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Citrus limon: a source of flavonoids of pharmaceutical interest

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Abstract

Some of the medicinal properties of lemons are due to the flavonoids they contain since they are involved in many biological activities and have many health-related functions. The levels of the principal flavanones and flavone found in different cultivars of *Citrus limon*, are analysed in an attempt to identify the most interesting as regards the content of such secondary compounds. The results show that the immature fruits from cultivars Lisbon and Fino-49 are ideal for obtaining the flavanone hesperidin, while the mature fruits of cultivar Fino-49 and the leaves of cultivar Eureka are the most interesting for obtaining the flavone diosmin and the flavanone eriocitrin.

Keywords: Flavanones; Flavones; Diosmin; Eriocitrin; Hesperidin; Fino-49; Eureka; Lisbón

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1. Introduction

The Lemon [*Citrus lemon* (L.) Burms.f] has many important natural chemical components, including citric acid, ascorbic acid, minerals and flavonoids. Although their health-related properties have always been associated with their content of vitamin C, it has recently been shown that flavonoids also play a role in this respect. Some authors suggest that flavonoids have different biological functions, including antioxidative, antiinflammatory, antiallergic, antiviral, antiproliferative, antimutagenic, and anticarcinogenic activities (Benavente-García, Castillo, Marín, Ortuño, & Del Río, 1997a; Elangovan, Sekar, & Govindasamy, 1994; Godeberg, 1994; Jean & Bodinier, 1994; Meyer, 1994; Midleton & Kandaswami, 1986,1992; Rice-Evans, Miller, Paganda, 1997). Therefore, although the new *Citrus* cultivars have mainly been selected and developed for fresh consumption, the particular characteristics of their flavonoid contents have led to their being used in the pharmacological and food technology area (Del Río, Fuster, Sabater, Porras, García-Lidón, & Ortuño, 1997a; Horowitz & Gentili, 1977; Ortuño et al., 1997).

Hesperidin, the principal flavanone in lemon and other *Citrus* species (Fuster, 1997), influences vascular permeability, increases capillary resistance and has analgesic and antiinflammatory properties. It is also an effective antioxidant since it is able to quench the oxygen free radicals which are involved in cancer (Berkarda, Koyuncu, Soybir, & Baykut, 1998; Galati, Monforte, Kirjavainen, Forestieri, Trovato, & Tripodo, 1994; Koyuncu et al., 1999; Monforte, Trovato, Kirjarainen, Forestieri, Galati, & LoCurto, 1995; Tanaka, Makita, & Mori, 1996).

Diosmin is an important flavonoid in *Citrus* (Fuster, 1997). This flavone has important pharmacological applications, being the active ingredient of certain drugs that are used in the treatment of several illnesses of the circulatory system. It improves muscular tone and vascular resistance to inflammatory processes, for which reason it is used against illnesses, such as chronic venous insufficiency and rheumatic arthritis. It possesses antihemorroidal, antioxidant and anti-lipid peroxidation properties, and protects against free radicals (Berqvist, Hallbrook, Lindblad, & Lindhagen, 1981; Damon et al.,

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1987; DaSilva Emim, Oliviera, & Lapa, 1994; Gábor, 1988; Galley & Thiollet, 1993; Jean & Bodinier, 1994; Loncampt, Guardiola, Sicot, Bertrand, Perdrix, & Duhault, 1989).

The flavone eriocitrin is abundant in lemon and lime but not in all Citrus fruits (Fuster, 1997). It is obtained from the *Citrus* peel and is used in numerous multivitaminic complexes, in which the antioxidant activity of the "bioflavonoids", for maintaining capillary integrity and peripheral circulation, is of note. The high stability of this antioxidant compound during processing and the storage of juices, means that it can be used in the preparation of many nutritional products. Eriocitrin has the greatest antioxidant activity of all the glycoside flavonoids present in lemon fruit (Miyake, Yamamoto, & Osawa, 1997a; Miyake, Yamamoto, Morimitsu, Osawa, 1997b; Miyake, Yamamoto, Morimitsu, & Osawa, 1998).

This study aims to identify lemon cultivars of potential interest for industrial application or fresh consumption due to their high flavonoid content.

2. Material and methods

2.1. Plant material

Young leaves, young stems, flowers, recently set fruit, immature fruit 30 days after anthesis and mature fruit 150 days after anthesis of different lemon (*Citrus* limon (L.) Burm.f.) cultivars were used. In some mature fruits (150 days after anthesis) the flavedo, albedo and pulp were separated. The selected cultivars were Fino-49, Eureka, and Lisbon, from the experimental plantation located in Centro de Investigación y Desarrollo Agroalimentario (CIDA), La Alberca, Murcia.

