Chocolate intake increases urinary excretion of polyphenol-derived phenolic acids in healthy human subjects

Laurent Y Rios, Marie-Paule Gonthier, Christian Rémésy, Isabelle Mila, Catherine Lapipe, Sheryl A Lazarus, Gary Williamson, and Augustin Scalbert

ABSTRACT

Background: Proanthocyanidins, the most abundant polyphenols in chocolate, are not depolymerized in the stomach and reach the small intestine intact, where they are hardly absorbed because of their high molecular weight. In vitro and in vivo studies using pure compounds as substrates suggest that proanthocyanidins and the related catechin monomers may be degraded into more bioavailable low-molecular-weight phenolic acids by the microflora in the colon.

Objective: The aim of the study was to estimate the amounts of phenolic acids formed by the microflora and excreted in the urine of human subjects after consumption of polyphenol-rich chocolate.

Design: After consumption of a polyphenol-free diet for 2 d and a subsequent overnight fast, 11 healthy subjects (7 men and 4 women) consumed 80 g chocolate containing 439 mg proanthocyanidins and 147 mg catechin monomers. All urine was collected during the 24 h before chocolate consumption and at 3, 6, 9, 24, and 48 h after chocolate consumption. Aromatic acids were identified in urine by gas chromatography–mass spectrometry and were quantified by HPLC–electrospray ionization tandem mass spectrometry.

Results: Chocolate intake increased the urinary excretion of the 6 following phenolic acids: m-hydroxyphenylpropionic acid, ferulic acid, 3,4-dihydroxyphenylacetic acid, m-hydroxyphenylacetic acid, vanillic acid, and m-hydroxybenzoic acid.

Conclusion: The antioxidant and biological effects of chocolate may be explained not solely by the established absorption of catechin monomers but also by the absorption of microbial phenolic acid metabolites. Am J Clin Nutr 2003;77:912–8.

KEY WORDS Cocoa, chocolate, polyphenols, flavonoids, proanthocyanidins, catechins, bioavailability, microbial metabolites

INTRODUCTION

Polyphenols are naturally occurring compounds that are widely distributed in higher plants and are therefore an integral part of the human diet. Fruit, vegetables, some beverages (tea, coffee, fruit juices, and red wine), and cocoa or chocolate are among the richest food sources (1). Cocoa or chocolate contains flavonoids that form a unique class of polyphenols, including monomers (epicatechin and catechin), oligomers, and polymers (proanthocyanidins) (2). Two main types of proanthocyanidins, which differ in their hydroxylation patterns, are known in plants: procyanidins and prodelphinidins. Only procyanidins are present in chocolate. The content of total polyphenols varies between 5 and 8.4 mg/g according to chocolate type and reaches 20 mg/g in cocoa powder (3). Higher values (up to 70 mg/g in cocoa powder) have also been reported (4, 5). Flavanol oligomers and polymers account for ≥90% of the total polyphenols, and catechin monomers account for only 5–10% (4, 6).

The dietary intake of catechin monomers in a representative sample of the Dutch population was estimated as 50 mg/d, with chocolate as a major food source (3–20% according to age) (7). The dietary intake of oligomers and polymers is still unknown because of the lack of accurate data on their content in food, but it may be as high as several hundred milligrams per day, as previously suggested (2). Proanthocyanidins may therefore account for a major fraction of the total polyphenols ingested in Western diets (1).

Polyphenols are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health in reducing the risk of cardiovascular diseases and cancers (2). Different in vitro studies showed that cocoa flavanols prevent LDL oxidation (3, 8, 9), suppress peroxynitrite-induced nitration of tyrosine (10, 11), enhance endothelium-dependent relaxation of isolated rabbit aorta (12), and modulate cytokine transcription in peripheral blood mononuclear cells (13). It is assumed in these in vitro studies that cocoa polyphenols are bioavailable and reach the target inner tissues. However, proanthocyanidins are poorly absorbed through the gut barrier because of their high molecular weight (14–18). It is possible that these biological effects, which were partially confirmed in vivo (5, 19–21), may not be due to a direct action of proanthocyanidins themselves but to an effect of some more readily absorbed low-molecular-weight metabolites.

