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Bioavailability and Metabolism of Orange Juice Flavanones in Humans: Impact of a Full-Fat Yogurt

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The bioavailability of dietary phytochemicals may be influenced by the food matrix in which they are consumed. In this study the impact of a full-fat yogurt on the bioavailability and metabolism of orange juice flavanones was investigated. Human plasma and urine were collected over a 24 h period after the consumption of 250 mL of orange juice containing a total of 168 μmol of hesperetin-7-*O*-rutinoside and 12 μmol of naringenin-7-*O*-rutinoside, with and without 150 mL of full-fat yogurt. The juice also contained 1 g of paracetamol and 5 g of lactulose. HPLC-MS^2 analysis revealed the accumulation of hesperetin-7-*O*-glucuronide, and an unassigned hesperetin-*O*-glucuronide metabolite in plasma reached a peak concentration (C_{max}) of 924 $\pm$ 224 nmol/L, 4.4 $\pm$ 0.5 h (T_{max}) after orange juice ingestion. The T_{max} is indicative of absorption in the colon. When the juice was consumed with yogurt, neither the C_{max} at 661 $\pm$ 170 nmol/L nor the T_{max} at 5.1 $\pm$ 0.4 h were significantly different from those obtained with juice alone. The two hesperetin glucuronides were also excreted in urine along with a third hesperetin-*O*-glucuronide, two hesperetin-*O*-glucuronide-*O*-sulfates, a hesperetin-*O*-diglucuronide, a naringenin-*O*-diglucuronide, and, tentatively identified, naringenin-7-*O*-glucuronide and naringenin-4′-*O*-glucuronide. This indicates the occurrence of substantial, postabsorption, phase II metabolism prior to urinary excretion. The quantity of flavanone metabolites excreted 0−5 h after orange juice ingestion was significantly reduced by yogurt, but over the full 0−24 h urine collection period, the amounts excreted, corresponding to ca. 7.0% of intake, were not affected by the addition of yogurt to the drink. Nor did yogurt have a significant effect on gastric emptying, as determined by plasma paracetamol levels, or on the mouth to cecum transit time of the head of the meal, assessed by measurement of lactulose-derived breath hydrogen. There is also a discussion of the merits of studies of the absorption and metabolism of flavanones based on direct analysis of metabolites by HPLC-MS and the more traditional indirect approach where samples are treated with a mollusc glucuronidase/sulfatase preparation prior to HPLC analysis of the released aglycones.

KEYWORDS: Orange juice; yogurt; bioavailability; flavanone rutinosides; glucuronide and sulfate metabolites; plasma pharmacokinetics; urinary excretion

INTRODUCTION

There has been much research on the potential health benefits of flavonoids derived from fruit and vegetable products (*1, 2*). From recent studies on a range of foods, it is clear that when considering their health benefits, it is essential to have more information on the exact form in which these compounds are absorbed into the body. It is also important that mechanistic studies should investigate the bioactivity of circulating glucuronide and sulfated metabolites instead of the parent compounds that may never reach target tissues (*3*). A further consideration is the food matrix as several properties of food components can influence the rate and extent to which flavonoids are absorbed. For instance, there may be a physiological interaction with fats that slows gastric emptying and small bowel transit time (*4, 5*). This would delay but not decrease absorption of flavonoids, as in the case of anthocyanin-rich strawberries eaten with cream (*6*). There may also be more complicated direct chemical

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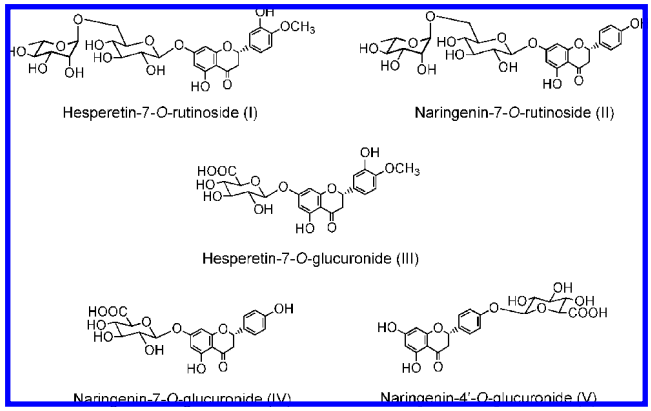

Figure 1. Structures of the flavanones hesperetin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside and their metabolites hesperetin-7-*O*-glucuronide, naringenin-7-*O*-glucuronide, and naringenin-3′-*O*-glucuronide.

interactions between flavonoids and milk (*7*), and there has been considerable debate, and disagreement, about the impact of milk on plasma flavan-3-ol and antioxidant levels after the consumption of tea and chocolate products (*8*−*16*).

Citrus fruits are rich sources of flavanones, a subgroup of flavonoids, and oranges (*Citrus sinensis*) contain principally hesperetin-7-*O*-rutinoside (aka hesperidin) (**I** in **Figure 1**) and naringenin-7-*O*-rutinoside (aka narirutin) (**II**) (*17*). The bioavailability of these flavanone conjugates and their aglycones in humans has previously been investigated by a number of groups who have collected plasma and urine after ingestion and cleaved putative hesperetin and naringenin glucuronide conjugates by treatment with glucuronidase before analysis of the released aglycones (*18*−*23*). These studies have shown that the time to reach the flavanone peak plasma concentration (T_{max}) is typically 5−7 h after intake. This indicates that the 7-*O*-rutinosides are not deglycosylated and absorbed in the small intestine but pass to the colon, where the aglycone, released by the action of bacterial enzymes such as α-rhamnosidase and *β*-glucosidases, is glucuronidated during transport through colonocytes in the gut wall en route to the circulatory system.

