In Vitro Availability of Flavonoids and Other Phenolics in Orange Juice

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Hand-squeezed navel orange juice contains 839 mg/L phenolics, including flavanones, flavones, and hydroxycinnamic acid derivatives. The flavanones are the main phenolics in the soluble fraction (648.6 mg/L) and are also present in the cloud fraction (104.8 mg/L). During refrigerated storage of fresh juice (4 °C), 50% of the soluble flavanones precipitate and integrate into the cloud fraction. Commercial orange juices contain only 81−200 mg/L soluble flavanones (15−33%) and the content in the cloud is higher (206−644 mg/L) (62−85%), showing that during industrial processing and storage the soluble flavanones precipitate and are included in the cloud. An in vitro simulation of orange juice digestion shows that a serving of fresh orange juice (240 mL) provides 9.7 mg of soluble hesperidin (4′-methoxy-3′,5,7-trihydroxyflavanone-7-rutinoside) and 4.7 mg of the *C*-glycosylflavone vicenin 2 (apigenin, 6,8-di-*C*-glucoside) for freshly squeezed orange juice, whereas pasteurized commercial juices provide 3.7 mg of soluble hesperidin and a higher amount of vicenin 2 (6.3 mg). This means that although orange juice is a very rich source of flavanones, only a limited quantity is soluble, and this might affect availability for absorption (11−36% of the soluble flavanones, depending on the juice). The flavanones precipitated in the cloud are not available for absorption and are partly transformed to the corresponding chalcones during the pancreatin−bile digestion.

Keywords: *Orange juice; flavonoids; processing; flavanones; flavonols; caffeic acid derivatives; availability; solubility*

INTRODUCTION

In recent years it has become evident that significant health risks and benefits are associated with dietary food choice. In this way, fruits and vegetables are an essential part of the Mediterranean diet, and these food products are important by their contribution to the prevention of chronic and acute diseases. Among fruit products orange juice must be highlighted, as this product is a major source of flavonoid intake in the diet of developed countries (*1*). Citrus flavonoids possess health-promoting properties (*2*−*4*). It is generally accepted that the development of certain cancers can be prevented by the consumption of a number of foods, and citrus flavonoids have been suggested as one of the possible cancer-preventing agents (*5*, *6*). Both in vitro and in vivo studies described anticarcinogenic, antitumor, and antimutagenic activities of these compounds (*7*−*12*). Citrus juice flavonoids also showed activity against acute myeloid leukemia (*13*). In addition, epidemiological studies corroborate the reduction of cancer risk by the consumption of orange and other vegetable and fruit products (*14*, *15*). Moreover, citrus flavonoids, hesperidin and diosmin, have shown a wide range of therapeutical properties for medical and clinical applications such as anti-inflammatory, antihypertensive, diuretic, analgesic, and hypolipidemic activities (*16*−*21*).

Oranges are a very rich source of flavanones (mainly hesperidin and narirutin) (*22*), and they also contain hydroxycinnamic acid derivatives (feruloyl-galactaric, *p*-coumaroyl-galactaric, sinapic and caffeic derivatives) (*23*, *24*) and polymethoxyflavones (nobiletin, tangeretin, sinensetin, etc.) (*25*). It has been shown that citrus flavanones, particularly hesperidin, are quite insoluble in acidic water solutions and form white crystals that precipitate. It is known that flavonoid crystals incorporate into the juice cloud (*26*, *27*). These normally form after juice is extracted and stored, but they may form in freeze-damaged fruit prior to extraction. The extent of flavonoid crystallization in juice is influenced by several factors, including fruit cultivar, juice extraction pressure, holding time before pasteurization, and pasteurization itself (*27*). Due to flavonoid crystallization the data available on flavonoid content of orange juices should be viewed with caution, as most of the analyses did not take into account the flavonoids that are insoluble in the juice and form part of the cloud. Thus, large quantitative variability for the hesperidin content of orange juices has been reported (100-800 mg/L) (*28*). The use of dimethyl sulfoxide−methanol mixtures for solubilization of the precipitated flavanones prior to analysis has proved to be very useful (*29*, *30*). Flavonoid precipitation might affect their availability, bioavailability, absorption, and biological activity.

The aim of the present work was first to evaluate the flavonoid content of orange juices produced by different processing techniques (hand-squeezed, pasteurized, mildly pasteurized, single strength, from concentrate, etc.) taking into account the soluble flavonoids and the flavonoids that are precipitated in the cloud. Changes in phenolic composition that take place during juice storage were studied. In addition, the availability of flavonoids in the different juices was evaluated by an

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in vitro method that simulates the gastric and small intestine conditions (*31*).