1 From the Laboratoire des Maladies Métaboliques et Micronutriments, INRA, Saint-Genès-Champanelle, France (LYR, M-PG, CR, and AS); the Laboratoire de Chimie Biologique, INRA, INRA-PG, Thiverval-Grignon, France (JM and CL); the Analytic and Applied Sciences Group, Mars, Inc, Hackettstown, NJ (SAL); and the Institute of Food Research, Norwich Research Park, Colney, Norwich, United Kingdom (LYR and GW).
2 Supported by a postdoctoral grant (to LR) from Mars, Inc.
3 Address reprint requests to A Scalbert, Laboratoire des Maladies Métaboliques et Micronutriments, INRA, 63122 Saint-Genès-Champanelle, France. E-mail: scalbert@clermont.inra.fr.

Received June 18, 2002.
Accepted for publication October 8, 2002.
Studies on the in vitro depolymerization of proanthocyanidins suggested that they might be depolymerized into catechins in the stomach or the small intestine (22, 23). However, we showed that chocolate proanthocyanidins were stable in the stomach of healthy volunteers (24) and were not cleaved into monomers when fed to rats (14). The presence of different pH values between experiments is a likely explanation of these opposing conclusions, because the pH in the stomach after chocolate consumption (pH 5) is higher than that used in the in vitro studies (pH 2.0). A strong influence of pH on the depolymerization of different pure dimers was also described in vitro, with minimal depolymerization at pH 4 (25).

Other low-molecular-weight metabolites may be formed by the microflora in the colon. We showed the formation of several phenolic acids from proanthocyanidins in vitro studies with human fecal microflora (26). These phenolic acids could be absorbed through the colon barrier and contribute to the biological effects of chocolate polyphenols observed in vivo. In the present study, we analyzed the phenolic acids recovered in the urine of human subjects collected for 2 d after the consumption of polyphenol-rich chocolate.

SUBJECTS AND METHODS

Subjects

Eleven healthy subjects [7 men and 4 women with a mean (±SD) age of 24 ± 3 y, a mean height of 172 ± 8 cm, and a mean weight of 67.3 ± 9.4 kg] with no gastrointestinal diseases participated in the study. They received no medication for ≥4 wk before the beginning of the study until the end of the study. Fruit and fruit-containing products, vegetables, whole-flour–based cereal products, chocolate, and beverages rich in polyphenols (tea, coffee, cocoa drinks, cider, wine, beer) were excluded from the diet. After an overnight fast, the subjects consumed 80 g flavanol-rich chocolate (Cocoapro; Mars, Inc, Hackettstown, NJ) with bread and water. The composition of the chocolate is shown in Table 1. The flavanol content was determined by normal-phase HPLC as previously described (6). Urine samples were collected for 24 h before chocolate intake. All urine was then collected at 3, 6, 9, 24, and 48 h after the test meal. An aliquot of each of these urine samples was mixed in proportion to their volume to provide the 0–24-h sample. Immediately after collection, urine was treated with sodium azide (1 g/L) and acidified with a 40-mmol HCl/L solution. Aliquots were kept at −20°C until analyzed.

Urinary creatinine was measured by using a modified version of Jaffé’s colorimetric method (27). The mean (±SEM) daily creatinine excretion was 17.9 ± 1.5 mg · kg body wt−1 · d−1.

Measurement of hippuric acid concentrations in urine by HPLC with a diode-array detector

Urine containing syringic acid (100 µmol/L; Sigma, St Louis) as an internal standard was centrifuged at 18 700 × g for 4 min at 15°C. The resulting supernatant fluid was analyzed by reversed-phase HPLC on a Hypersil BDS C18 column (5 µm, 150 mm × 4.6 mm; Life Sciences International, Cergy, France). Mobile phases consisted of, by vol, 10% methanol in 0.5% aqueous formic acid (solvent A) and 80% methanol in 0.5% aqueous formic acid (solvent B). The following elution conditions were applied: 0–10 min, 0% solvent B; 10–25 min, linear gradient to 57% solvent B; 25–40 min, linear gradient to 100% solvent B. The flow rate was 1 mL/min. Detection was carried out at 240 nm for hippuric acid (Sigma) and at 280 nm for the internal standard with a diode-array detector (Kontron, Milan, Italy). Hippuric acid was identified by comparing its retention time and ultraviolet spectrum with those of an authentic standard.

