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Food Chemistry 66 (1999) 217–220

Food Chemistry

www.elsevier.com/locate/foodchem

Evolution of polymethoxy flavones during development of tangelo Nova fruits

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Received 7 October 1998; received in revised form and accepted 1 December 1998

Abstract

The production of nobiletin, sinensetin, tangeretin, quercetogetin and heptamethoxyflavone by tangelo Nova fruits is described. The levels of these compounds were examined by high performance liquid chromatography–mass spectrometry. The results suggest that the highest levels of the nobiletin, sinensetin and tangeretin are associated with young developing states in fruits, while quercetogetin and heptamethoxyflavone come into full production towards the end of the lineal phase of growth. A study of the distributions of these secondary metabolites revealed that they were only located in the peel.

Keywords: Citrus; Heptamethoxyflavone; Nobiletin; Quercetogetin; Sinensetin; Tangeretin

1. Introduction

In previous papers it was demonstrated that tangelo Nova fruits produce high quantities of the flavanone, hesperidin, which represents about 40% (on a dry matter basis) in the immature fruit, making it an ideal candidate for commercial production (Del Río, Ortuño, Marín, García Puig, & Sabater, 1995; Ortuño et al., 1995).

Besides the flavanones glycoside, which are accumulated during specific stages of citrus fruit growth (Benavente-García, Castillo, & Del Río, 1993; Berhow & Vandercook, 1991; Castillo, Benavente, & Del Río, 1992, 1993; Del Río et al., 1992, 1997; Del Río & Ortuño, 1994; Jourdan, McIntosh, & Mansell, 1985; Ortuño et al., 1995, 1997; Vandercook & Tisserat, 1989), the citrus genus contains a series of characteristic polymethoxy flavones which are absent from or which occur in minimal concentrations in other plant genera. Such compounds are therefore of taxonomic interest (Gaydou, Bianchini, & Randriamihariosa, 1987) and can be analysed in an industrial context to detect the adulteration of citric juices (Ooghe, Ooghe, Detavernier, & Huyghebaert, 1994).

The polymethoxy flavones of citrus are also of potential interest from a pharmacological point of view. Sinensetin and nobiletin are effective in decreasing erythrocyte aggregation and the rate of blood cell sedimentation in humans (Robbins, 1976). Tangeretin induces leucocytes to partially inhibit the development of HL-60 leukemia cells (Hirano, Abe, & Oka, 1995). Tangeretin, nobiletin and heptamethoxyflavone have cytotoxic properties towards cancerous cells and play a role in the blood circulation of patients with coronary diseases (Kupchan, Knox, & Udayamurthy, 1965; Robbins, 1976). In addition, heptamethoxyflavone is a cardiotonic which has a positive inotropic effect on mouse heart tissue (Itoigawa, Takeya, & Furukawa, 1994).

Another possible role of these compounds is related to the defense mechanisms of the plant itself, since sinensetin, nobiletin and tangeretin have an antiviral and antimicrobial capacity which, together with the other components of the essential oil, confer a certain degree of resistance against microbial infections in citrus (Ben-Aziz, 1967; Huet, 1982; Del Río, Arcas, Benavente-García, & Ortuño, 1998a).

The purpose of this work was to study the polymethoxy flavone compositon of the fruits of tangelo Nova and to determine the developmental state of the fruit in which the greatest accumulation of these secondary metabolites occurs. The distribution of these compounds in the fruit is analysed with a view to using this plant material for their isolation on an industrial scale.

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0308-8146/99/$ - see front matter
PII: S0308-8146(99)00047-3

2. Materials and methods

2.1. Plant material

Tangelo Nova, a mandarin hybrid (*Citrus reticulata* B)×tangelo Orlando (*Citrus reticulata*×*Citrus paradisi* Macf.) trees, located in an experimental plantation in Alhama (Murcia, Spain) were used.

2.2. Isolation, chromatographic analysis and identification of polymethoxy flavones

Fruits of different ages were collected and immediately dried at 50°C to constant weight. The peel and pulp of some fruit were separated for the polymethoxy flavone distribution assays. The dried plant materials were ground and shaken with dimethylsulphoxide for 24 h in a proportion of 40 mg of dry weight/ml in order to extract the polymethoxy flavones. The corresponding extracts were filtered through a 0.45 μm nylon membrane before analysis in a Hewlett–Packard liquid chromatograph (Model HP 1050, USA) equipped with a diode array detector. The stationary phase was a (250×4 mm i.d.) C_{18} Spherisorb ODS column with a particle size of 5 μm thermostatted at 30°C. As solvent we used: tetrahydrofuran (A): water (B): acetonitrile (C) (Ooghe et al., 1994), optimized for our particular work conditions with a gradient profile of 12% (A), 68% (B) and 20% (C) for 20 min, and then changed to 12% (A), 18% (B) and 70% (C) in 20 min. At 45 min it began to change to its initial composition, a process which lasted 15 min. Eluent flow was 1 ml/min. The absorbance changes were recorded in the V/UV diode-array detector at 340 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 principal polymethoxy flavones present in these extracts were collected with a fraction collector (Pharmacia LKB Biotechnology, Sweden) for identification by means of a Hewlett–Packard mass spectometer (Model 5989).

