**Procedimientos actuales para la extracción y purificación de flavonoides cítricos**

**Current procedures for extraction and purification of citrus flavonoides**

**Título corto: Current extraction of citrus flavonoids**

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**Resumen**

En la industria alimenticia, los agentes microbicidas son usados para preservar la calidad y seguridad de los alimentos procesados. Los flavonoides encontrados en extractos cítricos han mostrado capacidad de inhibición del crecimiento celular de un gran grupo de microorganismos infecciosos, por lo tanto, éstos compuestos pueden ser útiles como agentes antivirales, antifúngicos y antibacteriales. Los flavonoides que se pueden encontrar principalmente en varias de las especies cítricas son hesperetina, hesperidina, luteolina, naringenina, naringina, narirutina, neohesperidina, nobiletina y tangeretina. A continuación se resumen los procesos utilizados recientemente para extraer, purificar y analizar los flavonoides principales en frutas cítricas.

Ya optimizado el medio de cultivo se procedió a realizar una cinética confirmatoria con base a las condiciones encontradas pero evaluando el crecimiento celular por conteo en cámara de Neubauer y la producción de etanol mediante método enzimático (Procedimiento de ensayo K-ETOH, Megazyme) para confirmar los valores.

**Palabras clave:** flavonoides, polifenoles, purificación, cítricos, microbicida.

**Abstract**

Numerosos estudios ecológicos se han realizado a lo largo de los años para conocer la dinámica, cuantificación y composición de la microflora responsable de las fermentaciones Numerosos estudios ecológicos se han realizado a lo largo de los años para conocer la dinámica, cuantificación y composición de la microflora responsable de las fermentacionesIn the food industry, antimicrobial agents are used for preserving the quality and safety of processed food. Flavonoids found in citrus extracts inhibit cell growth of a large group of infectious microorganisms, therefore, these compounds may be useful as antiviral, antifungal and antibacterial agents. Hesperetin, hesperidin, luteolin, naringenin, naringin, narirutin, neohesperidin, nobiletin and tangeretin are some of the main flavonoids found in various citrus fruits. The processes used in recent years to extract, purify and analyze typical flavonoids from citrus species are reviewed. Ya optimizado el medio de cultivo se procedió a realizar una cinética confirmatoria con base a las condiciones encontradas pero evaluando el crecimiento celular por conteo en cámara de Neubauer y la producción de etanol mediante método enzimático (Procedimiento de ensayo K-ETOH, Megazyme) para confirmar los valores.

**Key words:** flavonoids, polyphenol, purification, citrus, antimicrobial.

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**Introduction**

Flavonoids are a group of polyphenols that are found in fruit, vegetables, nuts, seeds, stems and flowers as well as tea, wine, propolis and honey (Tham & Liew, 2014). Some flavonoids are responsible for fruit coloration. Most of flavonoids are structured basically by 3 rings, two of them are aromatic benzene rings (called rings A and B), connected by an oxygen pyrane ring (called ring C), as shown in figure 1, and there is the characteristic presence of hydroxyl groups in one or more R positions.



**Figure 1.** Flavonoids general structure.

Especially abundant in citrus fruits, there are some flavonoids that can be found in almost all citrus fruits (table 1), like hesperidin, which consolidate a group called citrus flavonoids. Their concentration in peels is higher than in juice and seeds (Codoñer & Valls, 2010) as a result of flavonoids role for fruit coloration.

**Table 1**. Substitution in the general structure for some of the main citrus flavonoids (Barreca *et al.*, 2011a; Gonzalez *et al.*, 2010)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **R1** | **R2** | **R3** | **R4** | **R5** | **R6** | **R7** | **R8** |
| **Flavanones** |  |  |  |  |  |  |  |  |
| Hesperidin | H | OH | H | O-rut | H | OH | OCH3 | H |
| Naringin | H | OH | H | O-nh | H | H | OH | H |
| Neohesperidin | H | OH | H | O-nh | H | OH | OCH3 | H |
| Narirutin | H | OH | H | O-rut | H | H | OH | H |
| **Flavones** |  |  |  |  |  |  |  |  |
| Hesperetin | H | OH | H | OH | H | OH | OCH3 | H |
| Naringenin | H | OH | H | OH | H | H | OH | H |
| **Flavone Aglycon** |  |  |  |  |  |  |  |  |
| Luteolin | H | OH | H | OH | H | OH | OH | H |
| **Polymethoxyflavones** |  |  |  |  |  |  |  |  |
| Nobiletin | H | OCH3 | OCH3 | OCH3 | OCH3 | OCH3 | OCH3 | H |
| Tangeretin | H | OCH3 | OCH3 | OCH3 | OCH3 | H | OCH3 | H |

*rut: rutinose; nh: neohesperidose.*

Citrus flavonoids are well known for their antioxidant (Asikin *et al.*, 2015; Yu *et al.*, 2014; Barreca *et al.*, 2011a; Barreca *et al.*, 2011b; Pekal *et al.*, 2011; Procházková *et al.*, 2011; Ye *et al.*, 2011; Kelebek, 2010) antifungal (Buer *et al.*, 2010; Montes, 2009), and antimicrobial effect (Celiz & Audisio, 2011; Cushnie & Lamb, 2011; Vikram *et al.*, 2010), and even for accelerating wound and disease healing (Wang *et al.*, 2014; Arab & Liebeskind, 2010; Codoñer & Valls, 2010; Neves *et al.*, 2010).

For nourishment purposes, research has concluded that consumers in theory are ready to accept food rich in flavonoids, by informing them about the scientific benefits (Zang et al., 2014; Zhang *et al*., 2014a; Jung *et al.*, 2011; Lampila *et al.*, 2009). In Europe, an average adult person spends up to €454.7 per year in flavonoids contained in cardiovascular drugs (Sanfelix *et al.*, 2010).

