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Selection of citrus varieties highly productive for the neohesperidin dihydrochalcone precursor

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The levels of the flavanones, neohesperidin and naringin, and the neohesperidin/naringin ratio in immature and mature fruit of different varieties of *Citrus aurantium* and the *Citrus paradisi* Macf. × *Citrus depresssa* Hayata hybrid are compared, and the flavonic content is analysed for the first time. Fruits of the hybrid, which are used to obtain neohesperidin for industrial-scale transformation into the intensely sweet neohesperidin dihycrochalcone, have two advantages: (1) although the levels of neohesperidin in the hybrid are similar to those detected in the immature fruit of *Citrus aurantium*, the levels of naringin detected in the immature fruit of the hybrid are lower, which means that the neohesperidin/naringin ratio is greater and the need for costly neohesperidin purification processes correspondingly less; (2) unlike the mature fruit of *Citrus aurantium*, the mature fruits of the hybrid accumulate high levels of neohesperidin with a high neohesperidin/naringin ratio, so that these too can be used to obtain neohesperidin. © 1997 Published by Elsevier Science Ltd. All rights reserved

INTRODUCTION

The *Citrus* genus is characterized by the accumulation of large quantities of glycosylated flavanones, which are the first intermediaries in the flavonoid biosynthetic pathway (Maier & Hasegawa, 1970; Hasewaga & Maier, 1972). Depending on the particular species, the biosynthetic pathway tends towards the synthesis of: naringin, the 7-β-neohesperidoside of naringenin (4′,5,7-trihydroxyflavanone), which is the major flavonoid in *Citrus paradisi*; neohesperidin, the 7-β-neohesperidoside of hesperetin (3′,5,7-trihydroxy-4′-methoxy flavanone), which is the principal flavonoid in *Citrus aurantium*; or hesperidin, the 7-β-rutinoside of hesperetin, which is the principal flavonoid in *Citrus sinensis* (Albach *et al.*, 1969; Horowitz & Gentili, 1977; Kamiya *et al.*, 1979; Jourdan *et al.*, 1985; Berhow & Vandercook, 1989; Castillo *et al.*, 1992, 1993; Del Río *et al.*, 1992, 1994; Benavente García *et al.*, 1993; Ortuño *et al.*, 1995).

Besides the considerable pharmacological potential of these compounds (Bruckner & Szent-Györgyi, 1936; Gábor, 1988; Salvayre *et al.*, 1988; Francis *et al.*, 1989; Chen *et al.*, 1990; Guengerich & Kim, 1990; Galati *et al.*, 1994), it is known that naringin and neohesperidin have another important industrial application in that they can be chemically converted into their corresponding, intensely sweet, dihydrochalcones (Horowitz & Gentili, 1963; Krbechek *et al.*, 1968; Horowitz, 1986; Bär *et al.*, 1990; Borrego *et al.*, 1991), with values relative to sucrose of 1000 for neohesperidin dihydrochalcone and 300 for naringin dihydrochalcone.

Until now, the material used to obtain neohesperidin and naringin commercially has been the peel of *C. aurantium* and *C. paradisi* fruits, respectively, with the associated problem of having to submit the extracts to sometimes costly purification processes, since these flavanones are not the only ones to occur during fruit development.

The synthesis of neohesperidin dihydrochalcone from the extracted neohesperidin is a simple process involving hydrogenation in the presence of a catalyst under alkaline conditions. However, to synthesize naringin dihydrochalcone is a more complicated and costly process since naringin has to be converted to phloroacetophenone-4′-β-neohesperidoside. This intermediate product then has to be condensed with isovanillin to form neohesperidin before the final hydrogenation step (Krbechek *et al.*, 1968; Robertson *et al.*, 1974). This, and the fact that the sweetening power of naringin dihydrochalcone is less than that of neohesperidin dihydrochalcone, means that, from a commercial point of view, it would be more profitable if the latter were obtained.

This work aims to characterize the most suitable plant materials for the extraction of neohesperidin,

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bearing in mind concentration and purity, with the object of reducing costs and increasing the efficiency of the industrial process used in obtaining neohesperidin dihydrochalcone.

