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Polyphenols Distribution in Juices from *Citrus* Allotetraploid Somatic Hybrids and Their Sexual Hybrids

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The polyphenol profile of an interspecific allotetraploid somatic hybrid, achieved by protoplast fusion, the 'Valencia' sweet orange (*Citrus sinensis* L. Osbeck) + 'Femminello' lemon (*Citrus limon* L. Burm), and three sexual hybrids obtained by backcrosses between Femminello lemon and the allotetraploid somatic hybrid ('Valencia' + 'Femminello') was studied by liquid chromatography–ultraviolet–diode array detector–mass spectrometry (LC-UV-DAD-MS). The aim of the work was to evaluate whether superior traits and improved performance can be observed in these new genotypes. Ten flavonoids (TF), comprising seven flavanones and three flavones, and four hydroxycinnamic acids (HCA) have been characterized and quantified in all hybrids and compared with those of the respective parents. The 'Valencia' + 'Femminello' somatic hybrid shows an intermediate polyphenol composition with respect to those of the parents, with a slight prevalence of lemon influence. The three sexual hybrids show, instead, different and more complex chromatographic profiles.

KEYWORDS: ***Citrus sinensis* 'Valencia'; *Citrus limon* 'Femminello'; allotetraploid hybrid; sexual hybrid; polyphenol; flavonoid; hydroxycinnamic acid; LC-UV-DAD-MS**

INTRODUCTION

In the wide field of the natural polyphenols the flavonoids undoubtedly have a preeminent role. The continuous interest in these compounds is due to various reasons, ranging from their ecological and biological functions in the accumulating organisms to their exogenous biological activities to their use as chemotaxonomic and food quality markers as well as potential industrial exploitations (*1*–*3*). Among these, their pharmacological properties, or more properly their nutraceutical features (see functional food) are the most intriguing for humans. Recent American studies report that a consumer intakes 250 mg/day of flavonoids on average (*4*), whereas several epidemiological studies establish a positive relationship between the intake of food rich in these substances and the onset of numerous pathologies (*5, 6*).

It has been estimated that ca. 2% of all photosynthesized carbon on the planet by plants, amounting to 1×10^9 t/year, is converted into flavonoids and strictly correlated derivatives (*1*). This makes flavonoids one of the largest groups of natural products.

On the basis of their carbon skeleton (mainly the central ring), flavonoids can be subdivided in different subclasses: flavone, flavonol, flavanone, isoflavone, flavanonol, flavan, flavanol, chalcone, dihydrochalcone, flavan-3,4-diol, and anthocyanidin. Among these flavanones and flavones are those least represented in the plant kingdom, with an important exception, the *Citrus* genus. Flavanones, in fact, are the most important secondary metabolites of *Citrus* species and in many cases exclusive patrimony of this genus. Their content ranges between 1000 and 5000 mg/kg, being present almost exclusively as glycosides (*1*). Normally the glycosylation occurs at the 7-position by two disaccharides: rutinose or neohesperidose. The most important difference between these two kinds of glycosylation, also from a commercial point of view, is that the flavanone neohesperidosides are strongly bitter, whereas the corresponding rutinosides are tasteless. For a long time, a *Citrus* classification based on the presence of either glucoside has been accepted; today this clear separation has been refuted, because all species contain both, the difference being the predominance of either class, which justifies the presence of sweet and bitter citrus fruits.

Flavones are likewise not so widespread, whereas in *Citrus* they take on an important role (*1, 2*). The polymethoxyflavones are largely the most characteristic components, present in the external layer of *Citrus* peel, the flavedo; for this reason they are usually mixed with the essential oil from which they are easily separated. They are also present in the *Citrus* flesh as glycosides with the same modality as described above for

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flavanones. Finally, it should be emphasized that the less widespread flavone *C*-glycosides are also present in the *Citrus* species (*7*).

Hydroxycinnamic acids are the other class of polyphenol components present in the *Citrus* genus. These, as is well-known, are widespread throughout the plant kingdom, being present in *Citrus* species at lower levels than flavonoids (*1*).