MATERIALS AND METHODS

Orange Juice Samples. Two types of commercial orange juices were found on the Spanish market. One group of high-quality juices were produced by mild pasteurization (70 °C, 30 s) with aseptic packaging, have short sell-by dates (1−2 months), and should be stored under refrigeration (4 °C) (juices A−E). Juices B−E were single-strength juices, and juice A was obtained from concentrated juice. Another group includes standard-quality juices that are pasteurized (92−95 °C, 30 s) and can be kept at room temperature for up to 6 months (juices F−M). Juices F−J were obtained from concentrated juice, and juices K−M were single-strength juices. Navel oranges are a common source of orange juice in Spain. For this reason, fresh navel orange juice was prepared by hand-squeezing (domestic processing) for comparison purposes to evaluate the effect of industrial processing and storage on the orange flavonoids. The hand-squeezed juices were also used to follow the fate of the naturally occurring flavonoids during the juice storage at 4 °C for 72 h.

Sample Preparation and Analysis of Phenolic Compounds by HPLC. Orange juice cloud (insoluble fraction) was separated by centrifugation of 1 mL of juice at 12000 rpm in a Sigma 1-13 centrifuge (B. Braun Biotech International) with a rotor for Eppendorf tubes (10500*g*) for 5 min at room temperature. The supernatant was filtered through a 0.45 μm polyethersulfone filter and analyzed by HPLC. This filtration retains 7% of the soluble hesperidin. The sediment obtained after centrifugation was treated with 1 mL of dimethyl sulfoxide (DMSO) and sonicated for 2 min to extract the phenolic compounds. The solution was centrifuged at 12000 rpm as described above. The supernatant was then filtered through a 0.45 μm polyethersulfone filter and analyzed by HPLC. In this case no hesperidin was retained in the filter. Hydroxycinnamic acid derivatives, flavones, and flavanones (hesperidin and narirutin) were determined and quantified by HPLC. These analyses were performed with a Merck-Hitachi gradient liquid chromatograph with a pump model L-6200 and a diode array detector Merck-Hitachi model L-3000. Separations were achieved on a Licrochart column (Merck, Darmstadt, Germany) (RP-18, 12 × 0.4 cm; 5 μm particle size), using as mobile phase water/formic acid (95:5, v/v) (A) and methanol (B). The solvent flow rate was 1 mL min^{-1}, and a linear gradient starting with 10% B in A to reach 35% B in A in 25 min was used. Sample aliquots of 50 μL were injected, and soluble phenolic compounds were identified and quantified by comparison of peak areas with external standards. Chromatograms were recorded at 290 nm for flavanone quantitation and at 340 nm for hydroxycinnamic derivatives, chalcones, and flavones. A diode array detector was also used to record the UV spectra of the different compounds (spectra from 240 to 400 nm recorded every 0.64 s), and the spectra were used to identify the different compounds and test their purity. Hydroxycinnamic compounds were quantified as chlorogenic acid (5-caffeoylquinic acid) (Sigma, St. Louis, MO), flavones as vicenin 2 (previously isolated in our laboratory from lemon peel), flavanones as hesperidin (E. Merck AG Darmstadt, Germany), and chalcones as hesperidin-methyl chalcone (E. Merck AG Darmstadt). The results were expressed as milligrams per liter of juice. The flavanones hesperidin, narirutin, and hesperetin were identified by their UV spectra and chromatographic comparisons with authentic markers.

Availability Assay. An in vitro method was used to evaluate flavonoid and other phenolic compound availability in juices. The method was adapted from that reported by Miller et al. (*31*), to simulate human digestion and absorption of dietary iron from complex meals. Correlation analyses indicated significant agreement between the in vitro and human in vivo methods for estimating iron availability (*32*). However, there is no study on the correlation of in vitro and in vivo bioavailabilities of phenolic compounds, and the results obtained have to be considered with caution. The method consisted of a first pepsin−HCl digestion for 2 h (to simulate gastric digestion) and a pancreatin digestion with bile salts for 2.5 h at 37 °C (to simulate small intestine). For the pepsin−HCl digestion, samples of juice (100 mL) were added to 31500 units of pepsin (EC 3.423.1; Sigma, Steinheim, Germany) (1 unit of pepsin will produce a ΔA_{280} of 0.001 per min at pH 2.0 at 37 °C, measured as TCA-soluble products using hemoglobin as substrate), the pH was adjusted to 2 by addition of concentrated HCl, and the mixture was incubated in a 37 °C shaking water bath for 2 h. The pepsin digests were transferred to 250 mL beakers. Segments of dialysis tubing (cellulose membrane with molecular weight cutoff of 12000 Da; Sigma, Steinheim, Germany) containing 25 mL of water and the amount of $NaHCO_3$ equivalent to the titratable acidity measured previously (see below) were placed in the beaker. The beakers were sealed with parafine film (Parafilm, American National Can) and incubated in a 37 °C shaking water bath until the pH reached ∼5 (∼30 min). Five milliliters of pancreatin [4 g/L; 1.6 × 10^{-3} U.S. Pharmacopeia (USP) specifications (Sigma)]−bile extract mixture (25 g/L; Sigma) was added to each beaker, and incubation was continued for an additional 2 h (For the pancreatin, USP specifies amylase, protease, and lipase only. Pancreatin will convert not less than 25 times its weight of potato starch into soluble carbohydrates in 5 min in water at 40 °C, will digest not less than 25 times its weight of casein in 60 min at pH 7.5, at 40 °C, and will release not less than 2 μequiv of acid per minute per milligram of pancreatin from olive oil at pH 9.0 at 37 °C.) At the end of the incubation period the dialysis tubes were removed and rinsed with distilled water, and the dialysates were weighed and analyzed. Titratable acidity was determined on a 20 mL aliquot of the pepsin digest to which 5 mL of the pancreatin−bile extract mixture was added. Titratable acidity was defined as the number of equivalents of $NaHCO_3$ required to titrate the combined pepsin−digest pancreatin−bile extract mixture to pH 7.5 (0.5 N $NaHCO_3$ was used in the titration). The phenolics present in the soluble and cloud fractions of the pepsin−HCl digest of the juice and in the dialyzed and nondialyzed fractions of the pancreatin−bile extract treated digest were analyzed by HPLC. Samples were prepared (and extracted in the case of the cloud fraction) as described above.

