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Juice Components of a New Pigmented Citrus Hybrid *Citrus sinensis* (L.) Osbeck × *Citrus clementina* Hort. ex Tan.

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Fruit juice of a new pigmented citrus hybrid named Omo-31 and those of its parents clementine cv. Oroval (*Citrus clementina* Hort. ex Tan.) and Moro orange [*Citrus sinensis* (L.) Osbeck] were analyzed during fruit maturation to determine juice yield, total soluble solids (TSS), total acidity (TA), TSS/TA ratio (classical parameters of quality), and potential health beneficial components, such as vitamin C, flavanones, anthocyanins, and phenolic acids. Results showed that juice yield, TA, TSS, and TSS/TA ratio values of Omo-31 were similar to those of the Moro orange. Vitamin C content of the new hybrid was slightly higher than that of clementine and lower than that of the Moro orange, but at maturity stage no differences were observed among the three genotypes. The phenolic compounds content of the new hybrid and those of the parents and their evolution during maturation were studied. At maturity stage the amount of anthocyanins, flavanones, and hydroxycinnamic acids in Omo-31 was found to be notably higher than those of the parents. The high level of antioxidant substances makes this new fruit important for its nutritional benefits.

KEYWORDS: Citrus hybrid; blood orange; clementine; anthocyanins; flavanones; hydroxycinnamic acids

INTRODUCTION

In Italy 70% of sweet orange production is represented by three pigmented (blood) cultivars of *Citrus sinensis* (L.) Osbeck, Tarocco, Moro, and Sanguinello. These varieties differ from the blond orange group by the presence in the flesh, and sometimes in the rind, of red pigments belonging to anthocyanin classes (*1*−*3*). Another peculiar characteristic of pigmented oranges is the high concentration of vitamin C (*4*, *5*), flavanones, and hydroxycinnamic acids (*6*, *7*).

The interest in pigmented orange among consumers is due to different factors, including better taste and higher biological properties with respect to the blonde orange. In particular, Saija et al. (*8*) demonstrated that the inclusion of Moro orange juice in rat diets can induce a protective effect on blood vessel walls and gastric mucosa. A higher in vitro antioxidant activity was found in pigmented orange juices with respect to blonde orange juices, and their antioxidant efficiency appears to be correlated to the anthocyanin levels (*9*). Finally, an in vitro antioxidant activity and in vivo photoprotective effect on skin after exposure to UV-B radiations of a standardized pigmented orange extract were demonstrated (*10*).

Since 1973 the Istituto Sperimentale per l'Agrumicoltura has been working on a genetic improvement program of extant pigmented orange cultivars and on the production of new pigmented citrus hybrids with easy peeling, increased size, and new original organoleptic characteristics (*11*). New pigmented hybrids were obtained by crossing some clones of clementine with different cultivars of pigmented oranges (*12*). In the course of our phytochemical studies of new pigmented citrus hybrid essential oils, we have noted that some traits were different with respect to their parents, and in some cases an increase in the expression of some characteristics of progenies was observed. In particular, two new hybrids obtained by a cross between Monreal clementine and Tarocco orange, named A-146 and C-1867, showed the highest content of oxygenated monoterpenes and aliphatic aldehydes in the former, whereas the latter had a large predominance of linalool (*13*).

Recently, the chemical composition of the essential oil of another new pigmented hybrid, named Omo-31, from the parents Oroval clementine and Moro orange, was studied. It showed a lower content of monoterpene hydrocarbons and a higher amount of oxygenated monoterpenes than found in the parents. However, the most significant difference between the essential oil of this hybrid and those of the parents was the higher amounts of aliphatic aldehydes, such as octanal and decanal, which are 2-fold higher with respect to the orange parent (*14*).

The fruits of this new hybrid have organoleptic characteristics that recall the aroma of the Moro orange and the sweetness of the Oroval clementine. They have the shape and size of a clementine and are easily peeled, and the peel color is deep orange with red streaks at advanced maturity. The flesh is strongly pigmented due to the presence of anthocyanins.