Identification of aromatic acids in urine by gas chromatography–mass spectrometry

Urine samples were hydrolyzed by β-glucuronidase (EC 3.2.1.31; type H3, 1250 U/mL; Sigma) and sulfatase (EC 3.1.6.1; type V, 5 U/mL urine; Sigma) for 3 h at 37°C in acetate buffer (0.1 mol/L, pH 5). Urinary aromatic compounds were extracted on a C18 solid-phase cartridge (SepPak; Waters, Milford, MA) with methanol and acetonitrile. The extract was dried under vacuum and solubilized in acetonitrile. The solution was dried over sodium sulfate, and 10-µL aliquots were silylated with 50 µL bis-trimethylsilyl trifluoroacetamide (Fluka, Buchs, Switzerland) and 5 µL pyridine (Merck, Darmstadt, Germany) before gas chromatography–mass spectrometry (GC-MS) analysis. Trimethylsilylated compounds were analyzed with a Varian 3400 chromatograph (Varian, Les Ulis, France) on an SPB1 polydimethylsiloxane capillary column (0.25 µm, 30 m × 0.2 mm inside diameter; Supelco, Bellefonte, PA) with helium as the carrier gas (inlet pressure of 0.5 bar). The column temperature was increased 30°C/min from 40 to 110°C, then 2.5°C/min from 110 to 280°C. The detection of trimethylsilylated derivatives of aromatic acids [70-eV electronic impact; mass-to-charge ratio (m/z) range: 50–650; positive mode] was performed with a Saturn 2000 ion trap instrument (Varian). Compounds were identified by comparison of their MS spectra with those of the MS

<table>
<thead>
<tr>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (g)</td>
</tr>
<tr>
<td>Vanillin (mg)</td>
</tr>
<tr>
<td>Theobromine (g)</td>
</tr>
<tr>
<td>Caffeine (g)</td>
</tr>
<tr>
<td>Flavanols Monomers (mg)</td>
</tr>
<tr>
<td>Dimer (mg)</td>
</tr>
<tr>
<td>Trimers (mg)</td>
</tr>
<tr>
<td>Tetramers (mg)</td>
</tr>
<tr>
<td>Pentamers (mg)</td>
</tr>
<tr>
<td>Hexamers (mg)</td>
</tr>
<tr>
<td>Heptamers (mg)</td>
</tr>
<tr>
<td>Octamers (mg)</td>
</tr>
<tr>
<td>Nonamers (mg)</td>
</tr>
<tr>
<td>Decamers (mg)</td>
</tr>
<tr>
<td>Decamers (mg)</td>
</tr>
</tbody>
</table>

TABLE 1 Composition of the 80-g chocolate serving consumed by participants in the study
library NIST92 (Varian) and of authentic reference compounds. Ferulic acid (FE), phenylacetic acid (PAc), m-hydroxyphenylacetic acid (mHPac), 3,4-dihydroxyphenylacetic acid (diHPAc), m-hydroxybenzoic acid (mHBA), p-hydroxybenzoic acid (pHBA), m-hydroxyhippuric acid (mHHA), and hippuric acid (HA) were purchased from Sigma, and 3,4-dihydroxyphenylpropionic acid (diHPP) and p-hydroxyhippuric acid (pHHA) was a gift from RR Scheline, University of Bergen, Norway.