**Raw materials**

Citrus fruits contain a range of key nutrients such as vitamin C, folate, dietary fiber, minerals and phytochemicals, which attributes to their health-promoting properties (Ledesma & Luque, 2014). It is believed that vitamin C is a major contributor to the anti-oxidant capacity of citrus. However, the major contribution of citrus anti-oxidant activity comes from the combination of phytochemicals and from their synergistic action with vitamin C. The major phytochemicals in citrus fruits are the terpenes and phenolic compounds, which possess anti-inflammatory and anti-carcinogenic activity (Wang *et al.*, 2014; Natarajan *et al.*, 2011; Codoñer & Valls, 2010).

The main citrus fruits in flavonoids research have been orange (*C. sinensis*), lemon (*C. limon*), grapefruit (*C. paradisi*) and tangerine (*C. reticulata*), for at least the past 30 years. Nowadays, native varieties are of special interest. Tough *Citrus* species are harvested all around the world (Lorente *et al.*, 2014), some species are better developed than others, depending on climate conditions, and its availability varies from one country to another or even regions within the same country (Roussos, 2011). This influences the research on specific *Citrus* species (table 2), where the main citrus producing countries have more studies on a wider variety of species, exploring even the wild varieties found in unique locations.

**Table 2**. Citrus species used in recent flavonoid research.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Citrus species** | **Country** | **References** | **Citrus species** | **Country** | **References** |
| *C. aurantifolia* | Italy | Costa *et al.*, 2014 | *C. paradisi* | China | Xi *et al.*, 2014a  |
|  |  | Loizzo *et al.*, 2013 |  |  | Sun *et al.*, 2013 |
|  | Spain | Guimaraes *et al.*, 2010 |  |  | Zhang *et al.*, 2011 |
| *C. aurantium* | Algeria | Lagha & Madani, 2013 |  | Spain | Abad *et al.*, 2014 |
|  | China | Sun *et al.*, 2013 |  |  | Guimaraes *et al.*, 2010 |
|  | Italy | Barreca *et al.*, 2011a |  | Turkey | Kelebek, 2010 |
|  | Tunisia | Moulehi *et al.*, 2012 | *C. poonensis* | China | Xi et al., 2014b  |
| *C. bergamia* | Italy | DiDonna *et al.*, 2011 |  |  | Sun *et al.*, 2013 |
|  | Mauritius | Ramful *et al.*, 2011 |  |  | Ye *et al.,* 2011 |
| *C. clementina* | China | Xi et al., 2014b | *C. reticulata* | China | Xi et al., 2014b |
|  | Colombia | Alvarez *et al.*, 2012 |  |  | Zhang *et al*., 2014c |
| *C. daoxianensis* | Mauritius | Ramful *et al.*, 2011 |  | Colombia | Londoño *et al.*, 2010 |
| *C. erythrosa* | China | Ye *et al.,* 2011 |  | Croatia | Levaj *et al*., 2008 |
| *C. grandis*  | China | Duan *et al.*, 2014 |  | Italy | Barreca *et al.*, 2013 |
|  |  | Li *et al.*, 2014 |  | Mauritius | Ramful *et al.*, 2011 |
|  |  | Xi *et al.*, 2014 |  | Slovenia | Makovsek *et al.*, 2012 |
|  |  | Zhang *et al.*, 2014b  |  | Spain | Abad *et al.*, 2014 |
|  |  | Sun *et al.*, 2013 |  | Tunisia | Moulehi *et al.*, 2012 |
|  |  | Zhang *et al.*, 2011 |  | Turkey | Kelebek & Selli, 2014 |
|  | South Korea | Yoo *et al*., 2009 | *C. sinensis* | Algeria | Lagha & Madani, 2013 |
| *C. jambhiri* | Egypt | Hamdan *et al.*, 2011 |  | China | Pan *et al.*, 2014 |
| *C. junos*  | South Korea | Yoo *et al*., 2009 |  |  | Sun *et al.*, 2013 |
| *C. latifolia* | Colombia | Londoño *et al.*, 2010 |  | Colombia | Londoño *et al.*, 2010 |
| *C. limetta* | México | Rodriguez *et al.*, 2014 |  | Mauritius | Ramful *et al.*, 2011 |
|  | Italy | Barreca *et al.,* 2011c |  | Italy | Barreca *et al.*, 2014 |
| *C. limon* | China | Sun *et al.*, 2013 |  | Spain | Abad *et al.*, 2014 |
|  | Spain | Abad *et al.*, 2014 |  |  | Andreu *et al.*, 2010 |
|  |  | Gonzalez *et al*., 2010 |  |  | Guimaraes *et al.*, 2010 |
|  |  | Guimaraes *et al.*, 2010 |  | Taiwan | Chen *et al.*, 2011 |
| *C. maxima* | Mauritius | Ramful *et al.*, 2011 | *C. succosa* | China | Ye *et al.,* 2011 |
| *C. medica* | Italy | Menichini *et al.*, 2011a | *C. suavissima* | China | Ye *et al.,* 2011 |
|  |  | Menichini *et al.*, 2011b | *C. tardiferax* | China | Ye *et al.,* 2011 |
| *C. meyeri* | Mauritius | Ramful *et al.*, 2011 | *C. unshiu* | China | Xi et al., 2014b |
| *C. microcarpa* | Singapore | Cheong *et al.*, 2012 |  |  | Sun *et al.*, 2013 |
| *C. mitis* | Taiwan | Yu *et al.*, 2013 |  |  | Ye *et al.,* 2011 |
| *C. myrtifolia* | Italy | Protti *et al.*, 2015 |  | Croatia | Levaj *et al*., 2008 |
|  |  | Barreca *et al.*, 2011b |  | Korea | Jung *et al.*, 2011 |
|  |  | Scordino *et al.*, 2011 |  | Mauritius | Ramful *et al.*, 2011 |
|  |  | Barreca *et al.*, 2010 |  | South Korea | Yoo *et al*., 2009 |
|  |  |  |  | Spain | Abad *et al.*, 2014 |
|  |  |  |  | Turkey | Kelebek & Selli, 2014 |

**Raw material conditioning**

Citrus have been collected and extracted in early springtime or late winter (Sandoval *et al.*, 2012; Barreca *et al.*, 2010), and about 2-5 months after the flowering period (Barreca *et al.,* 2011a; Chen *et al.*, 2011; Yoo *et al.*, 2009). Even though most compounds are found in the peel, as indicated before, studies also have been made to obtain extracts rich in flavonoids from the juice and the seeds. In order to facilitate the extraction of the components, raw material must be ground to a small particle size, improving extract transport from the solid matrix towards the solvent phase.