The *Citrus* genus is one of the most widely cultivated crops in the world, leading to a huge consumption of several products, namely, fresh fruits, juices, soft drinks, and jams; at the same time, *Citrus* species accumulate the largest amount of polyphenols, mainly flavonoids, which, as previously described, have the most significant biological activities among natural products. Therefore, *Citrus* is one of the most important sources of nutraceutical compounds (*8–11*).

With this in mind, the study here reported deals with the evaluation of the polyphenols content in the juices of the somatic hybrid 'Valencia + Femminello', obtained by protoplast fusion of 'Valencia' sweet orange (*Citrus sinensis* L. Osbeck) and 'Femminello' lemon (*Citrus limon* L. Burm), and of some sexual hybrids coming from the backcrosses of 'Femminello' lemon and the aforesaid somatic hybrid, marked as F × (V+F) 5, F × (V+F) 7, and F × (V+F) 10.

Somatic hybridization appears to be very promising in the enhancement of *Citrus* germplasm, development of new cultivars, and rootstock improvement. The additive nature of cell fusion hybridization offers the possibility of transferring important traits the expression of which is controlled by either complex or dominant gene systems. As a result, amphidiploid somatic hybrids potentially maintain all of the genetic traits of their parents (*12*). Furthermore, the use of the aforesaid somatic hybrid in backcrosses with 'Femminello' lemon results in more complex situations due to the sexual relationship between the two parents, in which phenomena of segregation and rearrangement of characters are present.

MATERIALS AND METHODS

Plant Materials. The 'Valencia + Femminello' somatic hybrid has been obtained by symmetric protoplast fusion between heterozygous inbred lines 'Valencia' sweet orange (*Citrus sinensis* L. Osbeck) + 'Femminello' lemon (*Citrus limon* L. Burm) and subsequently characterized by means of isozymes and cytology (*13*).

This allotetraploid somatic hybrid has been used as male parent in sexual crosses with diploid 'Femminello' lemon. Pollination was carried out immediately after emasculation of the 'Femminello' lemon flowers. Pollen was applied by bringing dehisced anthers from just opened flowers into contact with stigmas of the seed parent (*14*). The small immature fruits were collected at 105 days after pollination. Seeds were extracted, and the embryo-rescue technique was applied in vitro to recover zygotic embryos (*15*). Plantlets were grown on Murashige–Tucker agar-solidified medium (*16*) supplemented with 500 mg L^{-1} malt extract and 50 g L^{-1}sucrose and grown at 25 ± 1 °C with a 16 h photoperiod at 300 μmol m^{-2} s^{-1} light intensity, successively transplanted to Jiffy pots, and transferred to a basal heating bench to stimulate vegetative growth. The hybrids were all grafted onto sour orange rootstock and planted as their parents at our Experimental Field Station in Lascari.

Fruit Collection, Juice Preparation, and Standards. Fruits of 'Valencia', 'Femminello', and their hybrid derivatives were collected in February 2005 from three trees for each genotype. The juice from fruits (four or five for each tree) was obtained by a domestic squeezer and used fresh or stored at −18 °C.

HPLC grade diosmin, vitexin, caffeic acid, eriocitrin, narirutin, naringenin 7-*O*-glucoside, naringin, hesperidin, and didymin were purchased from Extrasynthese Z.I. (Lyon Nord, France); sinapic acid and coumaric acid were obtained from Fluka Chemie (Buchs SG, Switzerland); and ascorbic acid, ethylenediaminetetraacetate disodium salt, neohesperidin, ferulic acid, and isoferulic acid were purchased from Sigma-Aldrich (St. Louis, MO).

Sample Preparation for Analyses of Hydroxycinnamic Acids. To 1 mL of filtered (0.45 μm) juice were added 1 mL of a 2 N NaOH solution (containing 10 mM EDTA and 57 mM ascorbic acid) and 10 μL of 1 mM isoferulic acid in MeOH as internal standard. The solution was maintained at 40 °C for 30 min under stirring at 300 rpm. Each sample was then adjusted to pH 7 with 2 N HCl and freeze-dried. After the residue had been suspended in methanol (1 mL) and filtered on a 0.45 μm filter, the solution was directly injected to a HPLC system.

