Selection of Hybrids and Edible *Citrus* Species with a High Content in the Diosmin Functional Compound. Modulating Effect of Plant Growth Regulators on Contents

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The purpose of this study is to identify species, hybrids, and cultivars of edible *Citrus* species with high contents of diosmin as a functional compound and also to identify the developmental progress of the fruit in which it reaches maximum levels; these findings would be useful for extraction purposes and for the modulating effect of plant growth regulators on diosmin content to increase the level of this flavone. The results obtained reveal that the highest contents of diosmin are present in immature fruits of certain varieties of citron (Buda's finger) and lemon (Meyer), whereas the contents in the edible parts of the fruits are irrelevant from a pharmacological point of view. Similarly, it is shown that it is possible to increase the content of this flavone using hormonal treatments (6-benzylaminopurine and 2,4-dichlorophenoxyacetic acid) during the early stages of fruit growth.

Keywords: *Citrus spp.; flavone; diosmin; neodiosmin; nutraceuticals*

INTRODUCTION

Nowadays, it is widely accepted that fruits and vegetables have many health-related properties. In epidemiological literature more than 200 works have been published that demonstrate the association between a lack of sufficient consumption of fruits and vegetables and cancer, circulatory problems, and cases of other illnesses such as degenerative diseases, cardiovascular problems, cataracts, and brain malfunction (*1*−*3*). At present, there is widespread interest in the putative contribution of dietary constituents or functional compounds such as flavonoids, carotenoids, and others to the beneficial properties of vegetables and fruits. Over the past few years their importance as possible chemoprotective agents against cancer and other degenerative diseases has attracted attention (*4*−*7*).

Besides flavanones, which are the major flavonoids found in citrus fruits (*8*), species of the *Citrus* genus also produce flavones. Some of these flavones, such as diosmin and neodiosmin, contain interesting properties as functional components, in the case of the former, and as taste modifiers in the food industry, in the case of the latter.

The flavone diosmin is widely used in the European Union and is routinely prescribed for the treatment of varicose problems in pregnant women. In fact, this flavone is the active ingredient of certain drugs that are used in the treatment of several illness of the circulatory system due to their properties which improve vascular tone (*9*, *10*) and inflammatory disorders (*11*). The flavone diosmin also produces a significant decrease in venous capacitance, venous distensibility, and emptying time (*12*). Similarly, it also relieves the symptoms of severe hemorrhoids (*13*, *14*). It has also been reported that diosmetin, the aglycon form of diosmin, is capable of inhibiting the enzymatic activity of the CYP1A1 gene and thus the activation of carcinogenesis caused by heterocyclic amines and other compounds, such as benzoanthracenes, which result from the daily cooking of foodstuffs (*15*). Despite the fact that diosmin does not present anticarcinogenic activity against processes activated by the heterocyclic amines and benzoanthracenes, its aglycon form (diosmetin) happens to be the predominant form in vascular circulation in human beings after the loss of sugars during digestion which make up the rutinoside (*16*).

Furthermore, neodiosmin may be used, mainly in the citrus juice industry, to reduce the bitter taste caused by limonin. To this effect, when neodiosmin is used at an adequate concentration, it allows a reduction in the threshold perception of the bitter taste caused by the limonin from an equivalent level of 15 ppm, a level which causes refusal, to a level of 4−5 ppm, at which the juice is accepted as not being bitter (*17*).

No exhaustive studies have been published regarding sources, levels in different species, hybrids, and varieties of *Citrus*, and/or distribution in the different tissues of the fruit. Only one work mentions the presence of diosmin and neodiosmin in some Rutaceae species (*18*), although the work is not exhaustive with regard to content and distribution in edible *Citrus*. Similarly, no studies have been performed in which changes in the concentration of diosmin during the growth of the fruit have been recorded, although some have been performed with neodiosmin in bitter orange (*19*), nor has the possible modulating effect of plant growth regulators on diosmin content been studied. On the other hand, both compounds can be synthesized chemically, but by

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very costly processes that also leave traces of organic solvents. Thus, the discovery of any varieties and/or species with a high content in these compounds would allow their use as a natural source. In this study, results are given regarding the distribution of these flavones in different species and hybrids of edible *Citrus*, the evolution of the content in fruits during development, and the changes that occur in diosmin content due to the effect of treatment with plant growth regulators.

MATERIALS AND METHODS

Plant Material. To quantify the contents of flavones under study, as well as their respective precursors, immature (between the first and second week after setting) and mature fruits of *Citrus* spp. were analyzed. All of the studied species, hybrids, and varieties were obtained from the *Citrus* collection of the CIDA (Centro de Investigaciones y Desarrollo Agroalimentario), La Alberca (Murcia), Spain; the *Citrus* collection of the IVIA (Instituto Valenciano de Investigaciones Agrarias), Moncada (Valencia), Spain; and commercial plantation orchards in Seville, Spain.

To study the changes produced during the development of the fruits, the contents in diosmin flavone and hesperidin flavanone were analyzed in fruits of the Buda's finger cultivar (*C. medica*), CIDA, La Alberca (Murcia), Spain, and the tangelo Nova hybrid, from an experimental plantation situated in Alhama de Murcia (Murcia), Spain, in different stages of development.