Statistical Analyses. The results were submitted to a factorial analysis of variance and the mean values compared using the least significance difference test (LSD) at the 5% level. The number of replicates for each analyzed sample was three.

RESULTS AND DISCUSSION

Flavonoid and Phenolic Compounds Contents of Orange Juice. HPLC analysis of orange juice showed the presence of narirutin (5,7,4′-trihydroxyflavanone-7-rutinoside), hesperidin (4′-methoxy-3′,5,7-trihydroxyflavanone-7-rutinoside), hesperetin (4′-methoxy-3′,5,7-trihydroxyflavanone), and other two unidentified minor flavanones. Several hydroxycinnamic acid derivatives (sinapic, *p*-coumaric, ferulic, and caffeic acid derivatives) were also detected by the HPLC-DAD analyses as these compounds show characteristic UV spectra. Eleven chromatographic peaks with hydroxycinnamic acid derivative spectra were detected. Only one peak showed a UV spectrum similar to a sinapic acid derivative (maximum at 329 nm and shoulder at 305), and another peak showed a *p*-coumaric acid derivative spectrum (maximum at 312 nm and shoulder at 298 nm). The other nine peaks showed UV spectra as caffeic (or ferulic) acid derivatives (UV maximum at 325 nm with a shoulder at 298 nm). These UV values were in agreement with previously reported data for these compounds (*33*). All of these hydroxycinnamic acid derivatives were quantified as chlorogenic acid (caf-

Table 1. Phenolics Content of the Soluble and Cloud Fractions of the Available Orange Juices (mg L^{-1})

type of processing	flavanones		total flavanones	hydroxycinnamic derivatives	*C*-glycosylflavone (vicenin 2)		total phenolics
	soluble fraction	cloud fraction	soluble + cloud fractions	soluble fraction	soluble fraction	cloud fraction	soluble + cloud fractions
mild pasteurization							
A	200.5 (33.1)	405.1 (66.9)	605.6	14.4	40.6	2.5	663.1
B	116.0 (15.3)	644.0 (84.7)	760.0	19.3	80.0	8.1	867.4
C	91.7 (17.8)	421.8 (82.2)	513.5	14.6	53.3	2.9	584.3
D	115.0 (30.1)	266.8 (69.9)	381.8	16.3	33.8	3.9	435.8
E	106.8 (30.2)	247.0 (69.8)	353.7	15.7	40.6	6.5	416.5
pasteurization							
F	105.6 (28.8)	261.0 (71.2)	366.6	13.5	35.8	nd[b]	415.9
G	126.8 (38.1)	205.9 (61.9)	332.6	16.0	27.8	nd	376.4
H	100.3 (34.7)	188.9 (65.3)	289.2	20.2	31.2	nd	340.6
I	157.1 (37.0)	267.8 (63.0)	424.9	20.7	39.5	nd	485.1
J	129.8 (33.8)	254.1 (66.2)	383.9	19.8	34.5	nd	438.2
K	80.9 (14.7)	469.4 (85.3)	550.3	13.2	32.1	nd	595.6
L	127.6 (28.2)	324.3 (71.8)	451.9	18.3	40.8	nd	511.0
M	88.1 (14.7)	509.7 (85.3)	597.9	14.9	51.0	nd	663.8
hand squeezed							
N	648.6 (86.1)	104.8 (13.9)	753.4	19.3	80.0	9.8	839.0
	(21.9)[a]	(45.2)[a]	(52.9)[a]	(8.3)[a]	(7.5)[a]	(2.0)[a]	(53.5)[a]

[a] LSD values. Values are means ($n = 3$). Percentages of flavanones are in parentheses. [b] nd, not detected.

feoylquinic acid), as they are hydroxycinnamoyl esters of galactaric and glucaric acids and esters of glucose and other sugars.

