ARTICLES

Variations of Nootkatone and Valencene Levels during the Development of Grapefruit

José A. del Río,*,† Ana Ortuño,† Damián García-Puig,† Ignacio Porras,‡ Angel García-Lidón,‡ and Francisco Sabater†

Departamento de Biología Vegetal, Facultad de Biología, Universidad de Murcia, Campus de Espinardo,
30071 Murcia, Spain, and Consejería de Agricultura Ganadería y Pesca de la Región de Murcia, Departamento
de Citricultura, Centro Regional de Investigaciones Agrarias, La Alberca, 30150 Murcia, Spain

The distribution of nootkatone and valencene in grapefruit was determined by gas–liquid chromatography–mass spectrometry (GLC–MS). Changes in the concentration of valencene and nootkatone were observed during the development of fruits, increases in these compounds coinciding with the linear and the maturation steps, respectively. The possible participation of valencene and 2-hydroxyvalencene as precursors in the biosynthetic pathway for nootkatone in grapefruit is discussed.

INTRODUCTION

The sesquiterpene nootkatone is a secondary metabolite, first found in grapefruit peel oil by MacLeod and Buigues (1964) and later detected in pummelo (Sawamura and Kuriyama, 1988; Porras et al., 1991) and in other *Citrus* fruits (Boelens and Jimenez, 1989; Sawamura et al., 1990; Del Río et al., 1991). This compound is an important constituent of commercial flavorings and fragances (Sinclair, 1972).

Although several precursors for sesquiterpenoid biosynthesis have been described (Loomis and Croteau, 1980), this biosynthetic pathway has not been confirmed for nootkatone. Some authors have reported that nootkatone may be formed from valencene, as revealed by experiments carried out by chemosynthesis (Hunter and Brogden, 1965; Schulte-Elte et al., 1974; Wilson and Shaw, 1978) or by in vitro assays using both *Citrus limon* cell suspension cultures (Drawert et al., 1984) and *Citrus paradisi* callus cultures (Del Río et al., 1991). However, there are no results that confirm either the existence or the degree of possible participation of valencene as an in vivo precursor of nootkatone in *Citrus*. On the other hand, recent results suggest that the levels of nootkatone may increase during the grapefruit processing season (Wilson and Shaw, 1980) or during pummelo storage (Sawamura et al., 1989), although until now the mechanisms involved in these concentration changes have not been described.

In the present investigation, we have analyzed the levels of nootkatone during grapefruit development to establish the correlation between physiological and biochemical changes in grapefruit associated with the expression of this secondary metabolite. The simultaneous analysis of valencene and 2-hydroxyvalencene evolution also provides information on the possible degree of participation of these sesquiterpenes in the biosynthetic pathway for nootkatone in grapefruit.

MATERIALS AND METHODS

Plant Material and Measurement of Growth. Twenty-four *C. paradisi* L. (cv. Star Ruby) trees on *C. aurantium* L.

---

† Universidad de Murcia.
‡ Centro Regional de Investigaciones Agrarias.

rootstocks, located in the experimental plantation of the Centro Regional de Investigaciones Agrarias (Murcia) in Orihuela (Alicante), were used. Between May 1990 and April 1991, fruits of these were used for different measurements. The equatorial diameters of the grapefruits left in the trees (40 per tree) were measured with a digimatic caliper (Mitutoyo, Tokyo) at 15-day intervals during this period. These measurements were repeated for four trees, and the mean values at each age were used to express the development of growth in the fruit. Fruits, representative of each age assayed, were collected from the remaining 20 trees for the ripening and sesquiterpenoid measurements. In some cases, at 25 and 36 weeks after anthesis, samples of fruit were picked and maintained at room temperature.

Ripening Measurement. At each age, samples of 20 freshly picked grapefruit were used to measure ripeness. Two parameters were used: total acidity (TA), as citric acid (grams per liter), and the index of ripeness expressed as the ratio between total soluble solids (TSS; percent at 20 °C) and the TA, both measured from the juice of the corresponding fruits according to the procedure described by Ortiz et al. (1987).

Isolation of the Sesquiterpenoids. Five grapefruit peels (flavedo plus albedo) from freshly picked fruits and the fruits picked at 25 and 36 weeks after anthesis were used in each assay. These samples were chopped into 0.5-cm pieces and mixed. Four grams of fresh weight (FW) of this mixture was used in each case for the corresponding isolation of the sesquiterpenoids. 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. Occasionally, flavedo, albedo, and pulp were used separately.