This paper describes an investigation of the bioavailability and metabolism of orange juice flavanones, hesperetin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside, and the impact of ingesting the juice with a full-fat natural yogurt, which represents a natural fat/protein matrix. Plasma and urine were collected at several time points over a 24 h period postingestion and, without prior enzymatic hydrolysis, analyzed by high-performance liquid chromatography (HPLC) with photodiode array (PDA) and mass spectrometric (MS) detection. This enabled pharmacokinetic profiles to be obtained on the individual flavanone glucuronide and sulfate metabolites that appeared in plasma and urine after supplementation. The orange juice supplements were ingested together with 1 g of paracetamol as monitoring paracetamol levels in plasma enabled the rate of gastric emptying to be assessed. Breath hydrogen was also measured to determine the mouth to cecum transit time.

MATERIALS AND METHODS

Orange Juice and Chemicals. Own brand full-fat natural yogurt (3.8% fat) and orange juice were purchased from a local supermarket (Somerfield's, Glasgow, U.K.). HPLC grade methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Borders, U.K.). Formic acid was purchased from Riedel-deHaen (Seelze, Germany) and acetic acid from BDH (Poole, U.K.), whereas L-(+)-ascorbic acid, quercetin-3-*O*-rutinoside, naringenin-7-*O*-rutinoside, and hesperetin-7-*O*-rutinoside were purchased from Extrasynthese (Genay, France). Hesperetin-7-*O*-glucuronide was synthesized according to methods described by Matsumoto and co-workers (*24*).

Study Design. The Glasgow Royal Infirmary Research Ethics Committee approved the study protocol. Four male and four female volunteers (21−50 years of age; mean body mass index = 22.6, range = 19.2−27.2), who were healthy, nonsmokers, and not on any medication, gave their written consent and participated in the study. They followed a diet low in flavonoids and phenolic compounds, which excluded fruits and vegetables, high-fiber products, and beverages such as tea, coffee, fruit juices, and wine, for two days before the study. After an overnight fast, volunteers consumed 1 g of paracetamol, 5 g of lactulose, and 250 mL of orange juice fortified with 131 μmol of hesperetin-7-*O*-rutinoside. Four weeks later, the same juice together with 150 mL of natural yogurt was ingested under identical conditions. Volunteers ate ham or turkey with white bread rolls 3 h after drinking the juice and thereafter remained on a low-flavonoid diet for a further 21 h until the final blood and urine samples were collected.

Twelve milliliters of venous blood was collected in heparinized tubes from all volunteers at 0, 0.5, 1, 2, 3, 4, 6, 8, 10, and 24 h postingestion and plasma separated by centrifugation at 4000*g* for 10 min at 4 °C. Two 1-mL aliquots of plasma were acidified to pH 3 with 15 μL of 50% aqueous formic acid and 50 μL of 10 mmol/L ascorbic acid, frozen in liquid nitrogen, and stored at −80 °C prior to analysis by HPLC-PDA-MS. For paracetamol analysis, venous blood samples, obtained 0, 15, 30, 45, 60, 75, 90, 105, and 120 min postingestion, were collected in EDTA tubes, and the plasma obtained was stored as two 1-mL aliquots at −80 °C prior to analysis.

Urine was collected prior to supplementation and over four time periods, 0−2, 2−5, 5−10, and 10−24 h, after the ingestion of the orange juice. The total volume for each period was recorded. After collection, urine samples were acidified to pH 3 with formic acid, and aliquots were stored at −80 °C prior to analysis by HPLC-PDA-MS.

End expiratory breath hydrogen levels were monitored every 15 min for 8 h after ingestion of the orange juice supplement. Measurements were made by blowing into an EC60 Breath Hydrogen Monitor (Bedfont Scientific Ltd., Rochester, Kent, U.K.). The time of first sustained rise in breath hydrogen was taken as the mouth to cecum transit time of the head of the meal.

Analysis of Orange Juice. Triplicate aliquots of orange juice (500 μL) were taken for quantitative analysis of their flavanone content. Each aliquot was mixed with an equal volume of methanol for 1 min using a Disruptor Genie mixer (Fisher Scientific, U.K.). The mixture was then centrifuged at 3000*g* at 4 °C for 15 min. The supernatant was collected and the flavanone content of 10 μL aliquots analyzed by HPLC-PDA-MS^2.

Extraction of Plasma. Triplicate plasma samples were extracted using a method developed by Day et al. (*25*). Two hundred and fifty microliters of plasma, to which had been added 100 ng of quercetin-3-*O*-rutinoside, as an internal standard, and 1.0 mL of acetonitrile, was vortexed for 30 s every 2 min over a 10 min period before the mixture was centrifuged at 4000*g* for 10 min at 4 °C. The supernatant was decanted and the pellet re-extracted with 1.0 mL of methanol, and after centrifugation, the two supernatants were combined and reduced to dryness under a stream of nitrogen at 38 °C. The dried samples were then resuspended in 40 μL of methanol plus 160 μL of 1% aqueous formic acid, and duplicate 50 μL aliquots were analyzed by HPLC-PDA-MS^2. Recoveries of the quercetin-3-*O*-rutinoside internal standard were typically 85%. In pilot studies we have previously checked the recovery of standards of quercetin-3′-*O*-sulfate and quercetin-3-*O*-glucuronide from plasma, and they were comparable with those obtained with quercetin-3-*O*-rutinoside and quercetin-3-*O*-glucoside. The use of quercetin-3-*O*-rutinoside as an internal standard is, therefore, unlikely to introduce an error into the quantitative estimates of the flavanone sulfate and glucuronide content of plasma and urine in the present study.