In addition, a compound with a flavone spectrum was detected (UV spectrum maxima at 272 and 335 nm). This spectrum was similar to those reported for apigenin *C*-glycosides (*34*). This compound eluted in the HPLC analyses with shorter retention times than a marker of vitexin (8-*C*-glucosylapigenin) and 2″-xylosylvitexin, two compounds previously reported in Swiss chard (*35*), and coincided with an apigenin di-*C*-glucoside (vicenin 2) recently isolated from lemon peel (F. A. Tomás-Barberán, unpublished results). This compound did not hydrolyze under standard hydrolysis conditions for flavonoid *O*-glycosides (*34*) and thus confirmed its *C*-glycosidic nature. In addition, no isomerization was observed during acid treatment, showing that this was a symmetrical di-*C*-glycoside. Its UV study showed free hydroxyls at the 5-, 7-, and 4′-positions (apigenin structure). Its ^{1}H NMR analysis confirmed that this was apigenin 6,8-di-*C*-glucoside (vicenin 2) [2H, anomeric protons of both glucoses, 4.94 ppm, broad singlet; 1H, H-3, singlet 6.79 ppm; 2H, H-3′ and H-5′, doublet 6.90 ppm ($J = 8.8$ Hz); 2H, H-2′ and H-6′, doublet 8.02 ppm ($J = 8.8$ Hz) (*36*)]. This was confirmed by chromatographic comparisons with a marker of vicenin 2 obtained in previous works from different Labiatae species (*37*). The presence of apigenin di-*C*-glucoside in citrus products has already been reported (*25*) as well as diosmetin (5,7,3′-trihydroxy-4′-methoxyflavone) 6,8-di-*C*-glucoside in the peel of lemons (*38*).

Freshly prepared hand-squeezed navel orange juice contained 648.6 mg/L flavanones, 80.0 mg/L 6,8-di-*C*-glucosyl apigenin (vicenin 2), and 19.3 mg/L hydroxycinnamic acid derivatives in the soluble fraction (Table 1). The values found for hydroxycinnamic acid derivatives (13−20 mg/L) were smaller than those values reported for Italian navel orange juices (65.5 mg/L) (*39*). This difference could be explained by the method of analysis used in this study: hydroxycinnamates were quantified after alkaline hydrolysis, and compounds bound to cell walls were therefore quantified. When the cloud was analyzed, smaller amounts of flavanones (104.8 mg/L) and vicenin 2 (9.8 mg/L) and no hydroxycinnamic acid derivatives were detected (Table 1). These results show that in freshly produced orange juice, as is the case of juices produced by domestic processing (hand-squeezing), the flavanones and other phenolic compounds are mainly in a soluble form. In commercial juices, both pasteurized and mildly pasteurized, the amount of soluble flavanones decreased dramatically to reach levels of ∼100 mg/L, whereas the precipitated flavanones reached to 644 mg/L in some samples. In freshly hand-squeezed juices, the percentage of soluble flavanones amounted to 86% of the total flavanones, whereas in commercial juices the percentage of soluble flavanones was between 15 and 38% (Table 1). However, some significant differences were found among the analyzed commercial samples. The pasteurized juice samples K and M contained only 15% of flavanones in soluble form, whereas samples G and I had ∼40%. This suggests that either the technological treatments or the characteristics of the oranges used for juice production (cultivar, environmental conditions, postharvest storage, etc.) can affect the precipitation of juice flavanones. The content of soluble flavanones in pasteurized juices ranged between 81 and 157 mg/L (Table 1). Similar values were observed in mildly pasteurized juices (92−200 mg/L), suggesting that the thermal treatment applied for juice production has a limited effect in flavanone precipitation. The contents of hydroxycinnamic derivatives of hand-squeezed juice (19.3 mg/L) and the available commercial juices (13−20 mg/L) were very similar, indicating a small effect of the processing technologies on these compounds. Regarding the flavones, some differences were observed. The vicenin 2 content of mildly pasteurized juices was in the same range (40.6−80.0 mg/L) as that observed in the hand-squeezed juice (80.0 mg/L). Smaller values were found in pasteurized juices (27.8−51.0 mg/L), showing that the pasteurization process at higher temperatures leads to a decrease in orange juice *C*-glycosyl flavones. This content was higher in single-strength pasteurized juices (samples L and M) than in the pasteurized juices obtained from concentrate, indicating that the concentration process degrades or transforms the *C*-glycosyl flavones to some extent (Table 1).

These results show that the flavanones precipitate from the soluble fraction to the cloud (Table 1). This process was so intense that the percentage of flavanones