Determination of important nonvolatile components of the Omo-31 fruit juice and those of the parents was carried out to evaluate if the expression of other desirable traits such as vitamin

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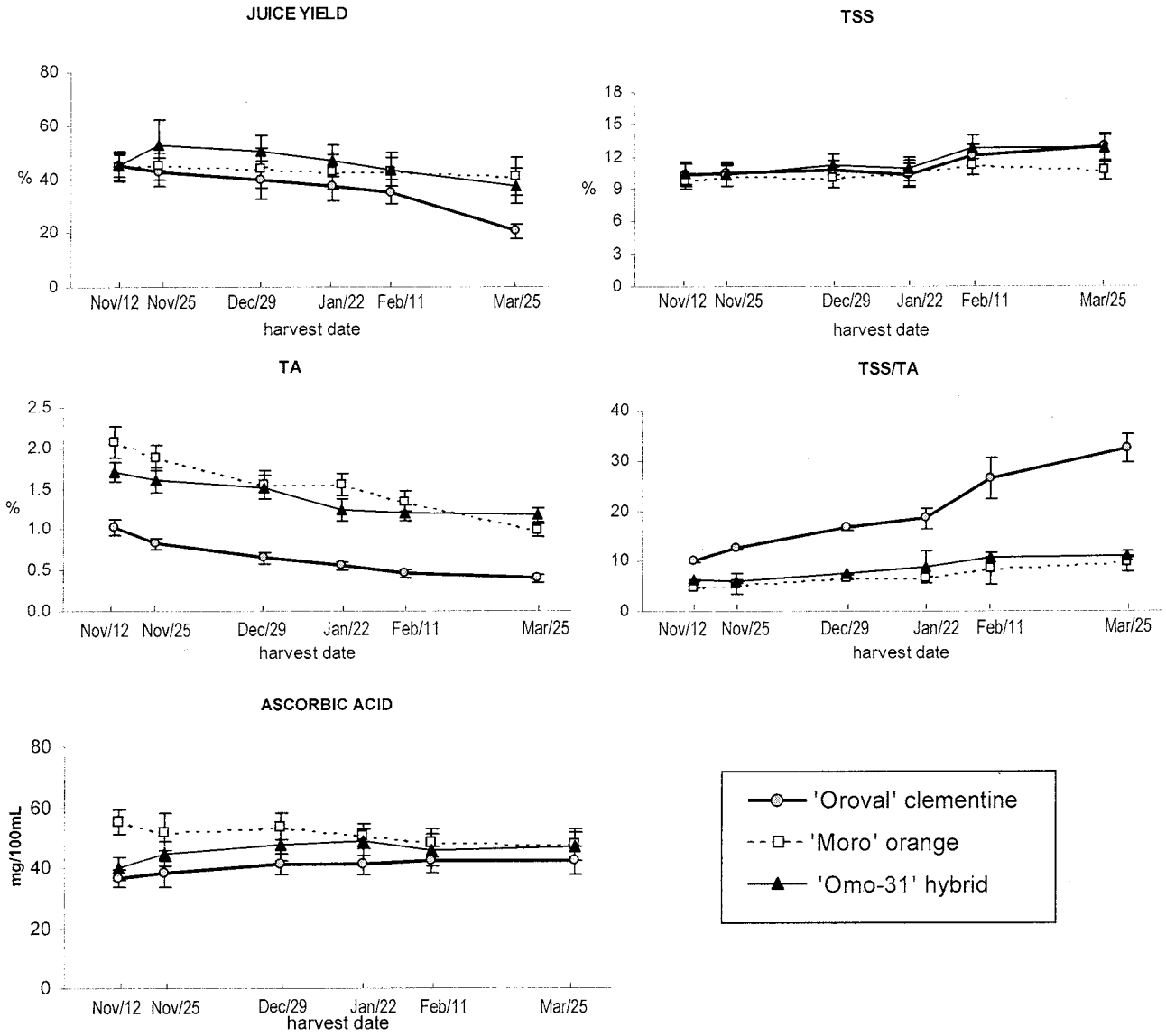

Figure 1. Evolution of juice yield, TSS, TA, TSS/TA ratio, and ascorbic acid content in juice of Oroval clementine, Moro orange, and Omo-31 hybrid, during fruit ripening.