HPLC with PDA and MS Detection. Samples were analyzed on a Surveyor HPLC system comprising a HPLC pump, a PDA detector scanning from 250 to 700 nm, and an autosampler cooled to 4 °C (Thermo Electron Corp., San Jose, CA). Separation was carried out using a 250 × 2 mm i.d., 4 μm, Synergi RP-Max column (Phenomenex,

Macclesfield, U.K.) maintained at 40 °C and eluted with a gradient over 30 min of 15−30% acetonitrile in 1% formic acid at a flow rate of 200 μL/min. After passing through the flow cell of the PDA detector, the column eluate was directed to a LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface. (Thermo Electron Corp.). Analyses utilized the negative ion mode as this provided the best limits of detection for flavanones. Each sample was analyzed using a two-segment selected ion monitoring (SIM) method looking for ions *m/z* 653, 623, and 609 from 0 to 14 min and at *m/z* 557, 477, and 447 from 14 to 30 min. Capillary temperature was 250 °C, sheath gas and auxiliary gas were 60 and 20 units, respectively, and the source voltage was 4 kV. Quantification of hesperetin-7-*O*-rutinoside and naringenin-7-*O*-rutinoside in the orange juice was based on standard curves obtained at 330 nm by HPLC of the appropriate rutinoside. In either the absence, or absence of sufficient quantities of naringenin and hesperetin metabolites, all quantitative estimates of flavanones in plasma and urine were based on selected ion monitoring (SIM) traces with data expressed as hesperetin-7-*O*-glucuronide equivalents with an on-column limit of detection of 5 pmol. Peak identifications were confirmed by full-scan MS^2 and/or selected reaction monitoring (SRM).

Analysis of Plasma Paracetamol. Plasma paracetamol was measured using an acetaminophen assay kit (Cambridge Life Sciences, Cambridge, U.K.) with amounts of standard adapted to predicted paracetamol concentrations from initial measurements in this study (*26*). An index of the half-time for gastric emptying was obtained by applying curve-fitting analysis to data on plasma paracetamol levels (*27*).

Pharmacokinetic Analysis of Hesperetin Glucuronides in Plasma. Maximum plasma concentration of the metabolites from 0 to 10 h postdose was defined as C_{max} with T_{max} being the time at which C_{max} was reached. The elimination half-life for the metabolites was computed by using the following formula: $T_{1/2} = 0.693/\mathrm{Ke}$, where Ke is the slope of the linear regression of the plasma metabolite concentrations. Area-under-the-curve (AUC) calculations were determined using a Kinetica software package (Thermo Electron Corp.).

Statistical Analysis. Data on flavanone metabolite levels are presented as mean value ± standard error (SE) ($n = 8$). When appropriate, data were subjected to statistical analysis using analysis of variance (ANOVA) and paired *t* test with Minitab software, version 13 (Minitab Inc., Addison-Wesley Publishing, Reading, MA). When data were not normally distributed, a Wilcoxon signed rank test (WSRT) was used, as indicated in the text.

RESULTS

Orange Juice. Gradient reverse phase HPLC with PDA detection and full-scan data-dependent MS^2 was used to identify and quantify the flavanone content of the orange juice. The main peak at 330 nm with a retention time (t_R) of 21.6 min was hesperetin-7-*O*-rutinoside (**I**) on the basis of cochromatography with a standard and a mass spectrum with a *m/z* 609 molecular ion ($[M - H]^-$) that fragmented with a 308 amu loss of a rutinoside moiety to produce a *m/z* 301 hesperetin daughter ion. A smaller peak with a t_R of 20.1 min was identified as naringenin-7-*O*-rutinoside (**II**) on the basis of cochromatography with a standard and a mass spectrum with a $[M - H]^-$ at *m/z* 579, which fragmented with a loss of 308 amu, yielding a MS^2 naringenin ion at *m/z* 271. The hesperetin-7-*O*-rutinoside content of the orange juice drink was 168 μmol per 250 mL, of which 131 μmol was added to the juice so that the quantities ingested would be similar to anthocyanin, flavonol, and flavan-3-ol intake in feeding studies previously carried out with strawberries (*6*), onions (*28*), tomato juice (*29*), and green tea (*30*). The naringenin-7-*O*-rutinoside content of the juice was 12 μmol/250 mL. The orange juice did not contain detectable quantities of the aglycones hesperetin and naringenin or their respective glucoside conjugates.

Qualitative Analysis of Plasma and Urine. Plasma extracts and urine samples collected after ingestion of 250 mL of orange juice with and without yogurt were analyzed by HPLC-PDA-MS^2. **Figure 2** illustrates typical HPLC-SIM and 330 nm absorbance profiles, which together with MS^2 data led to the identification of hesperetin-7-*O*-glucuronide and the tentative identification of eight other metabolites, as presented in **Table 1**. The basis of these proposed identifications is summarized as follows.

Plasma contained two detectable flavanone metabolites, peaks 6 and 7 (t_R 26.4 and 27.4 min)m which had the same $[M - H]^-$ and MS^2 fragmentation patterns with *m/z* 477 undergoing a 176 amu loss and cleavage of a glucuronide unit, to produce *m/z* 301. Further fragmentation of the ion at *m/z* 301 produced a spectrum matching that of hesperetin. Cochromatography with a reference compound established that peak 6 is hesperetin-7-*O*-glucuronide (**III**), whereas peak 7 is an unassigned hesperetin-*O*-glucuronide. These metabolites were also detected in urine along with a third hesperetin-*O*-glucuronide (peak 8, t_R 27.9 min). After the C-7 position, the remaining potential sites for *O*-glucuronidation of hesperetin are through the hydroxyl groups at C-3′ and C-5. Hesperetin-3′-*O*-glucuronide has been detected in rat plasma after oral administration of hesperetin (*24*) but, to date, there are no reports of C-5 conjugates of hesperetin. An alternative explanation for at least one of the two glucuronide metabolites could be the conversion of hesperetin to homoeriodictyol. This has been reported in vitro using rat liver microsomes (*31*) and also in vivo in rat plasma (*24*). However, the MS fragmentation pattern of homoeriodictyol is different from that of hesperetin. so this possibility can be excluded.

All three hesperetin-*O*-glucuronides were detected in urine along with five other flavanone metabolites (**Table 1**). Peak 1 (t_R 9.2 min) had a $[M - H]^-$ at *m/z* 653 and fragmented in MS^2 to produce ions at *m/z* 477, a loss of 176 amu, indicative of cleavage of a glucuronide moiety, and *m/z* 301, the aglycone hesperetin after the loss of a second glucuronide unit. The presence of the *m/z* 477 ion indicates the two glucuronide units are attached to different positions on the aglycone. This peak is, therefore, a hesperetin-*O*-diglucuronide.