To study the effect of different plant growth regulators on the content of diosmin in fruits, adult tangelo Nova trees were used from an experimental plantation in Alhama de Murcia (Murcia), Spain.

Chromatographic Study. For isolation, chromatographic analysis, and identification of the flavonoids, whole immature fruits and occasionally separately flavedo, albedo, and pulp were collected and immediately dried in a forced air oven at 50 °C to constant weight. The dried material was ground to a fine powder and extracted with dimethyl sulfoxide (DMSO) in the ratio of 40 mg of dry weight/mL for 24 h. The corresponding extracts were filtered through a 0.45 μm nylon membrane before analysis in a Hewlett-Packard liquid chromatograph, model HP 1050, with a diode array detector (range scanned = 220−500 nm). Reversed phase chromatographic separation was performed with a C18 Spherisorb ODS-1 analytical column (250 × 4.6 mm i.d.), with an average particle size of 5 μm. As solvent we used water/methanol/acetonitrile/acetic acid (15:2:2:1) (A) and acetonitrile (B) with a gradient profile of 100% (A) for 30 min and then changed to 20% (A) in 10 min and at 50 min changed to 100% (A) in 15 min. The flow rate was 1 mL/min at 30 °C. The absorbance changes were recorded in the vis−UV diode array detector at 350 nm for the glycosylated flavones diosmin and neodiosmin and at 280 nm for the primary metabolic flavanones, hesperidin and neohesperidin, respectively. In these conditions the diosmin, neodiosmin, hesperidin, and neohesperidin standards showed retention times of 24.77, 29.03, 17.00, and 20.27 min, respectively. The flavones under study present in the plant extracts were collected with a fraction collector (Pharmacia LKB Biotechnology, Uppsala, Sweden), and the identities of these were confirmed by reference to their nuclear magnetic resonance spectrum (^{1}H MNR) (200 MHz) (Bruker, Germany) in hexadeutero-DMSO.

Chemicals. Diosmin (5,3′-dihydroxy-4′-methoxyflavone-7-*O*-hesperidoside), neodiosmin (5,3′-dihydroxy-4′-methoxyflavone-7-*O*-neohesperidoside), hesperidin (5,3′-dihydroxy-4′-methoxyflavanone-7-*O*-hesperidoside), and neohesperidin (5,3′-dihydroxy-4′-methoxyflavanone-7-*O*-neohhesperidoside) were obtained from Extrasynthese, Genay, France. Plant growth regulators 6-benzylaminopurine (6-BA) (20, 50, 100, and 250 ppm) and 2,4-dichlorophenoxyacetic acid (2,4-D) (5, 10, and 20 ppm) were obtained from Sigma, and etephon (50, 100, and 200 ppm) was from ETISA S.A. (Especialidades Técnico-Industriales, S.A.), (Barcelona, Spain). All of them were applied in a solution containing polyethylene glycol at 0.1% and by using an amount of 5 L/tree.

Plant Growth Regulator Treatments. Plant growth regulator treatments were applied by spraying on the whole tree at 90% of petal fall, coinciding with setting, as described by Del Río et al. (*20*).

RESULTS AND DISCUSSION

Presence and Distribution. The results obtained show that the flavone diosmin reveals a ubiquitous distribution pattern compared to that of neodiosmin. The presence of this latter flavone is restricted to the cultivars of bitter orange (*C. aurantium*) and the cv. Pero lemon (*C. limon*), in which neodiosmin is found in lower concentration than diosmin (Table 1). None of either flavone was found in grapefruits (*C. paradisi*) and pummelo (*C. grandis*) (data not shown). These results agree with the absence, or very low quantities, of 3′-hydroxylated and 4′-methoxylated flavones in the species (*C. paradisi* and *C. grandis*) analyzed (*21*).

In general, the contents of diosmin are higher in immature fruits than in mature ones. This finding is consistent with that previously observed for major flavonoids in *Citrus* fruits, in which the highest contents of these compounds are found in immature fruits (*22*). Contrary to what is observed for the content in diosmin and/or neodiosmin in dry weight, the ratio (expressed as a percentage of the content in flavones compared to the total amount of flavones plus flavanones) in the diosmin and neodiosmin flavones with respect to their putative precursors, the flavanones hesperidin and neohesperidin, is greater, in general, in mature fruits than in immature ones (Table 1). These results are consistent with those obtained previously for the evolution of the content in neodiosmin in the cv. Sevillano of bitter orange (*C. aurantium*) (*19*) and suggest that the greatest amount of synthesis of the diosmin and neodiosmin flavones is produced after that of the hesperidin and neohesperidin flavanones, because their relative ratios are greater in mature fruits than in immature ones.