C, anthocyanins, flavanones, and hydroxycinnamic acids contents, important for their biological activity, increased in the new hybrid with respect to the parents.

MATERIALS AND METHODS

Crosses. Omo-31 comes from a selection of 35 hybrids obtained by controlled pollination using standard citrus breeding methods (*11*), between diploid clementine cv. Oroval used as the female parent and a nucellar clone of Moro orange (NL 58-8D-1) as the male parent. Seeds obtained from the fruits of these crosses were transferred to soil, and the seedlings were grown in a greenhouse. After 2 years, some scions from the apical part of seedling were cut and grafted onto *Poncirus trifoliata* L. Raf. rootstocks. One year later, the grafted plants were transferred to the experimental farm "Palazzelli" (Siracusa, Italy) of the Istituto Sperimentale per l'Agrumicoltura.

Plant Material. Fruits of the Omo-31 hybrid, Oroval clementine, and Moro orange were harvested, at different stages of maturity, from three trees for each genotype.

Sample Preparation. A sample of 20 fruits per tree was collected at different ripening stages, from November 2000 to March 2001. The juice of the hybrid and its parents was extracted using a domestic squeezer and filtered through a 2 mm sieve. A portion was bottled in 500 mL glass bottles with screw plugs and stored at −18 °C for analysis.

Chemical Analysis. Total soluble solids (TSS), total acidity (TA), and juice yield were determined according to standard methods (*15*, *16*). Vitamin C was determined by using the 2,6-dichlorophenolindophenol titrimetric method modified by Rapisarda et al. (*4*). Total anthocyanin content was determined spectrophotometrically by the pH differential method of Rapisarda et al. (*17*, *18*). Samples for HPLC analysis were prepared as follows: an aliquot of centrifuged juice (5−10 mL) was poured into a 5 mL syringe fitted with a Sep-Pak C18 (Waters Associates, Milford, MA). After a wash with 5 mL of water, anthocyanins were eluted with 5 mL of MeOH containing 0.1% HCl. The alcoholic solution was evaporated at 35 °C under vacuum, and the residue was made up to 5−10 mL with B solvent of HPLC analysis.

Acid and alkaline hydrolysis of anthocyanins was performed with the method described by Maccarone et al. (*1*). Approximately 5 mg of purified pigments was dissolved in 2 N HCl (2 mL) and hydrolyzed in a stoppered vial at 100 °C for 30 min. After cooling, the anthocyanidins were extracted with 2 mL of *n*-amyl alcohol and then passed through a 0.45 μm filter before HPLC injection. For alkaline hydrolysis, 5 mg of the purified pigments was placed in a vial with 2 mL of 2 N KOH at 20 °C under nitrogen in the dark. After 30 min, the solution was acidified with 2 N HCl and filtered with a 0.45 μm filter before HPLC injection.

HPLC equipment was a Waters 600E pump (Waters Associates) equipped with a Waters 996 photodiode array detector (PAD). The

