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ARTICLE

Concentration and Solubility of Flavanones in Orange Beverages Affect Their Bioavailability in Humans

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Orange juice is a very rich source of dietary flavanones. The effect of flavanone concentration and solubility of orange beverages on their bioavailability has been studied in a crossover study with 10 healthy volunteers. Five different beverages with different flavanone concentrations were evaluated. Commercial orange juices (29.2−70.3 mg of flavanones/100 mL) were compared with experimental orange beverages in which the flavanone concentration was enhanced (110.2 mg/100 mL). Hesperetin and naringenin glucuronides and sulfates were detected and quantified in plasma and urine. The study shows that the solubility of the flavanones, and particularly that of hesperidin, in the juice is a key factor for the bioavailability as flavanone excretion and the C_{max} in plasma correlate well with the soluble flavanone concentration in the juice, whereas it has no correlation with the total flavanone intake. In addition, a large interindividual variation was observed, this being consistent for each individual after the intake of the different beverages, suggesting that flavanone bioavailability is also dependent on the occurrence of specific microbiota that is able to remove the rutinosides from the juice glycosides, which results in aglycones that are then absorbed from the gut.

KEYWORDS: Citrus; flavonoids; metabolism; absorption; extracts; juice enrichment

INTRODUCTION

Fruits of the genus *Citrus* such as oranges as well as their derived products (e.g., beverages, nutraceuticals) are rich in flavanones and are a major source of these flavonoids for humans (*1*). Although it is difficult to determine total dietary consumption of these compounds, the daily intake of flavanones has been estimated to range between 25 and 65 mg on the basis of the consumption of a serving of orange juice (*1*, *2*). These figures may be increased by additional consumption of fresh peeled oranges with surrounding white tissues (albedo) (*1*).

Numerous animal experiments and in vitro studies have shown that *Citrus* flavanones exhibit a wide range of biological activities, such as antioxidant, hypocholesterolemic, hypoglycemic, prevention of bone loss, or antitumor activities, which indicate that these compounds may exert beneficial effects against cardiovascular diseases, osteoporosis, or cancer (*3*). Significantly, much of the bioactivity of *Citrus* flavanones appears to affect blood, microvascular endothelial cells, and inflammation. Hesperidin, naringin, and naringenin have been reported to exert noticeable in vivo anti-inflammatory systemic effects in various animal models (*4*−*6*). Recent research indicates additional potential bioactive roles of the flavanones. Thus, naringenin has been shown to protect against lung fibrosis (*7*), hepatotoxicity (*8*), and oxidative renal dysfunction (*9*), whereas naringin can prevent alterations in mitochondrial lipid peroxides (*10*) and reduce genotoxicity (*11*). The mechanisms underlying all of these activities have not yet been elucidated, but it has been reported that some flavanones and flavanone-enriched orange extracts do exert gene expression modulatory effects with specific anti-inflammatory and antifibrotic effects (*12*). Despite the accumulated experimental evidence, human clinical trials are still scarce and inconclusive, with volunteers consuming several servings of orange juice or levels of flavanones, resulting in contradictory changes in plasma levels of cholesterol or triglycerides (*3*).

Many of the studies looking at the bioactivity of flavanones have been conducted using either the glycosides (naringin, hesperidin, neohesperidin), which are the forms most commonly found in the *Citrus* fruits (*1*), or the hydrolyzed corresponding aglycones (naringenin, hesperetin). For these compounds to exert their beneficial effects through the diet, it is essential that they are bioavailable and can reach their target organs in a bioactive form. Two essential factors influence flavanones' bioavailability: (i) the physical form in which they are ingested (e.g., juice, soluble extract, capsule); (ii) the transformations suffered during gastrointestinal digestion and metabolism (phase I, phase II, and microbiota metabolism). The flavanones are found in the orange fruit either solubilized within vesicles or in a solid form as part of the

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albedo. During orange juice processing, part of the extracted flavanones remain soluble but some precipitate out by self-aggregation or by combination with pectins or other macromolecules and become part of the haze or cloud that naturally sediments in the juice (*13*). In vitro studies have shown that flavanones from the soluble fraction are readily available to the body, whereas precipitated flavanones are quite water insoluble and are difficult to dissolve even in alcohol or hydroalcoholic solvents (*2*). Strong organic solvents such as dimethyl sulfoxide or dimethylformamide are needed to recover the flavanones present in the juice cloud (*1*). With regard to the bioavailability, orange juice flavanone rutinosides such as hesperidin are mostly absorbed in the large intestine after deglycosylation and release of the aglycone hesperetin, which is then absorbed and further metabolized by phase II enzymes, forming several glucuronide and sulfate conjugates (*14*). Additionally, there is research demonstrating that the aglycone can be further degraded by the colon microbiota to render hydroxyl-phenyl propionic acid derivatives and benzoic acid derivatives that are then absorbed and detected in plasma and urine (*15*).

Polyphenol-enriched functional juices are currently an important market and the subject of intense research. In relation to both critical aspects, the solubility and bioavailability of orange juice flavanones, it is not yet known whether increasing concentrations of the flavanones in the juice affect the proportion of soluble/insoluble compounds in the haze and how this may have an effect on the absorption and metabolism of these compounds. The aim of the present work is to evaluate whether the concentration and solubility of flavanones in orange beverages affect their bioavailability in humans.

MATERIALS AND METHODS

Orange Beverages. Three commercial orange juices (A, B, and C) were purchased in the market. The first two were regular juices produced from concentrate, and the last one (C) was a pulp-enriched juice. In addition, two experimental orange beverages (D and E) were prepared using an orange flavonoid extract (*16*). Beverage E was the extract dissolved in water. Beverage D was a flavanone extract enriched juice B.

Qualitative and Quantitative Analysis of Orange Beverage Flavanones and Other Flavonoids. Every drink that was given to the volunteers was analyzed by HPLC-DAD, and the content of the individual phenolic compounds was achieved using external standards of hesperidin for flavanones and rutin (quercetin-3-*O*-rutinoside) for vicenin-2. Briefly, a volume (10 mL) of each beverage was centrifuged at 4500*g* for 10 min, and both the supernatant and pellet were separated. An aliquot of the supernatant (1 mL) was filtered through a 0.45 μm PVDF Millex filter (Millipore, Tokyo, Japan) and directly injected (75 μL) in the HPLC equipment. The pellet was then extracted with dimethyl sulfoxide (2 mL), and an aliquot (1 mL) was filtered through a 0.45 μm PVDF Millex filter and analyzed by HPLC (10 μL). Samples from both supernatant and pellet were analyzed using an Agilent HPLC-MS-MS-DAD system (Agilent Technologies, Waldbronn, Germany) equipped with a binary pump G1312A, an autosampler G1313 A, a photodiode array detector G1315B, controlled by Agilent software v. A.08.03, and a degasser G1322A. Separations were achieved on a LiChroCART column (Merck, Darmstadt, Germany; ODS-18, 25 × 0.4 cm; 5 μm particle size). The mobile phase was water/formic acid (98:2 v/v) (A) and methanol (B). The flow rate was 1 mL/min, and a linear gradient started with 5% B at time 5 min, 8% B at 10 min, 13% B at 15 min to reach 15% B at 19 min, 40% B at 47 min, 65% B at 64 min and 98% B at 69 min for 5 min, and returned to the initial conditions (5% B). The different flavonoids were identified by their UV spectrum, molecular mass, daughter ions, and fragmentation pattern (naringenin-glucosyl-rutinoside (**1**) $[M - H]^-$ m/z 741, 579, 433, 271; vicenin-2 (**2**) $[M - H]^-$ m/z 593, 473, 383, 353; hesperetin-glucosyl-rutinoside (**3**) 771, 609, 463, 301; narirutin (**4**) $[M - H]^-$ m/z 579, 271; hesperidin (**5**) $[M - H]^-$ m/z 609, 301; didymin (**6**) $[M - H]^-$ m/z 593, 285 (**Figures 1** and **2**). Quantification was performed using UV detection at 290 nm for flavanones, with hesperidin as an external standard.

