Procedimientos actuales para la extracción y purificación de flavonoides cítricos

Current procedures for extraction and purification of citrus flavonoides

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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

In 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.

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

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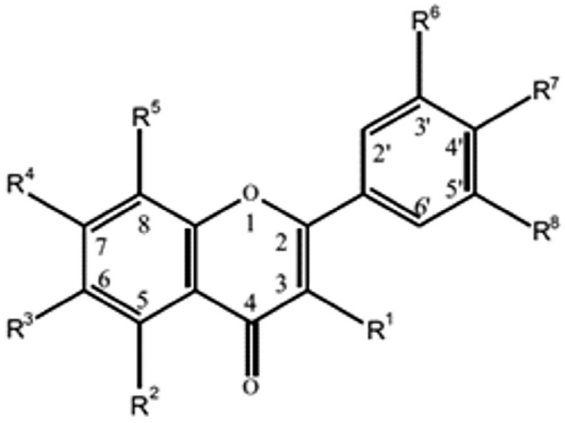

Figure 1. Flavonoids general structure.

characteristic presence of hydroxyl groups in one or more R positions.

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.

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

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

	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8
Flavanones								
Hesperidin	H	OH	H	O-rut	H	OH	OCH_3	H
Naringin	H	OH	H	O-nh	H	H	OH	H
Neohesperidin	H	OH	H	O-nh	H	OH	OCH_3	H
Narirutin	H	OH	H	O-rut	H	H	OH	H
Flavones								
Hesperetin	H	OH	H	OH	H	OH	OCH_3	H
Naringenin	H	OH	H	OH	H	H	OH	H
Flavone Aglycon								
Luteolin	H	OH	H	OH	H	OH	OH	H
Polymethoxyflavones								
Nobiletin	H	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	OCH_3	H
Tangeretin	H	OCH_3	OCH_3	OCH_3	OCH_3	H	OCH_3	H

rut: rutinose; nh: neohesperidose.

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.

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

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

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

Citrus species	Separation Technique	Solvents	References	Citrus species	Separation Technique	Solvents	References
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

Citrus species	Separation Technique	Solvents	References	Citrus species	Separation Technique	Solvents	References
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 H_2O. 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) H_2O / HCOOH (99.9:0.1, v/v), (B) H_2O / CH_3CN/ 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 H_2O, (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 H_2O, (B) CH_3CN	HPLCMS	Hd, Lu, Ni	Brito *et al.*, 2014
Pulp	Purospher star-C18 250 x 5 mm	(A) 10% HCOOH in H_2O, (B) CH_3CN	HPLCMS	Hd, Lu, Ni	Brito *et al.*, 2014
C. aurantium					
Juice	Discovery C_{18} 250x4.6 mm	CH_3CN / H_2O 0-100%	HPLCMS	Nr, Ni, Nh	Barreca *et al.*, 2011a
Seeds	Hypersil ODS C18 250x4.6 mm	(A) CH_3CN, (B) 0.2% H_2SO_4 in H_2O.	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 H_2O, (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 H_2O, (B) MeOH	UV-DAD, 283 – 367 nm	Nr, Hd, Nb	Xi *et al.*, 2014b