Gas Chromatography, Mass Spectrometry. The extracts were analyzed by a Hewlett-Packard 5890 gas–liquid chromatography (GLC), equipped 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 spec-

Table I. Distribution of Valencene and Nootkatone in *C. paradisi*[a]

part of fruit	valencene		nootkatone	
	µg/100 g of FW	%	µg/100 g of FW	%
flavedo	1960 ± 172	99.5	4800 ± 362	97
albedo	ND		58 ± 5	1
pulp	9 ± 0.5	0.5	92 ● 3	2

[a] Fruits 36 weeks after anthesis (recently picked) were used. Data represent mean values ± SE (n = 3). ND, not detected.
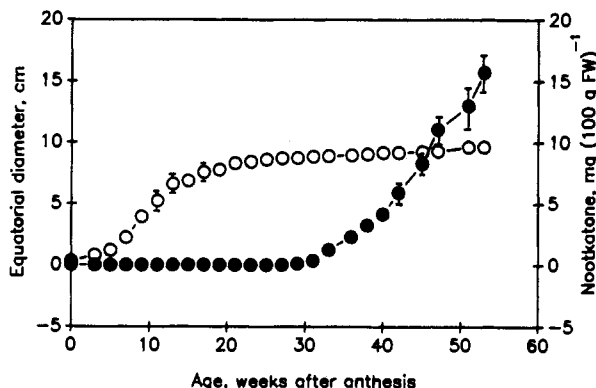

Figure 1. Growth and levels of nootkatone in grapefruit. For each age, the mean value of equatorial diameter (centimeters, O) and the nootkatone content (milligrams per 100 g of FW, ●) were determined. Growth data were obtained from 160 representative fruits. Nootkatone assays were repeated in triplicate with recently picked fruit. Vertical bars denote ±SE when larger than symbols.

trometry (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 samples in a previous paper (Porras et al., 1991). Quantitative determinations were based on the known amount of added standard.

Chemicals. Nootkatone and valencene are commercially available (Extrasynthèse, S.A.). 2-Hydroxyvalencene was synthesized from nootkatone as described by Wilson and Shaw (1978). The purity of all compounds was tested by GLC analysis.

RESULTS AND DISCUSSION

Distribution of Nootkatone and Valencene in Grapefruit. The highest levels of both nootkatone and valencene are found in the flavedo: on the order of 97 and 99.5%, respectively, of the totals found in the whole fruit (Table I). Both could be detected in the pulp of the fruit in low concentrations (2 and 0.5%, respectively), but only nootkatone was found in the albedo, in a lower concentration (1%). This distribution is similar to that described for mono- and sesquiterpenes in other *Citrus* fruits (Ting and Attaway, 1971).

Changes in Nootkatone Content during Development of Grapefruit. Grapefruit presents a sigmoid growth curve in which three phases can be detected. The logarithmic phase includes the first 3 weeks after anthesis, while the linear phase extends from 5 to 13 weeks after anthesis and the maturation phase begins 15–19 weeks after anthesis (Figure 1).

Synthesis of nootkatone could not be detected until 29 weeks after anthesis (Figure 1), after which time a rapid increase in concentration took place between weeks 33 and 53. The regression analysis corresponding to this period provides the calibration equation y = (0.74 ± 0.04)x - (24.55 ± 0.85), r^2 = 0.9755. From the experimentally obtained growth curve, we see that this linear stage of nootkatone production coincides with fruit
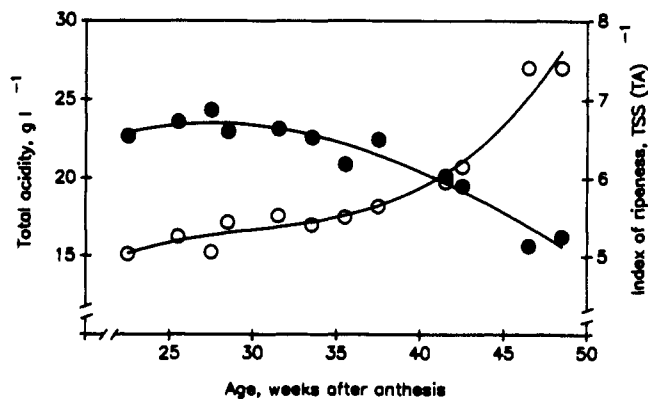

Figure 2. Development of index of ripeness (TSS/TA, O) and total acidity (grams per liter, ●) during the ripening of grapefruit. Experiments were repeated two times with similar results. The data presented correspond to one of these.
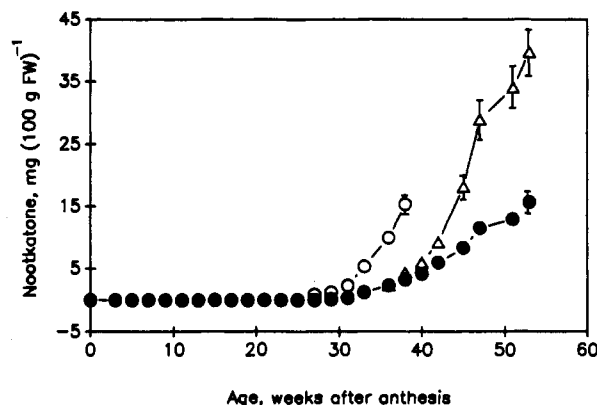