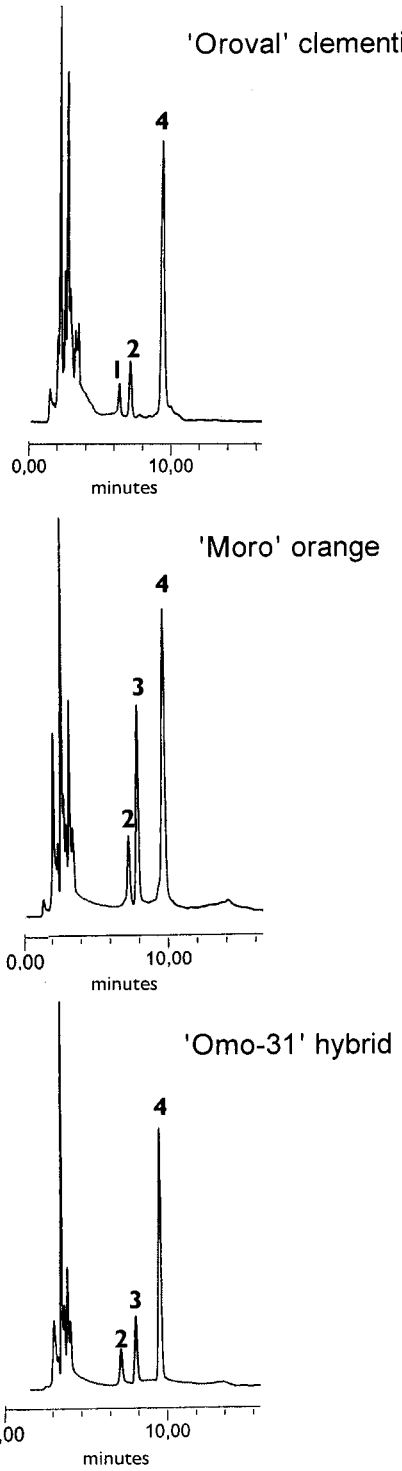

Figure 2. HPLC analysis of flavanone glycosides in Oroval clementine, Moro orange, and Omo-31 hybrid juices. Peak identification: (1) unidentified flavanone; (2) narirutin; (3) cinnamoyl-D-glucose; (4) hesperidin.

column was a 250 × 4 mm i.d., 5 μm Hypersil ODS (Phenomenex, Torrance, CA), and the solvent systems used were the following: A, H_2O/HCOOH (85:15); B, H_2O/HCOOH/CH_3CN (35:15:50). The percentage of B increased linearly from 10 to 30% in 30 min at a flow rate of 1 mL/min. The elution was monitored at 520 nm, and the column temperature was maintained at 35 °C.

Anthocyanin characterization was carried out by a Waters 1525 high-pressure pump interfaced with a Waters Micromass ZQ2000 detector. The same analytical conditions as in the above HPLC-PAD analysis were used. Flow to the mass spectrometer was split 1:4 to have a 0.25 mL/min flow rate in the interface. Mass spectrometry parameters were the following: polarity in positive ion mode; ion spray voltage, 3.0 kV; cone voltage, 30 V; source temperature, 80 °C; cone temperature, 20 °C; desolvation temperature, 150 °C; cone gas flow, 1.22 L/min; desolvation gas flow, 7 L/min. Spectra were acquired from *m*/*z* 105 to 700.

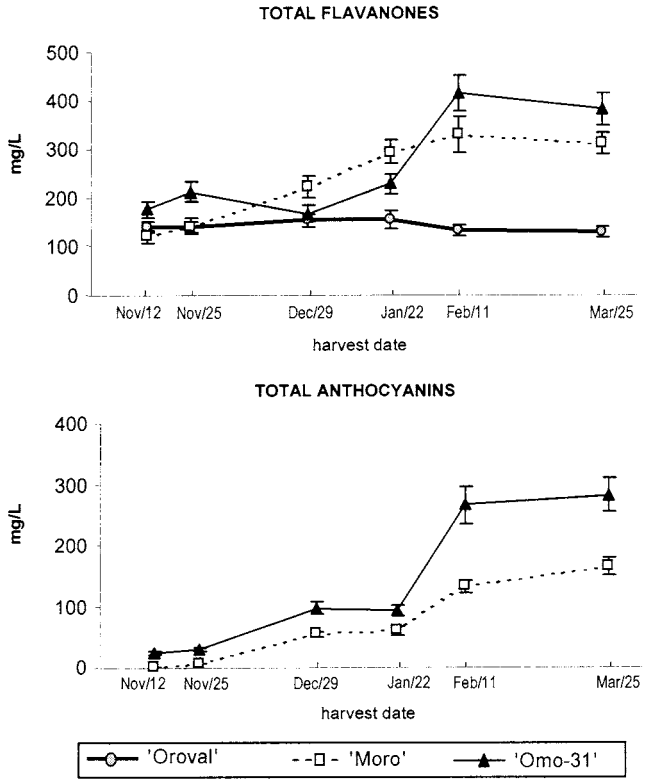

Figure 3. Change in total flavanone glycosides and in total anthocyanin content in juice of the three genotypes studied as a function of fruit ripening.

