Orange juice (poly)phenols are highly bioavailable in humans$^{1-3}$

Gema Pereira-Caro, Gina Borges, Justin van der Hooft, Michael N Clifford, Daniele Del Rio, Michael EJ Lean, Susan A Roberts, Michele B Kellerhals, and Alan Crozier

ABSTRACT

**Background:** We assessed the bioavailability of orange juice (poly)phenols by monitoring urinary flavanone metabolites and ring fission catabolites produced by the action of the colonic microbiota.

**Objective:** Our objective was to identify and quantify metabolites and catabolites excreted in urine 0–24 h after the acute ingestion of a (poly)phenol-rich orange juice by 12 volunteers.

**Design:** Twelve volunteers [6 men and 6 women; body mass index (in kg/m²): 23.9–37.2] consumed a low (poly)phenol diet for 2 d before first drinking 250 mL pulp-enriched orange juice, which contained 584 µmol (poly)phenols of which 537 µmol were flavanones, and after a 2-wk washout, the procedure was repeated, and a placebo drink was consumed. Urine collected for a 24-h period was analyzed qualitatively and quantitatively by using high-performance liquid chromatography–mass spectrometry (HPLC-MS) and gas chromatography–mass spectrometry (GC-MS).

**Results:** A total of 14 metabolites were identified and quantified in urine by using HPLC-MS after orange juice intake. Hesperetin-O-glucuronides, naringenin-O-glucuronides, and hesperetin-3′-O-sulfate were the main metabolites. The overall urinary excretion of flavanone metabolites corresponded to 16% of the intake of 584 µmol (poly)phenols. The GC-MS analysis revealed that 8 urinary catabolites were also excreted in significantly higher quantities after orange juice consumption. These catabolites were 3-(3′′-methoxy-4′′-hydroxyphenyl)propionic acid, 3-(3′′-hydroxy-4′′-methoxyphenyl)propionic acid, 3-(3′′-hydroxy-4′′-methoxyphenyl)hydroxycrylic acid, 3-(3′′-hydroxyphenyl)hydrazoylacetic acid, 3′′-methoxy-4′′-hydroxyphenylacetic acid, hippuric acid, 3′′-hydroxyhippuric acid, and 4′′-hydroxyhippuric acid. These aromatic acids originated from the colonic microbiota-mediated breakdown of orange juice (poly)phenols and were excreted in amounts equivalent to 88% of (poly)phenol intake. When combined with the 16% excretion of metabolites, this percentage raised the overall urinary excretion to ~100% of intake.

**Conclusions:** When colon-derived phenolic catabolites are included with flavanone glucuronide and sulfate metabolites, orange juice (poly)phenols are much more bioavailable than previously envisaged. In vitro and ex vivo studies on mechanisms underlying the potential protective effects of orange juice consumption should use in vivo metabolites and catabolites detected in this investigation at physiologic concentrations. The trial was registered at BioMed Central Ltd (www.controlledtrials.com) as ISRCTN04271658. Am J Clin Nutr 2014;100:1378–84.

INTRODUCTION

Citrus fruits are rich source of flavanones, which are a subgroup of flavonoids, and oranges (*Citrus sinensis*) and their derivatives such as orange juice contain mainly hesperetin-7-O-rutinoside (hesperedin) and naringenin-7-O-rutinoside (narinrutin). Prospective studies have revealed that the consumption of either oranges or orange juice is associated with reduced risk (19%) of ischemic stroke and acute coronary events in women (1) with an improvement of vascular function (2). In addition, a reduction of plasma lipid peroxidation has been observed in healthy women (3). These effects have been attributed mainly to the flavanone content of oranges. It has been reported that hesperetin and naringenin intakes reduce risk of cerebrovascular disease and asthma (4). Furthermore, the consumption of hesperetin-7-O-rutinoside for a period of 3 wk by subjects with metabolic syndrome has been shown to improve endothelial function, reduce circulating biomarkers of inflammation, and favorably alter lipid profiles (5).

Animal and in vitro studies have shown that hesperetin and naringenin exhibit a wide range of biological activities, such as antioxidant, anti-inflammatory (6), and antitumor activities (7, 8) and also are able to reduce plasma markers of endothelial dysfunction in high-cholesterol–fed rats (9), atherosclerosis plaque progression in high cholesterol-fed rabbits (10) and mice (11), and improved insulin sensitivity in diabetic rat models (12, 13).