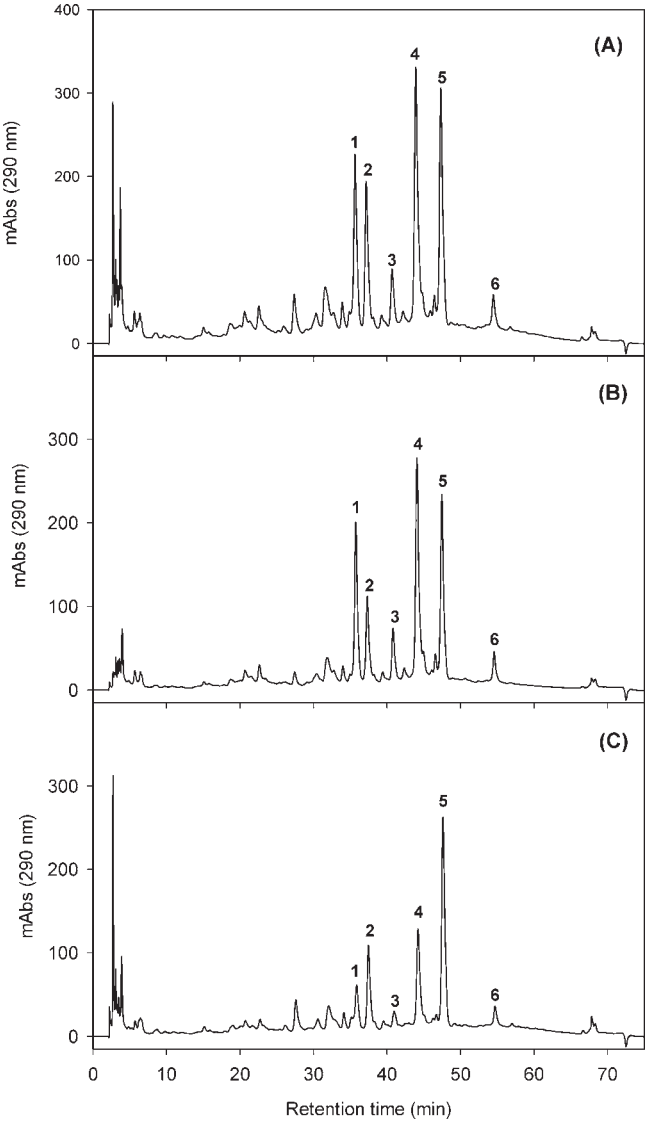

Figure 1. HPLC chromatograms (290 nm) of flavonoids in beverages (supernatants): (**A**) beverage D (commercial orange juice B enriched with E); (**B**) beverage E (extract in water); (**C**) commercial orange juice B, control juice. Peaks: (1) naringenin 7-rutinoside-4′-glucoside; (2) vicenin-2 (apigenin 6,8-di-*C*-glycoside); (3) hesperetin 7-rutinoside-4′-glucoside; (4) naringenin-7-rutinoside; (5) hesperetin 7-rutinoside; (6) naringenin-4′-methyl-ether 7-rutinoside (didymin).

Design of Human Intervention Study. The intervention was a crossover study. Volunteers were 20−30 years old. Five males (body weight of 68−102 kg with a mean value of 81 ± 14 kg and BMI of 25.2 ± 2.9 kg/cm^2) and 5 females (body weight of 48−69 kg with a mean value of 60.1 ± 9.3 kg and BMI of 21.8 ± 2.3 kg/cm^2) were selected for the assay. The volunteers selected for the study (n = 10) were healthy, nonsmokers, nonvegetarians, without a history of gastrointestinal disease, apparently without any disease such as cardiovascular disease, hematological alterations, or chronic diseases and had not been following body-weight loss diets. The health status of the volunteers was evaluated through a questionnaire. Before the intervention study was begun, the participants were informed of the background, objectives, methodology, advantages, and risks of the experiment, as well as the benefits and results that were expected to come out of the study. All participants were required to sign informed consent to participate in the study. In addition, the protocol of the study was approved by the Ethics Committee from UCAM (Universidad Católica S. Antonio of Murcia) before the start of the study.

The volunteers avoided any intake of citrus fruit derived foods prior to and during the experiment, starting 7 days before the first and including