Flavanone glycosides were determined by an HPLC procedure (*19*) using the HPLC-PAD equipment described above. A sample of centrifuged juice was diluted 1:5 with the mobile phase, filtered through a 0.45 μm filter, and injected directly into the column. The solvent system used was H_2O/CH_3CN/AcOH (79.5:20:0.5), and the flow rate was 0.8 mL/min. The column effluent was monitored at 280 nm.

Cinnamoyl-D-glucose was isolated from the purified sample by semipreparative HPLC using a 250 × 10 mm i.d. Hypersil ODS 10 μm column (Phenomenex). The same apparatus and analytical conditions of flavanone analysis were used. A portion of the fraction of interest, collected from semipreparative HPLC, was subjected to alkaline hydrolysis. The hydrolysate was acidified and passed through a 0.45 μm filter and analyzed by HPLC using the same procedure of flavanone analysis. The presence of cinnamic acid was confirmed by comparison with standard. The sugar component was identified as glucose with TLC on silica gel with acetone/H_2O (9:1, v/v) as eluent and detected with aniline−diphenylamine.

Hydroxycinnamic acids were extracted from juice by solid-phase extraction (SPE) after alkaline hydrolysis of hydroxycinnamic esters (*7*). Ten milliliters of centrifuged juice was added to 10 mL of 2 N NaOH and stored at room temperature in the dark. Complete hydrolysis of bound forms of hydroxycinnamic acids occurred in 4 h. The solution was then acidified with 2 N HCl to pH 2.5 and passed through a C18 Sep-Pak cartridge (Waters). Hydroxycinnamic acids were eluted with 0.1% HCl in MeOH. The alcoholic solution was filtered through a 0.45 μm filter, and 20 μL of this solution was analyzed by HPLC equipped as above. The eluents were solvent A, H_2O/AcOH (98:2), and solvent B, H_2O/MeOH/AcOH (68:30:2), with a gradient transition from 100 to 85% of solvent A during 40 min. The flow rate was 1 mL/min, and detection was performed at 300 mn.

Statistical Analysis. Data were expressed as mean ± SD, and the comparison among genotypes at each stage of ripening was subjected to analysis of variance and means separated by Tukey's test.