In order for orange flavanones to exert their health effects in vivo, it is essential that they are bioavailable and absorbed from the gastrointestinal tract into the circulatory system. Human bioavailability studies have shown that flavanones are absorbed...
as glucuronide and sulfate conjugates in amounts corresponding to only 5–15% of intake (14–17). Consumption studies with ileostomists who consumed a (poly)phenol-rich drink that contained 45.3 μmol hesperetin-3-O-rutinoside, detected 3 hesperetin-7-O-glucuronides, and a hesperetin-O-glucuronide-O-sulfate in urine, with 0–24 h excretion that amounted to 1.6 μmol, which is equivalent to 3.5% of intake (18). This amount is much less that the 11.9% of intake of hesperetin metabolites excreted by healthy volunteers with a functioning colon after drinking the same (poly)phenol-rich beverage (15). These figures indicate ～30% of hesperetin metabolites are absorbed in the small intestine and the remaining ～70% are absorbed in the colon. Plasma pharmacokinetic profiles of 2 hesperetin-O-glucuronides, one of which is hesperetin-7-O-glucuronide, obtained in healthy volunteers, exhibited a high nanomole per liter peak plasma concentration 3.7–4.4 h after intake in keeping with this proposal (14, 15).

Flavanones that reach the large intestine as well as being absorbed as sulfate and glucuronide metabolites are also broken down by the colonic microbiota that produce an array of phenolic catabolites (19). These catabolites are absorbed into the circulatory system before renal excretion and, as such, are an integral part of the overall bioavailability equation of dietary flavanones and may play a role in the protective effects of dietary flavanones (20, 21).

Building on existing research findings and by using a pulp-enriched orange juice, the current study investigated the urinary excretion of flavanone metabolites and their colonic microflora-mediated breakdown products. The study focused on the cumulative excretion of these compounds in urine because it provides a more-accurate quantitative assessment of absorption than do plasma pharmacokinetics. Although plasma profiles can yield useful information, they are not an accurate quantitative guide of absorption because the presence of flavonoid metabolites and catabolites in the circulatory system is transient as they are treated as xenobiotics and are rapidly removed from the bloodstream via renal excretion (21).

SUBJECTS AND METHODS

Chemicals and materials

Methanol and ethyl acetate were obtained from Rathburn Chemicals. Formic acid, N-methyl-N-(trimethylsilyl) trifluoroacetamide, pyridine, 2′,4′,5′-trimethoxy-cinnamic acid, 4-hydroxybenzoic acid, ferulic acid, 4′-hydroxyphenylacetic acid, 3′-methoxy-4′-hydroxyphenylacetic acid, hippuric acid, 3-(3′-methoxy-4′-hydroxyphenyl)propionic acid, and 3-(3′-hydroxy-4′-methoxyphenyl)propionic acid were purchased from Sigma-Aldrich. 4′-Hydroxyhippuric acid was obtained from Bachem (UK) Ltd. Hesperetin-7-O-rutinoside (hesperedin), 4′-O-methyl-naringenin-7-O-rutinoside (didymin), and naringenin-7-O-rutinoside (narirutin) were acquired from Extrasyntheses. 3-(3′-Hydroxyphenyl) hydrazinoc acid, hesperetin-7-O-glucuronide, naringenin-4′-O-glucuronide, and naringenin-7-O-glucuronide were purchased from Toronto Research Chemicals Inc. Deuterated methanol (99.8% pure) was obtained from Goss Scientific Instruments Ltd. HPLC-grade methanol was purchased from Fisher Scientific.

The study was approved by the University of Glasgow Royal Infrmary Ethics Committee (project 2011017) and registered with BioMed Central Ltd (http://www.controlled-trials.com; ISRCTN04271658). Twelve healthy volunteers (6 men and 6 women) between 23 and 60 y old and with BMI (in kg/m²) from 23.9 to 37.2 were recruited. Volunteers were nonsmokers and not taking medication. All subjects were informed about the purpose of the study and gave written informed consent before their inclusion in the trial. Volunteers were required to follow a diet low in (poly)phenolic compounds for 48 h before the study and 24 h after supplementation and avoid fruit, vegetables, high-fiber products, and beverages such as tea, coffee, fruit juice, and wine. On the day of consumption, after an overnight fast, each subject drank 250 mL pulp-enriched orange juice. A light breakfast that consisted of white bread, cheese, ham, and milk was provided 2 h after orange juice intake. After a washout period of 2 wk, volunteers ingested 250 mL placebo drink. After supplementation, all urine was collected over 5 time periods (0, >0–2, >2–5, >5–10, and >10–24 h). The volume of urine excreted was measured, and aliquots were stored at −80 °C before analysis by using high-performance liquid chromatography–photodiode array–mass spectrometry and gas chromatography–mass spectrometry (GC-MS).

Extraction of orange juice

The pulp-enriched orange juice and placebo drink used in the feeding study were extracted by using the following procedure. Briefly, 5-mL aliquots of juice, which were previously homogenized by using an Ultraturrax homogenizer, were extracted twice with 5 mL methanol for 2 min and centrifuged at 2800 × g for 15 min at 4 °C. The pellet was extracted in the same manner with 2 mL methanol. The 2 supernatant fluids were pooled and reduced to dryness in vacuo, redissolved in 6 mL 50% aqueous methanol, and stored at −80 °C before analysis.