2.3. Chemicals

Sinensetin and tangeretin were purchased from Extrashynthèse S.A. (Genay, France). Quercetogetin, heptamethoxyflavone and nobiletin were obtained by TLC of citrus essential oils and identified by MS (Del Río et al., 1998a).

3. Results and discussion

3.1. Identification of the polymethoxy flavones present in tangelo Nova fruit

The polymethoxy flavone composition of tangelo Nova fruit has not been described. The chromatographic analysis of immature fruit extracts revealed the presence of a major compound with a retention time coinciding with that of the polymethoxy flavone, nobiletin (R_t = 16.08 min). The other polymethoxy flavones present were: sinensetin (R_t = 12.68 min), tangeretin (R_t = 25.14 min), heptamethoxyflavone (R_t = 17.50 min) and quercetogetin (R_t = 13.71 min) (Fig. 1).

The absorption spectra of these compounds, obtained by means of a V/UV diode array detector, have the following maxima when eluted in the same solvent as described above: 245, 271 and 331 nm for nobiletin, 237 (shoulder), 267 (shoulder) and 325 nm for sinensetin, 271 and 324 nm for tangeretin, 254, 269 (shoulder) and 341 nm for heptamethoxyflavone, and 266 and 334 nm for quercetogetin.

The mass spectra obtained for the sinensetin and tangeretin compounds were identical to those obtained for the corresponding standards, and those obtained for nobiletin, heptamethoxyflavone and quercetogetin were identical to the mass spectra obtained previously for *Citrus aurantium* fruits (Del Río, Arcas, Benavente, Sabater, & Ortuño, 1998; Del Río et al., 1998a,b).

3.2. Evolution of polymethoxy flavones during the development of tangelo Nova fruit

The highest concentrations of nobiletin, sinensetin and tangeretin were associated with the exponential phase of fruit growth followed by a fall in production during the linear phase of fruit growth (Fig. 2). These results agree with those obtained by us and other authors for flavanones in different citrus species (Berhow & Vandercook, 1991; Castillo et al., 1992; Del Río & Ortuño, 1994; Del Río et al., 1995; Hasagawa & Maier, 1981; Jourdan et al., 1985; Ortuño et al., 1995, 1997; Vandercook & Tisserat, 1989), and for the polymethoxy flavones detected in fruit of *Citrus aurantium* (Del Río et al., 1998b). Thus, the levels of nobiletin, sinensetin and tangeretin reach 7.7, 4.3 and 2.4 mg/100 g of dry weight, respectively, in recently set fruit, but fall to 4.2, 1.9 and 0.8 mg/100 g dry weight, in 100-day old fruit, which coincides with the end of the lineal phase of growth in this plant material (Del Río et al., 1995).

The flavones quercetogetin and heptamethoxyflavone, behave somewhat differently to the above flavanones, since we recorded levels of 1.5 and 4.4 mg/100 g of dry weight in recently set fruit. These concentrations were

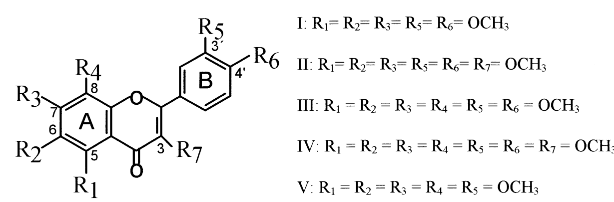

Fig. 1. Sinensetin (I), quercetogetin (II), nobiletin (III), heptamethoxyflavone (IV) and tangeretin (V), structures.