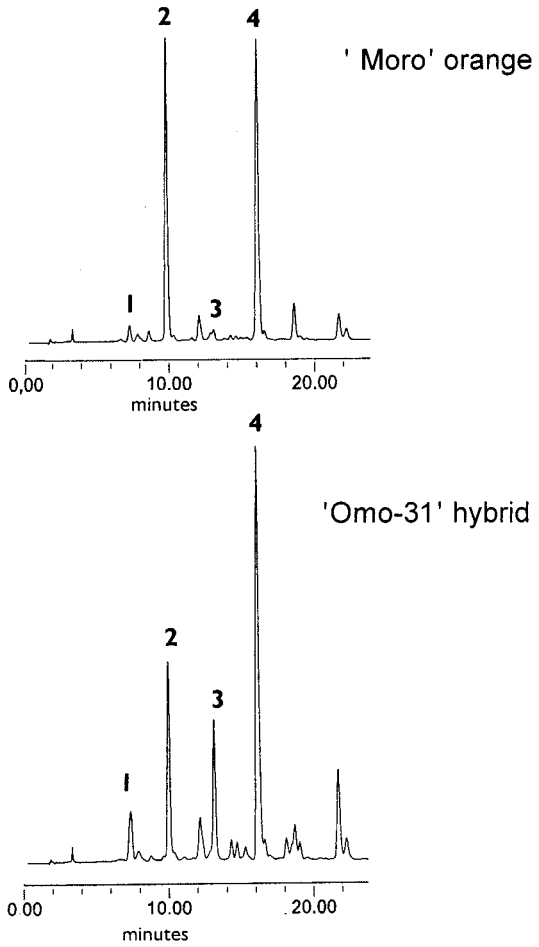

Figure 4. HPLC analysis of anthocyanins in Moro orange and Omo-31 hybrid juices. Peak identification: (1) delphinidin 3-glucoside; (2) cyanidin 3-glucoside; (3) delphinidin 3-(malonylglucoside); (4) cyanidin 3-(6″-malonylglucoside).

RESULTS AND DISCUSSION

Evolution of juice yield, TSS, TA, TSS/TA ratio, and vitamin C of Omo-31 hybrid and those of its parents, during fruit maturation, is shown in **Figure 1**. The juice yield values of the three studied genotypes decreased slightly with ripening. Similar values were observed in all stages between the Moro orange and Omo-31 fruits, whereas the juice content of clementine was slightly lower in all samples with statistically significant differences only in the last sampling. TSS determined in Omo-31 and clementine juices ranged from 10.4 in November to 12.8 in March; similar values were shown by Moro orange juice during maturation with the exception of the last stage, for which this parameter was slightly lower.

The TA levels in the juice of the new hybrid and of the Moro orange, at all sampling stages, remained higher with respect to those of the clementine. This agrees with previous research that highlighted how high acidity can be a persistent trait in sexual crosses (*20*). However, it was possible to note that in January TA values of the hybrid reached a commercially acceptable level (∼1.2%) (*3*). The TSS/TA ratio represents an important quality index for citrus juices, because it is related to the maturity and to the organoleptic characteristics of the fruit. During maturation, perfectly superimposable values of TSS/TA in the Moro orange and Omo-31 hybrid were found. It was a further demonstration that the juice of the new hybrid, with regard to sweetness and sourness balance, has characteristics similar to those of the male parent.

Vitamin C level in the juice of the new hybrid was intermediate between those of orange and clementine; only at maturity stage did it reach values similar to those of the orange at ∼47−48 mg/100 mL. Vitamin C content of mandarin or clementine is generally lower than for oranges; thus, apparently the introduction of orange parentage enhanced the vitamin C level in this hybrid (*21*).

Orange and clementine fruits mainly accumulate the tasteless flavanone rutinosides narirutin and hesperidin (*22*). Therefore, in crosses between these two species the progeny will contain only flavanone rutinosides (*23*, *24*). **Figure 2** shows the HPLC chromatogram of flavanones in the juice of the Omo-31 hybrid and those of the parents. Four main peaks were detected. Peaks 2 and 4 were identified, respectively, as narirutin and hesperidin by on-line UV spectra and cochromatography with authentic standards, whereas peak 1, present in traces only in clementine, has not been identified. Hesperidin was the most predominant flavanone glycoside in the three genotypes. Narirutin was the other major flavanone glycoside in clementine, whereas in orange and Omo-31 juices peak 3 was present in higher concentrations after hesperidin.

The UV spectra of peak 3, monitored during on-line HPLC, showed absorption maxima of 284 nm, similar to that of cinnamoyl-β-D-glucopyranoside found by Mouly et al. (*25*) in Moro orange juice. Alkaline hydrolysis of this compound yielded cinnamic acid and glucose, identified, respectively, by cochromatography with autentic standard and by TLC on silica gel. Therefore, this peak was assigned as cinnamoyl-D-glucose.

The concentration of total flavanone glycosides (**Figure 3**) changed during ripening, reaching a maximum in January for clementine and in February for orange juice. Significant differences have been observed in the Omo-31 hybrid, which showed flavanone concentrations similar to those of the clementine and orange at the former three stages of maturation, followed by a sharp increase until the fifth stage when it reached a level of 418.72 mg/L.