Limes (*C. aurantifolia* and *C. limettioides*) and citron (*C. medica*) (Table 1) are characterized by presenting the flavone diosmin as the major flavone with contents greater than those found for sweet oranges (*C. sinensis*) and mandarins. The different cultivars of lemon (*C. limon*) present, in immature fruits, contents similar to those found in limes (*C. aurantifolia* and *C. limettioides*) but with a higher level in diosmin, reaching maximum values of 15% in lemons as was seen in the Laphitos cultivar (Table 1). Among all of the different cultivars and hybrids of lemon that were analyzed, the Meyer hybrid should be brought to attention due to an exceptionally high content in diosmin, in immature fruits, surpassed only by the content found in the immature fruits of cv. Buda's finger citron (Table 1).

Citron has a special importance among all the species and hybrids analyzed due to the high ratio in diosmin found in this species (Table 1). The Buda's finger cultivar has ratios of 54.95 and 62.25% in immature and mature fruits, respectively. This cultivar shows the highest levels of diosmin of all the cultivars and hybrids analyzed in this study, being approximately 3.64 g/100 g of dry weight, in immature fruits. This means that the Buda's finger cultivar is the only *Citrus* species, of those under study, in which the major flavonoid in mature fruits, and during a lot of its development, is a

Table 1. Contents of the Flavones Diosmin and Neodiosmin and Ratio of Flavone with Respect to Flavanone Precursor, Expressed as a Percentage of Flavones with Respect to the Flavone−Flavanone Content, in Immature and Mature *Citrus* Fruits[a]

		flavone content (g/100 g of dry wt)		
cultivar and species	stage	diosmin	neodiosmin	ratio flavone/flavanone (%)
C. aurantium				
Afin 1	immature	ND	0.479 ± 0.037	1.50
	mature	ND	0.045 ± 0.014	3.98
Cajel	immature	ND	0.710 ± 0.080	2.74
	mature	ND	0.099 ± 0.012	9.23
Sevillano	immature	ND	0.896 ± 0.054	3.12
	mature	ND	0.050 ± 0.008	3.41
Calabacita	immature	ND	0.750 ± 0.122	2.87
	mature	ND	0.110 ± 0.034	4.22
C. sinensis				
Valencia Late	immature	0.246 ± 0.015	ND	0.57
	mature	ND	ND	0.00
Navelate	immature	0.041 ± 0.009	ND	0.09
	mature	ND	ND	0.00
Summer Navel	immature	0.099 ± 0.011	ND	0.28
	mature	ND	ND	0.00
Sanguinelli	immature	0.278 ± 0.043	ND	0.90
	mature	ND	ND	0.00
mandarins and hybrids				
Fortune	immature	1.024 ± 0.132	ND	6.29
	mature	0.064 ± 0.015	ND	2.32
Cleopatra	immature	0.359 ± 0.056	ND	1.31
	mature	0.064 ± 0.009	ND	2.00
Encore	immature	0.730 ± 0.052	ND	1.94
	mature	0.102 ± 0.021	ND	2.35
Ortanique	immature	0.401 ± 0.092	ND	1.31
	mature	0.154 ± 0.034	ND	4.01
Nova	immature	0.255 ± 0.066	ND	0.58
	mature	0.176 ± 0.032	ND	0.76
C. limon				
Verna	immature	1.981 ± 0.057	ND	7.06
	mature	0.235 ± 0.044	ND	11.76
Fino	immature	1.223 ± 0.102	ND	6.37
	mature	0.322 ± 0.087	ND	6.58
Pero	immature	1.949 ± 0.154	0.171 ± 0.023	6.86
	mature	0.166 ± 0.047	ND	9.76
Laphitos	immature	1.901 ± 0.104	ND	9.96
	mature	0.392 ± 0.037	ND	15.58
Meyer	immature	3.056 ± 0.325	ND	7.86
	mature	NA	NA	NA
C aurantifolia				
Mexicana	immature	1.773 ± 0.206	ND	5.44
	mature	0.077 ± 0.012	ND	6.31
C. limettioides				
De Palestina	immature	1.283 ± 0.141	ND	4.18
	mature	NA	NA	NA
C medica				
Diamond	immature	1.602 ± 0.142	ND	21.53
	mature	0.118 ± 0.074	ND	45.04
Buda's fingers	immature	3.644 ± 0.452	ND	54.95
	mature	0.285 ± 0.044	ND	62.25

[a] Data are means of three different samples ± SE. Whole immature and mature fruits were analyzed. ND, not detected. NA, not available.

glycosylated flavone (diosmin) instead of a glycosylated flavanone (hesperidin), as is the usual case in the *Citrus* genus.

In light of these results and in agreement with the contents and richness in the diosmin flavone, a group may be established in which a high content in diosmin may be found, and in which lemon (*C. limon*), limes (*C. aurantifolia* and *C. limettioides*),and citron (*C. medica*) can be included, and another group will contain fruits having a low content in diosmin in which sweet oranges (*C. sinensis*), mandarins, and hybrids may be included. Lemon, limes, and citron present as the common characteristic the expression of high levels of diosmin in immature fruits. These values are between 1.2 g/100 g of dry weight for the Fino lemon cutivar (*C. limon*) and the De Palestina lime cultivar (*C. limettioides*) and 3.6 g/100 g of dry weight for the Buda's finger citron cultivar (*C. medica*). In the group with a low content in diosmin (sweet oranges and mandarins) the contents found are below 1.2 g/100 g of dry weight, the highest being that found in the Fortune mandarin cultivar (Table 1).