Figure 3. Changes in nootkatone production in recently picked grapefruits (●) and those picked 25 (O) or 36 weeks (△) after anthesis. Vertical bars denote ±SE (n = 3), when larger than symbols.

ripening. In addition, simultaneous with the linear increase of nootkatone described above, a large increase in the index of ripeness of the grapefruit (TSS/TA, see Materials and Methods) was observed from 33 to 48 weeks after anthesis (Figure 2). This increase in the index of ripeness with the age of grapefruits is caused by a fall in their total acidity (Figure 2), since the total soluble solids remained practically constant during the period of analysis ($\simeq$12% at 20 °C).

Fruit was picked to accelerate ripening, and Figure 3 illustrates the effect that this has on the capacity for nootkatone production. Picking, whether at 25 or 36 weeks after anthesis, produced an increase in nootkatone concentration when compared with the levels in fruit left on the tree. The regression analysis corresponding to both assays, at 25 and 36 weeks, provides the following calibration equations: y = (1.81 ± 0.17)x - (54.24 ± 0.94), r^2 = 0.9814; and y = (2.67 ± 0.22)x - (101.47 ± 2.55), r^2 = 0.9721, respectively. A steeper slope in relation to that obtained when the fruits were left on the trees can be clearly seen in both cases.

Thus, since changes in nootkatone concentration were concomitant with changes associated with the ripening process, we propose that the nootkatone might be seen as an indicator of maturity in grapefruit. These results contrast with those obtained by Coggins et al. (1969) for valencene content and its relation with degree of maturity or senescence in oranges. However, bearing in mind that nootkatone has not been described in navel orange, we are of the opinion that this accumulation of valencene could be due to the absence or inhibition of the enzymatic system
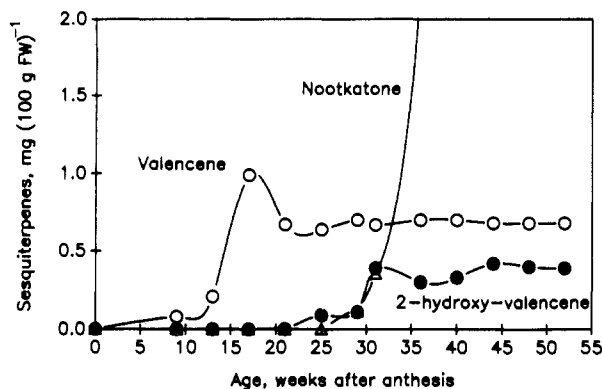
1490 *J. Agric. Food Chem.*, Vol. 40, No. 9, 1992

del Río et al.

Figure 4. Levels of valencene, 2-hydroxyvalencene, and nootkatone as functions of the age of grapefruit. Experiments were repeated three times. The data presented correspond to one of these.

that transforms valencene into nootkatone (nootkatone synthase) in this plant material.

Evolution of Valencene and 2-Hydroxyvalencene in Correlation with Nootkatone in Grapefruit. Valencene synthesis (Figure 4) begins 9 weeks after anthesis and coincides with the linear stage of growth (see Figure 1), reaching its maximum level 15–19 weeks after anthesis ($\simeq 1$ mg/100 g of FW) when the fruit enters the maturation phase (see Figure 1). The level falls after this time and remains practically constant (around 0.7 mg/100 g of FW) between 21 and 53 weeks after anthesis.

Five weeks after the level of valencene has fallen (by 0.3 mg/100 g of FW), 2-hydroxyvalencene begins to be detected, but 25 weeks after anthesis it can only be detected in trace amounts (Figure 4). After 31 weeks, its level is about the same (approximately 0.39 mg/100 g of FW) as the amount by which the valencene has declined. These results, together with the imbalance between valencene decline (19 weeks after anthesis) and the appearance of nootkatone (29 weeks after anthesis), suggest that 2-hydroxyvalencene might be an intermediary in the biosynthetic pathway for nootkatone in grapefruit, generated from the valencene, in accordance with the results described for *C. limon* cell suspension (Drawert et al., 1984). On the other hand, the presence of a lag in each of the curves (Figure 4) seems to suggest that the system operates with various coupled enzymes (García-Carmona et al., 1981). In addition, when the linear phase of nootkatone synthesis is reached (Figure 4), the valencene and 2-hydroxyvalencene levels are stabilized, suggesting that the system has reached steady state and that the nootkatone accumulated is the final product of this biosynthetic pathway.