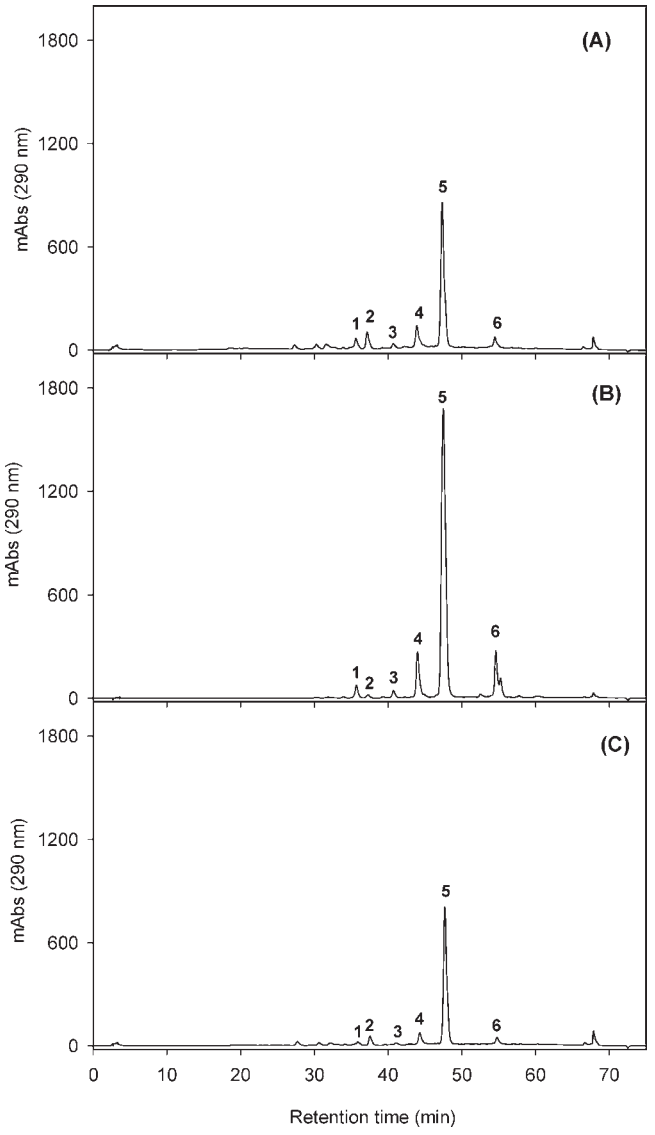

Figure 2. HPLC chromatograms (290 nm) of flavonoids in beverages (pellets): (**A**) beverage D (commercial orange juice B enriched with E); (**B**) beverage E (extract in water); (**C**) commercial orange juice B, control juice. Peaks: (1) naringenin 7-rutinoside-4′-glucoside; (2) vicenin-2 (apigenin 6,8-di-*C*-glycoside); (3) hesperetin 7-rutinoside-4′-glucoside; (4) naringenin-7-rutinoside; (5) hesperetin 7-rutinoside; (6) naringenin 4′methyl-ether 7-rutinoside (didymin).

the wash-out periods between the intakes. In the same way they had restricted the consumption of fruits and plant-derived food such as chocolate, tea, beer, wine, and fruit juices. The intake of these products was forbidden starting the day before every assay. During the whole study, the volunteers wrote down in a notebook that was provided any fruit of plant-derived food intake.

The beverage intake was accompanied by a controlled amount of food intake. Every meal component provided was weighed. In the crossover experiments the volunteers had exactly the same meal both quantitatively and qualitatively. Breakfast consisted of two slices of toast with margarine and plain low-fat dairy-based dessert (non-yogurt) and 400 mL of the corresponding experimental drink. Breakfast was done in a maximum time of 20 min. The juice was consumed at the end of the breakfast intake. Lunch consisted of dishes such as rice or spaghetti, grilled fish and/or meat, low-fat plain dairy-based dessert (non-yogurt), and water. At 6 p.m. a light dinner based on milk, yogurt, and biscuits was provided.

Volunteers gathered in a room specifically designed for this purpose for the duration of the study. A television and magazines were provided in the room to keep the participants occupied during the sampling procedure.

Sampling Procedure. Blood (5 mL) was collected in heparinized tubes from the antecubital arm vein. A basal sample was taken before breakfast and every hour during the following 11 h. One baseline and 10 postingestion blood samples per volunteer at predetermined times were obtained in the five independent assays (one for each beverage and with 2 week wash-up intervals).

The volunteers were given special containers for urine collection. They collected three urine samples, that is, the morning urine of the day of the assay before breakfast (control urine), as well as all of the urine produced during the next 24 h and from 24 to 48 h after beverage consumption.

Triplicate plasma samples were extracted as previously described (*14*) with slight modifications. Two hundred and fifty microliters of plasma was thoroughly mixed with 100 ng of rutin as an internal standard. The mixture was extracted with 1 mL of acetonitrile by vortexing for 30 s every 2 min over a 10 min period and then was centrifuged at 14000*g* for 5 min in a Sigma 1-13 microcentrifuge (Braun Biotech International, Melsungen, Germany), and the supernatant obtained was kept at 4 °C. The pellet was further extracted with 1 mL of methanol and centrifuged as described above. The two supernatants were combined and reduced to dryness in a Speedvac concentrator set at 35 °C (Thermo Fisher Scientific SPD121P, Worcester, MA) coupled to a refrigerated vapor trap at −95 °C (ThermoFisher Scientific RVT4104). The dried samples were then dissolved in 20 μL of methanol plus 80 μL of 1% aqueous formic acid, filtered through a 0.45 μm PVDF Millex filter, and placed in polypropylene inserts C4010-630P (National Scientific, Rockwood, TN). A 4 μL aliquot was injected into LC-MS/MS equipment. Recoveries of rutin internal standard were 85%.

Thawed urine samples (250 μL) were centrifuged at 14000*g* for 5 min in the microcentrifuge at 4 °C. The supernatant was then filtered through a 0.45 μm Millex filter and analyzed (5 μL) by LC-MS/MS.

HPLC-DAD-MS-MS Analysis of Flavanone Metabolites in Urine and Plasma. Flavanone metabolites were analyzed using a HPLC-DAD-MS system (1200 series, Agilent Tecnologies) equipped with a HTC Ultra mass detector in series (Brüker Daltonics, Bremen, Germany). The mass detector was an ion-trap mass spectrometer equipped with an ESI system (capillary voltage, 4 kV; dry temperature, 350 °C). Mass scan (MS) and MS/MS daughter spectra were measured from m/z 100 to 800 using the Ultra scan mode (26000 m/z/s). Collision-induced fragmentation experiments were also performed using helium as collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode. HPLC separations were carried out on a 150 × 0.5 mm i.d., 5 μm, reverse phase SB C18 Zorbax column (Agilent) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 10 μL/min. The gradient started with 5% B in A to reach 40% B at 30 min and 90% B at 36 min for 5 min and returned to the initial conditions (5% B). The different metabolites in plasma and urine were identified by their UV spectrum, molecular mass, daughter ions, and fragmentation pattern: naringenin-glucuronide-sulfate (a) $[M - H]^-$ m/z 527, 447, 271; naringenin-glucuronide (b and c) $[M - H]^-$ m/z 447, 271; hesperetin-glucuronide (d and e) $[M - H]^-$ m/z 477, 301; hesperetin-sulfate (f) $[M - H]^-$ m/z 381, 301 (**Figure 3**). Quantification of plasma metabolites was performed using UV detection at 290 nm, with hesperidin as external standard. The quantitative determination of hesperetin and naringenin metabolites in urine was carried out after enzymatic degradation of the conjugates to improve the quantification method. Thus, glucuronide and sulfate metabolites were hydrolyzed by incubating 100 μL of urine in 50 μL of 0.1 M sodium acetate buffer (pH 5.2) with β-glucuronidase (88500 U/mL) and sulfatase (404 U/mL) (aqueous solution from *Helix pomatia*, type H-2, G-0876, EC 3.2.1.31, Sigma) at 37 °C for 18 h. The reaction mixture was extracted with 300 μL of ethyl acetate, vortexed, and centrifuged at 1000*g* for 1 min. The supernatant fraction was dried under N_2 and the residue dissolved in 100 μL of methanol. To evaluate if the enzyme hydrolysis was complete, the remaining water phase was HPLC-DAD-MS-MS analyzed and 10% of the original flavanone sulfates remained unhydrolyzed. No flavanone glucuronides were detected after the enzymatic treatment.

Pharmacokinetic Analysis. Total flavanone plasma concentration−time data were analyzed by noncompartmental pharmacokinetic analysis. Pharmacokinetic parameters were estimated using the WinNonlin software package (WinNonlin Professional version 5.2.1, Pharsight Corp., Mountain View, CA). WinNonlin model 200 was used for the analysis.