Raw material can be used either fresh or dry (Ye *et al.*, 2011), the use of fresh raw materials involves the presence of an aqueous phase in the extract, and a further separation, like decantation, must be carried out.

When dry raw material is used, it must be conditioned first, in order to permit cells to stretch back to their original size and shape, allowing the components to transfer through the cell’s structure into solvent bulk. Juices, peels and other tissues can be separated manually (Barreca *et al.*, 2011a) or using commercial extractors.

**Preliminary Separation**

Organic solvents are often used for the extraction of citrus compounds. There are two main operations to extract the compounds from the citrus matrix. One is simple maceration, with solvents extracting the compounds by diffusion from the citrus matrix (Yoo *et al.*, 2009), where methanol is a frequently used solvent (table 3). The second one is centrifugation of the juices, eliminating aqueous phases (Barreca *et al.*, 2011a).

**Table 3**. Solvents used for flavonoids extraction in citrus species.

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Citrus species** | **Separation Technique** | **Solvents**  | **References** | **Citrus species** | **Separation Technique** | **Solvents**  | **References** |
| *C. aurantifolia* |  |  |  | *C. myrtifolia* |  |  |  |
| Juice | Centrifuged, filtered and evaporated | EASSE | Costa *et al.*, 2014 | Albedo | Centrifuged and filtered | DMF | Barreca *et al.,* 2011b |
|  | Lyophilized, disolved in water | Water | Guimaraes *et al.*, 2010 | Flavedo | Centrifuged and filtered | DMF | Barreca *et al.,* 2011b |
| Peel | Lyophilized, stirred and filtered | MOH | Guimaraes *et al.*, 2010 | Juice | Centrifuged and filtered | DMF | Barreca *et al.,* 2010 |
|  | Macerated, filtered and evaporated | MWC | Brito *et al.*, 2014 |  |  |  | Barreca *et al.,* 2011b |
|  | Maceration | MWH | Loizzo *et al.*, 2013 |  | Stirred, centrifuged and filtered | MFA | Scordino *et al.*, 2011 |
| Pulp | Macerated, filtered and evaporated | MWC | Brito *et al.*, 2014 | Membrane | Centrifuged and filtered | DMF | Barreca *et al.,* 2011b |
| *C. aurantium* |  |  |  | Pulp | Stirred, centrifuged and filtered | MFA | Scordino *et al.*, 2011 |
| Juice | Centrifuged and filtered | DMF | Barreca *et al.,* 2011a | Seeds | centrifuged and filtered | DMF | Barreca *et al.,* 2011b |
| Peels | Macerated, centrifuged and filtered | MAW | Lagha & Madani, 2013 | Whole | Dried, vortexed, centrifuged and evaporated | MOH | Protti *et al.*, 2015 |
| Seeds | Ground, macerated, filtered and evaporated | MOH | Moulehi *et al.*, 2012 | *C. paradisi* |  |  |  |
| Whole | Dried, UB30, centrifuged | MDS | Sun *et al.*, 2013 | Juice | Centrifuged and filtered | None | Kelebek, 2010 |
| *C. bergamia* |  |  |  |  | Freeze-dried, centrifuged and filtered | MWA | Abad *et al.*, 2014 |
| Albedo | MWE | Water | DiDonna *et al.*, 2011 |  | Lyophilized, disolved in water | Water | Guimaraes *et al.*, 2010 |
| *C. daoxianensis* |  |  |  |  | Macerated, disolved | EAM | Zhang *et al*., 2011 |
| Peel & Pulp | Dried, UB30, centrifuged | MOH | Xi et al., 2014b | Peel | Dried, UB30, centrifuged | MOH | Xi et al., 2014a |
| *C. erythrosa* |  |  |  |  | Lyophilized, stirred and filtered | MOH | Guimaraes *et al.*, 2010 |
| Whole | Dried, UB30, centrifuged | MDS | Ye *et al.,* 2011 | Pulp | Dried, UB30, centrifuged | MOH | Xi et al., 2014a |
| *C. grandis*  |  |  |  |  | Ground, macerated, centrifuged, disolved | MOH | Zhang *et al*., 2011 |
| Epicarp | Dried, UB30, centrifuged | MOH | Li *et al.*, 2014 | Whole | Dried, UB30, centrifuged | MDS | Sun *et al.*, 2013 |
| Flavedo | UB30, centrifuged, evaporated | MOH | Zhang *et al.*, 2014b | *C. poonensis* |  |  |  |
| Juice | Macerated, disolved | EAM | Zhang *et al*., 2011 | Peel | Dried, UB30, centrifuged | MOH | Xi et al., 2014b |
|  | UB30, centrifuged, evaporated | MOH | Zhang *et al.*, 2014b | Pulp | Dried, UB30, centrifuged | MOH | Xi et al., 2014b |
| Peel | Dried, UB30, centrifuged | MOH | Xi et al., 2014a | Whole | Dried, UB30, centrifuged | MDS | Sun *et al.*, 2013 |
| Pulp | Dried, UB30, centrifuged | MOH | Xi et al., 2014a |  |  |  | Ye *et al.,* 2011 |
|  | Ground, macerated, centrifuged, disolved | MOH | Zhang *et al*., 2011 | *C. reticulata* |  |  |  |
| Whole | Dried, UB30, centrifuged | MDS | Sun *et al.*, 2013 | Juice | Centrifuged and filtered | DMF | Barreca *et al.,* 2013 |
|  |  | MOH | Duan *et al.*, 2014 |  | Filtered | None | Kelebek & Selli, 2014 |
|  |  |  | Li *et al.*, 2014 |  | Freeze-dried, centrifuged and filtered | MWA | Abad *et al.*, 2014 |
| *C. jambhiri* |  |  |  | Peel | Dried, macerated and evaporated | WEA | Makovsek *et al.*, 2012 |
| Peel | Rectificated | MWPCE | Hamdan *et al.*, 2011 |  | Dried, UB30, centrifuged | MOH | Xi et al., 2014b |
| Seeds | Dried, deffated, rectificated | PMWD | Hamdan *et al.*, 2011 |  | Dried, ultrasonic bath | Water | Londoño *et al.*, 2010 |
| *C. junos*  |  |  |  |  | Lyophilized, macerated, centrifuged | MOH | Zhang *et al*., 2014c |
|  | Freeze-dried, UB30, filtered | MDS | Yoo *et al*. 2009 | Pulp | Dried, UB30, centrifuged | MOH | Xi et al., 2014b |
| *C. latifolia* |  |  |  |  | Freeze-dried, macerated and centrifugated | MOH | Ramful *et al.*, 2011 |
| Peel | Dried, ultrasonic bath | Water | Londoño *et al.*, 2010 | Seeds | Ground, macerated, filtered and evaporated | MOH | Moulehi *et al.*, 2012 |
| *C. limetta* |  |  |  | *C. sinensis* |  |  |  |
| Juice | Centrifuged and filtered | DMF | Barreca *et al.,* 2011c | Juice | Centrifuged and filtered | DMF | Barreca *et al.,* 2014 |
| Peels | Macerated, filtered and evaporated | MOH | Rodriguez *et al.*, 2014 |  | Filtered and diluted | Water | Andreu *et al.*, 2010 |
| *C. limon* |  |  |  |  | Freeze-dried, centrifuged and filtered | MWA | Abad *et al.*, 2014 |
| Juice | Freeze-dried, centrifuged and filtered | MWA | Abad *et al.*, 2014 |  | Lyophilized, disolved in water | Water | Guimaraes *et al.*, 2010 |
|  | Lyophilized, disolved in water | Water | Guimaraes *et al.*, 2010 | Pulp | Freeze-dried, macerated and centrifugated | EAA | Pan *et al.*, 2014 |
| Peel | Lyophilized, stirred and filtered | MOH | Guimaraes *et al.*, 2010 |  |  | MOH | Ramful *et al.*, 2011 |
|  | Macerated, filtered and evaporated | MWC | Brito *et al.*, 2014 | Peel | Dried, stirred and filtered | MDS | Chen *et al.*, 2011 |
| Pulp | Macerated, filtered and evaporated | MWC | Brito *et al.*, 2014 |  | Dried, ultrasonic bath | Water | Londoño *et al.*, 2010 |
| Whole | Dried, UB30, centrifuged | MDS | Sun *et al.*, 2013 |  | Lyophilized, stirred and filtered | MOH | Guimaraes *et al.*, 2010 |
| *C. maxima* |  |  |  | Peels | Macerated, centrifuged and filtered | MAW | Lagha & Madani, 2013 |
| Pulp | Freeze-dried, macerated and centrifugated | MOH | Ramful *et al.*, 2011 | Whole | Dried, UB30, centrifuged | MDS | Sun *et al.*, 2013 |
| *C. medica* |  |  |  | *C. suavissima* |  |  |  |
| Endocarp | Stirred, rotavaporated | EOH | Menichini *et al.*, 2011b | Whole | Dried, UB30, centrifuged | MDS | Ye *et al.,* 2011 |
| Mesocarp | Stirred, rotavaporated | EOH | Menichini *et al.*, 2011b | *C. tardiferax* |  |  |  |
| Peel | Stirred, rotavaporated | EOH | Menichini *et al.*, 2011a | Whole | Dried, UB30, centrifuged | MDS | Ye *et al.,* 2011 |
| *C. meyeri* |  |  |  | *C. unshiu* |  |  |  |
| Pulp | Freeze-dried, macerated and centrifugated | MOH | Ramful *et al.*, 2011 | Juice | Filtered | None | Kelebek & Selli, 2014 |
| *C. mitis* |  |  |  |  | Freeze-dried, centrifuged and filtered | MWA | Abad *et al.*, 2014 |
| Peels | Ground, macerated, filtered and evaporated | EAW | Yu *et al.*, 2013 |  |  |  |  |

