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Flavanone and Nootkatone Levels in Different Varieties of Grapefruit and Pummelo

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The effects of variety, rootstock, and geographical location were studied as to their effects on secondary metabolite production in grapefruit and pummelo. The concentration of the flavanones narirutin, naringin, and neohesperidin and of the sesquiterpene nootkatone, which is principally responsible for the grapefruit's aroma, varies during fruit development. The highest flavanone levels are detected during the juvenile stages of fruit development, while nootkatone expression is associated with the processes of maturation and senescence. The possibility of increasing the levels of these metabolites by regulating the associated processes of growth and cell differentiation is discussed.

Keywords: *Citrus grandis; Citrus paradisi L.; flavonoids; naringin; narirutin; neohesperidin; nootkatone; sesquiterpene*

INTRODUCTION

Several studies have revealed a limited range of plants which produce a series of organic compounds known as secondary metabolites. These compounds not only play an important physiological and ecological role but are also of commercial interest. For example, it is known that grapefruit and pummelo accumulate naringin as flavonone glycoside in their fruit, leaves, and juice, and, to a lesser extent, they also produce narirutin, prunin, hesperidin, and neohesperidin (Albach and Redman, 1969; Albach et al., 1981; Albach and Wutscher, 1988; Berhow and Vandercook, 1989, 1991; Del Río and Ortuño, 1994; Jourdan et al., 1985; Kamiya et al., 1979; Kanes et al., 1993; Robertson and Nisperos, 1983; Rouseff et al., 1987; Shaw et al., 1991; Sinclair, 1972).

Commercial interest in these products is considerable since, pharmacologically, hesperidin can affect vascular permeability (Bruckner and Szent-Györgyi, 1936; Gábor, 1988) and naringin can act as antioxidant (Chen et al., 1990), protecting against lipid peroxidation (Salvayre et al., 1988; Guengerich and Kim, 1990), and is an antimutagenic (Francis et al., 1989).

In addition, naringin and neohesperidin have important industrial applications since they can be converted into their corresponding dihydrochalcones with a strong sweetening capacity (Horowitz and Gentili 1963; Krbechek et al., 1968; Horowitz, 1986; Bär et al., 1990; Borrego et al., 1991).

Although it is known that components such as volatile aldehydes (Keterson et al., 1971) and several acetate esters (Moshonas, 1971; Wilson and Shaw, 1980; Shaw and Wilson, 1981) are important contributors to the flavor and aroma of grapefruit, it appears that the sesquiterpene nootkatone (MacLeod and Buigues, 1964) is an even greater contributor, and it has also been described in pummelo (Sawamura and Kuriyama, 1988; Porras et al., 1991). This sesquiterpene is widely used

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as an industrial flavoring and in fragrances (Furia and Bellanca, 1975).

In accordance with the above, it is clear that the commercial outlook for grapefruit is bright and that it can be used much more widely than for the present purposes of producing juice, pectins, and peel oil. The present work attempts to identify the most productive varieties and to ascertain the effect of variety, rootstock, and cultivation area on the levels of these metabolites.

MATERIALS AND METHODS

Plant Material. The following varieties of *Citrus paradisi* L. were used in the different experiments: Star Ruby grafted onto sour orange (*Citrus aurantium*) and grown in Orihuela (Alicante, Spain); Star Ruby, Marsh, Shambar, and Red Blush grafted onto Cleopatra mandarin orange, grown in La Alberca (Murcia, Spain); Garner, Davis Seedless, and Pink Foster grafted onto sour orange (*C. aurantium*); CRC 343, Thompson, and Reed grafted onto Citrange Troyer, grown in Alcanar (Tarragona, Spain); Red Blush, Shambar, and Marsh grafted onto Citrumelo, grown in Játiva (Valencia, Spain); Star Ruby, Marsh, Shambar, and Red Blush grafted onto Cleopatra mandarin orange and Citrange Troyer, grown in La Palma (Murcia, Spain).

The varieties of *Citrus grandis* were Shaddock CAS 668 and Rose Ruby grafted onto sour orange (*Citrus aurantium*), grown in Alcanar (Tarragona), and Chandler grafted onto Citrumelo, grown in Játiva, (Valencia, Spain).