The area under the plasma concentration−time curve (AUC_{last}) from time 0 to the last point (t_{last}) with measurable concentration (C_{last}) was estimated using a linear/log trapezoidal approximation. The time to reach peak concentration (t_{max}) and peak concentration (C_{max}) were obtained directly from the plasma concentration−time curves. Other pharmacokinetic parameters obtained were lag time (t_{lag}), time prior to the first measur-able (nonzero) concentration, and mean residence time (MRT_{last}), which is defined as the average time for all drug molecules to reside in the body calculated from the time of dosing to the time of the last measurable concentration.

Statistical Analysis. Differences between pharmacokinetic parameters were tested by Mann−Whitney's *U* test. A *P* value of < 0.05 was considered to be significant.

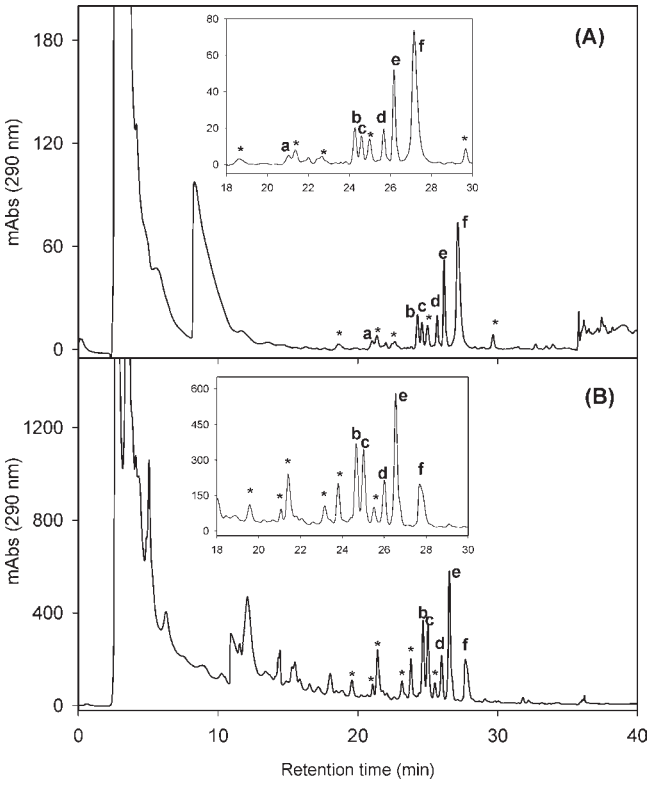

Figure 3. HPLC chromatograms (290 nm) of plasma (**A**) and 24 h urine (**B**) (volunteer 6, plasma sample 7 h after intake of beverage D). Peaks: (a) hesperetin-sulfoglucuronide; (b) naringenin 7-*O*-glucuronide; (c) naringenin 4′-*O*-glucuronide; (d) hesperetin 7-*O*-glucuronide; (e) hesperetin 3′-*O*-glucuronide; (f) hesperetin sulfate (two isomers). Asterisks designate peaks that are not related to flavanone intake.

RESULTS AND DISCUSSION

Flavanone Content in Different Orange Beverages. The flavonoids present in the supernatant and pellet phases were analyzed by HPLC-DAD-MS-MS. The supernatant chromatograms (**Figure 1**) were characterized by the presence of a number of flavanone-glycosides and one flavone derivative. The flavanones were identified by the MS-MS spectra as glycosidic conjugates of naringenin (aglycone fragment at m/z^- 271); hesperetin (aglycone fragment at m/z^- 301), and naringenin 4′-methyl ether (aglycone fragment at m/z^- 285). The main flavanone conjugates were the rutinosides (rhamnosyl(1→6)glucosides), namely, narirutin (**4**), hesperidin (**5**), and didymin (**6**). In addition, the glucosyl-rutinosides of naringenin (**1**) and hesperetin (**3**) were also detected. Vicenin-2 (apigenin 6,8-di-*C*-glucosyl-flavone) (**2**) was also a relevant peak in the chromatograms of the soluble phase. When the regular control juice (B) with the orange flavanone extract dissolved in water (E) and the flavanone-enriched juice (D) were compared, the HPLC chromatograms of the soluble fraction of the last two showed a flavonoid profile in which the flavanones **1**, **3**, **4**, and **5** increased with respect to the control B. This means that the enriched juice had a higher proportion of the glucosyl rutinosides of hesperetin and naringenin, the most water-soluble flavonoids, and also increased the content of narirutin (**4**) and hesperidin (**5**). The HPLC chromatograms of the pellet fractions (**Figure 2**) were characterized by a prominent peak of hesperidin in all cases, whereas the other flavonoids were present in smaller amounts. The flavanone content of the different juices used in the study is shown in **Table 1**. Both flavanones in the soluble and pellet fractions were quantified. Commercial juices A and B showed a flavanone concentration in the range of previously reported commercial juices (29.2 and 53.8 mg/100 mL, respectively), with a similar content in the soluble fraction (*17*). Commercial juice C, labeled as a pulp-enriched juice, had a higher total content (> 70 mg/100 mL), but a flavanone content in the soluble fraction similar to that found in the other commercial juices (A and B). This showed that pulp enrichment increased the total flavanone content of the juice, although it had little effect on the soluble fraction, as most of the additional flavanones of the pulp-enriched juice went to the haze fraction. The quantitative data matched what was observed in the HPLC chromatograms, as the addition of the orange flavanone extract increased the flavanone content of the soluble fraction as well as increased the total flavanone content. Hesperidin was one of the less soluble flavonoids in orange juice and represented the main flavanone in the haze of the different juices.

Identification of Flavanone Metabolites in Plasma. The flavanone metabolites were evaluated in plasma samples at different times after juice intake. In all cases, the maximal concentration was found between 6 and 7 h after intake, suggesting that

Table 1. Flavanone and Vicenin-2 Content of the Orange Juice Beverages Used in This Study[a]

	A (commercial juice 1)			B (commercial juice 2)			C (pulp-enriched juice)			D (B enriched with E)			E (extract in water)		
flavanones + flavones	supernatant	pellet	total	supernatant	pellet	total	supernatant	pellet	total	supernatant	pellet	total	supernatant	pellet	total
Nar-7-rut, 4′-glc (**1**)	1.6 (0.0)	0.2 (0.0)		1.7 (0.3)	0.5 (0.0)		2.6 (0.0)	0.5 (0.0)		8.3 (0.4)	2.3 (0.3)		6.6 (0.1)	0.5 (0.0)	
vicenin-2 (**2**)	3.6 (0.0)	0.2 (0.0)		3.9 (0.0)	0.5 (0.0)		1.5 (0.0)	0.3 (0.0)		6.9 (1.1)	1.5 (0.2)		3.7 (0.0)	0.1 (0.0)	
Hesp-7-rut, 3′glc (**3**)	0.8 (0.0)	0.1 (0.0)		0.8 (0.0)	0.3 (0.0)		1.0 (0.0)	0.3 (0.0)		2.6 (0.0)	1.0 (0.2)		2.2 (0.0)	0.2 (0.0)	
Nar-7-rut (**4**)	3.9 (0.0)	0.9 (0.1)		4.0 (0.0)	2.2 (0.2)		3.7 (0.0)	2.6 (0.1)		12.7 (0.1)	6.0 (0.9)		9.5 (0.0)	1.6 (0.1)	
Hesp-7-rut (**5**)	5.4 (0.2)	11.4 (0.1)		5.7 (0.3)	32.3 (3.5)		5.7 (0.2)	49.8 (0.1)		11.2 (0.1)	53.3 (6.4)		6.6 (0.0)	18.6 (1.3)	
didymin (**6**)	0.7 (0.0)	0.4 (0.0)		0.8 (0.1)	1.1 (0.1)		0.6 (0.0)	1.6 (0.0)		1.5 (0.0)	2.8 (0.3)		1.0 (0.0)	1.2 (0.1)	
total flavonoids	15.9 (0.1)	13.3 (0.4)	29.2 (0.3)	16.9 (0.1)	36.9 (1.3)	53.8 (0.8)	15.1 (0.1)	55.1 (0.4)	70.3 (0.3)	43.2 (0.4)	66.8 (2.3)	110.2 (1.6)	29.5 (0.0)	22.2 (0.5)	51.8 (0.3)