2.2. Extraction, identification and quantification of flavonoids

To extract the flavonoids from the different plant materials they were first dried at 50 °C (Hosoda & Noguchi, 1988), in a forced air oven, to constant weight. The dried plant material was ground to a fine powder and extracted for 30 min with dimethyl sulfoxide (DMSO) in a ratio of 6 mg of dry weight/ml. The corresponding extracts were filtered through a 0.45 μm nylon membrane before analysis in a Hewlett-Packard liquid Chromatograph, model HP1050 (USA) coupled to a quaternary pump and automatic injector with a diode array detector. Reversed phase chromatographic separation was performed with a C_{18} μ-Bondapak (Waters Associates, Milford, MA, USA) analytical column with an average particle size of 5 μm (250×4 mm i.d.) at 35 °C, eluting in an isocratic gradient of water/methanol/acetonitrile/acetic acid (15:2:2:1) at a constant flow of 1 ml min^{-1}. Changes in absorbance were recorded in the vis-UV diode array detector at 280 nm. These compounds were quantified by HPLC under the chromatographic conditions described above, and the response obtained was compared with the corresponding external standards. The main flavanones and flavones under study were collected with a fraction collector (Pharmacia LKB Biotecnology, Uppsala, Sweden) at the exit of the HPLC column and their identities were confirmed by reference to their nuclear magnetic resonance spectra (^{1}H NMR and ^{13}C NMR) (200 MHz) (Bruker, Germany) in hexadeutero-DMSO.

2.3. Chemicals

Hesperidin, eriocitrin and diosmin were purchased from SIGMA (USA).

3. Results and discussion

3.1. Flavonoid levels in leaves, stems and flowers of Citrus limon (cv. Lisbón, cv. Eureka, cv. Fino-49)

The HPLC study of different extracts of *Citrus limon* revealed the presence of one principal compound (compound 2) with a retention time which coincided with that of the flavanone rutinoside, hesperidin (Rt = 15.5 min) and two minor compounds (compound 1 and 3) with retention times of 4.6 and 21.5 min, coinciding with those of the flavanone eriocitrin and the flavone diosmin, respectively (Fig. 1). The absorption spectra of these compounds obtained by means of a V/UV diode array detector showed a maximum at 280 nm for compounds 1 and 2, and two maxima, at 280 and 350 nm for compound 3. These data are consistent with the compounds having flavanone skeletons corresponding to eriocitrin and hesperidin (compounds 1 and 2) and a flavone skeleton corresponding to diosmin (compound 3).

Compounds 1–3 were isolated by the method described in Section 2. The ^{1}H NMR and ^{13}C NMR spectra of these compounds were identical to those obtained for the eriocitrin, hesperidin and diosmin standards, respectively.

The quantities obtained in young leaves, young stems and flowers for the three lemon cultivars are shown in Table 1. The accumulation of these flavonoids in the different organs analysed is a result of traslocation and *in situ* synthesis, as has been described for other *Citrus* sp. (Castillo, Benavente-García, & Del Río, 1992; Del Río, Castillo, Benavente-García, Fuster, Sabater, & Ortuño, 1997b).

Hesperidin was the main flavanone in all the analysed organs of all three lemon cultivars (Table 1). The levels of this flavanone in the flowers of the three cultivars analysed were similar (around 5 g/100 g dry weight),