Analysis of orange juice and urine by using HPLC with photodiode array and MS detection

Samples were analyzed by using a Surveyor HPLC system comprised of an HPLC pump, a photodiode array detector scanning from 200 to 600 nm and an autosampler operating at 4 °C (Thermo Electron Corp). Reverse-phase separations were carried out by using a 150 × 4.6-mm inside diameter 5-μm 10-A C18 Kinetex column (Phenomenex) maintained at 40 °C and eluted at a flow rate of 1.0 mL/min with a 1) 60-min gradient of 5–50% methanol in 0.1% aqueous formic acid for juice extracts and 2) 25-min gradient of 15–45% methanol in 0.1% aqueous formic acid for urine samples. After passing through the flow cell of the photodiode array detector, the column eluate was split, and 0.2 mL/min was directed to an LCQ Advantage ion-trap mass spectrometer fitted with an electrospray interface operating in negative-ionization mode (Thermo Electron Corp). The capillary temperature was 300 °C, heath gas was 60 units, auxiliary gas was 40 units, and source voltage was 3 kv. Identifications

Abbreviations used: GC-MS, gas chromatography–mass spectrometry; HPLC-MS, HPLC–mass spectrometry; MS, mass spectrometry.
were based on full-scan, data-dependent MS scanning from 100 to 800 \( m/z \), MS-MS fragmentation, and comparisons of the retention time and absorption \( \lambda_{\text{max}} \) with available standards.

(Poly)phenols in orange juice were quantified on the basis of chromatographic peak areas acquired at 290 nm and expressed relative to standards of hesperetin-7-O-rutinoside, naringenin-7-O-rutinoside, 4'-O-methyl-naringenin-7-O-rutinoside, and ferulic acid. Hesperetin-O-glucosyl-O-rutinoside was quantified as hesperetin-7-O-rutinoside equivalents, and naringenin-O-glucosyl-O-rutinoside and eriodictyol-7-O-rutinoside (ericroitin) were quantified as naringenin-7-O-rutinoside equivalents. The quantification of metabolites in urine was carried out by using HPLC–mass spectrometry (HPLC-MS) in selected ion monitoring mode by reference to ferulic acid, hesperetin-7-O-glucuronide, naringenin-4'-O-glucuronide, or naringenin-7-O-glucuronide calibration curves. Remaining metabolites, for which authentic standards were not available, were quantified in hesperetin-7-O-rutinoside and naringenin-7-O-rutinoside equivalents.

All calibration curves were prepared from reference compounds dissolved in methanol at concentrations ranging from 0.1 to 100 ng/\( \mu \)L. A linear response was obtained for all the standard curves as checked by using a linear regression analysis (\( R^2 > 0.999 \)). Limits of detection (range: 0.1–0.9 ng), limits of quantification (1.7–3.0 ng), and the precision of the assay (as the coefficient of intraassay variation, which ranged from 2.8% to 5.0%) were considered acceptable allowing the quantification of flavonoids and their metabolites.

### Analysis of urine by gas chromatography with MS detection

Urine samples were prepared as described by Roowi et al (19) with some modifications. Briefly, 1 mL of urine sample was added to 3 mL 0.2 mol/L HCl that contained 60 \( \mu \)g of 2',4',5'-trimethoxycinnamic acid as an internal standard. Styrene divinylbenzene solid-phase extraction cartridges (Strata SDB-L 200 mg; Phenomenex.) were used for sample purification. Before loading with the acidified urine samples, the cartridge was activated with 3 mL ethyl acetate and conditioned with 3 mL methanol followed by 3 mL 0.1 mol/L HCl. The acidified sample was added, the cartridge was washed with 3 mL 0.1 mol/L HCl, and the cartridge was dried with nitrogen for 20 min after which phenolic catabolites were eluted with 3 mL ethyl acetate. The ethyl acetate eluate was transferred to an amber glass vial and reduced to dryness under a stream of nitrogen gas at 35 \( ^\circ \)C. The dried extract was silylated by using 300 \( \mu \)L of a mix of pyridine and \( N \)-methyl-(\( N \)-trimethylsilyl) trifluoraceticamide (1:4, vol:vol) heated at 80 \( ^\circ \)C for 20 min on a heating block. Samples were cooled in vacuo in a desiccator before analysis by using GC-MS.