Peak 2 (t_R 10.1 min) had a $[M - H]^-$ at *m/z* 623 and fragmented in the same way as peak 1, producing the ion of the aglycone naringenin at *m/z* 271. Peak 2 is, thus, a naringenin-*O*-diglucuronide.

Peak 3 (t_R 22.3 min) and peak 4 (t_R 23.5 min) both had a $[M - H]^-$ at *m/z* 447, which fragmented on MS^2 with a loss of 176 amu to produce an ion at *m/z* 271. Further fragmentation of the *m/z* 271 ion produced a spectrum of ions matching that of an authentic standard of naringenin. In view of the identification of naringenin-7-*O*-glucuronide (**IV**) and naringenin-4′-*O*-glucuronide (**V**) in human plasma and urine after orange juice consumption and the fact that the 7-*O*-glucuronide elutes from a reversed phase HPLC column before its 4′-isomer (*32*), peak 3 is tentatively identified as naringenin-7-*O*-glucuronide and peak 4 as naringenin-4′-*O*-glucuronide.

Peak 5 (t_R 25.7 min) had a $[M - H]^-$ at *m/z* 557, and MS^2 fragmentation yielded an ion at *m/z* 477, an 80 amu loss of a sulfate group. Additional ions were present at *m/z* 381, a loss of 176 amu from cleavage of glucuronide moiety, and *m/z* 301, the aglycone hesperetin. This peak is, therefore, a hesperetin-*O*-glucuronide-*O*-sulfate. As illustrated in **Figure 2**, peak 5 is broader than other peaks in the HPLC profile. Further investigation using a 60 min rather than a 30 min HPLC gradient revealed that there were two hesperetin-*O*-glucuronide-*O*-sulfates in peak 5.

HPLC-SIM analysis at *m/z* 271 and 301 revealed that none of the plasma or urine samples contained detectable quantities

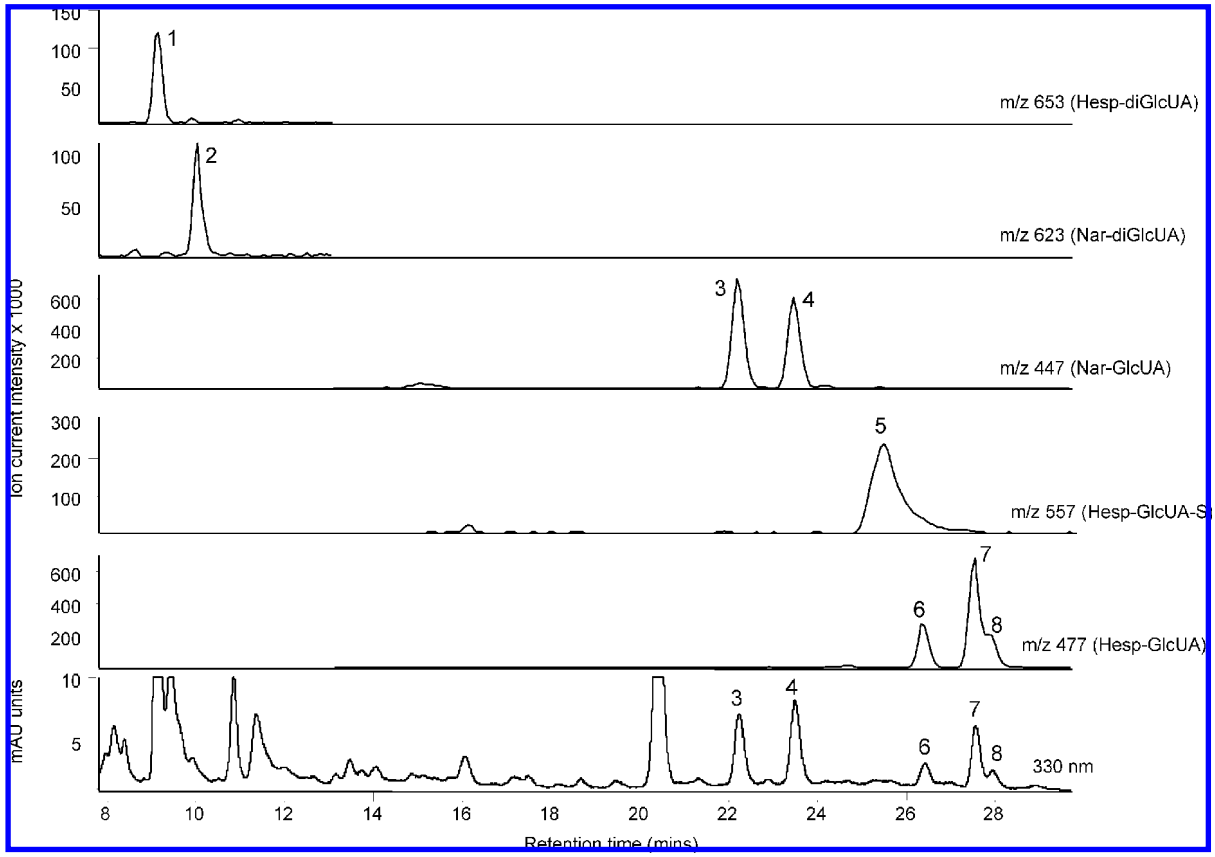

Figure 2. HPLC-PDA-MS analysis of flavanone metabolites in human urine. Chromatograms represent gradient reversed phase HPLC analysis of urine collected 2−5 h after the ingestion of 250 mL of orange juice with detection of hesperetin and naringenin metabolites by SIM at *m*/*z* 653 (hesperetin-*O*-diglucuronide), *m*/*z* 623 (naringenin-*O*-diglucuronide), *m*/*z* 447 (naringenin-*O*-glucuronides), *m*/*z* 557 (hesperetin-*O*-glucuronide-*O*-sulfates, and *m*/*z* 477 (hesperetin-*O*-glucuronides) and with a PDA detector at 330 nm. For identity of peaks 1−8, see **Table 1**.