Measurement of aromatic acid concentrations in urine by HPLC-electrospray ionization tandem MS

Urine samples (35 μL) were diluted with 4 volumes sodium acetate buffer (pH 5). Syringic acid was added as an internal standard (3 μmol/L). The solution was acidified to pH 4.9 with 20 μL of a 0.58-mol acetic acid/L solution and incubated at 37°C for 45 min in the presence of a Helix pomatia extract containing 1100 U β-glucuronidase and 42 U sulfatase (Sigma). After further acidification to pH 2 with a 6-mol HCl/L solution, the urine was extracted twice with ethyl acetate and centrifuged at 2400 × g for 10 min at room temperature. The resulting supernatant fluid was dried under nitrogen and redissolved in 500 μL 25% (by vol) aqueous methanol. HPLC-electrospray ionization tandem MS analyses were performed on a Hewlett-Packard HPLC system equipped with tandem MS detection (API 2000; Applied Biosystems, Foster City, CA). The column was a Hypersil BDS C18 column (5 μm, 150 mm × 2.1 mm; Touzart & Matignon, Les Ulis, France), and the mobile phases consisted of, by vol, 5% acetonitrile in 0.1% aqueous formic acid (solvent A) and 40% acetonitrile in 0.1% aqueous formic acid (solvent B). The following gradient was applied: 0–15 min, linear gradient from 0% to 100% solvent B. The flow rate was 0.2 mL/min. Detection by electrospray ionization was carried out at 450°C in negative mode with a nebulizer pressure of 620 kPa (90 psi), a drying gas flow of 11 L/min, a fragmentor voltage of 20 V, and a capillary voltage of 4000 V. The MS data were collected in multiple reaction monitoring mode, in which parent and product ions specific for each compound were monitored with a dwell time of 500 ms. The phenolic acids were quantified by using the following parent and product ions, respectively: mHBA, m/z 137 and 93; pHBA, m/z 137 and 93; pHHA, m/z 194 and 100; VA, m/z 167 and 123; syringic acid, m/z 197 and 123; PAc, m/z 135 and 91; mHPac, m/z 151 and 107; diHPac, m/z 167 and 123; mHPP, m/z 165 and 121; diHPP, m/z 181 and 59; FE, m/z 193 and 134.

Statistics

Results are presented as group means ± SEMs. Statistical tests were performed with the GRAPHPAD INSTAT statistical package version 3.00 (GraphPad, San Diego). Values were log transformed before statistical analysis to compensate for unequal variance. Analysis of variance was used to test for any significant differences between the groups. If the result of the analysis of variance was found to be significant (P < 0.05), Tukey’s test was used to determine specific differences between group means. Differences were considered significant at P < 0.05.