Derivatized phenolic catabolites in urine were analyzed on a 6890 gas chromatograph equipped with a 7683S autosampler, a 5973 mass spectrometer (Agilent Technologies), and a ZB-5MS Zebron 30 m \( \times \) 0.25 mm \( \times \) 0.25-\( \mu \)m (inside diameter) capillary column (Phenomenex) with helium as a carrier gas (1.2 mL/min). GC-MS conditions were as follows: injection volume (1 \( \mu \)L), split ratio (1:25), initial temperature of 40\( ^\circ \)C and raised to 160\( ^\circ \)C at 20\( ^\circ \)C/min, 200\( ^\circ \)C at 1.5\( ^\circ \)C/min, and 250\( ^\circ \)C at 10\( ^\circ \)C/min, and a final temperature of 300\( ^\circ \)C at 40\( ^\circ \)C/min held for 5 min. The injector temperature was maintained at 220\( ^\circ \)C, MS transfer line was 150\( ^\circ \), and ion source was 230\( ^\circ \)C. Mass spectra were scanned at \( m/z \) 50–470 with an ionization energy of 70 eV. Phenolic compounds were identified according to their retention time and mass spectra of authentic standards. When standards were not commercially available, an identification was achieved through the integrated National Institute of Standards and Technology mass spectral library, with confidence \( \geq 95 \% \). Quantification was based on calibration curves of the ratio between the target ion (\( m/z \)) of the standard of interest and the target ion of the 2',4',5'-trimethoxycinnamic acid internal standard.

### Results

#### Statistical analysis

Results are expressed as means \( \pm \) SEMs of 2 measurements obtained from urine samples from 12 volunteers. Data were assessed for homogeneity of variances by using Levene’s test and subjected to a nonparametric 1-factor analysis of variance (Friedman’s test for related samples) with the SPSS Statistics program (version 22; IBM). Differences were considered to be significant at \( P < 0.05 \).

### RESULTS

#### Identification and quantification of (poly)phenols in orange juice

The HPLC-MS identified 8 compounds (see Supplemental Table 1 under “Supplemental data” in the online issue) in quantifiable amounts in the pulp-enriched orange juice. These compounds were flavanones naringenin-7-O-rutinoside (114 \( \mu \)mol/250 mL), hesperetin-7-O-rutinoside (329 \( \mu \)mol), 4'-O-methyl-naringenin-7-O-rutinoside (also known as isosakuranetin-7-O-rutinoside and didymin) (19 \( \mu \)mol), naringenin-7-O-rutinoside-4'-O-glucoside (51 \( \mu \)mol) hesperetin-7-O-rutinoside-3'-O-glucoside (19 \( \mu \)mol), and eriodictyol-7-O-rutinoside (5 \( \mu \)mol) along with the flavone apigenin-6,8-C-diglucoside (vicenin-2) (42 \( \mu \)mol) and hydroxycinnamate ferulic acid-4'-O-glucoside (5 \( \mu \)mol) (see Supplemental Figure 1 under “Supplemental data” in the online issue for structures). In total, this amounted to 584 \( \mu \)mol/250 mL of the 8 (poly)phenolic compounds of which 537 \( \mu \)mol/250 mL were flavanones. The placebo drink ingested by volunteers did not contain detectable quantities of any of the (poly)phenols that were present in the pulp-enriched orange juice.

#### Identification of flavanone metabolites in urine

After acute intake of 250 mL orange juice, 14 flavanone metabolites were identified or partially identified by an HPLC-MS analysis of urine collected from 12 volunteers over a 24-h post-ingestion period. See Supplemental Figure 2 and Supplemental Table 2 under “Supplemental data” in the online issue for a summary of the basis of identifications. Identified flavanone metabolites were hesperetin-3'-O- and 7-O-glucuronides, hesperetin-3',7',O-, 5,7-O- and 3',5-O-diglucuronides, hesperetin-3'-O-sulfate, a hesperetin-5'-glucuronide-3'-O-sulfate, naringenin-4'-O- and 7-O-glucuronides, naringenin-4',5'-O-, 5,7-O- and 4',7'-O-diglucuronides, and an eriodictyol-5'-sulfate (see Supplemental Figure 3 under “Supplemental data” in the online issue for structures of full identified compounds).
Quantification of flavanone and flavone metabolites in urine

Quantitative data on the excretion of flavanones metabolites in urine 0–24 h after orange juice intake are presented in Table 1. No metabolites were detected in urine collected over a 12-h period before ingestion of the juice. Likewise, no metabolites were detected in the 0–24-h urine of any of the volunteers after the consumption of the placebo drink.

Hesperetin-O-glucuronides were the main flavanone metabolites to appear in urine after orange juice consumption followed by naringenin-O-glucuronides and hesperetin-3′-O-sulfate. Hesperetin-O-diglucuronides and hesperetin-O-sulfate-O-glucuronide were excreted in lower amounts. Small quantities of naringenin-O-diglucuronides, hesperetin-O-sulfate-O-glucoside, and eriodictyol-O-sulfate were also quantified (Table 1). There was a 61 ± 7 μmol excretion of hesperetin metabolites. A total of 21 ± 2 μmol naringenin metabolites was excreted. The sole eriodictyol metabolite, which is an eriodictyol-O-sulfate, was excreted in much lower amounts at 0.26 ± 0.15 μmol.