Table 1. Tentative HPLC-MS^2 Identifications of Flavanone Metabolites in Plasma and Urine Collected after the Ingestion of Orange Juice by Human Volunteers[a]

peak	t_R (min)	$[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	flavanone metabolite	location
1	9.2	653	477, 301	hesperetin-*O*-diglucuronide	urine
2	10.1	623	447, 271	naringenin-*O*-diglucuronide	urine
3	22.3	447	271	naringenin-7-*O*-glucuronide	urine
4	23.5	447	271	naringenin-4′-*O*-glucuronide	urine
5	25.7	557	477, 381, 301	hesperetin-*O*-glucuronide-*O*-sulfates	urine
6	26.4	477	301	hesperetin-7-*O*-glucuronide	plasma, urine
7	27.4	477	301	hesperetin-*O*-glucuronide	plasma, urine
8	27.9	477	301	hesperetin-*O*-glucuronide	urine

[a] For peaks 1−8 see **Figure 2**. t_R, retention time; $[M-H]^-$, negatively charged molecular ion; MS^2, daughter ion produced by fragmentation of $[M-H]^-$.

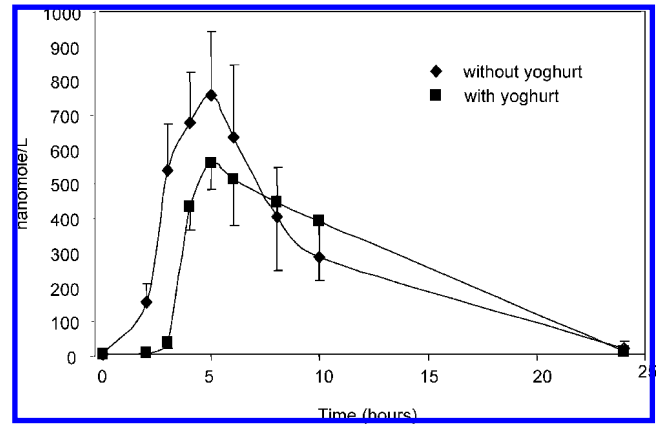

Figure 3. Combined concentration of hesperetin-7-*O*-glucuronide and an unassigned hesperetin-*O*-glucuronide (peak 7) (see **Table 1**) in the plasma of eight human subjects 0−24 h after ingesting 250 mL of orange juice with and without 150 mL of yogurt. Data expressed as nmol/L are presented as mean values ± standard errors depicted by vertical bars (n = 8).

of the aglycones naringenin and hesperetin. Likewise, analysis at *m*/*z* 579 and 609 did not detect either hesperetin-7-*O*-rutinoside or naringenin-7- *O*-rutinoside (data not shown).

Quantitative Analysis of Flavanone Metabolites in Plasma. Only hesperetin-7-*O*-glucuronide and peak 7, the unassigned hesperetin-*O*-glucuronide, were present in quantifiable amounts in the plasma of all the subjects. The plasma profiles over the 24 h period following ingestion of the orange juice with and without yogurt, presented in **Figure 3**, and the pharmacokinetic data summarized in **Table 2** are based on the sum of the two metabolites. The metabolites did not appear in plasma until 2 h after supplementation. The C_{max} without yogurt was 922 ± 224 nmol/L and with yogurt was 661 ± 170 nmol/L, with the difference not being significant. Likewise, the respective T_{max} times, 4.4 ± 0.5 and 5.1 ± 0.4 h, were also not statistically different. After reaching C_{max}, the levels of the two flavanone metabolites declined with $T_{1/2}$ values 3.6 ± 1.3 h for the orange juice alone and 3.8 ± 0.8 h when the juice was consumed with yogurt. Yogurt also had no significant effect on the other pharmacokinetic parameters, namely, AUC, C_{max}/dose, and C_{max}/AUC (**Table 2**). With the exception of samples from two subjects which contained trace quantities, no metabolites were detected in plasma collected 24 h after supplementation.

Table 2. Pharmacokinetic Parameters of Hesperetin-7-*O*-glucuronide and an Unidentified Hesperetin-*O*-glucuronide (Peak 7) in the Plasma of Eight Human Subjects after the Consumption of 250 mL of Orange Juice, Containing 168 μmol of Hesperitin-7-*O*-rutinoside, with and without 150 mL of Yogurt[a]

ingested	C_{max} (nmol/L)	T_{max} (h)	$T_{1/2}$ (h)	AUC (μmol/L/h)	C_{max}/dose (nmol/L/μmol)	AUC/dose (nmol/L/h/μmol)
orange juice	922 ± 224	4.4 ± 0.5	3.6 ± 1.3	4.1 ± 2.9	5.5 ± 1.3	24.4 ± 17.3
orange juice with yogurt	661 ± 170	5.1 ± 0.4	3.8 ± 0.8	3.0 ± 2.6	3.9 ± 1.0	17.9 ± 15.4

[a] Data expressed as mean value ± standard error ($n = 8$). C_{max}, maximum postingestion plasma concentration; T_{max}, time to reach C_{max}; $T_{1/2}$, elimination half-life; AUC, area-under-the-curve (0−10 h).