MATERIALS AND METHODS

Plant material and analysis of juice

Several clones of *Citrus aurantium* (Hodgson, 1967; Gogorcena & Ortíz, 1988) from different parts of Sevilla and Murcia (Spain) were used: Calabacita or mamillata (Mairena de Alcor), Cajel or bittersweet orange (Villaverde and Brenes), Afín (La Alberca), Bouquet de Fleurs (La Alberca), Mirtyfolia (La Alberca). *Citrus depressa* Hayata, *Citrus paradisi* Macf. and the *Citrus paradisi* Macf.×*Citrus depressa* Hayata hybrid, were from La Alberca, Murcia, Spain.

Occasionally, the total acidity, expressed as anhydrous citric acid, and the total soluble solids were measured from the juice of the *C. paradisi* Macf.×*C. depressa* Hayata hybrid fruits, according to the procedure described by Ortiz *et al.* (1987).

Extraction, measurement and identification of flavonoids

For the flavonoid extraction and measurement, ten fruits were used in each experiment. The whole immature and mature fruits were dried (50°C in a forced-air oven). The dried material was ground to a fine powder, then 60 mg of this was used for extraction with 10 ml of dimethyl sulphoxide (DMSO) (6 mg of dry weight (DW) ml^{-1}). The extracts were filtered through a 0.45 μm nylon mesh before HPLC analysis with a Beckman liquid chromatograph, a Model 110 B solvent-delivery module and a System Gold Module 168 diode-array detector (range scanned 220–500 nm). A reverse-phase chromatographic separation was carried out on a μ-Bondapak C_{18} (250 mm×4 mm i.d.) analysis column (Waters Associates, Milford, MA). The particle size was 5 μm, and an isocratic separation was achieved using a mixture of water–methanol–acetonitrile–acetic acid (15:2:2:1, v/v) at a flow rate of 1.0 ml min^{-1} at 35°C. Changes in absorbance were recorded in the Vis/UV diode-array detector at 280 nm. The procedure used for the isolation of neohesperidin was similar to that described in a previous paper (Castillo *et al.*, 1992), and its identity was confirmed by its ^1H NMR (200 MHz) spectrum (Bruker, Germany) in hexadeutero-DMSO.

Chemicals

Hesperidin, naringin, neoeriocitrin and neohesperidin standards were obtained from EXTRASYNTHESE.

RESULTS AND DISCUSSION

Changes in the levels of naringin, hesperidin and neohesperidin with age of fruit in different clones of *Citrus aurantium*

Table 1 shows the levels of the flavanones, naringin (nar), hesperidin and neohesperidin (neo), found in the immature and mature fruits of *Citrus aurantium*. The results show that these flavanones are mainly synthesized during the early stages of fruit growth, which agrees with our published results and results from other *Citrus* species (Hasegawa & Maier, 1981; Jourdan *et al.*, 1985; Vandercook & Tisserat, 1989; Berhow & Vandercook, 1991; Castillo *et al.*, 1992, 1993; Del Río *et al.*, 1992; Benavente García *et al.*, 1993; Del Río & Ortuño, 1994; Ortuño *et al.*, 1995). Neohesperidin is the principal flavanone in this species and represents 70–80% of the total flavanone content identified in immature fruit.

Table 1. Levels of the flavanones naringin, hesperidin and neohesperidin in fruits of *Citrus aurantium*

Clones	Fruit	Flavanones (mg per 100 g DW)			
		Naringin (nar)	Hesperidin	Neohesperidin (neo)	Ratio neo/nar
1	A	9090 ± 378	210 ± 18	27440 ± 1270	3.02
	B	3220 ± 162	ND	220 ± 27	0.07
2	A	8930 ± 162	250 ± 12	23000 ± 980	2.57
	B	940 ± 36	1920 ± 210	1270 ± 334	1.35
3	A	8430 ± 175	430 ± 13	23110 ± 1910	2.74
	B	ND	2130 ± 193	840 ± 42	—
4	A	6090 ± 290	640 ± 35	27190 ± 1175	4.46
	B	1910 ± 182	80 ± 9	1050 ± 130	0.55
5	A	5200 ± 193	2950 ± 139	29290 ± 1430	5.63
	B	3810 ± 311	230 ± 21	3680 ± 160	0.96
6	A	7050 ± 387	290 ± 27	20100 ± 2240	2.85
	B	1410 ± 210	680 ± 42	950 ± 37	0.67