[a] Values are expressed as mg/100 mL of orange beverage. Standard deviation is reported in parentheses ($n = 3$). (**1**) Naringenin-7-rut-4′-glc; (**2**) vicenin-2; (**3**) hesperetin-7-rut-3′-glc; (**4**) naringenin-7-rut; (**5**) hesperetin-7-rut (hesperidin); (**6**) naringenin-4′-methyl-7-rut (didymin).

absorption takes place in the large intestine. In some volunteers, during the first 2 h, small amounts of hesperidin were detected in plasma, showing that a small proportion of nonmetabolized hesperidin can be absorbed in the stomach or in the first portion of the small intestine. Hesperidin was detected in plasma by HPLC-MS-MS. An $[M - H]^-$ ion was observed at m/z^- 609 that, after isolation with the ion trap and fragmentation, led to the loss of the rutinoside residue, leading to the corresponding aglycone (hesperetin at m/z^- 301).

A characteristic chromatogram of the flavanone metabolites detected in plasma after the orange beverage intake is shown in **Figure 3A**. Naringenin- and hesperetin-sulfoglucuronide (**Figure 3A**, peak a) were detected, as well as minor proportions of naringenin and hesperetin diglucuronides (these were detected only with the MS detector and were not quantified). The main chromatographic peaks were naringenin-7-*O*-glucuronide (peak b) and naringenin 4′-*O*-glucuronide (peak c), one naringenin sulfate (not quantified), hesperetin 7-*O*-glucuronide (peak e), hesperetin 3′-*O*-glucuronide (peak e), and a broad peak of hesperetin sulfate (peak f). Previous studies have shown the presence of these phase II metabolites of orange flavanones in human urine (*14*), but the sulfoglucuronide metabolites had not been reported earlier in plasma.

Pharmacokinetics of Orange Beverage Flavanones. The kinetics of plasma disposition of total flavanones showed maximum concentration times (t_{max}) between 6 and 8 h after the intake of the different orange beverages (**Figure 4**). These t_{max} values are longer than those reported by Erlund et al. (*18*), Manach et al. (*19*), and Mullen et al. (*14*) (5.5, 5.8, and 4.4 h, respectively). In two of these studies (*14*, *18*) the orange juice intake was not accompanied by any food intake, and this could explain a shorter t_{max}. In fact, in one of the studies, the juice intake together with yogurt delayed the t_{max} from 4.4 to 5.1 h (*14*). Therefore, the co-ingestion of the orange beverages with breakfast could partly explain the longer t_{max} values observed in the present study. The t_{max} values observed for beverages A and E are similar to those previously reported, but those of the other three beverages are higher, suggesting that the type of beverage (manufacturing and composition) can also have an effect on flavanone absorption. All of these t_{max} values reflected absorption in the large intestine, and this fact may confirm previous studies in which it was proposed that flavanones reach the colon intact, where they are hydrolyzed by the microbiota to release the aglycones that are then absorbed and metabolized by phase II enzymes in the intestinal wall to produce the corresponding glucuronides and sulfate conjugates (*14*). This phase II conjugation can also be completed in the liver.

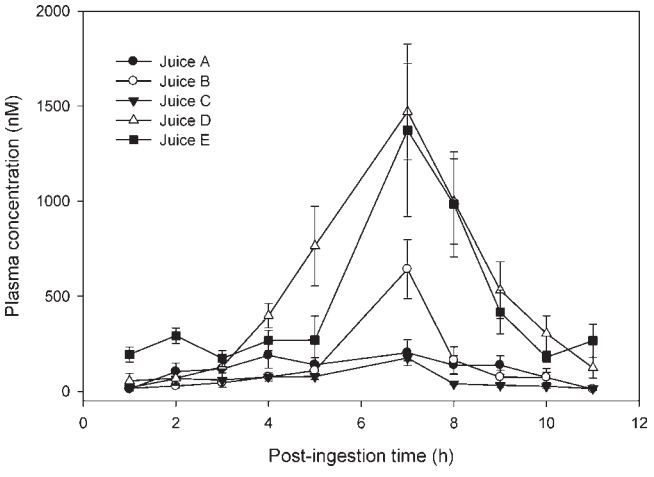

Figure 4. Plasma concentration−time data of total flavanones. Error bars are standard errors (n = 10).

The total flavanone plasma concentration−time curves after the intake of the different orange beverages reflected in general the total flavanone content of the juice. Thus, enrichment of juice B with the flavanone extract (flavanone-enriched juice D) leads to a larger concentration of flavanones in plasma. Interestingly, the pulp-enriched orange juice (C) with a relatively large flavanone content showed a rather poor concentration−time curve. The pharmacokinetic parameters were calculated for all 10 volunteers after the intake of the different juices and beverages (**Table 2**). The peak plasma concentrations (C_{max}) obtained for the total flavanone metabolites upon ingestion of the different commercial juices were in the range of those previously reported. Erlund et al. (*18*) and Manach et al. (*19*) reported plasma values between 2200 ± 1600 and 1300 ± 1600 nM hesperetin-derived metabolites, respectively, after the ingestion of orange juices providing 149 and 265 mg of flavanones, respectively. In addition, Mullen et al. (*14*) reported a C_{max} value of 900 nM hesperetin metabolites upon ingestion of an orange juice with 110 mg of flavanones, which is in the range of that obtained upon ingestion of orange juice B (680 ± 146 nM) containing 98.8 mg of flavanones. This last value was significantly enhanced for the orange flavanone extract E (1545 ± 408 nM) and the flavanone-enriched juice D (1473 ± 251 nM). The t_{max} ranged from 4.5 to 6.8 h, and the area under the concentration−time curve (AUC) increased significantly with flavanone enrichment of the juice (**Table 2**). The AUC/dose values, however, suggest that the values for the same soluble dose of hesperetin appear to be reducing in line with the amount of insoluble hesperetin present, suggesting that the insoluble fraction inhibits absorption.