Table 3. Quantities of Hesperetin-7-*O*-glucuronide and Other Putative Flavanone Metabolites in the Urine of Eight Human Subjects 0−24 h after the Consumption of 250 mL of Orange Juice with and without 150 mL of Yogurt[a]

	0−2 h		2−5 h		5−10 h		10−24 h		total	
flavanone metabolite (peak)	w/o	with	w/o	with	w/o	with	w/o	with	w/o	with
hesperetin-*O*-diglucuronide (1)	1.3 ± 0.7	nd	97 ± 30	43 ± 16	375 ± 179	237 ± 49	287 ± 189	370 ± 290	767 ± 361	647 ± 333
naringenin-*O*-diglucuronide (2)	2.6 ± 1.7	1.3 ± 1.3	27 ± 8.6	7.3 ± 3.9	44 ± 26	37 ± 14	24 ± 14	14 ± 14	98 ± 46	60 ± 31
naringenin-7-*O*-glucuronide (3)	67 ± 31	13 ± 4.3	395 ± 90	187 ± 30	342 ± 176	380 ± 156	199 ± 101	211 ± 150	1001 ± 344	789 ± 319
naringenin-4′-*O*-glucuronide (4)	59 ± 26	21 ± 8.6	288 ± 71	215 ± 25	277 ± 227	500 ± 152	230 ± 102	300 ± 170	976 ± 389	1034 ± 334
hesperetin-*O*-glucuronide-*O*-sulfates (5)	8.3 ± 5.7	nd	675 ± 217	261 ± 80	1341 ± 339	1140 ± 181	816 ± 349	1314 ± 660	2841 ± 699	2714 ± 776
hesperetin-7-*O*-glucuronide (6)	9.3 ± 7.3	nd	372	184 ± 65	651 ± 302	564 ± 99	340 ± 166	556 ± 327	1373 ± 471	1301 ± 411
hesperetin-*O*-glucuronide (7)	19 ± 13	nd	911 ± 267	389 ± 90	1699 ± 810	1405 ± 327	1036 ± 687	1667 ± 1193	3662 ± 1483	3459 ± 1473
hesperetin-*O*-glucuronide (8)	14 ± 7.8	nd	381 ± 111	210 ± 83	1139 ± 241	1052 ± 96	786 ± 209	1259 ± 407	2319 ± 420	2526 ± 410
total	180 ± 79a (1.4%)	35 ± 13b (0.3%)	3148 ± 798a (24%)	1497 ± 345b (12%)	5995 ± 2184 (46%)	5315 ± 899 (42%)	2947 ± 1784 (29%)	5693 ± 3169 (45%)	13040 ± 4064 (100%)	12540 ± 401 (100%)

[a] Data expressed as nmol ± standard error ($n = 8$). Figures in parentheses represent flavanone metabolites excreted as a percentage of the total excreted over the 0−24 h collection period. w/o, orange juice without yogurt; with, orange juice with yogurt. For flavanone metabolites and HPLC peaks refer to **Table 1** and **Figure 2**. For each collection period, values for total metabolites, with and without yogurt, that are significantly different are indicated by a different letter ($p < 0.07$, WSRT).

Quantitative Analysis of Flavanone Metabolites in Urine. Data on the excretion of flavanones in urine 0−2, 2−5, 5−10, and 10−24 h after the ingestion of the orange juice supplements are presented in **Table 3**. The main urinary metabolites were peak 7, a hesperetin-*O*-glucuronide, and the sulfo-glucuronyl conjugates of hesperetin (peak 5). Peak 8, a further hesperetin-*O*-glucuronide, was the next most abundant metabolite in urine. Hesperetin-7-*O*-glucuronide was the least abundant of the three hesperetin monoglucuronides. A substantial amount of peak 1, a hesperetin-*O*-diglucuronide, was also detected. The urinary plasma profile is, therefore, more complex than that of plasma, which contained only hesperetin-7-*O*-glucuronide and peak 7, a hesperetin-*O*-glucuronide, in detectable amounts.

Although the orange juice supplement contained 12 μmol of naringenin-7-*O*-rutinoside compared to 168 μmol of hesperetin-7-*O*-rutinoside, and no naringenin metabolites were detected in plasma, urine contained naringenin-4′-*O*-glucuronide and naringenin-7-*O*-glucuronide in similar quantities along with smaller amounts of the naringenin-*O*-diglucuronide (**Table 3**). The percentage of each of the urinary metabolites excreted with respect to flavanone intake is shown in **Table 4**.

Although there were no significant differences in plasma C_{max}, T_{max}, and AUC values (**Table 2**), there was a significantly lower 0−2 h ($p = 0.028$, WSRT) and 2−5 h ($p = 0.069$, WSRT) excretion of total flavanone metabolites in urine from volunteers who had consumed the orange juice with yogurt (**Table 3**). In contrast, between 10 and 24 h more flavanones were excreted by the subjects who consumed the orange juice with yogurt, but the difference was not significant. There was not a statistically significant difference in excretion of flavanones over the 0−24 h period, with 7.0 ± 2.3% of intake being with orange juice and 7.1 ± 2.3% when the juice was consumed with yogurt (**Table 4**). However, there were different recoveries for hesperetin and naringenin metabolites. The with and without yogurt recoveries of hesperetin metabolites were 6.3 ± 2.0 and 6.4 ± 2.0% of intake, respectively, whereas the comparable figures for naringenin metabolites were much higher at 17.7 ± 3.9 and 15.7 ± 3.4%.

Table 4. Quantities of Hesperetin-7-*O*-glucuronide and Other Putative Flavanone Metabolites in the Urine of Eight Human Subjects 0−24 h after the Consumption of 250 mL of Orange Juice with and without 150 mL of Yogurt[a]

	excretion as a percentage of intake	
flavanone metabolite (peak)	without yogurt	with yogurt
hesperetin-*O*-diglucuronide (1)	0.4 ± 0..3	0.4 ± 0.1
naringenin-*O*-diglucuronide (2)	0.9 ± 0.4	0.6 ± 0.3
naringenin-7-*O*-glucuronide (3)	8.5 ± 2.9	6.6 ± 2.7
naringenin-4′-*O*-glucuronide (4)	8.3 ± 3.3	8.6 ± 2.9
hesperetin-*O*-glucuronide-*O*-sulfates (5)	1.7 ± 0.4	1.6 ± 0.4
hesperetin-7-*O*-glucuronide (6)	0.9 ± 0.3	0.7 ± 0.3
hesperetin-*O*-glucuronide (7)	2.3 ± 0.3	2.2 ± 0.9
hesperetin-*O*-glucuronide (8)	1.4 ± 0.3	1.6 ± 0.3
% of hesperetin-7-*O*-rutinoside intake (1, 5−8)	6.3 ± 2.0	6.4 ± 2.0
% of naringenin-7-*O*-rutinoside intake (2−4)	17.7 ± 3.9	15.7 ± 3.4
% of total flavanone intake (all metabolites)	7.0 ± 2.3	7.1 ± 2.3

[a] Data expressed as flavanone metabolites excreted in urine over the 0−24 h collection period as a percentage of flavanone intake ± standard error ($n = 8$). For HPLC peaks refer to **Table 1** and **Figure 2**.