Regarding the distribution of the flavones diosmin and neodiosmin in the different tissues of the fruit (Table 2), both diosmin and neodiosmin present a heterogeneous distribution in the three main tissues of the fruit (flavedo, albedo, and pulp). When both flavones are present, they are found in greater concentrations and ratio in the flavedo than in the albedo and in the albedo than in the pulp, in which it is detected only in Laphitos lemon, Mexican lime, and Encore mandarin hybrid. A higher ratio is observed in Laphitos lime and

Table 2. Distribution of Diosmin and Neodiosmin in Different Tissues of the Mature Fruit and Ratio (*R*) in the Flavone Present with Respect to Its Flavanone Precursor Expressed as a Percentage of Flavones with Respect to the Flavone−Flavanone Content in Different Species and Hybrids[a]

species	flavedo flavone (*R*, %)	albedo flavone (*R*, %)	pulp flavone (*R*, %)
sweet orange (cv. Navelate)	ND	ND	ND
sour orange (cv. Calabacita)	0.154 ± 0.021 (2.59)[b]	0.074 ± 0.08 (1.07)[b]	ND[b]
mandarin hybrid (Encore)	0.109 ± 0.027 (2.84)	0.099 ± 0.031 (1.19)	0.021 ± 0.007 (0.52)
mandarin hybrid (Ortanique)	0.175 ± 0.015 (6.78)	0.097 ± 0.011 (2.05)	ND
lemon (cv. Laphitos)	0.375 ± 0.024 (17.89)	0.205 ± 0.037 (10.70)	0.043 ± 0.008 (12.32)
lime (cv. Mexicana)	0.092 ± 0.015 (10.8)	0.040 ± 0.010 (5.02)	0.006 ± 0.001 (6.28)

[a] Data express average content in different species and hybrids in g/100 g of dry weight ± SE ($n = 3$). ND, not detected. [b] Major flavone: neodiosmin.

Mexican lime, although there is a lower content in diosmin, in pulp instead of in albedo. When the flavone diosmin is present in pulp, the levels are between 5 and 6 times less than those found in albedo. Similarly, the levels found in albedo ranged from 50 to 90%, depending on the species or hybrid, of those found in flavedo. This distribution is consistent with what would be expected for low-polarity compounds, as happens with the flavones diosmin and neodiosmin, in comparison to their putative precursors, the flavanones hesperidin and neohesperidin, and would explain the higher ratio in tissues with a high content in essential oils and a greater apolar environment, as happens in the flavedo in relation to the albedo.

From the point of view of diosmin as a nutraceutical, the usually prescribed dose in medicine is between 1 and 3 g/day (*12*). In light of the previous results it is clear that the edible parts of the fruits of *Citrus* do not contain sufficient diosmin to justify a functional effect. Thus, we have calculated, using an Encore mandarin as an example, that to be able to reach the prescribed dose in medicine ∼280 fruits/day would have to be eaten. However, the content in the immediate precursor hesperidin (∼686 mg/fruit) suggests the possibility of modifying, by means of bioengineering, the expression of the metabolic pathway in its last downstream steps to increase the content in diosmin to the detriment of the putative precursor, and thus the possibility of reaching concentrations of diosmin, in fruits, compatible with the minimum levels needed to obtain a dosage equivalent to that prescribed in medicine.

Changes in the Content of Diosmin during the Development of the Fruit. To enable the identification of the stages of development in which the levels of diosmin attain their maximum concentration, the fruits were analyzed, from setting to maturity, of tangelo Nova (as a representative of the group of low diosmin content) and of the Buda's finger cultivar of citron (as an example of high diosmin content).

As can be seen in Figures 1 and 2, the highest levels of diosmin are attained in the earliest stages of development, after the setting of the fruit. This coincides with the greatest content in dry weight (*23*), and it is consistent with a previous study (*20*). A similar pattern is found for the evolution of the content of diosmin in tangelo Nova as well as in citron (Figures 1 and 2). This pattern consists of a maximum content of diosmin during the first stages of development and a decrease in the content of this flavone during growth of the fruit. These results are consistent with those observed for the behavior of the levels of several flavonoids, fundamentally flavanones, in different species of *Citrus* fruits (*24*−*26*) and in particular with those obtained for the hesperidin flavanone in fruits of tangelo Nova (*20*) and for the neodiosmin flavone in bitter orange (*19*).

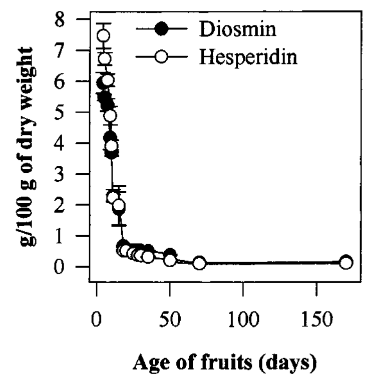

Figure 1. Evolution of the content in diosmin and hesperidin during the development of the fruits of cv. Buda's finger citron. The figures express an average content in diosmin and hesperidin in grams per 100 g of dry weight, the standard errors (±SE) being shown by vertical bars ($n = 3$), when these are greater than the symbols.