The correlation between AUC and total flavonoids ingested was lower than that observed for the soluble flavanones (**Figure 5**). This suggests that not only the flavanone content but also the solubility of the flavanones was relevant in terms of flavanone absorption in orange beverages. Food matrix effects were also observed when the pharmacokinetics of the orange flavanone extract (E) were compared with those of the orange juice enriched with the extract (D). Thus, C_{max} was highest in the case of the extract (beverage E), and the shortest t_{max} for juice A (the one with less pulp) and beverage E indicated that the orange juice matrix had an effect in delaying and extending the absorption process of the flavanones.

The differences between the pharmacokinetics of the main flavanone aglycones, naringenin and hesperetin, were also evaluated

Table 2. Pharmacokinetic Parameters of Total Flavanones[a]

beverage	C_{max} (nM)	t_{lag} (h)	t_{max} (h)	AUC_{last} (nmol h/L)	AUC_{last}/dose[b]	AUC_{last}/soluble	C_{max}/dose	MRT_{last} (h)
A	332.5 ± 63.6	1.4 ± 0.5	4.5 ± 0.7	1218.5 ± 275.3	12.0	24.8	3.7	5.7 ± 0.4
B	680.3 ± 146.2	1.3 ± 0.4	6.8 ± 0.6	1532.9 ± 298.3	7.8	29.5	3.4	6.3 ± 0.4
C	194.1 ± 22.3	0.9 ± 0.3	6.5 ± 0.5	695.9 ± 66.6	2.5	12.8	0.7	5.7 ± 0.2
D	1473.4 ± 250.9	0.9 ± 0.2	6.7 ± 0.3	5834.1 ± 1028.6	14.3	40.2	4.0	6.4 ± 0.3
E	1545.5 ± 407.9	0.1 ± 0.1	5.9 ± 0.7	4995.3 ± 1033.7	26.0	48.4	8.1	6.1 ± 0.4

[a] Values are expressed as mean ± standard deviation (n = 10). [b] The dose is expressed in milligrams of flavanones beverage. Pharmacokinetic parameters are defined under Materials and Methods.

(**Table 3**). The derived metabolites of both flavanones were detected in plasma of all subjects after the ingestion of the different juices. Hesperetin-derived metabolites reached the maximum concentrations in plasma between 4.6 and 7.3 h after beverage consumption and those derived from naringenin between 4.7 and 6.4 h, showing a similar behavior. In three of the five beverages, the t_{max} was shorter for naringenin than for hesperetin. However, times of maximum concentration (t_{max}) were very similar to those previously reported (*18*−*20*), which confirmed that hesperetin-rutinosides and naringenin-rutinosides are hydrolyzed by colonic microbiota, rendering aglycones that are further absorbed and metabolized in the large intestine. The AUC/dose for hesperetin ranged from 2.2 nmol h/L/dose in the case of juice C (the dose expressed in mg of flavanones/400 mL) to 37.5 nmol h/L/dose in beverage E. Juice C showed the lowest ($P < 0.05$) AUC/dose and C_{max}/dose values among the juices studied, despite juice C being one of the juices with higher content of hesperetin (56.8 mg/100 mL). These values were in accordance with the low (1%) hesperetin urinary excretion for juice C (**Table 4**), suggesting that the lowest oral bioavailability for hesperetin was that of juice C. The pulp-enriched juice C had the maximum percent of insoluble fraction of all studied juices (about 80%), which supported the lowest hesperetin oral bioavailability and consequently urinary excretion for juice C. Significant differences ($P < 0.05$) were found between commercial juice B and flavanone-enriched juice D, corroborating that hesperetin enrichment of juice B resulted in major AUC/dose and C_{max}/dose values.

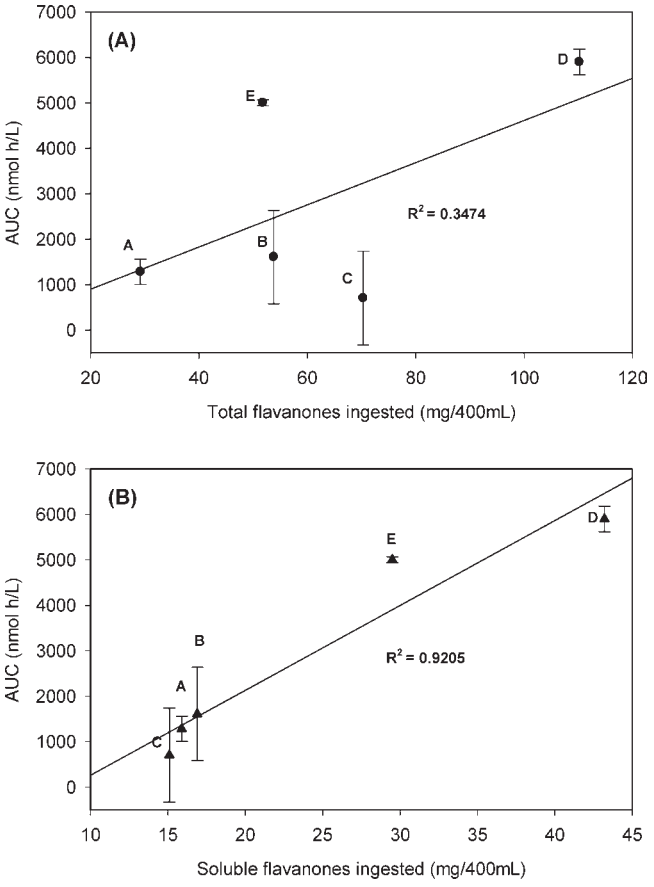

Figure 5. Linear correlation of the total (**A**) and soluble (**B**) flavanone intake and the area under the curve (AUC): (A) commercial juice from concentrate; (B) commercial juice from concentrate; C, pulp-enriched commercial juice; D, flavanone-enriched experimental juice; E, flavanone extract dissolved in water.

In the case of naringenin pharmacokinetics, the AUC/dose ranged from 2.2 nmol h/L/dose in juice A to 17.5 nmol h/L/dose in juice B, juice A being the one that significantly ($P < 0.05$) showed the lowest values in AUC/dose, C_{max}/dose, and AUC/soluble ratios. This contrasts with the excreted amounts of naringenin in urine (**Table 4**), which were higher for juice A than for juice B. This could indicate that juice B has some influence on the excretion of naringenin, forcing it to be excreted via the bile. When the kinetics parameters of juices B and D were compared, no significant differences were found, which suggested that the enrichment of juice B with the flavanone extract did not match an increase in naringenin bioavailability. When the means of the pharmacokinetics values of hesperetin and naringenin were compared, significant differences in their pharmacokinetic indices were found depending on the juice studied. In juice A the AUC/dose, C_{max}/dose, and AUC/soluble ratios were significantly higher for hesperetin than for naringenin, which agrees with Manach et al.'s (*19*) findings in commercial orange juice. However, pharmacokinetic parameters in juice C were significantly higher for naringenin, indicating a higher bioavailability of this compound. The data in the literature about differences in naringenin and hesperetin bioavailabilities are controversial. Higher bioavailability for naringenin was described by Gardana et al. (*20*) for blood orange juice, which agreed with a study carried out with a single oral administration of the aglycone flavanones (*21*). Mullen et al. (*14*) proposed a higher bioavailability for naringenin on the basis of the different chemical structures of both flavanones. In a comparative study of the bioavailability of naringenin in orange juice and grapefruit juice, large differences were found depending on the juice (*18*). The authors did not assign this effect to differences in absorption efficiency but to a dose-dependent renal clearance, different enzymes cleaving rutinosides and neohesperosides, or different affinities of the enzymes for the different