**DCF**: Diluted, centrifuged and filtered; **DMSO**: Dimethyl sulfoxide; **DMF:** dimethylformamide; **EAA**: Ethanol and ammonium acetate; **EAM**: Ethyl acetate and methanol; **EASSE**: Ethyl acetate, sodium sulfate and ethanol; **EAW**: Ethyl acetate and water; **EOH**: Ethanol; **MAW**: Methanol, acetone and water; **MDS**: Methanol and dimethyl sulfoxide;  **MFA**: Methanol and formic acid; **MOH**: Methanol; **MWE**: Microwave-assisted extraction; **MWA**: Methanol, water and acetic acid; **MWC**: Methanol, water and HCl; **MWH**: Methanol, water and n-hexane; **MWPCE**: Methanol, water, light petroleum, chloroform and ethyl acetate; **PMWD**: Light petroleum, methanol, water and dichloromethane; **UB30**: ultrasonic bath; **WEA**: Water, ethanol and acetone.

**Purification**

The mixtures obtained from extraction are quite complex, showing many species from the different tissues in the fruit. In order to obtain a higher concentration of some compounds, it’s necessary to carry out a further purification.

Compound purification has been carried out by column chromatography, allowing high single concentration of compounds (Levaj *et al.*, 2008). Purification through adsorption is versatile, simple and low cost, which makes it attractive for the selective recovery of a variety of phenolics and polyphenols. Adsorption shows other advantages like selectivity, environmental impact and toxicological effects. Studies on the characterization of the detailed interactions between resins and individual plant phenolics are needed for design (Soto *et al.*, 2011).