RESULTS

Identification of aromatic acids in urine

The following 11 aromatic acids were detected and identified by GC-MS in urine collected during the 24 h after consumption of the chocolate meal: diHPP, mHPP, FE, diHPAc, mHPac, PAc, VA, mHBA, pHBA, pHHA, and hippuric acid (Figure 1). These compounds were identified by comparing their MS spectra with those of a reference library and were confirmed by comparing their MS spectra with those of authentic reference standards (Table 2).
TABLE 2
Gas chromatography–mass spectrometry characteristics of trimethylsilylated derivatives of aromatic acids identified in urine after chocolate consumption.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>R&lt;sub&gt;t&lt;/sub&gt;</th>
<th>m/z</th>
<th>m/z of products&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHA</td>
<td>40.6</td>
<td>339</td>
<td>339 (M&lt;sup&gt;+&lt;/sup&gt;, 3), 324 (4), 294 (21), 193 (18), 147 (19), 73 (34)</td>
</tr>
<tr>
<td>HBA</td>
<td>21.2</td>
<td>282</td>
<td>282 (M&lt;sup&gt;+&lt;/sup&gt;, 19), 267 (100), 223 (20), 193 (23), 73 (24)</td>
</tr>
<tr>
<td>HPAc</td>
<td>20.4</td>
<td>296</td>
<td>296 (M&lt;sup&gt;+&lt;/sup&gt;, 30), 281 (29), 252 (21), 179 (6), 164 (28), 147 (63), 105 (6), 73 (100)</td>
</tr>
<tr>
<td>HA</td>
<td>26.4</td>
<td>312</td>
<td>312 (M&lt;sup&gt;+&lt;/sup&gt;; 35), 297 (100), 282 (15), 267 (53), 249 (15), 219 (20), 73 (66)</td>
</tr>
<tr>
<td>FE</td>
<td>38.1</td>
<td>308</td>
<td>308 (M&lt;sup&gt;+&lt;/sup&gt;; 100), 293 (87), 279 (10), 253 (43), 223 (25), 206 (23), 73 (66)</td>
</tr>
<tr>
<td>HPP</td>
<td>25.2</td>
<td>310</td>
<td>310 (M&lt;sup&gt;+&lt;/sup&gt;; 92), 295 (100), 280 (15), 267 (53), 249 (15), 219 (20), 73 (66)</td>
</tr>
<tr>
<td>mPAc</td>
<td>39.1</td>
<td>338</td>
<td>338 (M&lt;sup&gt;+&lt;/sup&gt;; 100), 323 (100), 293 (63), 279 (10), 249 (15), 219 (20), 73 (66)</td>
</tr>
<tr>
<td>diHPP</td>
<td>29.6</td>
<td>384</td>
<td>384 (M&lt;sup&gt;+&lt;/sup&gt;; 100), 369 (13), 341 (10), 280 (15), 267 (53), 249 (15), 219 (20), 73 (66)</td>
</tr>
<tr>
<td>diHPAc</td>
<td>20.4</td>
<td>296</td>
<td>296 (M&lt;sup&gt;+&lt;/sup&gt;; 30), 281 (29), 252 (21), 179 (6), 164 (28), 147 (63), 105 (6), 73 (100)</td>
</tr>
<tr>
<td>mHPP</td>
<td>25.2</td>
<td>310</td>
<td>310 (M&lt;sup&gt;+&lt;/sup&gt;; 92), 295 (100), 280 (15), 267 (53), 249 (15), 219 (20), 73 (66)</td>
</tr>
<tr>
<td>mPAc</td>
<td>39.1</td>
<td>338</td>
<td>338 (M&lt;sup&gt;+&lt;/sup&gt;; 100), 323 (100), 293 (63), 279 (10), 249 (15), 219 (20), 73 (66)</td>
</tr>
<tr>
<td>HPAc</td>
<td>20.4</td>
<td>296</td>
<td>296 (M&lt;sup&gt;+&lt;/sup&gt;; 30), 281 (29), 252 (21), 179 (6), 164 (28), 147 (63), 105 (6), 73 (100)</td>
</tr>
<tr>
<td>HA</td>
<td>26.4</td>
<td>312</td>
<td>312 (M&lt;sup&gt;+&lt;/sup&gt;; 35), 297 (100), 282 (15), 267 (53), 249 (15), 219 (20), 73 (66)</td>
</tr>
<tr>
<td>FE</td>
<td>38.1</td>
<td>308</td>
<td>308 (M&lt;sup&gt;+&lt;/sup&gt;; 100), 323 (100), 293 (63), 279 (10), 249 (15), 219 (20), 73 (66)</td>
</tr>
</tbody>
</table>

<sup>1</sup>R<sub>t</sub>, retention time; m/z, mass-to-charge ratio; C<sub>6</sub>C<sub>3</sub>, C<sub>6</sub>C<sub>2</sub>, C<sub>6</sub>C<sub>1</sub>, aromatic compounds with a 3-, 2-, or 1-carbon side chain, respectively; diHPP, 3,4-dihydroxyphenylpropionic acid; HPAc, 3,4-dihydroxyphenylacetic acid; mHPP, m-hydroxyphenylpropionic acid; mPAc, m-hydroxyphenylacetic acid; PAc, phenylacetic acid; VA, vanillic acid; mHBA, m-hydroxybenzoic acid; mHPP, m-hydroxyphenylpropionic acid; FE, ferulic acid.

<sup>2</sup>Numbers in parentheses are intensities relative to the intensity of the base peak.