Nootkatone Extraction and Measurement. Five grapefruit and pummelo were used in each experiment. The whole fresh fruit in the case of immature fruit and the whole fresh peel of mature fruit were cut into 0.5 cm sections and mixed. Four grams (fresh weight) of the resulting mixture was used in each assay for the isolation of nootkatone. For this, these samples were homogenized three times with *n*-pentane (1 g of FW/4 mL), adding 200 μg of internal standard lauric acid methyl ester. The homogenates were decanted, and the organic phase was dried with anhydrous Na_2SO_4 and then concentrated to 0.5 mL under nitrogen at room temperature before being analyzed. The extracts were analyzed by a Hewlett-Packard 5890 gas–liquid chromatography (GLC), equiped with a flame ionization detector (FID) and a glass capillary column coated with Carbowax 20 M (25 m × 0.25 mm i.d., 0.2 μm film thickness). The flow rate of carrier gas was 28 mL/min He. The injection volume was 0.5 μL, and the split ratio was 50/1. The injector and detector temperatures were 255 °C. The following column temperature-programming sequence was used: an initial temperature of 75 °C was maintained for 8 min before being increased to 255 °C at a rate of 4 °C/min. Each peak area on the gas chromatogram was calculated automatically with a Hewlett-Packard 3390A integrator. For capillary gas–liquid chromatography–mass spectrometry (GLC–MS) a Hewlett-Packard 5993 mass spectrometer was used with a column similar to that used above. The injector and transfer line temperature was 255 °C, ionization energy 70 eV, and scan time 1 s. Peak identification was confirmed by comparing the retention time and mass spectrum with those of authentic sample, as described previously (Del Río et al., 1992a). Quantitative determinations were based on the known amount of added standard.

Extraction and Measurement of Flavonoids. Five fruits were used in each experiment. In the case of immature fruit, the whole fruit (approximately 70% moisture) was dried, while only the peel of mature fruit (approximately 70% moisture) was dried (50 °C in a forced air oven). The dried material was ground to a fine powder, and then 60 mg of this was used for extraction with 10 mL of dimethyl sulfoxide (DMSO) (6 mg of dry weight/mL). The extracts were filtered through a 0.45 μm nylon mesh before HPLC analysis with a Beckman liquid chromatograph and 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 × 4 mm i.d.) analysis column (Waters Associates, Mildford, MA). The particle size was 5 μm, an isocratic separation was achieved using a mixture of water–methanol–acetonitrile–acetic acid (15:2:2:1 volume) at a flow rate of 1.0 mL/min at 35 °C. Changes in absorbance were recorded in the array of V/UV diodes detector at 280 nm. The procedure used for the isolation of naringin was similar to that described in a previous paper (Castillo et al., 1992), and its identity was confirmed by reference to its nuclear magnetic resonance spectrum (^{1}H NMR) (200 MHz) (Bruker, Germany) in hexadeuteriated dimethyl sulfoxide.

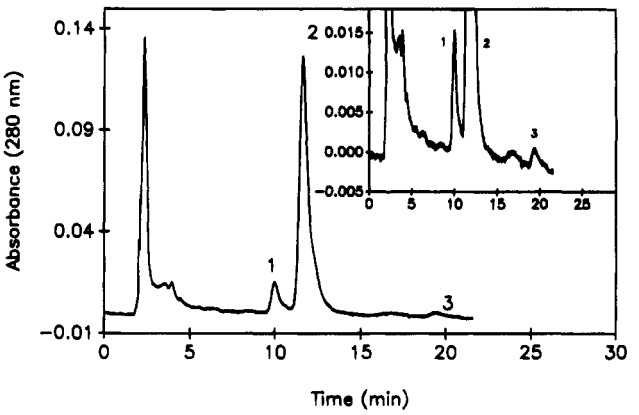

Figure 1. Representative HPLC elution profile of a dimethyl sulfoxide extract of immature grapefruit: (1) narirutin; (2) naringin; (3) neohesperidin.

Chemicals. Nootkatone was purchased from Extrasynthèse, S. A. (Genay, France), while narirutin, naringin, and neohesperidin were kindly donated by Zoster S. A. (Zeneta, Murcia, Spain).

RESULTS AND DISCUSSION

Levels of Narirutin, Naringin, Neohesperidin, and Nootkatone in Grapefruit and Pummelo Fruit. In grapefruit and pummelo, the presence of the sesquiterpene nootkatone was revealed by GLC–MS. In the chromatographic conditions used, the retention time (R_t) corresponding to this sesquiterpene was 41.70 min, and its mass spectrum was similar to that obtained in other works (Del Río et al., 1992a; Del Río and Ortuño, 1994).

In addition, the presence of the flavanones narirutin (compound **1**), naringin (compound **2**), and neohesperidin (compound **3**) was revealed by HPLC (Figure 1, R_t 10.1, 11.7, and 19.7 min, respectively). The absorption spectrum of these compounds obtained by means of a V/UV diode array detector is in accordance with that obtained for compounds with flavanone skeletons identical to narirutin, naringin and neohesperidin (maximums at a 283 and 326 nm for both compounds **1** and **2**; and 284 and 327 for compound **3**). Compound **2** was isolated by the procedure described in previous works (Castillo et al., 1992), and its identity was confirmed by reference to its ^{1}H NMR spectrum, which was identical to that obtained by us for naringin in immature fruit of *C. aurantium* (Castillo et al., 1992) and in callus cultures of *C. aurantium* (Del Río et al., 1992b) and *C. paradisi* (Del Río and Ortuño, 1994).