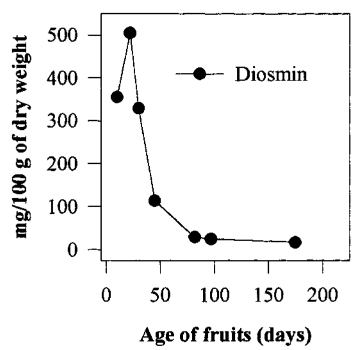

Figure 2. Evolution of the content in diosmin during the development of the fruits of tangelo Nova. The figures express an average content in diosmin and hesperidin in grams per 100 g of dry weight, the standard errors (±SE) being shown by vertical bars ($n = 3$), when these are greater than the symbols.

The results shown here suggest a dilution phenomenon, similar to that described in similar studies (*20*), which would explain the decrease in the levels of diosmin and hesperidin in citron and in tangelo Nova during the development of the fruit. Similarly, these results agree with those obtained for *C. aurantium* L. by Benavente-García et al. (*19*) and which may be explained due to cellular increase described for citrus fruits during the lineal phase of the growth curve (*27*).

The diosmin/hesperidin ratio, on the other hand, is modified quantitatively during the whole process of development of the fruit. Thus, in citron during the first stages of development, hesperidin presents a greater concentration than diosmin. When the levels of these flavonoids are at their maximum, the first one accounts for 55% of the sum of hesperidin and diosmin. This relation is equal at 50% when the fruit reaches a diameter of between 15 and 18 mm, which corresponds to 10−12 days of age (Figure 1), just before the content in dry weight becomes stabilized in the fruit (data not shown). From this moment onward the hesperidin/diosmin ratio is inverted and the flavone becomes the major component of this pair and reaches percentages, with respect to the hesperidin−diosmin group, of ∼58% in a 70-day-old fruit and ∼62% in a 170-day-old fruit. A similar pattern of decrease in the content in diosmin during the development of the fruit is observed in tangelo Nova, with the exception that in this hybrid the major flavonoid, during all stages of development, is the flavanone hesperidin.

Although degradation processes of flavonoids in plants of *Citrus* spp. during development have not been described to date, whereas they have for other genera (*28*, *29*), it has been suggested that the levels of flavonoids in a specific organ would fundamentally be determined by the processes of biosynthesis and/or transport (*30, 31*). This would explain the variations of the hesperidin−diosmin relation as a transport phenomenon in which the flavanone would be input with a lower speed than the diosmin flavone or by means of a synthesis phenomenon of the diosmin flavone from the flavanone precursor. This hypothesis would agree with that observed by Benavente-García et al. (*19*) for the flavone neodiosmin in relation to the flavanone neohesperidin in *C. aurantium* L.

Effect of Different Plant Growth Regulators on the Content of Diosmin. Previous studies performed by Del Río et al. (*20*) emphasized the effect of 6-BA, 2,4-D, and ethylene on the contents of the hesperidin flavone in tangelo Nova fruits. In these studies it is reported that the modulating effect on the contents in flavonoids is produced only when the different treatments are carried out immediately after the setting of the fruit, no effect existing after this stage. Under these conditions a similar stimulating effect is described of the auxins and cytokinins and an inhibiting effect of the ethylene.

The results obtained reveal an increase in the level of diosmin caused by treatment with auxines and cytokinins (Figures 3 and 4) and the lack of changes in the treatments carried out with ethylene (data not shown). Figure 3 shows the effect of different doses of 2,4-D (5, 10, and 20 ppm) on the content in diosmin in tangelo Nova fruits. In this graph it can be seen how all of the concentrations of 2,4-D tested produce increases in the levels of diosmin in relation to the control. Of the three concentrations of 2,4-D tested, 20 ppm produced the greatest increases in the concentration of diosmin, 14 and 30% compared to the control after 8 and 17 days of treatment, respectively.

When 6-BA was used for treatment (Figure 4), it was observed that all of the doses applied produced an increase in the synthesis and/or accumulation of diosmin. Of these, the 50 and 100 ppm doses produced the greatest increases in this glycosylated flavone after 8 days of treatment. Thus, when 50 and 100 ppm of 6-BA were applied, the increases in the concentration of diosmin with respect to the control were 33 and 32%, respectively, whereas doses of 20 and 250 ppm produced increases in the concentration of diosmin, compared to the control, of 13 and 22%, respectively. However, after 17 days, the 100 ppm dose produced the greatest increase in the levels of diosmin, 39% compared to the control, whereas the corresponding increases for doses of 20, 50, and 250 ppm were 17, 25, and 19%, respectively.