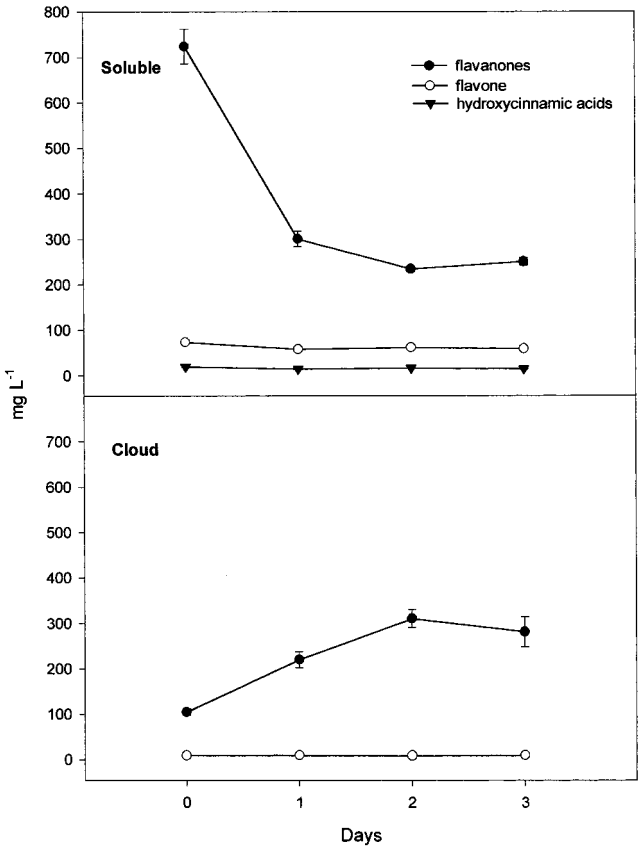

Figure 1. Evolution of the flavanone, flavone (vicenin 2), and hydroxycinnamic acid derivatives content in both soluble and cloud fractions of navel orange juice obtained by hand-squeezing and stored for 72 h at 4 °C (values are mg L^{-1}). Error bars are standard deviations.

changed thoroughly in the soluble fraction with respect to the cloud for whole juices, inverting totally the initial percentage found in hand-squeezed orange juice (Table 1).

The single-strength pasteurized juices showed a higher content of flavanones in the cloud fraction than the corresponding juices produced from concentrate, showing that the concentration process leads to a decrease in total flavanones (Table 1). The pasteurization process decreases the vicenin 2 content, as this compound was not detected in the cloud fraction of pasteurized juices. On the contrary, small amounts of this flavone were found in the cloud layer of mildly pasteurized orange juices. In the cloud fraction of both types of juices no hydroxycinnamic acid derivatives were detected (Table 1). In general, the total content of phenolic compounds (soluble plus cloud) decreased in pasteurized juices when compared with the content of hand-squeezed juice (Table 1).

Effect of Storage on Precipitation of Phenolic Compounds in Orange Juices. Hand-squeezed orange juice was prepared and stored for 72 h at 4 °C, and the changes in flavonoid composition of the soluble fraction and cloud were determined. The main changes were observed during the first 24 h of storage, as the initial concentration of soluble flavanones decreased in this case from 724 to 360 mg/L (Figure 1). During the remaining storage period, the flavanone content remained rather constant in the two fractions, and the percentage of flavanones was ~45% in the soluble fraction and ~55% in the cloud (Figure 1). However, a slow precipitation continued during the following days to invert the percentage in both soluble and cloud fractions (data not shown). This precipitation must continue during juice storage for longer periods as the analyses of commercial orange juice samples show that only 15% of the total flavanones are present as soluble compounds. On the other hand, vicenin 2 and hydroxycinnamic acid derivatives were mainly present in the soluble fraction of hand-squeezed juice and did not undergo any variation during the 3 days of storage (Figure 1). Although some vicenin 2 was found in the cloud layer, hydroxycinnamic acid derivatives were not detected. The vicenin 2 content in both soluble and cloud fractions remained unchanged (Figure 1). Therefore, the flavanones are mainly responsible for the variation in the content of total phenolic compounds in the soluble and cloud fractions.

In Vitro Flavonoid Availability in Orange Juices. Both hand-squeezed and pasteurized commercial orange juices (sample F) were submitted to an in vitro simulation of human digestion and absorption, and the flavonoids present in soluble and precipitated forms were analyzed. The hand-squeezed juice prepared for this experiment contained 370 mg/L flavanones (hesperidin plus narirutin plus hesperetin plus traces of other two flavanones) and 106 mg/L of vicenin 2 in soluble form and 36 mg/L flavanones (mainly hesperidin) and only traces of vicenin 2 in the cloud fraction. When the pepsin digest was analyzed, no significant variations in the flavonoid content of the juice were detected, and the percentage in soluble and precipitated forms remained unchanged, showing that the treatment with pepsin and the acidification to pH 2 (gastric conditions) did not increase flavanone precipitation. After the pancreatin−bile salt digestion, dialyzed and nondialyzed fractions were obtained and analyzed. In the dialyzed fraction both hesperidin and narirutin were observed. The dialyzed flavanone fraction was 16% of the soluble fraction of the juice, whereas the rest remained in the nondialyzed fraction. Wtih regard to vicenin 2, the dialyzation rate was higher as 20% of the soluble flavonoids of the juice dialyzed under these conditions (Tables 2 and 3). When the cloud flavanones were analyzed, after the pancreatin digestion, the nondialyzed fraction contained 50% hesperidin and 50% hesperidin chalcone, produced under the alkaline conditions of the pancreatin digestion (pH 7.5). Only trace amounts of narirutin were detected in the cloud, as could be expected for a flavanone that has a higher water solubility than hesperidin. No chalcone was detected in the soluble fraction, showing that this compound is even more insoluble under these conditions than the corresponding flavanone from which it originates.