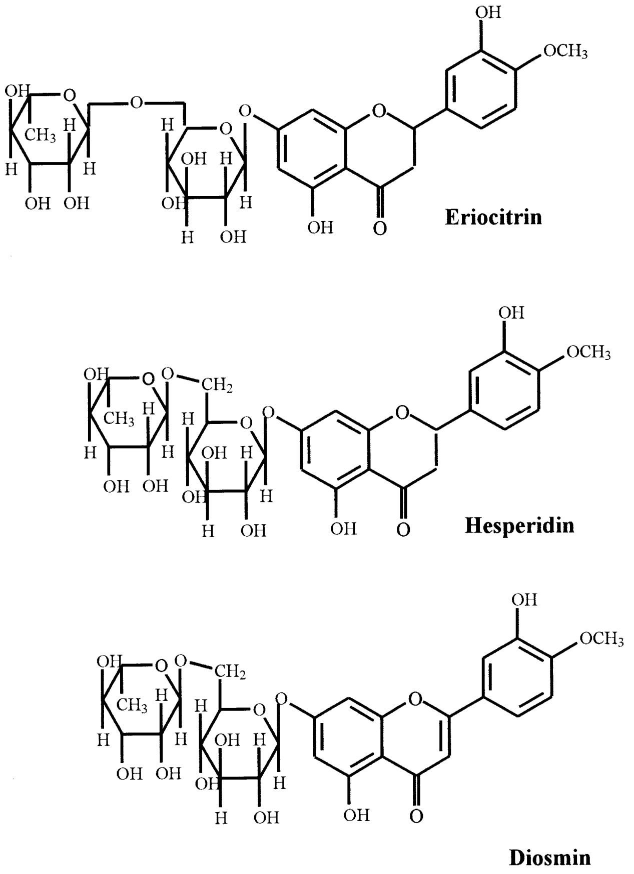

Fig. 1. Chemical structures of the flavanones, eriocitrin, hesperidin, and the flavone, diosmin.

Table 1
Hesperidin, diosmin and y eriocitrin levels in young leaves, young stems, and flowers, of Citrus limon cv. Lisbón, cv. Eureka and cv. Fino-49[a]

		Flavonoids (g/100 g dry weight)		
Citrus limon		Hesperidin	Diosmin	Eriocitrin
cv. Lisbon	Young leaves	6.98±1.83	1.79±0.09	0.37±0.11
	Young stems	7.00±0.86	0.50±0.05	0.17±0.02
	Flowers	5.53±0.05	0.34±0.03	0.06±0.01
cv. Eureka	Young leaves	9.23±2.16	1.91±0.45	0.51±0.20
	Young stems	6.94±1.00	0.60±0.12	0.18±0.03
	Flowers	5.95±0.60	0.37±0.04	0.08±0.01
cv. Fino-49	Young leaves	4.99±3.35	1.61±0.76	0.29±0.11
	Young stems	5.46±2.34	0.44±0.13	0.15±0.04
	Flowers	5.23±0.62	0.33±0.04	0.07±0.01

[a] Data are expressed in g/100g of dry weight±SE ($n=3$).

while the levels of diosmin and eriocitrin were lower, although also similar in all three cultivars (around 0.3 and 0.07 g/100 g dry weight, respectively).

A study of the flavonoid levels in young stems and young leaves of the three cultivars showed that the hesperidin levels in stems were similar to or slightly lower than those in leaves, while levels of diosmin and eriocitrin were lower in stems than in leaves (Table 1). The highest levels of diosmin (1.91 g/100 g dry weight) and eriocitrin (0.51 g/100 g dry weight) were found in the leaves of the cultivar Eureka.

These results are in agreement with the results obtained by other authors who also demonstrated the existence in lemon of flavanones and flavones (Gottlieb, 1975; Horowitz & Gentili, 1977; Park, Avery, Byers, & Nelson, 1983; Fuster, 1997).

3.2. Distribution of flavonoids in fruits

A study of the distribution of the flavanoids hesperidin, eriocitrin and diosmin was carried out on the flavedo, albedo and pulp of mature fruits from cv. Fino-49. The results pointing to an irregular distribution of flavanones and flavones in fruits. Thus, the flavanones (hesperidin and eriocitrin) are found in higher concentrations in the albedo (1.25 g/100 g dry weight and 1.59 g/100 g dry weight, respectively) followed by the flavedo (0.58 g /100 g dry weight and 0.66 g/100 g dry weight, respectively) and pulp (0.28 g/100 g dry weight and 0.25 g/100 g dry weight, respectively). The levels in the albedo, then, are 2-fold higher than in the flavedo and 4–6-fold higher than the levels detected in pulp (Table 2). These results agree with those obtained by other authors in relation to the hesperidin and eriocitrin distribution in fruits of other lemon cultivars (Miyake, Yamamoto, Morimitsu, & Osawa, 1998).

However, diosmin (flavone) is distributed differently, the levels in the flavedo (0.33 g/100 g dry weight) and albedo (0.20 g/100 g dry weight) (Table 2) being similar while only trace levels of diosmin (around 0.04 g/100 g dry weight) are found in the pulp since diosmin is a less soluble molecule than the others (more apolar). These results agree with the results obtained by us in other *Citrus* species (Fuster, 1997).

The results are in accordance with those obtained for other *Citrus* sp., in which it has been shown that these compounds are principally located in the peel (Del Río, Arcas, Benavente-García, Sabater, & Ortuño, 1998a; Kanes, Tisserat, Berhow, & Vandercook, 1992; Ortuño, Arcas, Benavente-García, & Del Río, 1999), supporting the idea that this superficial localization plays a role in protecting the fruit from possible pathogenic attacks (Arcas, Botía, Ortuño, & Del Río, 2000; Del Río, Arcas, Benavente-García, & Ortuño, 1998b; Wollenweber, 1994).