Moro orange is the richest in anthocyanin variety of the blood orange group (*2*). As previously reported (*1*, *26*), in Moro orange juices, only two anthocyanins were predominant, cyanidin 3-glucoside and cyanidin 3-(6″-malonylglucoside), the former with slightly higher concentrations with respect to the latter. Anthocyanin profiles of Omo-31 and Moro orange are shown in **Figure 4**. Even though the pattern of the anthocyanins present in Moro orange and Omo-31 was similar, a different distribution of the individual anthocyanins was noted. In Omo-31, cyanidin 3-(6″-malonylglucoside) (peak 4) was roughly double compared to cyanidin 3-glucoside (peak 2). With use of spectroscopic data, of the results of acid and alkali hydrolysis and HPLC−mass spectrometry, another anthocyanin (peak 3), present in notable concentrations in the hybrid, was identified for the first time in a pigmented citrus species and was assigned the structure of delphinidin 3-(malonylglucoside). In fact, the UV−vis spectrum of this peak was perfectly superimposable on that of peak 1 (delphinidin 3-glucoside). Saponification of the purified pigment obtained by semipreparative HPLC yielded delphinidin 3-glucoside. In addition, this identity was confirmed by the mass spectrum, which revealed a molecular ion with *m*/*z* values of 551 corresponding to the malonyl ester of delphinidin (*27*). Total anthocyanin concentration in the juice of the new hybrid remained higher than that found in Moro in all samplings (**Figure 3**). In particular, in February it was almost 2-fold (265.96 mg/L) that of the male parent (132.42 mg/L).

Citrus fruits contain esters and glycosides of hydroxycinnamic acids (*28*). Recent studies have shown that hydroxycinnamic