High speed countercurrent chromatography (HSCC) is also used to extract and purify flavonoids using two-phase solvent systems, flowing simultaneously in opposite directions. In addition, HSCC also realizes multiple forms of the gradient elution process; thus it can be used not only to remove impurities from crude extract of raw materials but also to purify the final product. Moreover, some pure compounds can even be obtained through one step from crude extract without sample pretreatment (Duo *et al.*, 2011).

Even tough, there is few available data for citrus flavonoids purification processes, i.e. the one used for purifying hesperidin, naringin, and narirutin with a Zorbax C18 column and a mobile phase of citric acid and ammonium acetate in water and methanol 60:40 (Levaj *et al.*, 2008). Also, preparative high performance liquid chromatography (HPLC) using an instrument equipped with a UV–vis detector has not been employed widely in the isolation of flavonoid compounds. Most mobile phases consisted of a linear gradient of acetonitrile in H2O. Crude juice is diluted with DMF, flavonoids are collected in the HPLC course range time of 5–30 min. The fractions collected are joined, evaporated to dryness in a rotary evaporator and redissolved to regenerate the original concentration of analytes in crude juice (Barreca *et al.*, 2011d).

**Analysis methods**

Once the extract is obtained, it’s important to analyze it, to know if the procedure was correct and the present species were separated as expected.

**Qualitative methods**

Thin layer chromatography (TLC) continues to be an important method for qualitative investigation of plant compounds because of its inherent advantages— many samples can be analyzed simultaneously and quickly and multiple separation techniques and detection procedures can be applied. TLC is one of the most powerful and general analytical tools used for qualitative purposes, indicating the presence of specific flavonoids in a simpler way than HPLC. It follows from numerous publications that, owing to large polarity differences, it is difficult to find a TLC system which separates similar structure molecules on a single chromatogram.

Most common stationary phase is silica gel, using a mobile phase of mixtures ethyl acetate – methanol – formic acid (Mohammad *et al.*, 2010). Conventional separation on silica gel with moderately polar mobile phases consisting of small amounts of methanol with less polar solvents has been successfully used for the polyphenolic compounds. The retention factor (Rf) values of the different compounds reflects their polarity, given by the number of -OH groups, wich displays much more affinity for the stationary phase.

Using the Folin−Ciocalteu reaction, the phenolic compounds form blue complexes with the phosphomolybdic− phosphotungstic reagent at high pH. The analysis is simple, highly reproducible under carefully controlled conditions, and, therefore, widely used. The Folin method represents a classic approach to estimate total phenolic compounds in a variety of matrices. Although the method is nonspecific, it is frequently applied as a measure of total phenolics in biochemical, animal, and clinical trials (Soto *et al*., 2012). Fluorescence detection of the flavonoids is also used to identify the effective separation of individual flavonoid compounds (Andreu *et al.*, 2010).

**Quantitative methods**

High performance liquid chromatography (HPLC) is widely used to quantify the amount of flavonoid compounds in the obtained extracts, and there are several methods reported for HPLC sets (table 4). For every method, it must be considered the polarity of the species to be analyzed, so the correct column and mobile phase may be chosen. The hypothesis proposes that the difference in the orientation of the -OH could result in different affinities of the two isomers for the stationary phase and hence their separation. Good characterization of mobile-phase systems can be achieved by determination of relationships between retention and mobile-phase composition.