The data represent mean values ± SE ($n = 3$) of the secondary metabolites. In the experiments, whole immature (3–7 mm) fruits (A) and whole mature (50–70 mm) fruits (B) were used.
Clones: 1, Calabacita (Mairena de Alcor, Sevilla); 2, Cajel (Villaverde, Sevilla); 3, Cajel (Brenes, Sevilla); 4, Afín (La Alberca, Murcia); 5, Bouquet de Fleurs (La Alberca, Murcia); 6, Mirtyfolia (La Alberca, Murcia).
ND, not detected.

For this reason, and bearing in mind the neo/nar ratio for the different fruit of *C. aurantium* analysed (Table 1), it can be deduced that the immature fruit of the Bouquet de Fleur and Afín varieties of *C. aurantium* (with neohesperidin levels of 29 290 and 27 190 mg per 100 g DW and neo/nar ratios of 5.63 and 4.46, respectively) are the most promising for the commercial production of neohesperidin. The same results point to the impossibility of using the mature fruit for the same purpose, not only because of the lower neohesperidin levels but also because of the unfavourable neo/nar ratios, which would involve expensive purification procedures.

Characteristics of the *C. paradisi* Macf.×*C. depressa* Hayata hybrid

This is a medium-sized tree (larger than *C. depressa*), which is vigorous, highly productive, and with small thorns (5–7 mm). The leaves are medium-sized (7–10 cm long×4–5 cm wide), with large winged petioles (1.5–2.5 cm long×0.4–0.6 cm wide) articulated with the leaf blade. The small white flowers, with five petals and five sepals, normally occur in racemes. The fruit are of medium size (60–70 mm in diameter and 55–65 mm long) and weigh 130–140 g. They are globular and yellow with a thin peel and no mammilla or faintly furrowed nipple. The base is low-collared and rounded. The number of segments varies from 10 to 13 and the juice content of the yellow–green pulp exceeds 45%. There are 6–7 seeds per fruit; these are fusiform, smooth and measure 12 mm×6 mm×5 mm. They are monoembryonic with white cotyledons. The juice of the fruit is acidic (between grapefruit and lemon) with an acidity of 3.5% expressed as anhydrous citric acid. The total soluble solid content varies between 11 and 12 Brix.

Flavonic content in fruits of *C. paradisi* Macf.×*C. depressa* Hayata hybrid

A representative chromatogram of an extract of the immature fruits of this hybrid is shown in Fig. 1. In this chromatogram, compounds 1, 2, 3 and 4 have retention times (R_ts) identical to those of neoeriocitrin, naringin, hesperidin and neohesperidin, respectively. The absorption spectra of these compounds, obtained by means of a Vis/UV diode-array detector, each have two maxima when eluted with the same solvent as in Fig. 1: at 287 and 330 nm for compound 1; at 283 and 326 nm for compound 2; at 283 and 320 nm for compound 3; and at 284 and 327 nm for compound 4, respectively. These data are consistent with the compounds having flavanone skeletons identical to those of neoeriocitrin, naringin, hesperidin and neohesperidin. Compound 4 was isolated by the procedure described in a previous paper (Castillo *et al.*, 1992), and the corresponding ^{1}H NMR spectrum was identical to that obtained by us for neohesperidin in immature fruits and callus cultures of *C. aurantium* (Castillo *et al.*, 1992; Del Río *et al.*, 1992).

As can be appreciated from Table 2, the levels of neohesperidin in immature fruit of this hybrid (23 530 mg per 100 g DW) are about the same as those in sour orange fruit (compare Tables 1 and 2), with the advantage that the naringin levels are lower, which results in a high neo/nar ratio (5.22 for immature fruit; Table 2). An additional advantage is that this ratio remains high in the mature fruit (3.40; Table 2), which means that these fruit too can be used to obtain neohesperidin for subsequent transformation into neohesperidin dihydrochalcone. The presence of neoeriocitrin in this hybrid poses no problem for the extraction and purification of neohesperidin since the solubility of each compound is different; thus, neoeriocitrin can be easily removed during the industrial production of neohesperidin.