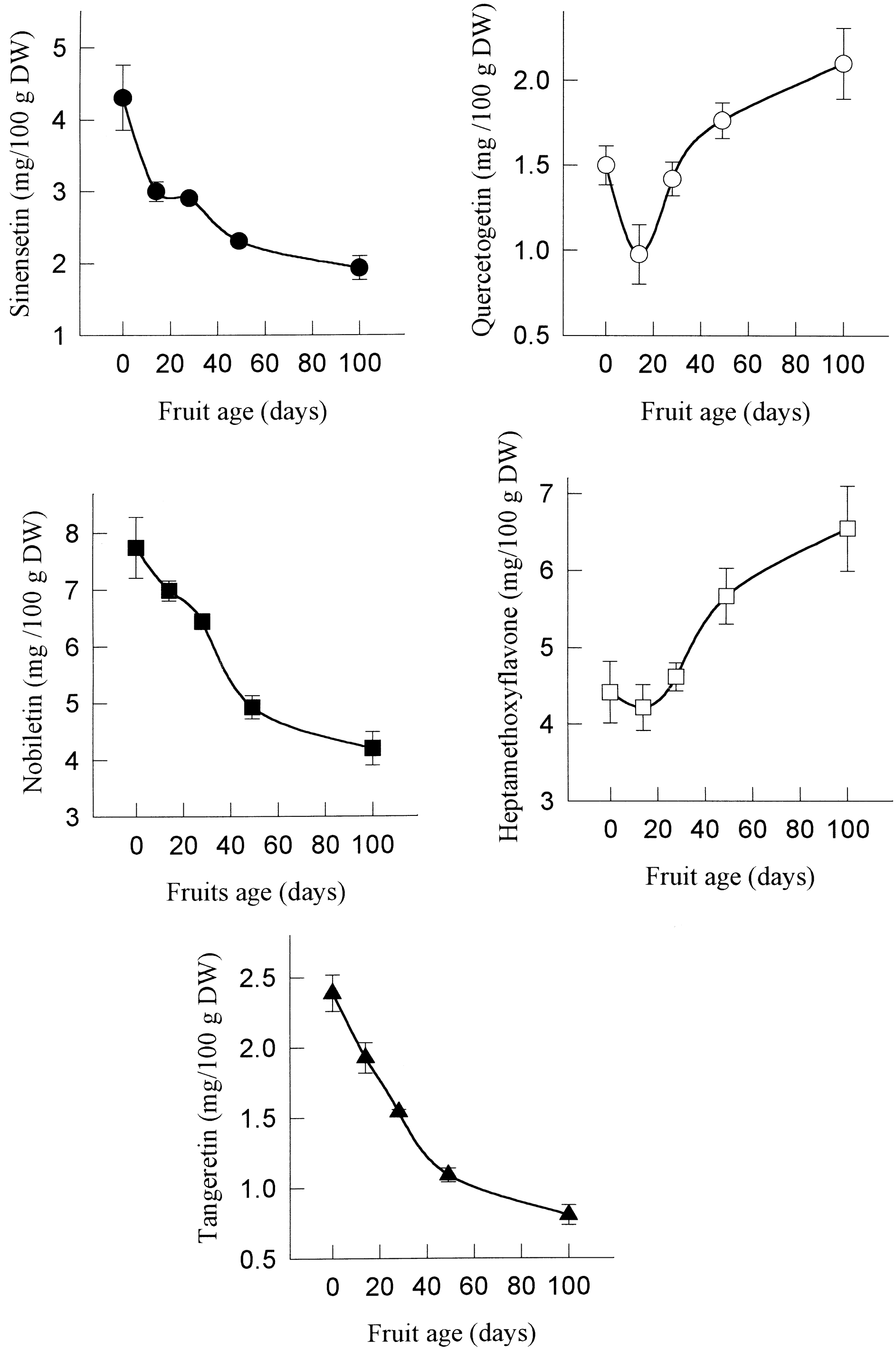

Fig. 2. Variation in the concentration of polymethoxylated flavones during the development of tangelo Nova fruit. The values represent the mean levels recorded (mg/100 g dry weight) ± SE ($n = 3$).

higher 25 days after anthesis and reached 2.1 and 6.5 mg/100 g of dry weight, respectively, 100 days after anthesis.

A study of the distributions of these secondary metabolites in tangelo Nova fruit revealed that they were only located in the peel (Table 1). These results agree with those of other authors in different citrus species (Cheng, Lee, Chou, & Chang, 1985; Machida & Osawa, 1989; Manthey & Grohmann, 1996) and support the idea that their superficial localisation is related with a possible protective role against pathogenic attack (Wollenweber, 1994; Del Río et al., 1998a).

Based on the results obtained, we postulate that the physiological activity of quercetogetin and heptamethoxyflavone might be related to the maturation phase of fruit, while the activity of nobiletin, sinensetin

Table 1
Polymethoxy flavone content in different tissues of tangelo Nova mature fruits

	Polymethoxy flavone (mg/100 g dry weight)		
	Peel	Pulp	Whole mature fruit
Sinensetin	8.9 ± 0.7	ND	1.9 ± 0.2
Quercetoget	10.3 ± 1.0	ND	2.1 ± 0.2
Nobiletin	19.1 ± 1.5	ND	4.2 ± 0.3
Heptametho.	29.2 ± 3.0	ND	6.5 ± 0.5
Tangeretin	3.6 ± 0.3	ND	0.8 ± 0.07

The data represent mean values ± SE ($n = 3$). ND, not detected.

and tangeretin is more probably related to the first stages of fruit developement. On the other hand, the delay observed in the processes of quercetogetin and heptamethoxyflavone synthesis and/or accumulation with respect to that of the other flavones may be related to the fact that sinensetin and nobiletin could be possible precursors of quercetogetin and heptamethoxyflavone, respectively. This is supported by the fact that the synthesis of quercetogetin and heptamethoxyflavone, coincides with the fall in sinensetin and nobiletin levels, respectively (Fig. 2).

Acknowledgements

This work was supported by a grant (ALI95-1015) from the CICYT, Spain. M.C.A. received a grant from the INFO, Murcia, Spain.

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