There was no excretion of metabolites of 4′-O-methyl-naringenin-7-O-rutinoside. Likewise, no urinary metabolites of the ingested 42 μmol of the flavone apigenin-6,8-C-diglucoside were detected, which was anticipated in view of the extremely low bioavailability of C-linked flavanone and dihydrochalcone glycosides noted in feeding studies with rooibos teas (22).

Urinary excretion of phenolic catabolites after orange juice intake

Urine collected 12 h before and 0–2, >2–5, >5–10, and >10–24 h after the ingestion of 250 mL orange juice or placebo drink by volunteers was analyzed by using GC-MS, which identified 10 phenolic catabolites (see Supplemental Figure 4 and Table 3 under “Supplemental data” in the online issue).

Quantitative data on the average level of excretion of catabolites are presented in Table 2. There was minimal excretion in the initial 0–2-h collection period. In samples collected thereafter, 4 compounds [3-(3′-hydroxy-4′-methoxyphenyl)hydroxycarbonylic acid, 3-(3′-hydroxy-4′-methoxyphenyl)propionic acid (dihydroferulic acid), 3-(3′-methoxy-4′-hydroxyphenyl)propionic acid (dihydroferulic acid), and 3′-hydroxyhippuric acid] appeared in urine after orange juice consumption but not after intake of the placebo drink. 3-(3′-hydroxyphenyl)hydroxycarboxylic acid, 4-hydroxybenzoic acid, 3-methoxy-4-hydroxyphenylacetic acid, 4-hydroxyphenylactic acid, hippuric acid, and 4′-hydroxyhippuric acids were detected in urine excreted after the ingestion of both drinks. However, the excretion of 3-(3′-hydroxyphenyl)hydroxycarbonylic acid, 3-methoxy-4-hydroxyphenylacetic acid, hippuric acid, and 4′-hydroxyhippuric acid was significantly higher after orange juice intake (Table 2) (see Supplemental Figure 5 under “Supplemental data” in the online issue for structures). These results indicated that these compounds, together with those that appeared only after orange juice intake, were derived directly (or indirectly in the case of the hippuric acids) from colonic microflora-mediated flavanone catabolism. Note that 3′-(3′-hydroxy-4′-methoxyphenyl)propionic acid, which is likely to be a breakdown product of hesperetin, was detected, for the first time to our knowledge, in human urine after orange juice intake.

Note that, in the absence of a reference compound, the identification of 3-(3′-hydroxy-4′-methoxyphenyl)hydroxycarbonylic acid after purification by HPLC was confirmed by the nuclear mass resonance spectroscopy analysis (see Supplemental Figure 6 under “Supplemental data” in the online issue for an explanation). The purified sample was subsequently used for the quantifications of 3-(3′-hydroxy-4′-methoxyphenyl)hydroxycarbonylic acid by using GC-MS.

The 0–24-h excretion of phenolic catabolites after the ingestion of orange juice and the placebo drink is summarized in Table 3. 3-(3′-Hydroxy-4′-methoxyphenyl)hydroxycarbonylic acid is a key indicator of orange juice consumption because it was excreted in substantial amounts (72 μmol) but was absent in urine collected after the consumption of the placebo. The 2 propionic acids and 3′-hydroxyhippuric acid were similarly absent in placebo urine and accumulated after orange juice intake but to a much lesser extent than was 3-(3′-hydroxy-4′-methoxyphenyl)hydroxycarbonylic acid. Although present in trace amounts after intake of the placebo, orange juice consumption was associated with...

### Table 1

Quantities of flavanone metabolites excreted in urine 0–24 h after consumption of 250 mL orange juice containing glycosides of hesperetin (348 μmol), naringenin (165 μmol), and eriodyctiol (5 μmol)