Sample Preparation for Analyses of Flavanones and Flavones. The juice was filtered on a 0.45 μm filter and directly injected to the HPLC system. Flavonoid quantification was achieved from the recorded absorbance in the chromatogram compared with its external standard curve. Diosmetin 6,8-di-*C*-glucoside and vicenin-2, for which reference samples were not available, were quantified against vitexin by assuming a response factor equal to 1.

HPLC-UV and HPLC-MS-ESI Analyses. HPLC analyses were performed on a thermostated (40 °C) Phenomenex Luna C18 250 × 4.6 mm (5 μm) column at a 1 mL/min flow rate using water/formic acid 9:1 (v/v) as eluant A and acetonitrile/formic acid 9:1 (v/v) as eluant B with the following gradient of composition: t_{0min} B (5%), t_{5min} B (5%), t_{20min} B (15%), t_{28min} B (15%), t_{38min} B (30%), t_{50min} B (100%), t_{55min} B (100%); injection volume =20 μL.

Qualitative analyses with simultaneous ESI-MS and UV-DAD detection were carried out on a Waters 1525 pump (Waters Associates) equipped with a Waters 996 photodiode array detector and a Waters Micromass ZQ2000 mass spectrometer detector. ESI-MS detection was performed in negative mode setting with a capillary voltage of 3.5 kV, a cone voltage of 40 V, a vaporizer temperature of 250 °C, a carrier gas flow (nitrogen) of 500 L/h, and mass acquisition between 100 and 1500 Da. DAD analyses were carried out in the range between 200 and 700 nm, with the detector set at 280 nm for flavanones, 340 nm for flavones, and 320 nm for hydroxycinnamic acids. The identification of juice constituents was carried out by comparison of the spectral properties (UV and ESI-MS) of analytes with those of reference samples (see **Table 1**).

Quantitative determination of the citrus juice composition was carried out on a Dionex HPLC system equipped with a P680 pump, a UV-170U detector, and an ASI 100 autosampler. All determinations were carried out in triplicate, using the internal standard method for hydroxycinnamic acids and the external standard method for flavanones and flavones.

Statistical Analysis. SPSS software, version 14.1, was used to carry out statistical analysis of the data. ANOVA and Duncan's multiple-range test were applied to the data to determine significant differences between the analyzed components; the model was statistically significant with a value of $P \leq 0.01$. Multivariate analyses using stepwise discriminant analysis (11 steps) were carried out to estimate the contribution of parents to the hybrid polyphenol composition.

RESULTS AND DISCUSSION

Table 1 lists the polyphenols, ten flavonoids (TF) and four hydroxycinnamic acids (HCA), reported in **Figure 1**, analyzed in this study with their spectral features utilized for characterization and quantitative determination. **Table 2** reports the results on the content of the aforesaid components of the two parents: 'Valencia' sweet orange (*Citrus sinensis* L. Osbeck) and 'Femminello' lemon (*Citrus limon* L. Burm), of somatic allotetraploid hybrid V + F, and of the three sexual hybrids F × (V+F)5, F × (V+F)7, and F × (V+F)10, obtained by backcrosses between 'Femminello' lemon with V + F.

The profile of 'Valencia' sweet orange (V) is characterized by the predominance of two flavanones, hesperidin and narirutin, which together constitute 93% of this subclass, whereas vicenin-2 is the main and almost unique flavone. Among HCA, ferulic acid is the main one, sinapic and coumaric acid follow after a considerable gap, and caffeic acid is found at trace level.