Citrus species	Column	Mobile phase	Detection	Flavonoids	Authors
C. erythrosa					
Whole	TSK-gel ODS-80TS	H_3PO_4: 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 H_2O (v/v)	HPLCMS	Ni, Ne	Li *et al.*, 2014
Flavedo	Zorbax SB C-18 250 x4 mm	(A) H_2O / AcOH (99:1, v/v), (B) CH_3CN / AcOH (99:1,v/v).	HPLCMS	Ni, Nh	Zhang *et al.*, 2011
	Zorbax SB C-18 250 x4 mm	(A) H_2O / AcOH (99:1, v/v), (B) CH_3CN / AcOH (99:1,v/v).	HPLCMS	Ni	Zhang *et al.*, 2014b
Juice	Zorbax SB C-18 250 x4 mm	(A) H_2O / AcOH (99:1, v/v), (B) CH_3CN / AcOH (99:1,v/v).	HPLCMS	Ni	Zhang *et al.*, 2011
	Zorbax SB C-18 250 x4 mm	(A) H_2O / AcOH (99:1, v/v), (B) CH_3CN / AcOH (99:1,v/v).	HPLCMS	Ni	Zhang *et al.*, 2014b
Peels	Acquity UPLC BEH C18 100x2.1 mm	(A) 0.2% AcOH in H_2O, (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 H_2O, (B) MeOH	UPLC-PDA	Nr, Ni, Nh, Ne	Xi *et al.*, 2014a
Whole	Diamonsil C18 250x4.6 mm	(A) MeOH, (B) 4% AcOH in H_2O	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 H_2O, (B) CH_3CN	HPLCMS	Nr, Ni, Ne	Duan *et al.*, 2014
	Phenomenex Kinetex 100x2.1 mm	(A) MeOH, (B) 0.1% HCOOH in H_2O (v/v)	HPLCMS	Ni, Ne	Li *et al.*, 2014
C. jambhiri					
Peel	LiChro CART 250x 4 mm	(A) H_2O – HCOOH (99.5: 0.5, v/v), (B) CH_3CN	HPLCMS	Nr, Ni, Hd, Nh	Hamdan *et al.*, 2011
C. junos					
Juice	Hypersil GOLD C_{18}	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 H_2O, (B) CH_3CN, 75% A and 25% B.	HPLCMS	Hd, Nh	Londoño *et al.*, 2010
C. limetta					
Juice	Diamonsil C_{18}	MeOH : CH_3CN:PBS (10:40:39, v/v)	UV-Vis, 210 nm	Hd	Barreca *et al.*, 2011c
Peels	ProntoSIL C18Aq 250x2.00 mm	(A) H_2O, (B) CH_3CN	HPLCMS	Hd	Rodriguez *et al.*, 2014
C. limon					
Juice	Phenomenex Luna C18(2) 150x4.6 mm	(A) AcOH – H_2O (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 H_2O, (B) CH_3CN	HPLCMS	Hd, Lu, Ni	Brito *et al.*, 2014
Pulp	Purospher star-C18 250 x 5 mm	(A) 10% HCOOH in H_2O, (B) CH_3CN	HPLCMS	Hd, Lu, Ni	Brito *et al.*, 2014
Whole	Diamonsil C18 250x4.6 mm	(A) MeOH, (B) 4% AcOH in H_2O (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) H_2O – CH_3CN (90:10, v/v), (B) CH_3CN	UV-DAD, 280-330 nm	Ni, Nh, Nr	Ramful *et al.*, 2011

Citrus species	Column	Mobile phase	Detection	Flavonoids	Authors
C. medica					
Endocarp	Phenomenex Luna C18, 250x 4.60 mm	(A) 0.1% HCOOH in H_2O, (B) MeOH	UV-Vis, 280 nm	Hd,	Menichini *et al.*, 2011b
Mesocarp	Phenomenex Luna C18, 250x 4.60 mm	(A) 0.1% HCOOH in H_2O, (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 H_2O, (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 H_2O (v/v), (B) 0.5% AcOH in H_2O / CH_3CN (1:1, v/v)	UV-DAD, 220 – 350 nm	Ni, Hd, Nb, Tg	Yu *et al.*, 2013
C. myrtifolia					
Albedo	Discovery C_{18} 250x4.6 mm	CH_3CN / H_2O 0-100%	HPLCMS	Nr, Ni, Nh, Nb, Tg	Barreca *et al.*, 2011b
Flavedo	Discovery C_{18} 250x4.6 mm	CH_3CN / H_2O 0-100%	HPLCMS	Nr, Ni, Nh, Nb, Tg	Barreca *et al.*, 2011b
Juice	Discovery C_{18} 250x4.6 mm	CH_3CN / H_2O 0-100%	HPLCMS	Nr, Nh, Ni	Barreca *et al.*, 2010 Barreca *et al.*, 2011b
	Phenomenex Luna C18, 250x 4.60 mm	(A) 0.3% HCOOH in H_2O, (B) 0.3% formic acid in CH_3CN	HPLCMS	Ni, Nh, Nb, Tg	Scordino *et al.*, 2011
Membrane	Discovery C_{18} 250x4.6 mm	CH_3CN / H_2O 0-100%	HPLCMS	Nr, Ni, Nh	Barreca *et al.*, 2011b
Pulp	Phenomenex Luna C18, 250x 4.60 mm	(A) 0.3% HCOOH in H_2O, (B) 0.3% formic acid in CH_3CN	HPLCMS	Ni, Nh, Nb, Tg	Scordino *et al.*, 2011
C. paradisi					
Flavedo	Zorbax SB C-18 250 x4 mm	(A) H_2O / AcOH (99:1, v/v), (B) CH_3CN / AcOH (99:1,v/v).	HPLCMS	Ni, Hd, Nh	Zhang *et al.*, 2011
Juice	Beckman Ultrasphere ODS 205x4.6mm	(A) H_2O / HCOOH (95:5; v/v), (B) CH_3CN /(A) (60:40; v/v)	UV-DAD, 200 – 600 nm	Nr, Ni, Hd, Nh	Kelebek, 2010
	Zorbax SB C-18 250 x4 mm	(A) H_2O / AcOH (99:1, v/v), (B) CH_3CN/ AcOH (99:1,v/v).	HPLCMS	Ni, Hd, Nh	Zhang *et al.*, 2011
	Phenomenex Luna C18(2) 150x4.6 mm	(A) AcOH – H_2O (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 H_2O, (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 H_2O, (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 H_2O (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 H_2O, (B) MeOH	UV-DAD, 283 – 367 nm	Nr, Hd, Nb	Xi *et al.*, 2014b
Whole	TSK-gel ODS-80TS	H_3PO_4: 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 H_2O (v/v)	UV-DAD, 200 – 400 nm	Nr, Hd, Nb, Tg	Sun *et al.*, 2013
C. reticulata					
Juice	Discovery C_{18} 250x4.6 mm	CH_3CN / H_2O 0-100%	HPLCMS	Nr, Hd, Nb, Tg	Barreca *et al.*, 2010 Barreca *et al.*, 2011b