<table>
<thead>
<tr>
<th>Metabolites (no. of isomers)</th>
<th>0–2 h</th>
<th>&gt;2–5 h</th>
<th>&gt;5–10 h</th>
<th>&gt;10–24 h</th>
<th>Total excreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol</td>
<td>μmol</td>
<td>μmol</td>
<td>μmol</td>
<td>μmol</td>
</tr>
<tr>
<td>Hesperetin-O-diglucuronide (3)</td>
<td>ND²</td>
<td>1.9 ± 0.8</td>
<td>4.9 ± 1.5</td>
<td>3.1 ± 1.1</td>
<td>9.9 ± 3.4</td>
</tr>
<tr>
<td>Hesperetin-O-sulfate-O-glucuronide</td>
<td>ND</td>
<td>2.2 ± 0.6</td>
<td>4.4 ± 1.6</td>
<td>1.1 ± 0.4</td>
<td>7.7 ± 2.6</td>
</tr>
<tr>
<td>Hesperetin-O-sulfate-O-glucoside</td>
<td>ND</td>
<td>0.4 ± 0.1</td>
<td>0.9 ± 0.4</td>
<td>0.3 ± 0.2</td>
<td>1.6 ± 0.7</td>
</tr>
<tr>
<td>Hesperetin-7-O-glucuronide</td>
<td>0.02 ± 0.01</td>
<td>2.2 ± 0.8</td>
<td>2.0 ± 0.7</td>
<td>0.5 ± 0.1</td>
<td>4.7 ± 1.6</td>
</tr>
<tr>
<td>Hesperetin-3′-O-glucuronide</td>
<td>0.05 ± 0.02</td>
<td>6.6 ± 2.1</td>
<td>9.5 ± 3.2</td>
<td>2.9 ± 1.2</td>
<td>19.0 ± 6.5</td>
</tr>
<tr>
<td>Hesperetin-3′-O-sulfate</td>
<td>0.06 ± 0.02</td>
<td>6.6 ± 2.0</td>
<td>9.3 ± 4.5</td>
<td>2.2 ± 0.8</td>
<td>18.2 ± 7.3</td>
</tr>
<tr>
<td>Total hesperetin metabolites</td>
<td>0.13 ± 0.03</td>
<td>19.9 ± 3.2</td>
<td>31.0 ± 6.0</td>
<td>10.0 ± 1.9</td>
<td>61 ± 7 (17.5)</td>
</tr>
<tr>
<td>Naringenin-O-diglucuronide (3)</td>
<td>ND</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>2.5 ± 0.7</td>
</tr>
<tr>
<td>Naringenin-4′-O-glucuronide</td>
<td>0.3 ± 0.1</td>
<td>5.3 ± 1.5</td>
<td>2.9 ± 0.6</td>
<td>1.2 ± 0.6</td>
<td>9.7 ± 2.8</td>
</tr>
<tr>
<td>Naringenin-7-O-glucuronide</td>
<td>0.2 ± 0.1</td>
<td>4.7 ± 0.5</td>
<td>2.9 ± 0.8</td>
<td>1.4 ± 0.6</td>
<td>9.2 ± 2.1</td>
</tr>
<tr>
<td>Total naringenin metabolites</td>
<td>0.5 ± 0.1</td>
<td>10.8 ± 1.6</td>
<td>6.9 ± 1.0</td>
<td>3.2 ± 0.9</td>
<td>21 ± 2 (12.7)</td>
</tr>
<tr>
<td>Eriodyctiol-O-sulfate</td>
<td>ND</td>
<td>0.13 ± 0.04</td>
<td>0.1 ± 0.1</td>
<td>0.03 ± 0.01</td>
<td>0.26 ± 0.15 (5.2)</td>
</tr>
<tr>
<td>Total eriodyctiol metabolites</td>
<td>ND</td>
<td>0.13 ± 0.04</td>
<td>0.1 ± 0.1</td>
<td>0.03 ± 0.01</td>
<td>0.26 ± 0.15 (5.2)</td>
</tr>
<tr>
<td>Total metabolites</td>
<td>0.6 ± 0.1</td>
<td>31 ± 4</td>
<td>38 ± 6</td>
<td>13 ± 2</td>
<td>83 ± 10 (16.0)</td>
</tr>
</tbody>
</table>

² All values are means ± SEs; excretions as percentages of intakes are shown in parentheses. n = 12.

ND, not detected.
Hippuric acid OJ 37
3-(3′-Hydroxy-4′-methoxyphenyl)hydracrylic acid OJ 0.1
6
3-(3′-Hydroxyphenyl)hydracrylic acid OJ 0.1
6
3-(3′-Hydroxy-4′-methoxyphenyl)propionic acid OJ 0.7
6
3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid OJ ND 1.1
6
3′-Methoxy-4′-hydroxyphenylacetic acid OJ ND 1.1
6
4′-Hydroxyphenylacetic acid OJ 0.1
6
232
10–24 h after orange juice administration. The baseline excretion of hippuric acid was high at 232.

Table 4 their excretion after ingestion of the placebo drink intake in increased amounts after orange juice intake were compared with hippuric acid, that were excreted in urine in significantly in-

to 12 volunteers which presumably was a reflection of the varying microflora of vol-

crease in excretion ranged from 4 to 11 of 12 volunteers which presumably was a reflection of the varying microflora of vol-
unteers (Table 3).

The overall amounts of key phenolic catabolites, excluding hippuric acid, that were excreted in urine in significantly increased amounts after orange juice intake were compared with their excretion after ingestion of the placebo drink intake in Table 4. Most excretion was observed between >5–10 and >10–24 h after orange juice administration.