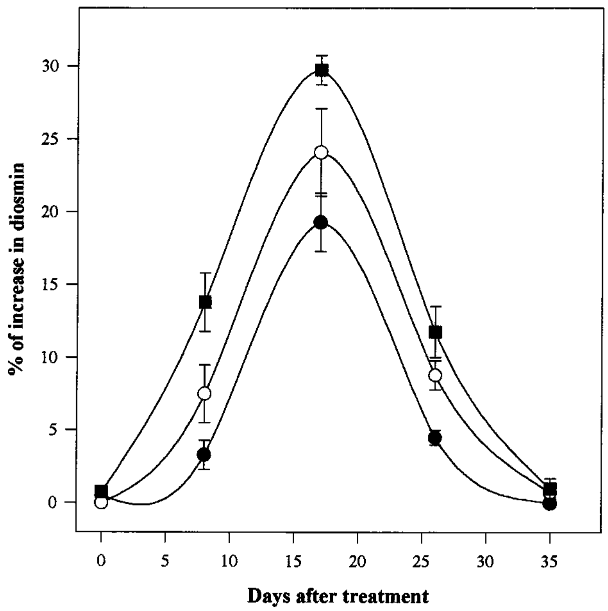

Figure 3. Effect of 2,4-D on the levels of diosmin in tangelo Nova fruits. Fruits were treated with 2,4-D at 5 (●), 10 (○), and 20 (■) ppm, respectively. The figures represent the average values of the percentage of increase in the concentration of diosmin with respect to the control, and the vertical bars indicate ±SE (n = 3), when these are greater than the symbols.

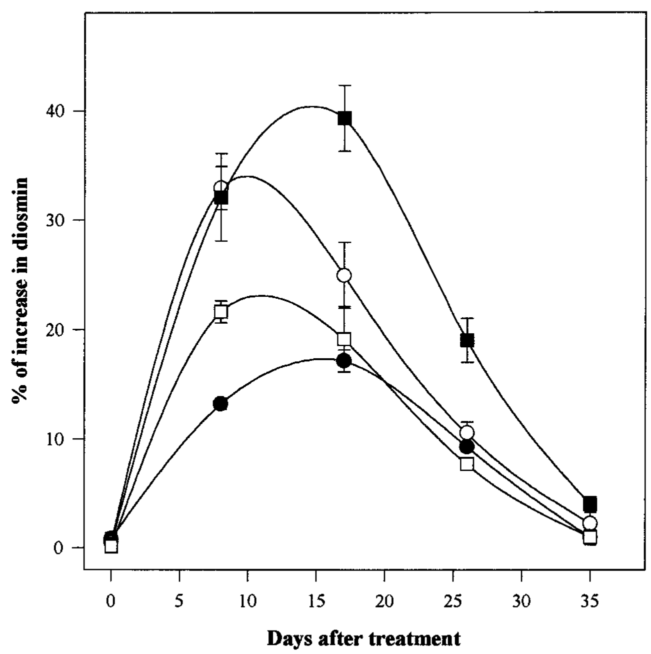

Figure 4. Effect of 6-BA on the levels of diosmin in tangelo Nova fruits. Fruits were treated with 6-BA at 20 (●), 50 (○), 100 (■), and 250 (□) ppm, respectively. The figures represent the average values of the percentage of increase in the concentration of diosmin with respect to the control, and the vertical bars indicate ±SE (n = 3), when these are greater than the symbols.

The effect produced by the different doses of 6-BA varies not only with regard to the increase produced in the contents of diosmin but also with respect to the moment at which the greatest difference is reached with respect to the control. Thus, applications of 50 and 250

Table 3. Percentage of Increase in Diosmin and Hesperidin with Respect to the Corresponding Controls, at the Different Doses of Treatment with 6-BA, 8 Days after Application

dosage of 6-BA (ppm)	% increase in diosmin	% increase in hesperidin
20	13	16
50	33	10
100	32	5
250	22	0

ppm reach their highest increase, with respect to the control, 8 days after treatment initiation, whereas with the 100 ppm application this happens 17 days from the start of treatment.

In these previous studies it was demonstrated that both the applications, with 2,4-D (5, 10, and 20 ppm) and 6-BA (20, 50, 100, and 250 ppm), under test conditions, do not present any effect on the growth of the fruit or on the cellular parameters regarding size and density (*20*). Thus, the results obtained cannot be correlated to possible changes in the processes of cellular growth and can only be related to modulating effects on the synthesis and/or transport of this flavone.

Table 3 shows the increases in the flavanone hesperidin and in the flavone diosmin as a function of the dose of 6-BA 8 days after treatment. It may be seen in the table that the increases of diosmin and hesperidin at doses of 20 ppm are similar (13 versus 16%). On the other hand, the doses of 6-BA that produce the maximum increases, compared to the control, of diosmin (50 and 100 ppm) are those in which the effect on the hesperidin is lower.

These data suggest that the increase, with respect to the control, produced by the treatment of 6-BA (20 ppm) 8 days after application might be a consequence of the increase of the concentration of the putative precursor hesperidin, which would provide more evidence supporting the fact that the glycosylated flavanone hesperidin could be a metabolic precursor of the flavone diosmin. This agrees with proposals by McIntosh et al. (*32*), McIntosh and Mansell (*33*), and Benavente-García et al. (*19*), who suggested that the glycosylated flavones, at least in *Citrus* fruits, are formed after the glycosylation and rhamnosylation of the flavanone skeleton; this is in contrast to proposals by other authors, who consider that the metabolic precursors of the flavones are the aglycon flavanones, being produced after the glycosylation of the flavone skeleton (*34*−*36*). On the other hand, doses of 50 and 100 ppm, for which the increase in diosmin compared to the control is maximum, would suggest that this increase is produced as a consequence of a stimulation of the flavone synthetase activity, becaused the increases observed in the hesperidin do not explain the increases in diosmin (Table 3).

