The most studied cores in organic chemistry are cores of 1H and 13C. The 1H has a high natural abundance (99.98%), which favors obtaining 1H NMR spectra (Connor et al., 2019). On the other hand, the 13C cores has a low natural abundance (1.1%) and obtaining of the corresponding NMR spectra requires more sample and / or experimentation time (Giraudeau, 2020). In the case of flavonoids, the 1H NMR spectrum contributes with essential information on the type of substitution in the ring's aromatics and the structural characteristics of the C ring, which allow define the type of flavonoid studied. The most informative region of the spectrum is the region between 6-9 ppm (Lovatti et al., 2020).

Other characterization techniques

In addition to these flavonoid characterization methods, two other methods, such as infrared spectroscopy and Raman spectroscopy, have been used more often in recent years. These methods have the advantage of being inexpensive compared to those described above and simple to use since no pretreatment of the sample or the use of any extra chemicals is required (Geraldes, 2020). The interpretation of the spectra obtained by this pair of techniques is not so simple, since each compound has a spectral band with a different and unique pattern and therefore requires an extensive literature review, with the help of these methods can identify different functional groups and structural bonds of the compounds so that with these techniques can be identified as well as quantify different types of compounds (Gniadecka et al., 1997). The structure of the molecules is in most cases very important and very influential in the properties of the different compounds and their biological activity, such as in the case of flavonoids where their molecular structure is directly related to their biological properties (antibacterial, antiviral & antifungal). (Brown et al., 1998). Therefore, these techniques allow you to observe the molecular structure of the compounds and the different anchored functional groups responsible for the different properties (Krysa et al., 2022).

In recent years, even transmission electron microscopy and scanning electron microscopy have been used to characterize flavonoid extracts, either directly observing the morphology of certain solids to identify compounds (Kanaze et al., 2006), or indirectly using the extracts in the synthesis of various metals, thus observing the form of these metals (Kashkouli et al., 2018: Veeramani et al., 2022).

Also techniques to observe the crystalline structure of materials such as X-ray diffraction have been used to observe the crystalline structure of different flavonoid complexes that can be used for the treatment of various diseases (Kim, 2020: Zhou et al., 2022).

## Conclusions

Due to the potential and diverse applications of bioactive compounds, researchers have focused their attention on extraction methods and conditions for these compounds for various analytical, formulation and industrial purposes. In the past few years, various extraction methods, which are referred to as conventional extraction methods in this paper, have been developed, however these methods have several disadvantages among which are: low yields, long extraction times, degradation of some compounds of interest, operating costs as well as the use of large quantities of organic solvents and the problems this brings with it. This is why in recent years new extraction techniques have emerged, which we call unconventional methods in this work, these techniques aim to increase the extraction efficiency of the compounds of interest by reducing or eliminating the use of organic solvents or replacing them with green solvents, which in turn results in shorter extraction times and increased extraction yields. Therefore, several research projects with these new methods have been developed in recent years. However, due to the nature and complexity of the different plant matrices used, it is agreed that there is no single and standardized method for each extractable material or type of flavonoid.

It is because of these different extraction methods that the aforementioned characterization methods become relevant to improve the use of these extracts. This type of characterization helps us understand how flavonoids have different health-promoting properties and how they can be improved. Therefore, this review describes the main characterization methods used and their recent adaptations.

For this reason, researchers continue to develop and apply different characterization methods for these important compounds which are beneficial to human health, and providing a reference point for current methods will enable the development of new characterization methods.

The future in the extraction, purification and characterization of catalysts is focused as mentioned in the development of these characterization techniques especially in the adsorption and excretion of these molecules, especially because there is not enough information about the chronic consumption of these compounds, also to discover different flavonoids that can contribute in the treatment of different diseases and which has a very large field of application. Different studies have recommended the realization of different in vivo researches to observe the reaction of different organisms to the treatment of different diseases with flavonoids.

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