3.3. Variation in flavanone and flavone content during fruit maturation

The levels of hesperidin, diosmin and eriocitrin present in the different development states of lemon fruits (cv. Fino-49, Eureka, and Lisbón) are shown in Table 3.

Table 2
Hesperidin, eriocitrin and diosmin distribution in different mature fruit tissues of cultivar Fino-49[a]

Tissue	Hesperidin	Eriocitrin	Diosmin
Flavedo	0.58±0.12	0.66±0.13	0.33±0.08
Albedo	1.25±0.27	1.59±0.15	0.20±0.06
Pulp	0.28±0.06	0.25±0.08	0.04±0.001

[a] Data express the average content in g/100 g dry weight±SE ($n=3$).

Table 3
Levels of hesperidin, diosmin and eriocitrin flavonoids during the growth of *Citrus limon* (cv. Lisbón, Fino-49 and Eureka) fruit[a]

	State I	State II	State III
Flavonoids (g/100 g dry weight) cv. Lisbon			
Hesperidin	18±1.2	39.6±2.3	0.81±0.01
Diosmin	1.02±0.3	2.02±0.1	0.16±0.03
Eriocitrin	0.18±0.01	0.22±0.05	0.56±0.06
Flavonoids (g/100 g dry weight) cv. Fino-49			
Hesperidin	15.6±1.1	32.24±1.5	0.59±0.03
Diosmin	0.94±0.05	1.49±0.4	1.20±0.2
Eriocitrin	0.16±0.02	0.13±0.01	0.79±0.03
Flavonoids (g/100 g dry weight) cv. Eureka			
Hesperidin	15.5±0.9	28.7±1.5	0.74±0.02
Diosmin	0.92±0.04	1.78±0.2	0.28±0.03
Eriocitrin	0.14±0.03	0.33±0.01	0.65±0.02

[a] Data are expressed in g/100 g dry weight±SE ($n=3$). States of fruit growth: recently set fruit (state I); immature fruit 30 days after anthesis (state II) and mature fruit 150 days after anthesis (state III).

Hesperidin and diosmin behave similarly in the three cultivars although the levels differ (hesperidin levels usually being about 10 or more times higher than those of diosmin) (Table 3). This parallelism between the two flavonoids is not surprising because, as is known, hesperidin is the precursor of diosmin (Benavente-García, Castillo, & Del Río, 1993).

From fruit set (state I) until the formation of immature fruits (state II) the levels of the main flavanone, hesperidin, increase to reach a maximum of more than 30% of the dry weight in cv. Lisbón and cv. Fino-49; but slightly less in cv. Eureka. However, as the fruit grows to maturity (state III), hesperidin levels decrease to reach minimum values of 0.6% of the dry weight (Table 3).

Diosmin behaves similarly in the three cultivars, increasing during the first stages of the fruit formation and growth (states I and II) until it reaches a maximum level (around 1.5–2% of the immature fruit dry weight), and decreasing slightly during the transition to the state III in the case of cv. Fino-49 (1.2% of mature fruit dry weight), but more sharply in the case of cv. Eureka and Lisbón (around 0.2–0.5% of the mature fruit dry weight, respectively) (Table 3).

Eriocitrin shows different behaviour since it tends to accumulate during the fruit maturation process (from state I to III) in all three cultivars (Table 3). In cv. Fino-49, eriocitrin increases in concentration from 0.16 g/100 g dry weight in recently set fruit (state I) to reach a maximum of 0.8 g/100 g dry weight in mature fruit (state III), while the mature fruit of cv.Eureka and cv. Lisbón levels reach about 0.6 g/100 g dry weight (Table 3).

The greatest increase in eriocitrin levels coincides with decreases in hesperidin and diosmin levels, both processes originating during state II (immature fruits) (Table 3). Eriocitrin is synthesized earlier than hesperidin and diosmin. These results suggest that the decrease in the levels of hesperidin and diosmin is due to the inactivation of the final enzymes in the biosynthetic pathway (4′-O-methyltransferase), while the activated enzymes of the lateral pathways, such as glucosyltransferase, produce eriocitrin (Benavente-García, Castillo, & Del Río, 1993; Benavente-García, Castillo, Sabater, & Del Río, 1997b).

Based on the results described and taking into consideration that the weight of immature Fino fruit to fall in Spain in 2000 was of 5,552,000 kg (Porras, personal communication), there is a potential for obtaining 536,000 kg of hesperidin, 24,000 kg of diosmin and 2000 kg of eriocitrin annually.

The results show the potential of lemon (cv. Fino) for providing flavonoids of pharmaceutical interest, either from the immature fallen fruit (inedible) or from the by-products of industrial processing.

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