Table 2 also shows the naringin, hesperidin and neohesperidin levels for the parent stock of the hybrid. As can be seen, *C. depressa* Hayata mainly accumulates the hesperidin, this rutinoside representing 98% of the total flavonic content. The other parent, *C. paradisi* Macf., principally accumulates the neohesperidoside, naringin, which represents 97% of its total flavonic content. The hybrid expresses 79% neohesperidin and 15% naringin, as percentages of the total flavonic content, suggesting that the hybrid receives the high hydroxylating and methoxylating capacity (3′-hydroxylase and 4′-*O*-methyltransferase) of *C. depressa* Hayata and the rhamnosylating capacity in the 2′-position (neohesperidosides) from *C. paradisi* (Bar-Peled *et al.*, 1993).

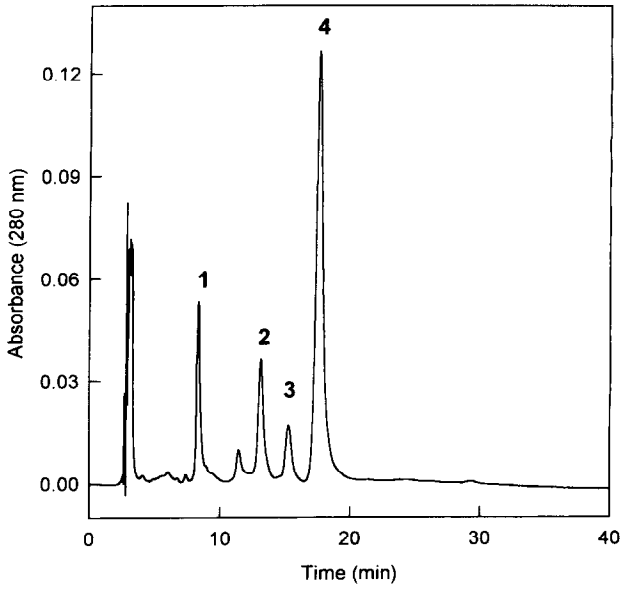

Fig. 1. Representative HPLC elution profile of a dimethylsulphoxide extract of immature fruits of *C. paradisi* Macf.×*C. depressa* Hayata hybrid. The column was eluted with water–methanol–acetonitrile–acetic acid (15:2·2:1, v/v) with a flow rate of 1 ml min^{-1}, at 35°C. Eluent was monitored at 280 nm. Identification: 1, neoeriocitrin (R_t = 8.22 min); 2, naringin (R_t = 12.96 min); 3, hesperidin (R_t = 15.33 min); 4, neohesperidin (R_t = 17.84 min).

Table 2. Levels of the flavanones neoeriocitrin, naringin, hesperidin and neohesperidin in fruits of *Citrus paradisi*, *Citrus depressa* Hayata, and the *Citrus paradisi* Macf.×*Citrus depressa* Hayata hybrid

Species	Fruit	Flavanones (mg per 100 g DW)				
		Neoeriocitrin	Naringin (nar)	Hesperidin	Neohesperidin (neo)	Ratio neo/nar
1	A	ND	37820 ± 7500	328 ± 40	639 ± 30	0.01
	B	ND	7210 ± 110	60 ± 9	40 ± 7	—
2	A	ND	240 ± 20	27500 ± 4100	90 ± 20	0.37
	B	ND	50 ± 4	1190 ± 311	30 ± 5	—
3	A	15816 ± 1340	4510 ± 190	1580 ± 175	23530 ± 2872	5.22
	B	2110 ± 220	1120 ± 180	80 ± 20	3810 ± 190	3.40

The data represent mean values ± SE ($n = 3$) of the secondary metabolites. Whole immature (3–7 mm) fruits (A) and whole mature (70–80 mm) fruits (B) were used in the experiments.
Species: 1, *C. paradisi* Macf.; 2, *C. depressa* Hayata; 3, *C. paradisi* Macf.×*C. depressa* Hayata hybrid.
ND, not detected.

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