When the fruits were picked at 25 and 36 weeks after anthesis, the levels of valencene and 2-hydroxyvalencene were similar to those obtained for fruits left on the tree (data not shown; see Figure 4). The fact that the former showed higher final concentrations of nootkatone than the latter (Figure 3) might be due, among other reasons, to the activation of some of the enzymatic systems involved in this biosynthetic pathway by the act of separation from the tree (this is the object to our present research). However, these results do not discount the possibility of another biosynthetic pathway for nootkatone in grapefruit besides the one described, as has been suggested for pummelo (Sawamura et al., 1990).

LITERATURE CITED

Boelens, M. H.; Jimenez, R. The chemical composition of the peel oils from unripe and ripe fruits of bitter orange, *Citrus aurantium* L. ssp. amara Engl. *Flavour Fragance J.* 1989, *4*, 139–142.

Coggins, C. W., Jr.; Scora, R. W.; Lewis, L. N.; Knapp, J. C. F. Gibberellin-delayed senescence and essential oil changes in the navel orange rind. *J. Agric. Food Chem.* 1969, *17*, 807–809.

Del Río, J. A.; Ortuño, A.; García Puig, D.; Iborra, J. L.; Sabater, F. Accumulation of the sesquiterpenes nootkatone and valencene by callus cultures of *Citrus paradisi*, *Citrus limonia* and *Citrus aurantium*. *Plant Cell Rep.* 1991, *10*, 410–413.

Drawert, F.; Berger, R. G.; Godelmann, R. Regioselective biotransformation of valencene in cell suspension cultures of *Citrus* sp. *Plant Cell Rep.* 1984, *3*, 37–40.

García-Carmona, F.; García-Cánovas, F.; Lozano, J. A. Optimizing enzyme assays with one or two coupling enzymes. *Anal. Biochem.* 1981, *113*, 286–291.

Hunter, G. L. K.; Brogden, W. B., Jr. Conversion of valencene to nootkatone. *J. Food Sci.* 1965, *30*, 876–878.

Loomis, W. D.; Croteau, R. Biochemistry of Terpenoids. In *The Biochemistry of Plants*; Stumpf, P. K., Ed.; Academic Press: London, 1980; ISBN 0-12-675404-7, Vol. 4, pp 363–418.

MacLeod, W. D.; Buigues, N. Sesquiterpenes I. Nootkatone, a new grapefruit flavor constituent. *J. Food Sci.* 1964, *29*, 565–568.

Ortiz, J. M.; Tadeo, J. L.; Estellés, A. Physicochemical characteristics of Navelina, Washinton Navel and Navelate and their evolution during maturation. *Fruits* 1987, *42*, 435–441.

Porras, I.; Ortuño, A.; García Puig, D.; Sabater, F.; García Lidón, A.; García-Legaz, M. F.; Del Río, J. A. Nootkatone and valencene biosynthesis in grapefruit and pummelos. Influence of cultive zone and time of harvest. *Levante Agrícola* 1991, *30*, 32–38.

Sawamura, M.; Kuriyama, T. Quantitative determination of volatile constituents in the pummelo (*Citrus grandis* Osbeck forma Tosa-buntan). *J. Agric. Food Chem.* 1988, *36*, 567–569.

Sawamura, M.; Tsuji, T.; Kuwahara, S. Changes in the volatile constituents of pummelo (*Citrus grandis* Osbeck Tosa-buntan) during storage. *Agric. Biol. Chem.* 1989, *53*, 243–246.

Sawamura, M.; Kuwahara, S.; Shichiri, K.; Aoki, T. Volatile constituents of several varieties of pummelos and a comparison of the nootkatone levels in pummelos and other *Citrus* fruits. *Agric. Biol. Chem.* 1990, *54*, 803–805.

Schulte-Elte, K. H.; Fracheboud, M. G.; Ohloff, G. Nootkatone alcohols as perfumes. Swiss Pat. 553141, 1974.

Sinclair, W. B. Grapefruit By-Products. In *The Grapefruit. Its Composition, Physiology and Products*; University of California, Division of Agricultural Sciences: Berkeley, 1972; pp 502–551.

Ting, S. V.; Attaway, J. A. Citrus Fruits. In *The Biochemistry of Fruits and their Products*; Hulme, A. C., Ed.; Academic Press: London, 1971; ISBN 12-361202-0, Vol. 2, pp 107–169.

Wilson, C. W., III; Shaw, P. E. Synthesis of nootkatone from valencene. *J. Agric. Food Chem.* 1978, *26*, 1430–1432.

Wilson, C. W., III; Shaw, P. E. Glass capillary gas chromatography for quantitative determination of volatile constituents in cold-pressed grapefruit oil. *J. Agric. Food Chem.* 1980, *28*, 919–922.