**Table 4**. HPLC sets for flavonoids in citrus species.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Citrus species** | **Column** | **Mobile phase** | **Detection** | **Flavonoids** | **Authors** |
| *C. aurantifolia* |  |  |  |  |  |
| Juice | Ascentis Express C18 50x4.6 mm | (A) H2O / HCOOH (99.9:0.1, v/v), (B) H2O / CH3CN/ 2-propanol/ HCOOH (39.9:20:40:0.1, v/v) | UV-DAD, 190 - 370 nm | Hd | Costa *et al.*, 2014 |
| Peel | Phenomenex Luna C18, 250x 4.60 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-Vis, 280 nm | Ne, Ni, Ht, Hd, Nb | Loizzo *et al.*, 2013 |
|  | Purospher star-C18 250 x 5 mm | (A) 10% HCOOH in H2O, (B) CH3CN | HPLCMS | Hd, Lu, Ni | Brito *et al.*, 2014 |
| Pulp | Purospher star-C18 250 x 5 mm | (A) 10% HCOOH in H2O, (B) CH3CN | HPLCMS | Hd, Lu, Ni | Brito *et al.*, 2014 |
| *C. aurantium* |  |  |  |  |  |
| Juice | DiscoveryC18 250x4.6 mm  | CH3CN / H2O 0-100% | HPLCMS  | Nr, Ni, Nh | Barreca *et al.*, 2011a |
| Seeds | Hypersil ODS C18 250x4.6 mm | (A) CH3CN, (B) 0.2% H2SO4 in H2O. | UV-Vis, 280 nm | Ni, Hd, Nh | Moulehi *et al.*, 2012 |
| Whole | Diamonsil C18 250x4.6 mm | (A) MeOH, (B) 4% AcOH (v/v) | UV-DAD, 200 – 400 nm | Nr, Ni, Hd, Nb, Tg | Sun *et al.*, 2013 |
| *C. bergamia* |  |  |  |  |  |
| Albedo | Luna C18 (2) 250 x 4.6 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-Vis, 280 nm | - | DiDonna *et al.*, 2011 |
| *C. daoxianensis* |  |  |  |  |  |
| Pulp | Zorbax SB-C18, 250×4.6 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-DAD, 283 – 367 nm | Nr, Hd, Nb | Xi et al., 2014b |
| *C. erythrosa* |  |  |  |  |  |
| Whole | TSK-gel ODS-80TS | H3PO4: MeOH (80:20 – 55:45)% | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Ye *et al.* 2011 |
| *C. grandis* |  |  |  |  |  |
| Epicarp | Phenomenex Kinetex 100x2.1 mm | (A) MeOH, (B)0.1% HCOOH in H2O (v/v) | HPLCMS | Ni, Ne | Li *et al.*, 2014 |
| Flavedo | Zorbax SB C-18 250 x4 mm | (A) H2O / AcOH (99:1, v/v), (B) CH3CN / AcOH (99:1,v/v). | HPLCMS | Ni, Nh | Zhang *et al*., 2011 |
|  | Zorbax SB C-18 250 x4 mm | (A) H2O / AcOH (99:1, v/v), (B) CH3CN / AcOH (99:1,v/v). | HPLCMS | Ni | Zhang *et al.*, 2014b |
| Juice | Zorbax SB C-18 250 x4 mm | (A) H2O / AcOH (99:1, v/v), (B) CH3CN / AcOH (99:1,v/v). | HPLCMS | Ni | Zhang *et al*., 2011 |
|  | Zorbax SB C-18 250 x4 mm | (A) H2O / AcOH (99:1, v/v), (B) CH3CN / AcOH (99:1,v/v). | HPLCMS | Ni | Zhang *et al.*, 2014b |
| Peels | Acquity UPLC BEH C18 100x2.1 mm | (A) 0.2% AcOH in H2O, (B) MeOH | UPLC-PDA | Nr, Ni, Nh, Ne | Xi *et al.*, 2014a |
| Pulp | Acquity UPLC BEH C18 100x2.1 mm | (A) 0.2% AcOH in H2O, (B) MeOH | UPLC-PDA | Nr, Ni, Nh, Ne | Xi *et al.*, 2014a |
| Whole | Diamonsil C18 250x4.6 mm | (A) MeOH, (B) 4% AcOH in H2O | UV-DAD, 200 – 400 nm | Nr, Ni, Hd, Nh, Nb, Tg | Sun *et al.*, 2013 |
|  | Agilent Zorbax SB-C18 50x4.6 mm | (A) 0.1% HCOOH in H2O, (B) CH3CN  | HPLCMS | Nr, Ni, Ne | Duan *et al.*, 2014 |
|  | Phenomenex Kinetex 100x2.1 mm | (A) MeOH, (B) 0.1% HCOOH in H2O (v/v) | HPLCMS | Ni, Ne | Li *et al.*, 2014 |
| *C. jambhiri* |  |  |  |  |  |
| Peel | LiChro CART 250x 4 mm | (A) H2O – HCOOH (99.5: 0.5, v/v), ( B) CH3CN | HPLCMS | Nr, Ni, Hd, Nh | Hamdan *et al.*, 2011 |
| *C. junos* |  |  |  |  |  |
| Juice | Hypersil GOLD C18 | MeOH:9% HAc aqueous, (5:95-40:60)% | UV-Vis, 280 nm | Hd, Ni, Nh, Ne, Lu, Ht | Yoo *et al*. 2009 |
| *C. latifolia* |  |  |  |  |  |
| Peel | Hypersil BDS(C8) 250x 4.6 mm | (A) 0.1% HCOOH in H2O, (B) CH3CN, 75% A and 25% B. | HPLCMS | Hd, Nh | Londoño *et al.*, 2010 |
| *C. limetta* |  |  |  |  |  |
| Juice | Diamonsil C18 | MeOH : CH3CN:PBS (10:40:39, v/v) | UV-Vis, 210 nm | Hd | Barreca *et al.*, 2011c  |
| Peels | ProntoSIL C18Aq 250x2.00 mm | (A) H2O, (B) CH3CN | HPLCMS | Hd | Rodriguez *et al.*, 2014 |
| *C. limon* |  |  |  |  |  |
| Juice | Phenomenex Luna C18(2) 150x4.6 mm | (A) AcOH – H2O (0.5:99.5, v/v), (B) MeOH | UV-DAD, 280 – 370 nm | Ht, Ne | Abad *et al.*, 2014 |
| Peel | Purospher star-C18 250 x 5 mm | (A) 10% HCOOH in H2O, (B) CH3CN | HPLCMS | Hd, Lu, Ni | Brito *et al.*, 2014 |
| Pulp | Purospher star-C18 250 x 5 mm | (A) 10% HCOOH in H2O, (B) CH3CN | HPLCMS | Hd, Lu, Ni | Brito *et al.*, 2014 |
| Whole | Diamonsil C18 250x4.6 mm | (A) MeOH, (B) 4% AcOH in H2O (v/v) | UV-DAD, 200 – 400 nm | Nr, Hd, Nb | Sun *et al.*, 2013 |
| *C. maxima* |  |  |  |  |  |
| Pulp | Waters Spherisorb ODS-2 150x4.6 mm | (A) H2O – CH3CN (90:10, v/v), (B) CH3CN | UV-DAD, 280-330 nm | Ni, Nh, Nr | Ramful *et al.*, 2011 |
| *C. medica* |  |  |  |  |  |
| Endocarp | Phenomenex Luna C18, 250x 4.60 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-Vis, 280 nm | Hd, | Menichini *et al.*, 2011b |
| Mesocarp | Phenomenex Luna C18, 250x 4.60 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-Vis, 280 nm | Ni, Hd | Menichini *et al.*, 2011b |
| Peel | Phenomenex Luna C18, 250x 4.60 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-Vis, 280 nm | Ni, Ht, Nb | Menichini *et al.*, 2011a |
| *C. mitis* |  |  |  |  |  |
| Peel | Merck RP-18 250x4.6 mm | (A) 2% AcOH in H2O (v/v), (B) 0.5% AcOH in H2O / CH3CN (1:1, v/v) | UV-DAD, 220 – 350 nm | Ni, Hd, Nb, Tg | Yu *et al.*, 2013 |
| *C. myrtifolia* |  |  |  |  |  |
| Albedo  | DiscoveryC18 250x4.6 mm  | CH3CN / H2O 0-100% | HPLCMS  | Nr, Ni, Nh, Nb, Tg | Barreca *et al.*, 2011b |
| Flavedo | DiscoveryC18 250x4.6 mm  | CH3CN / H2O 0-100% | HPLCMS  | Nr, Ni, Nh, Nb, Tg | Barreca *et al.*, 2011b |
| Juice | DiscoveryC18 250x4.6 mm  | CH3CN / H2O 0-100% | HPLCMS  | Nr, Nh, Ni | Barreca *et al.*, 2010Barreca *et al.*, 2011b |