Table 1 shows the levels of the flavanones narirutin, naringin, and nehoesperidin and those of the sesquiterpene nootkatone found in the immature and mature fruit of grapefruit and pummelo. It can be seen that these flavanones are mainly synthesized during the early stages of fruit growth, which agrees with our published results and results from other authors for different *Citrus* species (Hasegawa and Maier, 1981; Jourdan et al., 1985; Berhow and Vandercook, 1991; Castillo et al., 1992, 1993; Del Río et al., 1992b;

Table 1. Levels of the Flavanones Narirutin, Naringin, and Neohesperidin and of the Sesquiterpene Nootkatone in Grapefruit (*Citrus paradisi* L. Cv. Star Ruby) and Pummelo (*Citrus grandis* Cv. Chandler)[a]

	flavanones (mg/100 g FW)			sesquiterpene (mg/100 g FW)
	narirutin	naringin	neohesperidin	nootkatone
grapefruit[b]				
immature	1188 ± 220	12102 ± 2310	274 ± 35	ND[c]
mature	231 ± 63	2195 ± 339	17 ± 9	5 ± 0.5
pummelo[d]				
immature	12 ± 2	14775 ± 1892	14 ± 3	ND
mature	10 ± 7	569 ± 65	17 ± 3	0.9 ± 0.04

[a] The data represent mean values ± SE (n = 3) of the secondary metabolites (mg/100 g FW). Peel (flavedo + albedo) of grapefruit (80–90 mm) and pummelo fruit (130–140 mm) in the case of mature fruit and whole fruit (9–11 mm, in both species), and in the case of immature fruit was used in the experiments. [b] Orihuela (Alicante, Spain). [c] ND, not detected. [d] Jativa (Valencia, Spain).

Table 2. Effect of the Variety on the Levels of Narirutin, Naringin, Neohesperidin, and Nootkatone in Grapefruit and Pummelo[a]

	flavanones (mg/100 g FW)			sesquiterpene (mg/100 g FW)
variety	narirutin	naringin	neohesperidin	nootkatone
grapefruit				
Star Ruby[b]	137 ± 21	1882 ± 290	17 ± 7	0.3 ± 0.02
Marsh[b]	160 ± 43	2100 ± 193	14 ± 9	4.2 ± 0.31
Shambar[b]	146 ± 39	1432 ± 311	12 ± 3	3.7 ± 0.42
Red Blush[b]	119 ± 18	2017 ± 221	13 ± 7	2.1 ± 0.29
Garner[c]	118 ± 10	1364 ± 175	13 ± 2	0.7 ± 0.03
Davis Seedless[c]	215 ± 13	2397 ± 143	22 ± 4	0.2 ± 0.05
Pink Foster[c]	108 ± 21	1349 ± 210	13 ± 7	1.6 ± 0.4
CRC 343[c]	1042 ± 180	28 ± 9	12 ± 3	0.2 ± 0.02
Thompson[c]	174 ± 41	2509 ± 190	11 ± 3	1.0 ± 0.5
Reed[c]	195 ± 37	2181 ± 335	15 ± 6	0.1 ± 0.02
pummelo				
Shaddock CAS 668[c]	8 ± 0.4	537 ± 37	31 ± 7	2.1 ± 0.6
Rose Ruby[c]	22 ± 5	1270 ± 230	28 ± 4	5.4 ± 0.9

[a] The data represent mean values ± SE (n = 3) of these secondary metabolites (mg/100 g FW). The peel (flavedo ± albedo) of grapefruit (80–100 mm diameter) and pummelo (106–160 mm diameter) was used in the different experiments. [b] La Alberca (Murcia, Spain). [c] Alcanar (Tarragona, Spain).

Benavente-García et al., 1993; Del Río and Ortuño, 1994), the principal flavanone detected in both grapefruit and pummelo being naringin. This flavanone represents about 89% of the total flavanone content identified both in immature and mature grapefruit, while in pummelo it represents 99.8% or 96% of the immature and mature fruit, respectively.

As can be seen in Table 1, only the mature fruits of grapefruit and pummelo show any capacity to accumulate the sesquiterpene nootkatone, since the expression of this secondary metabolite is linked to processes of cell differentiation, as has been revealed by callus culture (Del Río et al., 1991) and, in fruit, by the modulation of the processes of maturation (Del Río et al., 1992a). Similarly, the need for cells to differentiate morphologically has also been suggested by the expression of monoterpenes (Paupardin, 1976; Brown and Charlwood, 1986).