### DISCUSSION

In the feeding study by Borges et al (18) in which ileostomists ingested a (poly)phenol-rich drink, the analysis of ileal fluid indicated that, in healthy volunteers with a functioning colon, substantial amounts of hesperetin-7-O-rutinoside passed from small to large intestines where, after cleavage of the rutinose moiety, the hesperitin would be subjected to phase II metabolism and absorbed into the portal vein as sulfated and glucuronide conjugates. In addition, a portion of the hesperetin undergoes colonic microbiota-mediated ring fission and yields low molecular weight phenolic compounds, which also enter the circulatory system, where some of the compounds may undergo additional metabolism in the liver and kidney before renal excretion.

### TABLE 3

<table>
<thead>
<tr>
<th>Phenolic and aromatic acids</th>
<th>Placebo drink</th>
<th>Orange juice</th>
<th>No. of volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-(3′-Hydroxy-4′-methoxyphenyl)hydracrylic acid</td>
<td>ND</td>
<td>72 ± 10^4</td>
<td>9 of 12</td>
</tr>
<tr>
<td>3-(3′-Hydroxyphenyl)hydracrylic acid</td>
<td>2.5 ± 0.8</td>
<td>31 ± 4^4</td>
<td>8 of 12</td>
</tr>
<tr>
<td>3-(3′-Hydroxy-4′-methoxyphenyl)propionic acid</td>
<td>ND</td>
<td>4.7 ± 0.8^4</td>
<td>8 of 12</td>
</tr>
<tr>
<td>3-(3′-Methoxy-4′-hydroxyphenyl)propionic acid</td>
<td>ND</td>
<td>6.3 ± 1.0^4</td>
<td>6 of 12</td>
</tr>
<tr>
<td>3′-Methoxy-4′-hydroxyphenylacetic acid</td>
<td>4.7 ± 0.7</td>
<td>7.3 ± 0.8^4</td>
<td>6 of 12</td>
</tr>
<tr>
<td>4′-Hydroxyphenylacetic acid</td>
<td>30 ± 4</td>
<td>41 ± 4</td>
<td>4 of 12</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>3.1 ± 0.6</td>
<td>3.6 ± 0.4</td>
<td>4 of 12</td>
</tr>
<tr>
<td>Hippuric acid</td>
<td>232 ± 21</td>
<td>610 ± 48^4</td>
<td>11 of 12</td>
</tr>
<tr>
<td>3′-Hydroxyhippuric acid</td>
<td>ND</td>
<td>0.8 ± 0.3^4</td>
<td>9 of 12</td>
</tr>
<tr>
<td>4′-Hydroxyhippuric acid</td>
<td>0.2 ± 0.1</td>
<td>19 ± 3^4</td>
<td>11 of 12</td>
</tr>
</tbody>
</table>

1 All values are means ± SEMs. n = 12.
2 No. of volunteers with a significant increase.
3 ND, not detected.
4 Values that increased significantly after orange juice supplementation (P < 0.05) were obtained by using Friedman’s test.
In the current study, the excretion of hesperetin and narigenin glucuronide and sulfate metabolites occurred mainly 2–10 h after ingestion of orange juice (Table 1), which was in keeping with earlier findings that flavanones are absorbed in both the small and large intestines (15, 18). The excretion of hesperitin metabolites corresponded to 17.5% of the total hesperetin intake, whereas narigenin metabolites were excreted in amounts equivalent to 12.7% of ingested narigenin. The excretion of eriodictyol-O-sulfate was 5.2% of eriodictyol-7-O-rutinoside intake. This result indicated that eriodictyol with a 3',4'-dihydroxy B ring was absorbed less effectively than the 4'-monohydroxylated narigenin. This effect is similar to that of anthocyanins whereby the pelargonidin is absorbed more readily than corresponding cyanidin glycosides (14, 23, 24). The amount of excretion of hesperitin metabolites compared with narigenin indicated that the methylation of 4'-hydroxyl group may have slightly enhanced absorption. The combined excretion of flavanones metabolites at 83 ± 10 μmol was equivalent to 16% of intake (Table 1).

The overall amounts of phenolic catabolites, excluding hippurinic acid, excreted in urine in significantly increased amounts after orange juice intake are compared with their excretion after ingestion of the placebo drink intake in Table 4. Note that, over the 24-h collection period, the amount of these phenolic catabolites excreted after drinking the placebo was 5.6 μmol, and with the consumption of orange juice, this amount increased by 135 to 141 μmol, which corresponded to 23% of (poly)phenolic intake. The addition of the 135 μmol to the 83 μmol hesperetin and narigenin glucuronide and sulfate metabolites made a total of 37% of the 584 μmol (poly)phenol intake.