|  | Phenomenex Luna C18, 250x 4.60 mm | (A) 0.3% HCOOH in H2O, (B) 0.3% formic acid in CH3CN | HPLCMS  | Ni, Nh, Nb, Tg | Scordino *et al.*, 2011 |
| Membrane | DiscoveryC18 250x4.6 mm  | CH3CN / H2O 0-100% | HPLCMS  | Nr, Ni, Nh | Barreca *et al.*, 2011b |
| Pulp | Phenomenex Luna C18, 250x 4.60 mm | (A) 0.3% HCOOH in H2O, (B) 0.3% formic acid in CH3CN | HPLCMS  | Ni, Nh, Nb, Tg | Scordino *et al.*, 2011 |
| *C. paradisi* |  |  |  |  |  |
| Flavedo | Zorbax SB C-18 250 x4 mm | (A) H2O / AcOH (99:1, v/v), (B) CH3CN / AcOH (99:1,v/v). | HPLCMS | Ni, Hd, Nh | Zhang *et al*., 2011 |
| Juice | Beckman Ultrasphere ODS205x4.6mm | (A) H2O / HCOOH (95:5; v/v), ( B) CH3CN /(A) (60:40; v/v) | UV-DAD, 200 – 600 nm | Nr, Ni, Hd, Nh | Kelebek, 2010 |
|  | Zorbax SB C-18 250 x4 mm | (A) H2O / AcOH (99:1, v/v), (B) CH3CN/ AcOH (99:1,v/v). | HPLCMS | Ni, Hd, Nh | Zhang *et al*., 2011 |
|  | Phenomenex Luna C18(2) 150x4.6 mm | (A) AcOH – H2O (0.5:99.5, v/v), (B) MeOH | UV-DAD, 280 – 370 nm | Ht, Ne | Abad *et al.*, 2014 |
| Peel | Acquity UPLC BEH C18 100x2.1 mm | (A) 0.2% AcOH in H2O, (B) MeOH | UPLC-PDA | Nr, Ni, Hd, Nh, Ne, Ht | Xi *et al.*, 2014a |
| Pulp | Acquity UPLC BEH C18 100x2.1 mm | (A) 0.2% AcOH in H2O, (B) MeOH | UPLC-PDA | Nr, Ni, Hd, Nh, Ne, Ht | Xi *et al.*, 2014a |
| Whole | Diamonsil C18 250x4.6 mm | (A) MeOH, (B) 4% AcOH in H2O (v/v ) | UV-DAD, 200 – 400 nm | Nr, Ni, Hd, Nh, Nb, Tg | Sun *et al.*, 2013 |
| *C. poonensis* |  |  |  |  |  |
| Pulp | Zorbax SB-C18, 250×4.6 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-DAD, 283 – 367 nm | Nr, Hd, Nb | Xi et al., 2014b |
| Whole | TSK-gel ODS-80TS | H3PO4: MeOH (80:20 – 55:45)% | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Ye *et al.* 2011 |
|  | Diamonsil C18 250x4.6 mm | (A) MeOH , (B) 4% AcOH in H2O (v/v ) | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Sun *et al.*, 2013 |
| *C. reticulata* |  |  |  |  |  |
| Juice | DiscoveryC18 250x4.6 mm  | CH3CN / H2O 0-100% | HPLCMS  | Nr, Hd, Nb, Tg | Barreca *et al.*, 2010Barreca *et al.*, 2011b |
|  | Phenomenex Luna C18(2) 150x4.6 mm | (A) AcOH / H2O (0.5:99.5, v/v), (B) MeOH | UV-DAD, 280 – 370 nm | Ht, Ne | Abad *et al.*, 2014 |
|  | Beckman UltrasphereODS 250x4.6 mm | (A) H2O / HCOOH (95:5; v/v), (B) CH3CN /(A) (60:40; v/v) | UV-DAD, 200-600 nm | Nr, Hd | Kelebek & Selli, 2014 |
| Peel | Hypersil BDS(C8) 250x 4.6 mm | (A) 0.1% HCOOH in H2O, (B) CH3CN, 75% A and 25% B. | HPLCMS | Hd, Nh | Londoño *et al.*, 2010 |
|  | Chromsep SS C-18 250×4.6 mm | (A) MeOH, (B) 2% AcOH in H2O (v/v) | UV-DAD, 282-330 nm | Hd, Nb, Tg | Makovsek *et al.*, 2012 |
|  | Zorbax SB-C18, 250×4.6 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-DAD, 283 – 367 nm | Nr, Ni, Hd, Nh, Ne, Lu, Nb, Tg | Zhang *et al*., 2014c |
| Pulp | Waters Spherisorb ODS-2 150x4.6 mm | (A) H2O – CH3CN (90:10, v/v), (B) CH3CN | UV-DAD, 280-330 nm | Hd, Nr | Ramful *et al.*, 2011 |
|  | Zorbax SB-C18, 250×4.6 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-DAD, 283 – 367 nm | Nr, Ni, Hd, Nh, Nb | Xi et al., 2014b |
| Seeds | Hypersil ODS C18 250x4.6 mm | (A) CH3CN, (B) 0.2% H2SO4 in H2O | UV-Vis, 280 nm | Ni, Hd | Moulehi *et al.*, 2012 |
| *C. sinensis* |  |  |  |  |  |
| Juice | Onyx monolithic C18, 100x4.6 mm | Ternary mixture of 0.15 mol L-1 acetic buffer, pH 4.0, CH3CN and MeOH. | SLM Aminco AB2 luminescence spectrometer, 585-625 nm  | Ni, Hd, Ne | Andreu *et al.*, 2010 |
|  | Phenomenex Luna C18(2) 150x4.6 mm | (A) AcOH – H2O (0.5:99.5, v/v), (B) MeOH | UV-DAD, 280 – 370 nm | Ht, Ne | Abad *et al.*, 2014 |
|  | DiscoveryC18 250x4.6 mm  | CH3CN / H2O 0-100% | HPLCMS  | Nr, Hd | Barreca *et al.,* 2013 |
| Peel | Hypersil BDS(C8) 250x 4.6 mm | (A) 0.1% HCOOH in H2O, (B) CH3CN, 75% A and 25% B. | HPLCMS | Hd, Nh, Nb, Tg | Londoño *et al.*, 2010 |
|  | Hypersil GOLD C18 250x 4.6 mm |  (A) MeOH, (B) 9% AcOH in H2O | UV–Vis, 280 nm | Ni, Nh | Chen *et al.*, 2011 |
| Pulp | Waters Spherisorb ODS-2 150x4.6 mm | (A) H2O – CH3CN (90:10, v/v), (B) CH3CN | UV-DAD, 280-330 nm | Hd, Nr | Ramful *et al.*, 2011 |
|  | Agilent Eclipse XDB-C18 150x2.1 mm | (A) 1 mM NH4F in H2O, (B) MeOH | HPLCMS | Hd, Nh, Ni, Nb | Pan *et al.*, 2014 |
| Whole | Diamonsil C18 250x4.6 mm | (A) MeOH, (B) 4% AcOH in H2O (v/v) | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Sun *et al.*, 2013 |
| *C. suavissima* |  |  |  |  |  |
| Whole | TSK-gel ODS-80TS | H3PO4: MeOH (80:20 – 55:45)% | UV-DAD, 200 – 400 nm | Ni, Nr, Hd, Nb, Tg | Ye *et al.* 2011 |
| *C. succosa* |  |  |  |  |  |
| Whole | TSK-gel ODS-80TS | H3PO4: MeOH (80:20 – 55:45)% | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Ye *et al.* 2011 |
| *C. tardiferax* |  |  |  |  |  |
| Whole | TSK-gel ODS-80TS | H3PO4: MeOH (80:20 – 55:45)% | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Ye *et al.* 2011 |
| *C. unshiu* |  |  |  |  |  |
| Juice | Phenomenex Luna C18(2) 150x4.6 mm | (A) AcOH – H2O (0.5:99.5, v/v), (B) MeOH | UV-DAD, 280 – 370 nm | Ht, Ne | Abad *et al.*, 2014 |
|  | Beckman UltrasphereODS 250x4.6 mm | (A) H2O / HCOOH (95:5; v/v), (B) CH3CN /(A) (60:40; v/v) | UV-DAD, 200-600 nm | Nr, Hd | Kelebek & Selli, 2014 |
| Peel | SunFire C18 column 250x4.6 mm | (A) MeOH, (B) 0.5% AcOH in H2O | UV-DAD, 280 nm | Nr, Hd, Ni, Ne, Ht | Jung *et al.*, 2011 |
| Pulp | Zorbax SB-C18, 250×4.6 mm | (A) 0.1% HCOOH in H2O, (B) MeOH | UV-DAD, 283 – 367 nm | Nr, Hd, Nb | Xi et al., 2014b |
| Whole | TSK-gel ODS-80TS | H3PO4: MeOH (80:20 – 55:45)% | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Ye *et al.* 2011 |
|  | Diamonsil C18 250x4.6 mm | (A) MeOH, (B) 4% AcOH in H2O (v/v) | UV-DAD, 200 – 400 nm | Nr, Hd, Nb, Tg | Sun *et al.*, 2013 |