Effect of Yogurt on Gastric Emptying and Mouth to Cecum Transit Time. Drinking the orange juice with and without yogurt did not affect the half-time of gastric emptying, which was 1.8 ± 0.1 h in both instances. Yogurt, therefore, did not delay gastric emptying, nor did it affect significantly the mouth to cecum transit time, the time for the head of the test meal to reach the colon, with values of 2.1 ± 1.3 h for orange juice and 2.3 ± 1.0 h for orange juice plus yogurt being obtained.

DISCUSSION

In contrast to our previous study on the effect of cream on the absorption and metabolism of strawberry anthocyanins (*6*), the full-fat yogurt had little effect on the bioavailability of the orange juice flavanones. Nor did it have a significant effect on either gastric emptying or mouth to cecum transit time, both of

which were delayed in the strawberry study. This would appear to be related to the cream containing 48% fat compared to the 3.8% of the yogurt. The fat in the cream would stimulate both duodenal and ileal fat receptors and thereby inhibit gastric emptying and the mouth to cecum transit time of the head of the meal (*4, 5*). There was probably not enough fat in the yogurt to affect these processes. The initial significant delay in urinary excretion of flavanone metabolites when the orange juice was consumed with yogurt (**Table 3**) may be due to a slower delivery of the bulk of the meal, as opposed to the head, reaching the colon, in which case the yogurt may have stimulated the fat receptors in the ileum.

The plasma T_{max} values of the two hesperetin-*O*-glucuronides (**Table 2**), in keeping with those obtained in other studies (*18*−*22*), indicate absorption in the large rather than the small intestine. This implies that the disaccharide glycoside moiety of hesperetin-7-*O*-rutinoside is cleaved by colonic bacteria releasing hesperetin, which is acted upon by UDP-glucuronosyltransferses in the colonocytes, producing hesperetin-7-*O*-glucuronide and the unknown hesperetin-*O*-glucuronide, peak 7, and that the two isomers pass into the portal vein. These two hesperetin glucuronides were also excreted in urine, along with a third hesperetin-*O*-glucuronide and six additional metabolites, tentatively identified as two hesperetin-*O*-glucuronide-*O*-sulfates and two hesperetin-*O*-sulfate-*O*-glucuronides, a hesperetin-*O*-diglucuronide, a naringenin-*O*-diglucuronide, and tentatively identified naringenin-7-*O*-glucuronide and naringenin-4′-*O*-glucuronide (**Table 1**). Some of these metabolites, especially the sulfoglucuronides, were present in sizable quantities (**Table 3**). This demonstrates that the flavanone metabolites absorbed into the bloodstream undergo substantial further metabolism prior to urinary excretion, the main modifications being sulfation and further glucuronidation. The hesperetin-*O*-glucuronide-*O*-sulfates and the unassigned hesperetin-*O*-glucuronide (peak 8), in particular, accumulated in urine in substantial amounts (**Table 3**), but were not detected in the circulatory system. Arguably, this implies that the kidneys, rather than the liver, may be involved in much of the phase II metabolism. The medullary and cortical tissues of kidneys are known to contain UDP-glucuronosyltransferases (*33*), as are hepatic cells (*34*).

Like hesperetin-7-*O*-rutinoside, the flavonol quercetin-3-*O*-rutinoside is also absorbed in the large intestine but seemingly less efficiently than the flavanone. Jaganath et al. (*29*) fed tomato juice containing 176 μmol of quercetin-3-*O*-rutinoside to human volunteers and quercetin- and isorhamnetin-*O*-glucuronides appeared in plasma at a T_{max} of ca. 5.0 h but with a C_{max} of only 16 nmol, which is 57-fold lower than the C_{max} for the hesperetin glucuronides. These figures are reflected in the C_{max}/dose value of 0.1 for the quercetin metabolites compared with 5.1 for the hesperetin glucuronides (**Table 2**). This suggests that the hesperetin-7-*O*-rutinoside is converted to glucuronides in the large intestine more efficiently than quercetin-3-*O*-rutinoside, perhaps because it is less prone to degradation by colonic bacteria. The flavonol glucuronides may also be absorbed less effectively than the hesperetin glucuronides. Overall urinary excretion of hesperetin metabolites as a percentage of hesperetin-7-*O*-rutinoside intake with orange juice was 6.3% (**Table 4**) compared to 0.02% obtained with the excretion of quercetin-3-*O*-rutinoside metabolites following the ingestion of tomato juice (*29*). Thus, both the plasma and urine data indicate that hesperetin-7-*O*-rutinoside is much more bioavailable than quercetin-3-*O*-rutinoside.