The variations observed in the content of diosmin as a consequence of the treatments with 2,4-D and 6-BA in tangelo Nova (Figures 3 and 4) affect only the metabolism of the flavonoids when they are carried out immediately after the setting (*20*). On the other hand, increases in the content of diosmin are not permanent; after ∼35 days from the moment of treatment, the contents of diosmin in the treated specimens and in the controls tend to equal themselves out (Figures 3 and 4). Thus, it may be said that although it is possible to increase the content of diosmin, in fruits of tangelo Nova, this increase is maintained only for a short period of time, which would allow the use of some hormonal treatments in order to increase the performance in diosmin in extractive processes in which immature *Citrus* fruits are used as raw material but not to increase the functional value (in diosmin) in mature fruits.

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LITERATURE CITED

(1) Key, T. J. A.; Thorogood, M.; Appleby, P. N.; Burr, M. L. Dietary habits and mortality in 11,000 vegetarians and health conscious people: results of a 17 year follow up. *Br. Med. J*. **1996**, *313*, 775−779.
(2) Ames, B. N.; Gold, L. S. The prevention of cancer. *Drug Metab*. **1998**, *30*, 201−223.
(3) Lipkin, M.; Reddy, B.; Newmark, H.; Lamprecht, S. A. Dietary factors in human colorectal cancer. *Annu. Rev. Nutr*. **1999**, *19*, 545−586.
(4) Hertog, M. G. L.; Feeskens, E. J. M.; Hollman, C. H.; Katan, M. B.; Kromhout, D. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen elderly study. *Lancet* **1993**, *342*, 1007−1011.
(5) Hirano, T.; Gotoh, M.; Oka, K. Natural flavonoids and lignans are potent cytostatic agents against human leukemia HL-60 cells. *Life Sci*. **1994**, *55*, 1061−1069.
(6) Elangovan, V.; Sekar, N.; Govindasamy, S. Chemopreventive potential of dietary bioflavonoids against 20-methylcholanthrene-induced tumorigenesis. *Cancer Lett*. **1994**, *87*, 107−113.
(7) Marín, F. R.; Frutos, M. J.; Pérez-Alvarez, J. A.; Martinez-Sánchez, F.; Del Río, J. A. Flavonoids as nutraceuticals: Structural related antioxidant properties and their role on ascorbic acid preservation. In *Studies in Natural Products Chemistry*; Atta-Ur-Rahman, Ed.; Elsevier Science Publishers: Amsterdam, The Netherlands, 2001; in press.
(8) Benavente-García, O.; Castillo, J.; Marín, F. R.; Ortuño, A.; Del Río, J. A. Uses and properties of *Citrus* flavonoids. *J. Agric. Food Chem*. **1997**, *45*, 4505−4515.
(9) Tsouderos, Y. Venous tone: are the phlebotonic properties of a therapeutic benefit? A comprehensive view of our experience with Daflon 500 mg. *Z. Kardiol*. **1991**, *80*, S95−S101.
(10) Galley, P.; Thiollet, M. A double-blind placebo-controlled trial of a new veno-active flavonoid fraction in the treatment of symptomatic capillary fragility. *Int. Angiol*. **1993**, *12*, 69−72.
(11) Jean, T.; Bodinier, M. C. Mediators involved in inflammation: effects of Daflon 500 mg on their release. *Angiology* **1994**, *45*, 554−559.
(12) Gerolaukos, G.; Nicolaides, A. N. Controlled studies of Daflon 500 mg in chronic venous insufficiency. *Angiology* **1994**, *45*, 549−553.
(13) Godeberg, P. Daflon 500 mg in the treatment of hemorroidal disease: a demonstrated efficacy in comparison with placebo. *Angiology* **1994**, *45*, 574−578.
(14) Meyer, O. C. Safety and security of Daflon 500 mg in venous insufficiency and in hemorroidal disease. *Angiology* **1994**, *45*, 579−584.
(15) Ciolino, H. P.; Wang, T. T. Y.; Yeh, C. C. Diosmin and diosmetin are agonist of the aryl hydrocarbon receptor that differentially affect cytochrome P450 1A1 activity. *Cancer Res*. **1998**, *58*, 2754−2760.
(16) Cova, D.; De Angelis, L.; Giavarini, F.; Palladini, G.; Perego, R. Pharmacokinetics and metabolism of oral diosmin in healthy volunteers. *Int. J. Clin. Pharmacol. Ther. Toxicol*. **1992**, *30*, 29−33.
(17) Guadani, G. D.; Maier, V. P.; Turnbaugh, J. G. Effect of neodiosmin on threshold and biterness of limonin in water and orange juice. *J. Food Sci*. **1976**, *4*, 681−684.