The values described in Table 1, both for flavanones and the sesquiterpene nootkatone, can be substantially increased by prolonging the juvenile stage of cell growth (in preparation) in the first case or accelerating the processes of cell differentiation in the second (Del Río et al., 1993; García-Puig et al., 1993; Ortuño et al., 1993).

Influence of Variety on the Expression of These Secondary Metabolites in Grapefruit and Pummelo Fruit. The levels of nootkatone detected in Marsh, Shambar, and Red Blush grapefruit are higher than those in the remaining varieties (Table 2).

Regarding pummelo, Rose Ruby grafted onto sour orange produces the highest levels of nootkatone (Tables 2 and 1), which are very similar to the levels recorded for the most productive grapefruit. Thus, pummelo, too, must be considered an important source of this sesquiterpene.

The highest levels of naringin are produced by Marsh and Red Blush grapefruit on Cleopatra and Davis Seedless on sour orange and Thompson and Reed varieties on Citrange Troyer (Table 2).

The variety CRC 343 on Citrange Troyer behaves differently, since narirutin is the principal flavanone produced and naringin is produced in very low quantities (Table 2).

Generally speaking, the varieties of pummelo assayed show lower levels of naringin (about 50–70% of the levels recorded for grapefruit) except Rose Ruby on sour orange, which produces levels similar to those recorded for some of the combinations of grapefruit (Tables 2 and 1).

Influence of Rootstock and Cultivation Zone on the Expression of These Secondary Metabolites in Grapefruit and Pummelo Fruit. In the Marsh, Shambar, and Red Blush grapefruit varieties, it is the graft on Citrange Troyer rather than on Cleopatra or Citrumelo which is most productive of nootkatone (compare Tables 2 and 3). This is also true of the variety Star Ruby grafted onto Citrange Troyer rather onto Cleopatra (Tables 2 and 3), although in this case there is an ever greater increase in nootkatone levels when it is grafted onto sour orange (compare Tables 1, 2, and 3; Porras et al., 1991).

With regard to the levels of the flavanones, the rootstock used for grafting seems to play a role (Table 3) as does the area in which the trees are cultivated (compare Marsh and Red Blush on Cleopatra mandarin in Tables 2 and 3). The influence of the rootstock chosen

Table 3. Effect of Rootstock on the Levels of Narirutin, Naringin, Neohesperidin, and Nootkatone Found in Different Varieties of Grapefruit[a]

variety	rootstock and location[b]	flavanones (mg/100 g FW) narirutin	naringin	neohesperidin	sesquiterpene (mg/100 g FW) nootkatone
Star Ruby	1	134 ± 36	1402 ± 216	12 ± 2	
	2	177 ± 27	1366 ± 372	12 ± 6	4.1 ± 0.3
Marsh	1	114 ± 31	484 ± 132	9 ± 4	
	2	126 ± 19	1058 ± 163	7 ± 0.8	5.8 ± 0.2
	3	117 ± 18	1578 ± 139	21 ± 2	0.3 ± 0.01
Shambar	1	147 ± 22	1229 ± 335	7 ± 0.6	
	2				4.3 ± 0.2
	3	93 ± 10	1378 ± 160	13 ± 5	0.2 ± 0.02
Red Blush	1	144 ± 76	999 ± 164	1 ± 0.5	
	2	73 ± 19	407 ± 63	5 ± 0.6	3.7 ± 0.3
	3	98 ± 17	1245 ± 321	11 ± 3	0.5 ± 0.04

[a] The data represent mean values ± SE ($n = 3$) of these secondary metabolites (mg/100 g FW). The peel (flavedo + albedo) of grapefruit (80–90 mm diameter) was used in the different experiments. [b] Rootstock and location: (1) mandarin Cleopatra, La Palma (Murcia, Spain); (2) Citrange Troyer, La Palma (Murcia, Spain); (3) Citrumelo, Játiva (Valencia, Spain).

varies depending on the variety since Marsh produces higher levels of these flavanones when grafted onto Citrange Troyer than onto Cleopatra mandarin, while the opposite is true for the variety Red Blush. In some cases the rootstock seems to play very little part in flavanone production: see the results for Star Ruby on Cleopatra and Citrange Troyer (Table 3).

These results suggest that the biosynthesis of these compounds can be regulated by environmental, nutritional, and genetic factors. In addition, they point to new perspectives for grapefruit cultivation, since the immature fruit which fall in June can be used to obtain flavanones while the mature fruit can be used to obtain the essential oil with its high levels of nootkatone.

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