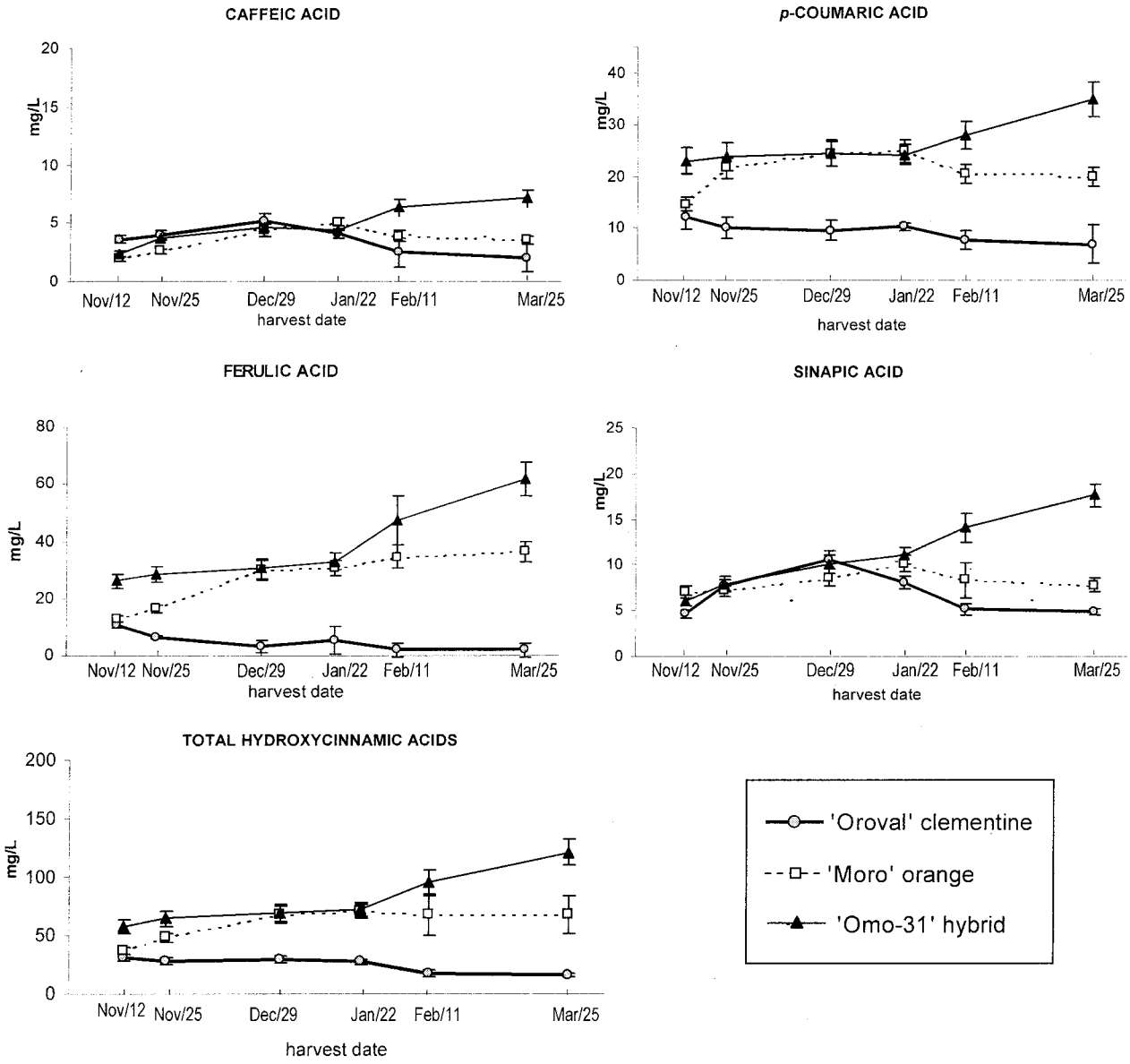

Figure 5. Change in caffeic, *p*-coumaric, ferulic, sinapic, and total hydroxycinnamic acids content in juice of the three genotypes studied as a function of fruit ripening.

acids were more abundant in Moro orange than in other blood varieties as well as in blond orange fruit juices, and the distribution of four acids (caffeic, sinapic, *p*-coumaric, and ferulic acids) was typical in each variety (*7*). **Figure 5** shows the evolution of concentration of individual and total hydroxycinnamic acids during maturation of the Omo-31 hybrid and its parents. Caffeic and sinapic acid contents determined in the three genotypes were similar in the first four stages of ripening. After that, these acids declined in clementine and orange and increased in Omo-31. Lower concentrations of *p*-coumaric and ferulic acids were observed in clementine compared to the orange and Omo-31 hybrid throughout the entire studied period. Similar values of *p*-coumaric and ferulic acids have been found in Moro orange and in the hybrid in the first four samplings, whereas after this period the concentrations of both acids increased in Omo-31 and remained stable or decreased slightly in the orange fruits. The evolution of total hydroxycinnamic acids in the three genotypes represents a mean of the values of the single acids determined at each stage of ripening. Also in this case, the concentration of these compounds found in the hybrid in March (121.18 mg/L) was roughly double that of the male parent (67.52 mg/L).

From the results obtained on the chemical composition of fruit juices of the Omo-31 hybrid and those of the parents, it is possible to make a number of conclusions on the fruit juice quality of the new hybrid. In particular, fruits of the Omo-31 hybrid have, in the entire period studied, values of juice yield, TSS, TA, TSS/TA ratio, and vitamin C content very similar to those of Moro orange. Thus, the moment of physiological maturity of fruits can be considered to be the same as that of the male parent (January−March). Significant differences in the polyphenol content were, instead, found in the latter part of the maturity stage, when the hybrid has a much higher content of these compounds than the parents. This trait is important because flavanones, phenolic acids, and anthocyanins influence the quality of citrus fruits and related processed products, as well as the antioxidant capacity. In fact, their roles in the defense mechanism of the fruits (*29*) and sensory qualities of the fruits and juices have already been widely demonstrated (*3*, *30*). In addition, these components play an important role as antioxi-

dants (*9*, *10*) and cancer-preventing agents (*31*). Therefore, the Omo-31 hybrid may be considered to be an excellent source of phytochemicals with potential health benefits for dietary supplementation.

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