In addition to 168 μmol of hesperetin-7-*O*-rutinoside, the ingested orange juice contained 12 μmol of naringenin-7-*O*-rutinoside. No naringenin metabolites were detected in plasma, but naringenin-7- *O*- and 4′-*O*-glucuronides, along with trace amounts of a naringenin-*O*-diglucuronide, did accumulate in urine (**Table 3**). Despite the presence of 7- and 4′-hydroxyl groups as potential sites for *O*-sulfation, no sulfated metabolites of naringenin were detected in urine, although *O*-glucuronide-sulfates were major urinary metabolites of hesperetin (**Table 3**). Arguably, this could be a consequence of the specificity of the hesperetin sulfuryltransferase. There is evidence, albeit indirect, that in vivo there is substantial specificity of human flavonoid sulfuryltransferases. Quercetin-3-*O*-rutinoside, like hesperetin-7-*O*-rutinoside, passes to the large intestine, where it undergoes cleavage through the action of colonic bacteria. The released aglycone, quercetin, appears in the bloodstream as quercetin-3-glucuronide and its methyl derivative, isorhamnetin-3-glucuronide, which are further metabolized to a variety of methylated diglucuronide and glucosyl products, which are excreted in urine (*29*). However, unlike the hesperetin glucuronides, the quercetin metabolites formed in the colon do not undergo sulfation. In contrast, the principal plasma quercetin metabolite derived from quercetin glucosides in the small intestine is quercetin-3′-sulfate (*28*), indicating the presence of sulfuryltranserase activity in the enterocyte rather than the liver.

A further point of difference in the fate of individual flavonoids occurring after ingestion is the extent to which phase II metabolism occurs. This appears to be independent of whether absorption occurs in the small or the large intestine. Consumption of onions containing quercetin-4′-glucoside and quercetin-3,4′-diglucoside is followed by absorption in the small intestine. The plasma and urinary profiles of quercetin metabolites are very different, indicating substantial postabsorption metabolism (*28*), arguably to an even greater extent than observed with the orange juice flavanones in the present study. In marked contrast, with other flavonoids there is little evidence of phase II metabolism as after the consumption of strawberries containing pelargonidin-3-glucoside, the human plasma and urinary metabolite profiles are very similar (*6*) and, likewise, after the ingestion of green tea rich in flavan-3-ol monomers (*30*).

The excretion of hesperetin metabolites corresponded to 6.3% of intake, whereas urinary naringenin metabolites were equivalent to ca. 17.7% of the amount ingested (**Table 4**), a trend that has been observed in some but not all flavanone feeding studies (*35*). Although the different levels of excretion relative to intake could be a reflection of a dose effect, and the substantially higher intake of the hesperetin conjugate, it is probably due to naringenin-7-*O*-rutinoside being more bioavailable than hesperetin-7-*O*-rutinoside. The differences in structure are that naringenin has a 4′-hydroxyl group, whereas hesperetin has 3′-hydroxyl and 4′-methoxy groups (see **Figure 1**). There is evidence with other flavonoids that the presence of substituent groups at the 3′- and 4′-positions can influence absorption. The 4′-hydroxylated anthocyanin pelargonidin-3-*O*-glucoside is absorbed much more readily than its 3′,4′-dihydroxy analogue cyanidin-3-*O*-glucoside (*6*), whereas kaempferol with a 4′-hydroxyl function is more bioavailable than quercetin, which has a 3′,4′-dihydroxy structure (*36, 37*). The exception to this trend is (−)-epicatechin, which has a 3′,4′-dihydroxy structure and is highly bioavailable with urinary excretion of metabolites corresponding to ca. 30% of intake. However, (−)-epigallocatechin, which has an additional hydroxyl group at the C-5′-position, is absorbed less efficiently, especially when ingested at higher doses (*30, 38*). It would be interesting to compare the bioavailability of the 4′-hydroxy flavan-3-ol (−)-epiafzelchin with that of (−)-epicatechin. However, there is no information

on the absorption of (−)-epiafzelchin, presumably because it is not known to be a major component in any food source.

In previous investigations into the bioavailability of citrus flavanones, plasma and urinary metabolites have been analyzed indirectly, as aglycones released by treating samples with a mollusc glucuronidase/sulfatase preparations (*18*−*22*). HPLC-SIM was used as the method of analysis in the present study because it provides detailed information on postabsorption flavanone metabolism that far exceeds anything produced by analysis of the aglycones after enzyme hydrolysis. Without standards of all the flavanone glucuronide and sulfoglucuronide metabolites, HPLC-SIM calibration curves for quantitative estimates were based on hesperetin-7-*O*-glucuronide. There is, therefore, a potential source of error in the SIM-based quantitative estimates, and there is a view that quantitative estimates based on enzyme hydrolysis are, therefore, much more accurate. We do not share this opinion. The glucuronidase/sulfatase preparations contain a mixture of enzyme activities, and there can be substantial batch-to-batch variation in their specificity (*39*). There are no reports of flavonoid bioavailability studies using glucuronidase/sulfatase preparations in which information on the identity, number, and quantity of the individual sulfate and glucuronide conjugates in the samples of interest has been obtained. As a consequence, there are no direct data on the efficiency with which the enzymes hydrolyze the individual metabolites and release the aglycone. This introduces a varying, unmeasured error factor. The accuracy of quantitative estimates based on the use of glucuronidase/sulfatase preparations is, therefore, probably no better, and possibly much worse, than those based on HPLC-SIM. The fact that enzyme hydrolysis results in very reproducible data is irrelevant as reproducibility is a measure of precision, although it is frequently mistaken for accuracy (*40*). These shortcomings of analyses based on enzyme hydrolysis apply not just to flavanones but to bioavailability studies with all dietary flavonoids and, in this context, it is interesting to note that the use of enzyme hydrolysis results in the underestimation of isoflavone metabolites in rat tissues (*41*).

In conclusion, there is substantial metabolism of orange juice flavanones in the gastrointestinal tract, most notably the large intestine, where colonic bacteria play a major role. There was little impact of yogurt on the postingestion fate of the orange juice flavanones, in contrast to the affect of cream on the absorption of strawberry anthocyanins (*6*) and, arguably, the influence of milk on tea and chocolate flavan-3-ol bioavailability (*8*−*16*). There is a need for detailed investigation of the complexities of food matrix effects as they will almost certainly have a substantial bearing on the absorption of flavonoids when they are consumed as part of a real meal as opposed to the ingestion of a single food item on an empty stomach.

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