Table 3. Pharmacokinetic Parameters of Individual Flavanones

beverage	flavanone aglycone	t_{max} (h)	C_{max} (nM)	AUC_{last} (nmol h/L)	AUC_{last}/dose	AUC_{last}/soluble	C_{max}/dose
A	hesperetin	4.6 ± 0.7	324.5 ± 65.1	1184.0 ± 276.7	16.7	47.7	4.6
	naringenin	4.7 ± 1.1	37.0 ± 10.1	66.7 ± 27.2	2.2	2.7	1.2
B	hesperetin	6.4 ± 0.7	366.0 ± 69.8	945.6 ± 231.4	6.1	36.4	2.3
	naringenin	5.7 ± 0.7	443.5 ± 109.3	735.2 ± 179.5	17.9	28.3	10.8
C	hesperetin	6.0 ± 0.7	151.5 ± 25.7	503.6 ± 67.1	2.2	18.8	0.7
	naringenin	5.0 ± 0.6	67.3 ± 7.1	200.6 ± 32.5	4.3	7.3	1.5
D	hesperetin	7.3 ± 0.2	1155.9 ± 185.8	4434.4 ± 909.1	16.3	80.3	4.2
	naringenin	5.9 ± 0.6	539.3 ± 125.3	1464.8 ± 384.6	10.9	16.3	4.0
E	hesperetin	6.3 ± 0.6	1482.8 ± 419.2	4145.1 ± 970.8	37.6	117.8	13.4
	naringenin	6.4 ± 1.1	443.2 ± 107.4	961.1 ± 193.0	11.8	14.1	5.4

Table 4. Evaluation of Urinary Excretion after Hydrolysis with Glucuronidase and Sulfatase

		total urinary excretion[a]		relative urinary excretion	
beverage	flavanone	μmol	mg	percentage of the total intake	percentage of the soluble intake
A	naringenin	4.8 ± 0.9	1.3 ± 0.3	2.6 ± 0.5	5.3 ± 1.0
	hesperetin	9.0 ± 2.1	2.7 ± 0.6	5.4 ± 1.2	11.1 ± 2.5
B	naringenin	2.6 ± 0.8	0.7 ± 0.2	0.7 ± 0.2	2.7 ± 0.8
	hesperetin	5.7 ± 1.5	1.7 ± 0.4	1.7 ± 0.4	6.6 ± 1.7
C	naringenin	2.3 ± 1.2	0.6 ± 0.3	0.5 ± 0.2	2.3 ± 1.2
	hesperetin	4.7 ± 2.2	1.4 ± 0.7	1.0 ± 0.5	5.2 ± 2.5
D	naringenin	18.7 ± 3.8	5.1 ± 1.0	2.5 ± 0.5	7.0 ± 1.4
	hesperetin	30.8 ± 6.9	9.3 ± 2.1	4.6 ± 1.0	12.8 ± 2.9
E	naringenin	18.9 ± 5.3	5.1 ± 1.5	5.4 ± 1.5	10.0 ± 2.8
	hesperetin	28.3 ± 9.2	8.6 ± 2.8	8.9 ± 2.9	16.6 ± 5.4

[a] The experimental conditions used for enzymatic hydrolysis leave 10% of the original flavanone sulfates unhydrolyzed. These values do not include the 10% of the flavanone sulfates that were not hydrolyzed.

flavanone glycosides (*18*). In our study, we did not find significant differences in hesperetin and naringenin bioavailability for juice B in accordance with previous work on orange juice (*18*). Analysis of juices D and E showed higher AUC/soluble ratios for hesperetin compared to naringenin. This suggests that the matrix of the juice differentiated the bioavailability of hesperetin and naringenin.

When the correlation of hesperetin and naringenin intake (total and soluble) with the flavanones excreted in urine was plotted (results not shown), a correlation for naringenin ingested and naringenin excreted (R^2 = 0.7565) was observed. However, this correlation was not observed in the case of hesperetin (R^2 = 0.0235). In addition, the correlation improved when the naringenin and hesperetin contents of the soluble fraction were compared with the naringenin (R^2 = 0.9156) and hesperetin (R^2 = 0.2575) amounts excreted in urine, respectively.

Urinary Excretion and Percentage of Absorption and Excretion. The flavanone metabolites were evaluated in urine for 48 h. The HPLC analyses showed that flavanone excretion occurred during the first 24 h, as almost no flavanone metabolites were detected during the second 24 h period. The HPLC-DAD chromatogram of a characteristic urine sample is shown in **Figure 3B**. The different metabolites were identified by HPLC-MS-MS, and they corresponded with those found in plasma. In this case peak f is rather broad and shows a shoulder, suggesting peak overlapping. This is expected for flavonoid sulfates that have poor resolution in HPLC. As the same UV and mass spectra are observed throughout the peak, this suggests the occurrence of two hesperetin sulfates coeluting under peak f. Thus, naringenin and hesperetin glucuronides and sulfates were detected. The quantification after glucuronidase-sulfatase hydrolysis is shown in **Table 4**. The percentage of excretion was rather variable between the different volunteers. The relative urinary excretion, compared with the intake, was calculated. This ranged from 1.4% for the pulp enriched juice (C) to 13.3% for the beverage obtained with the orange flavanone extract dissolved in water (E), with values around 6.5% for the flavonoid-enriched juice (D). This showed again an effect of the food matrix affecting the absorption of the orange juice flavanones, as the juice components, other than flavanones, decreased the absorption and excretion of flavonoids as is shown when the flavonoid-enriched juice is compared with the flavanone extract dissolved in water.

When the flavanones excreted in urine were plotted to evaluate their correlation with the total flavanone content in the orange beverages, no correlation was observed (**Figure 6A**; R^2 = 0.2593),