Table 1. Spectral Properties of Studied Compounds

elution order	RT (min)	compound	λ_{max} (nm)	*m/z* (M – H)$^-$	*m/z* aglycon (M – H)$^-$
		Flavonoids			
1	14.95	vicenin-2	270, 338	593	
2	18.62	diosmetin 6,8-di-*C*-glucoside	270, 348	623	
3	20.07	eriocitrin	283, 328	595	287
4	24.76	narirutin	282, 330	579	271
5	25.45	naringenin 7-*O*-glucoside	283, 326	433	271
6	26.69	naringin	284, 328	579	271
7	31.15	hesperidin	283, 328	609	301
8	33.35	diosmin	348	607	299
9	33.89	neohesperidin	286, 332	609	301
10	40.80	didymin	282, 329	593	285
		Hydroxycinnamic Acids			
1*	10.12	caffeic acid	326, 232	179	
2*	16.15	coumaric acid	310, 215	163	
3*	20.87	ferulic acid	323, 234	193	
4*	22.49	sinapic acid	323, 237	223	

'Femminello' lemon (F) shows a greater amount of the TF than 'Valencia' orange, hesperidin and eriocitrin being the most important flavanones (97% of total subclass); among flavones diosmetin 6,8-di-*C*-glucoside is the main component, but also vicenin-2 and diosmin are present in appreciable amounts. Concerning HCA, also in this case ferulic is the main acid, unlike 'Valencia' orange, sinapic and coumaric are present in higher amounts, and caffeic is still at trace level. The composition of both parents does not present significant differences with the average composition of this species as reported in the literature (*8–11, 17, 18*).

The compositional profile of the allotetraploid somatic hybrid V + F is listed in **Table 2**. From a quali-quantitative viewpoint the polyphenol composition is intermediate between those of parents, with a slight prevalence of lemon influence. In fact, hesperidin and eriocitrin are the most important flavanones (see lemon), but narirutin is not negligible (see orange). Analogously, diosmetin 6,8-*C*-glucoside, as in lemon, is the main flavone. Concerning the HCA, a closer behavior to 'Valencia' orange is instead observed.

The composition of the sexual hybrids is rather variegated. The hybrid F × (V+F)5 is the sole sample of this study that shows a slight predominance of flavones with respect to flavanones. The latter are present to a lesser degree than in either parent and with the peculiarity, not noted in any other sample of this study, of the predominance of eriocitrin with respect to hesperidin. The quantitative profile of flavones is intermediate between those of the parents. The other two hybrids of this series, F × (V+F)7 and F × (V+F)10, show a higher level of flavanones than both parents, with the usual predominance of hesperidin over eriocitrin; however, unlike the previous hybrid a higher amount of naritutin, heritage of the V + F parent, is observed. The behavior of flavones in the two hybrids is entirely different; F × (V+F)7 has a lower concentration of these components than both parents, whereas F × (V+F)10 has an intermediate amount, with the exception of diosmin, the amount of which is decidedly higher than in either parent. Finally, the HCA of F × (V+F)5 and F × (V+F)10 are comparable to those of lemon, whereas those of F × (V+F)7 are much more similar to those of V + F.

The similarities and differences between the juices examined were, however, difficult to elaborate on the simple comparison of quantitative data of each sample as reported in **Table 2**; therefore, a statistical treatment of all data was carried out. Statistically significant differences were found between the average content of all components in the six samples by ANOVA and Duncan's multiple-range test (**Table 2**), with the exception of neohesperidin. In particular, the most important components in differentiating samples were didymin and eriocitrin among flavanones, diosmetin 6,8-di-*C*-glc among flavones, and ferulic acid among HCA.

With the aim of obtaining a better differentiation of all species involved in the production of these new hybrids, TF and HCA of each sample were investigated by means of a multivariate analysis. This proves to be a very effective tool in recognizing differences and similarities among the sample analyzed and has been successfully applied elsewhere in the differentiation of citrus juices as well as peel and leaf citrus oils (*19–23*).

The regression analysis was carried out to select the most significant flavonoids and HCA in differentiating samples; forward discriminant analysis (11 steps) afforded the separation shown in **Figure 2**. Functions 1 (94.1%) and 2 (3.7%) represented 97.8% of the total variance. Furthermore, **Table 3** reports the statistical weight and therefore the importance of the 11 variables obtained by regression analysis as the most important components in differentiating samples; diosmin, hesperidin, didymin, and naringin are the main components for the first function and hesperidin, narirutin, eriocitrin, and naringin for the second one.