Citrus species	Column	Mobile phase	Detection	Flavonoids	Authors
	Phenomenex Luna C18(2) 150x4.6 mm	(A) AcOH / H_2O (0.5:99.5, v/v), (B) MeOH	UV-DAD, 280 – 370 nm	Ht, Ne	Abad *et al.*, 2014
	Beckman Ultrasphere ODS 250x4.6 mm	(A) H_2O / HCOOH (95:5; v/v), (B) CH_3CN /(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 H_2O, (B) CH_3CN, 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 H_2O (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 H_2O, (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) H_2O – CH_3CN (90:10, v/v), (B) CH_3CN	UV-DAD, 280-330 nm	Hd, Nr	Ramful *et al.*, 2011
	Zorbax SB-C18, 250×4.6 mm	(A) 0.1% HCOOH in H_2O, (B) MeOH	UV-DAD, 283 – 367 nm	Nr, Ni, Hd, Nh, Nb	Xi *et al.*, 2014b
Seeds	Hypersil ODS C18 250x4.6 mm	(A) CH_3CN, (B) 0.2% H_2SO_4 in H_2O	UV-Vis, 280 nm	Ni, Hd	Moulehi *et al.*, 2012
C. sinensis					
Juice	Onyx monolithic C_{18}, 100x4.6 mm	Ternary mixture of 0.15 mol L^{-1} acetic buffer, pH 4.0, CH_3CN 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 – H_2O (0.5:99.5, v/v), (B) MeOH	UV-DAD, 280 – 370 nm	Ht, Ne	Abad *et al.*, 2014
	Discovery C_{18} 250x4.6 mm	CH_3CN / H_2O 0-100%	HPLCMS	Nr, Hd	Barreca *et al.*, 2013
Peel	Hypersil BDS (C8) 250x 4.6 mm	(A) 0.1% HCOOH in H_2O, (B) CH_3CN, 75% A and 25% B.	HPLCMS	Hd, Nh, Nb, Tg	Londoño *et al.*, 2010
	Hypersil GOLD C_{18} 250x 4.6 mm	(A) MeOH, (B) 9% AcOH in H_2O	UV–Vis, 280 nm	Ni, Nh	Chen *et al.*, 2011
Pulp	Waters Spherisorb ODS-2 150x4.6 mm	(A) H_2O – CH_3CN (90:10, v/v), (B) CH_3CN	UV-DAD, 280-330 nm	Hd, Nr	Ramful *et al.*, 2011
	Agilent Eclipse XDB-C_{18} 150x2.1 mm	(A) 1 mM NH_4F in H_2O, (B) MeOH	HPLCMS	Hd, Nh, Ni, Nb	Pan *et al.*, 2014
Whole	Diamonsil C18 250x4.6 mm	(A) MeOH, (B) 4% AcOH in H_2O (v/v)	UV-DAD, 200 – 400 nm	Nr, Hd, Nb, Tg	Sun *et al.*, 2013
C. suavissima					
Whole	TSK-gel ODS-80TS	H_3PO_4: 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	H_3PO_4: 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	H_3PO_4: 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 – H_2O (0.5:99.5, v/v), (B) MeOH	UV-DAD, 280 – 370 nm	Ht, Ne	Abad *et al.*, 2014
	Beckman Ultrasphere ODS 250x4.6 mm	(A) H_2O / HCOOH (95:5; v/v), (B) CH_3CN /(A) (60:40; v/v)	UV-DAD, 200-600 nm	Nr, Hd	Kelebek & Selli, 2014

Citrus species	Column	Mobile phase	Detection	Flavonoids	Authors
Peel	SunFire C18 column 250x4.6 mm	(A) MeOH, (B) 0.5% AcOH in H_2O	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 H_2O, (B) MeOH	UV-DAD, 283 – 367 nm	Nr, Hd, Nb	Xi *et al.*, 2014b
Whole	TSK-gel ODS-80TS	H_3PO_4: 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 H_2O (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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