**AcOH**: acetic acid, **Hd**: Hesperidin, **HPLCMS**: High performance liquid chromatography coupled with ESI-MS/MS detection, **Ht**: Hesperetin, **MeOH**: Methanol, **Ni**: Naringin, **Nh**: Neohesperidin, **Ne**: Naringenin, **Nr**: Narirutin, **Nb**: Nobiletin, **Lu**: Luteolin, **Tg**: Tangeretin, **UPLC-PDA**: Ultra performance liquid chromatography with photodiode array detector, **UV-DAD**: Ultraviolet diode array detector, **UV-Vis**: Ultraviolet and visible detector.

Gas chromatography is also used, but due to its characteristics, volatile samples are required (Cheong *et al.*, 2012). In addition, water samples are not allowed, only the species that are soluble in volatile solvent should be measured.

Liquid chromatography has been the most used technique to analyze the obtained extracts from citrus fruits (Jiang *et al.*, 2011), performing tests at different pH levels and using a huge variety of detection methods. High performance liquid chromatography (HPLC) combined with ultraviolet (UV) detection and mass spectrometric (MS), electrospray ionization (ESI), and/or two mass spectrometer tandem (MS/MS) measurement provides the most useful techniques currently available to identify specific classes and structures of food phenolics (Barreca *et al.*, 2013; He *et al.*, 2011). The differences in ultraviolet spectra are an important tool in determining which wavelengths to monitor for detection and quantification by HPLC (Soto *et al*., 2012; González *et al.*, 2010).

**Conclusions**

Since citrus fruits are original from Asia, most of the varieties on the current literature were found and studied in Far East countries. It doesn’t mean that others countries are not interested in studying citrus flavonoids, only that they don’t have so much of wild or endemic citrus species. Most of the studies used grounded dry raw material for extraction, from peels and whole fruit. Methanol mixtures are the main solvent used in the extraction of citrus flavonoids.

There’s few literature found about purification of single flavonoids, since few details of purification behavior of single flavonoid compounds have been provided in most of the publications dealing with their isolation and structural elucidation, and, in some cases, inadequate information is supplied, there is an entire opportunity field for new research in purification techniques, and their efficiencies in flavonoids isolation.

High performance liquid chromatography has been used as the best analysis technique to quantify and identify structures of the obtained flavonoids and thin layer chromatography provides a quick method for qualitative identification of the compounds along the experimental process.

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