The graphic representation in the two functions shows the expected large differentiation between 'Valencia' orange and 'Femminello' lemon. The somatic hybrid V + F is placed between both parents according to the two functions, being closer to the 'Femminello' parent in both cases. The three sexual hybrids, F × (V+F)5, F × (V+F)7, and F × (V+F)10, show a more irregular behavior, confirming the previous considerations. It should be emphasized that in this case their parents, namely, F and V + F, are closer than the previous two parents V and F, in particular for the first and more significant function.

All three sexual hybrids are closer to the V + F parent, according to the first function, F × (V+F)5 being the closest. However, as previously mentioned, given that the differences between the two parents are not so great, the contribution of 'Femminello' in the constitution of the new hybrid appears to be consistent. According to the second function of the discriminant analysis, all three sexual hybrids place fairly distant from both parents, the most consistent being the F × (V+F)10 hybrid. Furthermore, a significant closeness between F × (V+F)5 and F × (V+F)7, more marked for the latter and 'Valencia orange', should be noted.

The results of this study show, analogously to many similar ones, just how difficult it is to foresee the possible accumulation

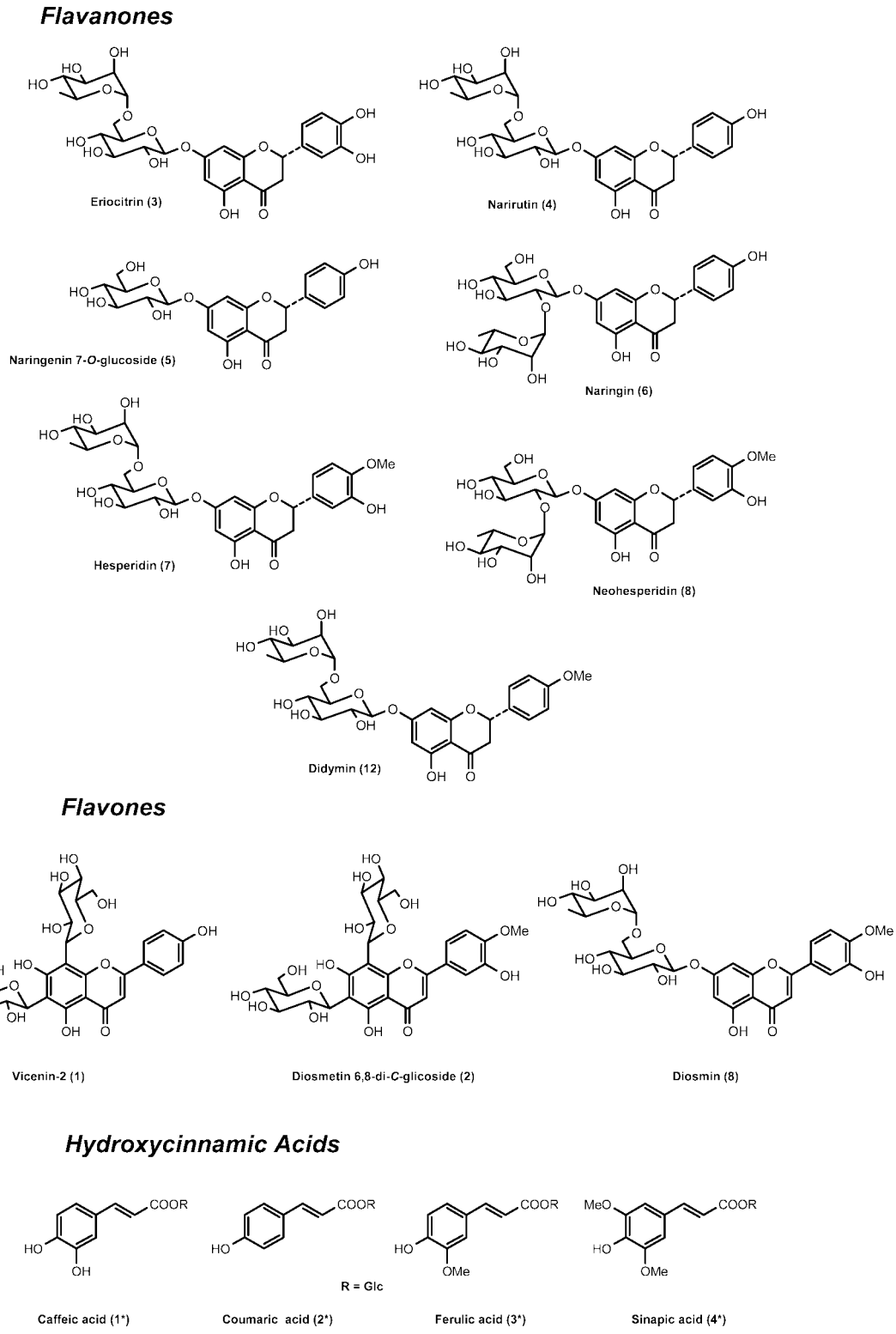

Figure 1. Polyphenols from *Citrus* juices.

of secondary metabolites throughout various breeding methodologies of *Citrus* species. This is probably due to the complexity and genetic changeability of this genus (*24, 25*).

The attempt, in this case, to produce new cultivars with a more pronounced amount of polyphenols did not achieve the desired goals. However, the results allow confirmation that the metabolic pattern of a somatic hybrid, as already observed for the essential oils (*26–29*), lies in an intermediate position between those of the parents also for nonvolatile components, such as polyphenols. Much more complex is the case of sexual hybrids, which normally show a more or less marked genetic diversity, due to phenomena of segregation and rearrangement of characters. Furthermore, in the case in hand, the genetic variability due to gamic factors is emphasized, because one of the parents is a somatic hybrid, the genoma of which is the sum of two different chromosomal patterns belonging to different species, 'Valencia' orange and 'Femminello' lemon.

Further studies on other hybrids as well as on other different breeding procedures are in progress to attain new data for the comprehension of this complex process.

Table 2. Polyphenols (Milligrams per Liter) in 'Valencia' Orange (V), 'Femminello' Lemon (F), and Their Hybrids[a]