The urinary amount of hippuric acid was not included in the calculated 37% recovery. The quantity of hippuric acid excreted in 0–24-h urine was 232 μmol after the consumption of the placebo compared with 610 μmol after the ingestion of the orange juice. The 2 figures were significantly different (P < 0.05) (Table 3). Exactly how much of the 378-μmol increase in hippuric acid (Table 3), which is primarily of hepatic origin being formed from glycination of benzoic acid (25), was derived from the orange juice (poly)phenols was difficult to ascertain because of the high person-to-person variation in amounts produced coupled with the high baseline amounts, which were a consequence of hippuric acid also being derived from other compounds in the body unrelated to orange juice intake (25–28). If hippuric acid was included in the calculation, the overall flavone and flavanone recovery increased from 37% to ~100%. Although high, this was clearly an approximation and a more-accurate estimate is unlikely to be obtained until feeds with its main potential precursor in the juice (ie, narigenin) are carried out with the flavanone labeled with either 13C or 14C in A and B rings. This method would enable an HPLC-MS analysis to distinguish between hippuric acid derived from narigenin and that produced by other unrelated pathways.

When only urinary flavanone glucuronide and sulfate metabolites are analyzed, flavanones, like most other dietary flavonoids, have only a low bioavailability. This report establishes that, when colon-derived phenolic and aromatic acids are included in the calculation, it is evident that flavanones are highly bioavailable with a recovery >100% of the ingested bolus as urinary metabolites and catabolites. This result is in keeping with data obtained in a human feeding study with [13C5]cyanidin-3-O-glucoside (29). Anthocyanins are considered to be very poorly absorbed with typically <1% of intake being excreted in urine as the parent compound or glucuronide and sulfate metabolites degradation. However, in the study with [13C5]cyanidin-3-O-glucoside, which took into account metabolites and colonic catabolites as well as 13CO2 output and fecal products, the <1% recovery increased to ~50%, which showed that cyanidin-3-O-glucoside is also subject to extensive metabolism and catabolism and was absorbed much-more extensively than previously envisaged (29).

An additional point of importance is that in vitro studies with cell-based test systems intended to investigate mechanisms by which flavonoids and related compounds might exert protective effects should not use parent dietary compounds but, instead, should use their metabolites and catabolites at physiologic doses that correspond to concentrations at which they occur in vivo. In this context, Chanet et al (30) investigated the impact of narigenin and hesperitin metabolites on monocyte adhesion to TNF-α-activated human umbilical vein endothelial cells and gene expression. At a concentration of 2 μmol/L, hesperetin-3'-O-sulfate, hesperetin-3'-O-glucuronide, and narigenin-4'-O-glucuronide significantly attenuated monocyte adhesion. These effects could have been a consequence of the modulation of genes involved in atherogenesis, such as those involved in inflammation, cell adhesion, and cytoskeletal organization and could be one of the mechanisms through which flavanone-rich diets might protect the human vascular system (30).

We thank the volunteers who participated in the study. The authors’ responsibilities were as follows—GP-C: conducted most of the experimental work and contributed to the draft of the manuscript; GB, JvdH, and DDR: contributed to the experimental work and helped revise the manuscript; MNC: helped design the study and contributed to the drafting of the manuscript; MEJL: obtained ethical permission for the study and contributed to the drafting of the manuscript; SAR and MBK: contributed to the design of the study and revision of the manuscript; and AC: designed the study, supported the experimental work, and contributed to the drafting of the manuscript and its revisions. SAR and MBK are employees of The Coca-Cola Company. GP-C, GB, JvdH, MNC, DDR, MEJL, and AC had no competing financial interests.

### Table 4

Quantities of key phenolic catabolites excreted in urine 0–24 h after ingestion of 250 mL orange juice or a placebo drink.

<table>
<thead>
<tr>
<th>Drink</th>
<th>0–2 h</th>
<th>&gt;2–5 h</th>
<th>&gt;5–10 h</th>
<th>&gt;10–24 h</th>
<th>Total (0–24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orange juice</td>
<td>1.3 ± 0.5</td>
<td>15 ± 2</td>
<td>72 ± 13.7</td>
<td>52 ± 14</td>
<td>141 ± 20</td>
</tr>
<tr>
<td>Placebo drink</td>
<td>0.6 ± 0.1</td>
<td>1.7 ± 0.6</td>
<td>1.4 ± 1.2</td>
<td>1.9 ± 0.4</td>
<td>5.6 ± 1.4</td>
</tr>
</tbody>
</table>

1 All values are means ± SEMs. n = 12. Quantification was based on the combined amounts of 3-(3′-hydroxy-4′-methoxyphenyl)hydracrylic acid, 3-(3′-hydroxyphenyl)hydracrylic acid, 3-(3′-hydroxy-4′-methoxyphenyl)-propionic acid, 3′-(4′-hydroxy-3′-methoxyphenyl)propionic acid, 3′-methoxy-4′-hydroxyphenylacetic acid, 3′-hydroxyhippuric acid, and 4′-hydroxyhippuric acid presented in Table 2.

2 Values were significantly different (P < 0.05) from those obtained after placebo drink consumption (Friedman’s test).
REFERENCES