When the same in vitro absorption experiment was carried out on a commercially pasteurized orange juice (sample F), similar results were obtained for the pepsin digestion, as no changes were observed in the flavonoid content of the soluble and cloud fractions. The dialyzed flavonoids of the pancreatin digestion reached 37% of hesperidin (30% of total flavanones), showing that ~10−15 mg/L of hesperidin and ~26 mg/L of vicenin 2 can be available for absorption in pasteurized juices. The analysis of the cloud fraction of the nondialyzed pancreatin digest shows that in this case 63% of the hesperidin present in the juice has been transformed to the corresponding chalcone. This chalcone, as occurred in the hand-squeezed juice, remains in the cloud, and it is not detected in the soluble fraction (Tables 4

Table 2. Soluble Flavonoids in Different Digestion Phases of Orange Juice Prepared by Hand-Squeezing (mg L^{-1})[a]

digestion phase	narirutin	hesperidin	total flavanones	*C*-glycosylflavone (vicenin 2)
initial	36.0	250.6	369.3	105.7
pepsin digest	36.3	251.9	386.0	104.7
pancreatin digest				
dialyzed fraction	3.8	40.6	44.4	19.7
nondialyzed fraction	37.2	214.9	341.0	80.2
	(1.9)	(17.8)	(22.3)	(8.8)

[a] Values are means ($n = 3$). LSD values are in parentheses.

Table 3. Cloud Flavonoids in Different Digestion Phases of Orange Juice Prepared by Hand-Squeezing (mg L^{-1})[a]

digestion phase	narirutin	hesperidin	total flavanones	hesperidin chalcone
initial	tr[b]	36.3	36.3	nd[b]
pepsin digest	tr	26.2	26.2	nd
pancreatin digest				
nondialyzed fraction	tr	34.7	34.7	37.2
		(12.2)	(12.2)	(13.9)

[a] Values are means ($n = 3$). LSD values are in parentheses. [b] tr, traces. [c] nd, not detected.

Table 4. Soluble Flavonoids in Different Digestion Phases of a Commercial Pasteurized Orange Juice (F) (mg L^{-1})[a]

digestion phase	narirutin	hesperidin	total flavanones	*C*-glycosylflavone (vicenin 2)
initial	32.8	41.0	120.1	87.9
pepsin digest	33.5	45.8	129.3	83.7
pancreatin digest				
dialyzed fraction	10.2	15.3	43.0	26.3
nondialyzed fraction	17.1	33.2	84.7	36.8
	(9.1)	(9.2)	(12.2)	(1.1)

[a] Values are means ($n = 3$). LSD values are in parentheses.

Table 5. Cloud Flavonoids in Different Digestion Phases of a Commercial Pasteurized Orange Juice (F) (mg L^{-1})[a]

digestion phase	narirutin	hesperidin	total flavanones	*C*-glycosylflavone	hesperidin chalcone
initial	13.9	295.3	323.6	6.5	nd[b]
pepsin digest	13.3	261.5	288.7	8.6	nd
pancreatin digest					
nondialyzed fraction	4.8	109.5	119.8	4.4	185.7
	(2.7)	(62.2)	(67.1)	(2.1)	(19.4)

[a] Values are means ($n = 3$). LSD values are in parentheses. [b] nd, not detected.

and 5). This behavior deserves comment as flavanones can be dissolved in water under strong alkaline conditions where they are transformed into the corresponding chalcones. In these experiments we have shown, however, that under mild alkaline conditions, such as those reached during the small intestine digestion, part of the flavanones are transformed into chalcones, but these compounds are not soluble under these conditions and remain precipitated in the cloud fraction.

These results show that in an orange juice serving [240 mL, according to the FDA (*40*)], of freshly prepared hand-squeezed juice, up to 9.7 mg of hesperidin and 4.7 mg of vicenin 2 are dialyzed during the in vitro simulation of digestion (available flavonoids). When the hand-squeezed juice is stored in the refrigerator (4 °C) for a few hours, the available hesperidin can be reduced ~50%. In the case of pasteurized juices, in which the concentration of soluble flavanones is smaller, one serving of juice provides only 3.7 mg of soluble hesperidin, which is available to dialyze in the in vitro experiment. However, the amount of vicenin 2 available for absorption is higher than in freshly hand-squeezed juices (6.3 mg/serving). The pancreatin digestion, which takes place in a mild alkaline medium (pH 7.5), transforms partly the flavanones in chalcones (50−60%). These compounds are even less soluble than the flavones at this pH, and they remain in the cloud fraction and therefore are not available for absorption under the in vitro conditions.

These absorption rates obtained in vitro are in the same range as those data reported for in vivo studies of hesperidin absorption (*41*); a low bioavailability (<25%) of hesperidin was reported when it was administered to humans as pure compounds or in orange juices.