no.	flavanones, flavones, hydroxycinnamic acids	Valencia	Femminello	Valencia + Femminello	F × (V+F)5	F × (V+F)7	F × (V+F)10
	flavanones	**108.03**	**199.84**	**182.38**	**79.81**	**235.25**	**261.79**
3	eriocitrin	0.65 (0.08)^{A}	70.74 (2.53)^{D}	54.22 (0.05)^{C}	43.45 (0.68)^{B}	82.46 (0.63)^{E}	112.12 (1.39)^{F}
4	narirutin	19.39 (1.10)^{E}	0.96 (0.26)^{A}	17.48 (0.27)^{D}	0.57 (0.33)^{A}	9.12 (0.17)^{C}	3.20 (0.27)^{B}
5	naringenin 7-*O*-glc	1.04 (0.31)^{BC}	2.69 (0.27)^{D}	1.52 (0.35)^{C}	0.38 (0.13)^{A}	0.99 (0.06)^{BC}	0.64 (0.13)^{AB}
6	naringin	1.82 (0.20)^{B}	0.08 (0.04)^{A}	1.30 (0.47)^{B}	0.02 (0.01)^{A}	0.13 (0.01)^{A}	0.12 (0.09)^{A}
7	hesperidin	81.50 (0.22)^{B}	124.58 (25.91)^{C}	107.35 (0.43)^{BC}	35.09 (0.48)^{A}	141.20 (0.31)^{C}	144.89 (7.16)^{C}
9	neohesperidin	0.64 (0.46)^{NS}	0.73 (0.20)^{NS}	0.41 (0.27)^{NS}	0.27 (0.01)^{NS}	0.76 (0.02)^{NS}	0.75 (0.02)^{NS}
10	didymin	2.99 (0.00)^{D}	0.06 (0.01)^{AB}	0.10 (0.00)^{B}	0.03 (0.00)^{A}	0.59 (0.02)^{C}	0.07 (0.05)^{AB}
	flavones	**27.31**	**146.06**	**60.77**	**87.97**	**32.04**	**118.64**
1	vicenin-2	26.50 (2.17)^{D}	13.46 (0.10)^{B}	25.21 (1.68)^{D}	10.27 (0.03)^{AB}	8.54 (1.60)^{A}	19.13 ((0.06)^{C}
2	diosmetin 6,8-di-*C*-glc	0.41 (0.17)^{A}	109.82 (0.39)^{F}	35.21 (1.25)^{C}	48.53 (0.02)^{D}	17.19 (0.39)^{B}	57.89 (0.17)^{E}
8	diosmin	0.40 (0.06)^{E}	22.78 (2.67)^{C}	0.35 (0.06)^{E}	29.17 (0.06)^{B}	6.31 (0.32)^{D}	41.62 (2.61)^{A}
	total flavonoids	**135.34**	**345.90**	**243.15**	**167.78**	**267.29**	**380.43**
	hydroxycinnamic acids	**16.51**	**11.45**	**15.12**	**10.25**	**17.35**	**7.78**
1*	caffeic acid	0.78 (0.00)^{D}	0.27 (0.01)^{A}	0.68 (0.00)^{C}	0.46 (0.04)^{B}	1.05 (0.00)^{E}	0.30 (0.01)^{A}
2*	coumaric acid	1.17 (0.00)^{A}	2.33 (0.03)^{C}	0.98 (0.00)^{A}	1.90 ((0.31)^{B}	1.97 (0.05)^{B}	0.97 (0.13)^{A}
3*	ferulic acid	13.05 (0.04)^{E}	6.59 (0.04)^{C}	11.72 (0.04)^{D}	5.31 (0.42)^{B}	12.94 (0.01)^{E}	3.84 (0.07)^{A}
4*	sinapic acid	1.51 (0.02)^{AB}	2.26 (0.08)^{C}	1.74 (0.02)^{B}	2.58 (0.22)^{D}	1.39 (0.00)^{A}	2.67 (0.04)^{D}

[a] Values represent averages of three determinations; standard deviation (± SD) is given in parentheses; different letters in the same row represent significant difference at $p \leq 0.01$ by Duncan's multiple-range test; NS = nonsignificant.

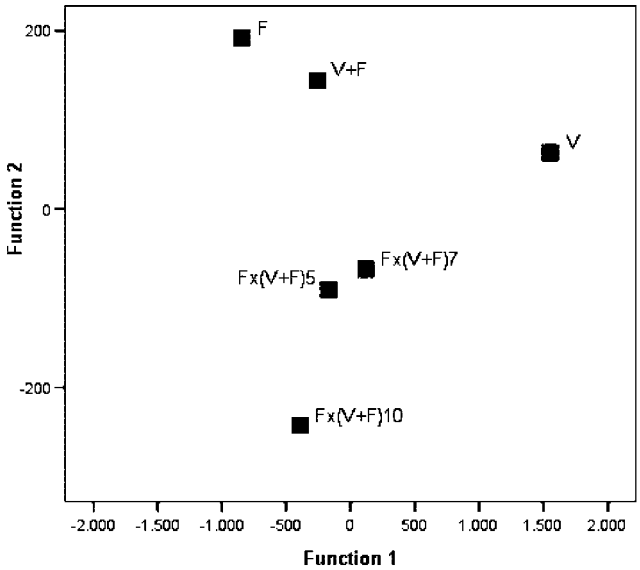

Figure 2. Forward stepwise discriminant analysis (11 steps) of the juices analyzed.

Table 3. Standardized Determinant Function Coefficient

compound	function 1	function 2
diosmetin 6,8-di-*C*-glucoside	−6.349	2.710
eriocitrin	−5.454	−4.490
narirutin	−8.099	4.832
naringenin 7-*O*-glucoside	−1.511	0.268
naringin	9.123	−4.059
hesperidin	18.719	6.823
diosmin	−20.338	0.943
neohesperidin	5.966	−3.024
didymin	9.795	−1.103
ferulic acid	−3.644	3.404
sinapic acid	7.149	−3.988

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