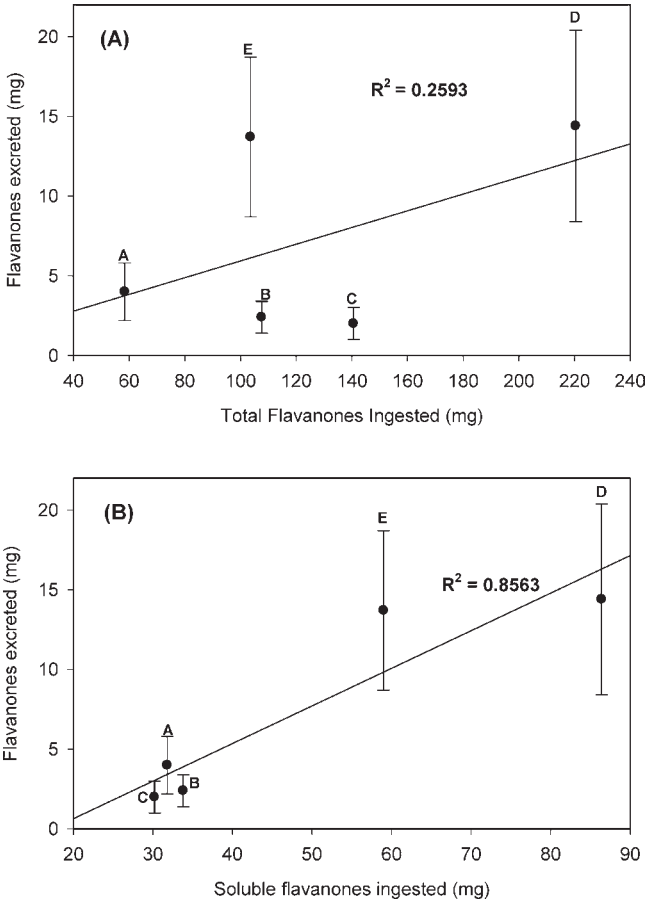

Figure 6. Linear correlation of the total (**A**) and soluble (**B**) flavanone intake and the excreted flavanones in urine. Letters from A to E designate the same beverages as in **Figure 5**.

but this correlation was improved (R^2 = 0.8563) when only the soluble flavanones were plotted (**Figure 6B**), showing that the soluble flavanones should be the ones to be considered when bioavailability is investigated. This was even better when the flavanone extract E was removed from the correlation, as this is not a juice but an orange extract dissolved in water. In this case the correlation coefficient R^2 improved to reach 0.9774, showing again the effect of the food matrix in the flavanone absorption and excretion. All of these results indicate that increasing

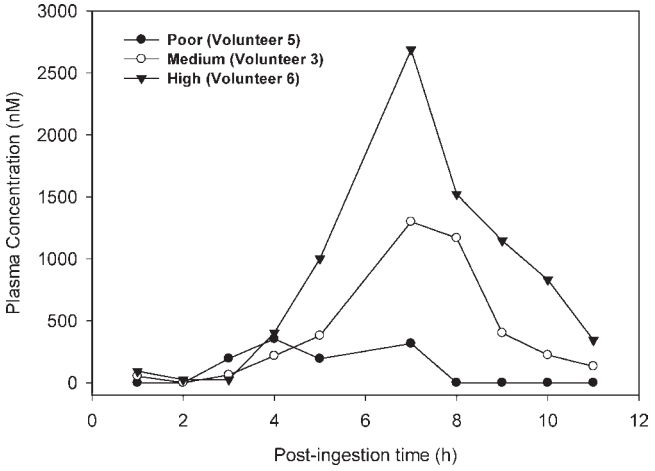

Figure 7. Interindividual differences in flavanone pharmacokinetics upon beverage D intake.

Table 5. Interindividual Urinary Excretion Variability of the 10 Volunteers[a]

	volunteer									
	sex									
	BMI (kg/m^2)									
	1	2	3	4	5	6	7	8	9	10
	F	F	M	M	M	M	M	F	F	F
beverage	(22.7)	(20.3)	(29.5)	(22.5)	(26.0)	(22.7)	(25.2)	(23.9)	(19.4)	(22.8)
A	2nd	1st	3rd	1st	3rd	1st	1st	2nd	2nd	3rd
B	1st	2nd	2nd	2nd	3rd	2nd	1st	1st	2nd	3rd
D	2nd	2nd	2nd	2nd	3rd	1st	1st	1st	2nd	3rd
E	1st	1st	2nd	2nd	3rd	1st	1st	2nd	3rd	3rd
score	M	M	M	M	L	H	H	M	M	L

[a] Values represent the thirtile where the different volunteers [(M) males; (F) females; BMI (kg/m^2)] are located after the intake of the different beverages. 1st, excretion values between 100% (the highest excretion) and 70% of the highest excretion; 2nd, excretion values between 70 and 30% of the highest excretion value; 3rd, excretion values between 30 and 10% of the highest excretion after the intake of each beverage. H, high excretor; M, medium excretor; L, low excretor. The values for beverage C are not included as the excretion was very low in all cases due to poor soluble flavanone concentration in this juice.

flavanone solubility in orange beverages leads to an increase in their bioavailability. This study confirm previous in vitro studies in which the orange juice flavanone availability for absorption was higher in juices with more soluble flavanones and that the flavanones precipitated in the cloud were not available for absorption (*2*, *17*, *22*).

Bioavailability and Excretion Interindividual Variability. Large interindividual variability was observed between the pharmacokinetics and the bioavailability and excretion of the different volunteers. Thus, high- and low-flavanone excretors were identified. **Figure 7** shows the pharmacokinetics of three different volunteers after the intake of the flavanone-enriched juice (D). Interestingly, this excretion behavior (high and low) was consistent for each volunteer (**Table 5**) after the intake of the different juices and beverages and, therefore, could not be attributed to specific physiological conditions of the day when the beverage was taken. This large variability could be due to differences in the colon microbiota or to epigenetic differences between the different volunteers. No correlation between flavanone absorption/metabolism and sex or BMI of the volunteers was observed (**Table 5**). These results support that native flavanones occurring in the orange juice are not absorbed in the stomach or small intestine, and they reach the colon intact. They need to be hydrolyzed to release the corresponding aglycones before absorption in the colon. Colon microbiota is responsible for this hydrolysis, and species of the genera *Bacteriodes* and *Eubacterium* have been suggested as responsible for this glycosidase activity (*23*). The absorbed aglycones are metabolized by phase II enzymes to yield the corresponding conjugates (glucuronides and sulfates), and they circulate in plasma and reach urine in these conjugate forms. One of the potential explanations of the differences found in absorption is that the native glycosides are not hydrolyzed to aglycones (lack of the necessary microbiota). On the other hand, they can also be further metabolized to produce the C-ring cleavage metabolites (phenyl propionic acid derivatives or benzoic acid derivatives). These last metabolites were detected in the HPLC-MS chromatograms, mainly as glucuronide derivatives in plasma and urine: 4-hydroxy-phenyl-propionic glucuronide, $[M - H]^-$ m/z 341; *trans*-cinnamic acid-glucuronide $[M - H]^-$ m/z 329; *p*-coumaroyl-glucuronide $[M - H]^-$ m/z 339; 4-hydroxy-benzoic acid-glucuronide, $[M - H]^-$ m/z 313; 3-methoxy-4-hydroxy-phenylacetic-glucuronide, $[M - H]^-$ m/z 357; hippuric acid-glucuronide $[M - H]^-$ m/z 354; 3-hydroxy-phenylacetic-glucuronide, and 4-hydroxy-phenylacetic glucuronide, $[M - H]^-$ m/z 326. Unfortunately, these were not quantified in the present work as HPLC-MS is not the method of choice for the quantification of these metabolites and GC-MS analyses after hydrolysis with glucuronidase and sulfatase should have been carried out. Further studies need to be carried out to evaluate differences in the low molecular weight phenolic metabolites and their relationship with bioavailability using GC-MS methodologies.

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