(18) Kanes, K.; Tisserat, B. T.; Berhow, M.; Vandercook, C. Phenolic composition of various tissues of Rutaceae species. *Phytochemistry* **1993**, *32*, 967−974.

(19) Benavente-García, O.; Castillo, J.; Del Río, J. A. Changes in neodiosmin levels during development of *Citrus aurantium* leaves and fruits. Postulation of neodiosmin biosynthetic pathway. *J. Agric. Food Chem*. **1993**, *41*, 1916−1919.

(20) Del Río, J. A.; Fuster, M. D.; Sabater, F.; Porras, I.; García-Lidón, A.; Ortuño, A. Effect of benzylaminopurine on the flavanones hesperidin, hesperetin-7-O-glucoside, and prunin in tangelo Nova fruits. *J. Agric. Food Chem*. **1995**, *43*, 2030−2034.

(21) Albach, R. F.; Redman, G. H. Composition and inheritance of flavanones in *Citrus* fruits. *Phytochemistry* **1969**, *8*, 127−143.

(22) Castillo, J.; Benavente-García, O.; Del Rio, J. A. Naringin and neohesperidin levels during development of leaves, flowers and fruits of *Citrus aurantium*. *Plant Physiol*. **1992**, *99*, 67−73.

(23) Salunkhe, D. K.; Desai, B. B. Citrus. In *Postharvest Biotechnology of Fruits*; CRC Press: Boca Raton, FL, 1984; Vol. I, pp 59−73.

(24) Jourdan, P. S.; McIntosh, C. A.; Mansell, R. L. Naringin levels in citrus tissues. II. Cuantitative distribution of naringin in *Citrus paradisi* Macfad. *Plant Physiol*. **1985**, *77*, 903−908.

(25) Vandercoock, C. E.; Tisserat, B. Flavonoids changes in developing lemons grown in vivo and in vitro. *Phytochemistry* **1989**, *28*, 799−803.

(26) Ortuño, A.; García-Puig, D.; Fuster, M. D.; Pérez, M. L.; Sabater, F.; Porras, I.; García-Lidón, A.; Del Río, J. A. Flavanone and nootkatone levels in different varieties of grapefruit and pummelo. *J. Agric. Food Chem*. **1995**, *43*, 1−5.

(27) Bain, J. M. Morphological, anatomical, and physiological changes in the developing fruit of Valencia orange (*Citrus sinensis* (L) Osb). *Aust. J. Bot*. **1958**, *6*, 1−25.

(28) Barz, W. Degradation of polyphenols in plants and plant cell suspension cultures. *Physiol. Veg.* **1977**, *15*, 261−277.

(29) Curir, P.; Van Sumere, C. F.; Termini, A.; Barthe, P.; Marchesini, A.; Dolci, M. Flavonoid accumulation is correlated with adventitious roots formation in *Eucalyptus gunni* Hook micropropagated through axillary bud stimulation. *Plant Physiol.* **1990**, *92*, 1148−1153.

(30) Castillo, J.; Benavente-García, O.; Del Río, J. A. Hesperetin 7-O-glucoside and prunin in *Citrus* species (*C. aurantium* and *C. paradisi*). A study of their quantitative distribution on immature fruits as immediate precursors of neohesperidin and naringin in *C. aurantium*. *J. Agric. Food Chem*. **1994**, *11*, 1920−1924.

(31) Del Río, J. A.; Castillo, J.; Benavente-García, O.; Fuster, M. D.; Sabater, F.; Ortuño, A. Flavanones biosynthesis and its modulation in *Citrus*. *Recent. Res. Dev. Plant Physiol*. **1997**, *1*, 55−56.

(32) McIntosh, C. A.; Latchinian, L.; Mansell, R. L. Flavanone specific 7-O-glucosyltransferase activity in *Citrus paradisii* seedlings. Purification and characterization. *Arch. Biochem. Biophys*. **1990**, *282*, 50−57.

(33) McIntosh, C. A.; Mansell, R. L. Biosynthesis of naringin in *Citrus paradisii*: UDP-glucosyltransferase activity in grapefruit seedlings. *Phytochemistry* **1991**, *29*, 1533−1538.

(34) Kochs, G.; Grisebach, H. Induction and characterisation of a NADPH-dependent flavone synthase from cell cultures of soybean. *Z. Naturforsch*. **1987**, *42C*, 343−348.

(35) Otani, K.; Takashashi, T.; Furuya, T.; Ayabe, S. Licodione synthase, a cytochrome P450 monooxygenase catalyzing 2-hydroxylation of 5-deoxyflavone, in cultured *Glycyrrhiza echinata* L cells. *Plant Physiol*. **1994**, *105*, 1427−1432.

(36) Akashi, T.; Aoki, T.; Takashashi, T.; Kameya, N.; Nakamura, I.; Ayabe, S. Cloning of cytochrome P450 cDNAs from cultured *Glycyrrhiza echinata* L cells and their transcriptional activation by elicitor treatment. *Plant Sci*. **1997**, *126*, 39−47.

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