We can conclude that although orange juice is a very rich source of flavanones (400−750 mg/L), the concentration of compounds that are in a soluble form, and thus available for absorption under the conditions of the small intestine, is probably much smaller (15−30% of the soluble flavanones depending on the juice). This does not mean that the ingested insoluble flavonoids have no role in health protection, as these compounds, if they are not absorbed in the small intestine, can reach the large intestine, where they can be transformed and/or degraded by the colon microflora (*42*). The metabolites obtained might have a beneficial effect on the large intestine cells and/or bacteria and also be absorbed and exert a biological action away from the large intestine (*28*).

ABBREVIATIONS USED

DMSO, dimethyl sulfoxide; FDA, U.S. Food and Drug Administration; HPLC, high-performance liquid chro-

matography; LSD, least significant difference test; USP, U.S. Pharmacopeia.

LITERATURE CITED

(1) Justesen, U.; Knuthsen, P.; Leth, T. Determination of plant polyphenols in Danish foodstuffs by HPLC-UV and LC-MS detection. *Cancer Lett.* **1997**, *114*, 165−167.

(2) Middleton, E.; Kandaswami, C. Potential health-promoting properties of Citrus flavonoids. *Food Technol.* **1994**, *11*, 115−119.

(3) Rouseff, F.; Nagy, S. Health and nutritional benefits of citrus fruits components. *Food Technol.* **1994**, *11*, 125−132.

(4) Benavente-García, O.; Castillo, J.; Marín, F. R.; Ortuño, A.; Del Río, J. A. Use and properties of Citrus flavonoids. *J. Agric. Food Chem.* **1997**, *45*, 4506−4515.

(5) Hertog, M. G. L.; Holmann, P. C. H.; Katan, M. B. Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruit consumed in The Netherlands. *J. Agric. Food Chem.* **1992**, *40*, 2379−2383.

(6) Stavric, B. Antimutagens and anticarcinogens in foods. *Food Chem. Toxicol.* **1994**, *32*, 79−90.

(7) Attaway, J. A. Citrus juice flavonoids with anticarcinogenic and antitumor properties. In *Food Phytochemicals for Cancer Prevention I*; Maple Press: York, PA, 1994; pp 240−248.

(8) Bracke, M. E.; Bruyneel, E. A.; Vermeulen, S. J.; Vennekens, K.; Van Marck, V.; Mareel, M. M. Citrus flavonoid effect on tumor invasion and metastasis. *Food Technol.* **1994**, *11*, 121−124.

(9) Obermeier, M. T.; White, R. E.; Yang, C. S. Effects of bioflavonoids on hepatic P450 activities. *Xenobiotica* **1995**, *25*, 575−584.

(10) Calomme, M.; Pieters, L.; Vlietinck, A.; Vanden Bergme, D. Inhibition of bacterial mutagenesis by Citrus flavonoids. *Planta Med.* **1996**, *62*, 222−226.

(11) Kuo, S. M. Antiproliferative potency of structurally distinct dietary flavonoids on human colon cancer cells. *Cancer Lett.* **1996**, *110*, 41−48.

(12) Tanaka, T.; Makita, H.; Kawabata, K.; Mori, H.; Kakumoto, M.; Satoh, K.; Hara, A.; Sumida, T.; Tanaka, T.; Ogawa, H. Chemoprevention of azoxymethane-induced rat colon carcinogenesis by the naturally occurring flavonoids, diosmin and hesperidin. *Carcinogenesis* **1997**, *18*, 957−965.

(13) Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. HL-60 Differentiating activity and flavonoid content of the readily extractable fraction prepared from Citrus juices. *J. Agric. Food Chem.* **1999**, *47*, 128−135.

(14) Wattenburg, L. Inhibition of carcinogenesis by minor nutrient constituents of the diet. *Proc. Nutr. Soc.* **1990**, *49*, 173−183.

(15) Hertog, M. G. L. Epidemiological evidence on potential health properties of flavonoids. *Proc. Nutr. Soc.* **1996**, *55*, 385−397.

(16) Galati, E. M.; Monforte, M. T.; Kirjavainen, S.; Forestieri, A. M.; Trovato, A. Biological effects of hesperidin, a Citrus flavonoid. (note I): antiinflamatory and analgesic activity. *Il Farmaco* **1994**, *49*, 709−712.

(17) Galati, E. M.; Trovato, A.; Kirjavainen, Forestieri, A. M.; Rossitto, A.; Monforte, M. T. Biological effects of hesperidin, a Citrus flavonoid. (note III): antihypertensive and diuretic activity in rat. *Il Farmaco* **1996**, *51*, 219−221.

(18) Da Silva, F. A.; Fraga, A.; Lapa, A. J. Pharmacological evaluation of the anti-inflammatory activity of a Citrus bioflavonoid, hesperidin, and the isoflavonoids, duartin and claussequinone, in rats and mice. *J. Pharm. Pharmacol.* **1994**, *46*, 118−122.

(19) Struckmann, M. D.; Nicolaides, M. S. A review of the pharmacology and therapeutic efficacy of Daflon 500 mg in patients with chronic venous insufficiency and related disorders. *J. Vasc. Dis.* **1994**, *45*, 419−428.

(20) Monforte, M. T.; Trovato, A.; Kirjavainen, S.; Forestieri, A. M.; Galati, E. M. Biological effects of hesperidin, a *Citrus* flavonoid. (note II): Hypolipidemic activity on experimental hypercholesterolemia in rat. *Il Farmaco* **1995**, *50*, 595−599.

(21) Ho, Y. H.; Foo, C. L.; Seow-Choen, F.; Goh, H. S. Prospective randomized controlled trial of a micronized flavonidic fraction to reduce bleeding after haemorrhoidectomy. *Br. J. Surgery* **1995**, *82*, 1034−1035.

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

(23) Risch, B.; Herrmann, K. Hydroxycinnamic acid derivatives in citrus fruit. *Z. Lebensm. Unters.-Forsch.* **1988**, *187*, 530−534.

(24) Clifford, M. N. Chlorogenic acids and other cinnamates—nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362−372.

(25) Park, G. L.; Avery, S. M.; Byers, J. L.; Nelson, D. B. Identification of bioflavonoids from citrus. *Food Technol.* **1983**, Dec, 98−105.

(26) Cameron, R. G.; Baker, R. A.; Grohmann, K. Citrus tissue extracts affect juice cloud stability. *J. Food Sci.* **1997**, *62*, 242−245.

(27) Baker, R. A.; Cameron, R. G. Clouds of citrus juices and juice drink. *Food Technol.* **1999**, *53*, 64−69.

(28) Tomás-Barberán, F. A.; Clifford, M. N. Flavanones, chalcones and dihydrochalcones—nature, occurrence and dietary burden. *J. Sci. Food Agric.* **2000**, *80*, 1073−1080.

(29) Manthey, J. A.; Grohmann, K. Concentrations of hesperidin and other orange peel flavonoids in citrus processing byproducts. *J. Agric. Food Chem.* **1996**, *44*, 811−814.

(30) Kawaii, S.; Tomono, Y.; Katase, E.; Ogawa, K.; Yano, M. Quantitation of flavonoid constituents in citrus fruits. *J. Agric. Food Chem.* **1999**, *47*, 3565−3571.

(31) Miller, D. D.; Schricker, B. R.; Rasmussen, R. R.; Van Campen, D. An in vitro method for estimation of iron availability from meals. *Am. J. Clin. Nutr.* **1981**, *34*, 2248−2256.

(32) Schricker, B. R.; Miller, D. D.; Rasmussen, R. R.; van Campen, D. A comparison of in vivo and in vitro methods for determining availability of iron from meals. *Am. J. Clin. Nutr.* **1981**, *34*, 2257−2263.

(33) Ibrahim, R.; Barron, D. Phenylpropanoids. In *Methods in Plant Biochemistry*; Harborne, J. B., Ed.; Academic Press: London, U.K., 1989; Vol. 1, pp 75−106.

(34) Mabry, T. J.; Markham, K. R.; Thomas, M. B. *The Systematic Identification of Flavonoids*; Springer: Berlin, Germany, 1970.

(35) Gil, M. I.; Ferreres, F.; Tomás-Barberán, F. A. Effect of modified atmosphere packaging on the flavonoids and vitamin C content of minimally processed Swiss chard (*Beta vulgaris* subspecies *cycla*). *J. Agric. Food Chem.* **1998**, *46*, 2007−2012.

(36) Markham, K. R.; Geiger, H. ^{1}H nuclear magnetic resonance spectroscopy of flavonoids and their glycosides in hexadeuterodimethylsulfoxide. In *The Flavonoids: Advances in Research since 1986*; Harborne J. B., Ed.; Chapman and Hall: London, U.K., 1994.

(37) Gil, M. I. Contribución al estudio fitoquímico y quimiosistemático de flavonoides en la familia Labiatae. Doctoral Thesis, University of Murcia, 1993.

(38) Miyake, Y.; Yamamoto, K.; Morimitsu, Y.; Osaka, I. Isolation of *C*-glucosylflavone from lemon peel and antioxidative activity of flavonoid compounds in lemon fruit. *J. Agric. Food Chem.* **1997**, *45*, 4619−4623.

(39) Rapisarda, P.; Carollo, G.; Fallico, B.; Tomaselli, F.; Maccarone, E. Hydroxycinnamic acids as markers of Italian blood orange juices. *J. Agric. Food Chem.* **1998**, *46*, 464−470.

(40) FDA (U.S. Food and Drug Administration). The food label. In The New Food Label; 1999; http://fda.gov/opacom/backgrounders/foodlabel/newlabel.html.

(41) Ameer, B.; Weintraub, R. A.; Johnson, J. V.; Yost, R. A.; Rouseff, R. L. Flavanone absortion after naringin, hesperidin, and citrus administration. *Clin. Pharm. Ther.* **1996**, *60*, 34−40.
(42) Miyake, Y.; Yamamoto, K.; Osawa, T. Metabolism of antioxidants in lemon fruit by human intestinal bacteria. *J. Agric. Food Chem*. **1997**, *45*, 3738−3742.

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