FIGURE 2. Chromatograms obtained by HPLC–electrospray ionization tandem mass spectrometry of a human urine sample collected 24 h after consumption of a chocolate meal. TIC, total ionic current; pHHA, p-hydroxyhippuric acid; diHPAc, 3,4-dihydroxyphenylacetic acid; VA, vanillic acid; diHPP, 3,4-dihydroxyphenylacetic acid; pHBA, p-hydroxybenzoic acid; mHBA, m-hydroxybenzoic acid; mHPAc, m-hydroxyphenylacetic acid; PAc, phenylacetic acid; FE, ferulic acid.

Measurement of aromatic acid excretion in urine after a chocolate meal

The urinary concentrations of the 11 aromatic acids detected by GC-MS were measured by HPLC with a diode-array detector (hippuric acid) and by HPLC–electrospray ionization tandem MS (Figure 2). These 2 methods have an advantage over GC-MS because they require a limited treatment of the samples before analysis. However, GC-MS was used for detection and identification because of the high interlaboratory reproducibility in fragmentation and the availability of a spectral library. The excretion of 6 of the 11 aromatic acids increased significantly after chocolate intake (Table 3):

TABLE 3
Urinary excretion of aromatic acids after chocolate consumption<sup>1</sup>

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>−24 to 0 h</th>
<th>0 to 24 h</th>
<th>24 to 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPP</td>
<td>3.1 ± 0.7</td>
<td>2.6 ± 0.6</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>mPAc</td>
<td>131 ± 59b</td>
<td>170 ± 32b</td>
<td>321 ± 99b</td>
</tr>
<tr>
<td>FE</td>
<td>15.8 ± 4.4a</td>
<td>38.8 ± 12.3b</td>
<td>38.4 ± 13.2b</td>
</tr>
<tr>
<td>mHPP</td>
<td>21.2 ± 3.5a</td>
<td>27.6 ± 5.8a</td>
<td>156 ± 54a</td>
</tr>
<tr>
<td>mPAc</td>
<td>70.1 ± 11.8b</td>
<td>30.5 ± 9.1a</td>
<td>36.8 ± 8.1a</td>
</tr>
<tr>
<td>VA</td>
<td>64.6 ± 25.55</td>
<td>228 ± 33b</td>
<td>99.7 ± 28.8a</td>
</tr>
<tr>
<td>mHBA</td>
<td>3.9 ± 1.2</td>
<td>8.93 ± 3.9</td>
<td>41.2 ± 10.4</td>
</tr>
<tr>
<td>pHBA</td>
<td>71.1 ± 13.6</td>
<td>63.6 ± 13.1</td>
<td>90.5 ± 21.3</td>
</tr>
<tr>
<td>HA</td>
<td>2493 ± 1923</td>
<td>974 ± 115</td>
<td>887 ± 94</td>
</tr>
</tbody>
</table>

<sup>1</sup>Means ± SEM; n = 11. ND, not detected; C<sub>6</sub>C<sub>3</sub>, C<sub>6</sub>C<sub>2</sub>, C<sub>6</sub>C<sub>1</sub>, aromatic compounds with a 3-, 2-, or 1-carbon side chain, respectively; diHPAc, 3,4-dihydroxyphenylpropionic acid; mHPP, m-hydroxyphenylpropionic acid; PAc, phenylacetic acid; FE, ferulic acid; diHPAc, 3,4-dihydroxyphenylacetic acid; mHPAc, m-hydroxyphenylacetic acid; PAc, phenylacetic acid; VA, vanillic acid; mHBA, m-hydroxybenzoic acid; pHBA, p-hydroxybenzoic acid; pHHA, p-hydroxyhippuric acid; HA, hippuric acid. Data were log transformed before statistical analysis. Values in the same row with different superscript letters are significantly different, P < 0.05 (Tukey’s test).
FIGURE 3. Mean (± SEM) urinary excretion of the following 6 aromatic acids in 11 subjects after consumption of a chocolate meal: m-hydroxyphenylpropionic acid, ferulic acid, 3,4-dihydroxyphenylacetic acid, vanillic acid, m-hydroxybenzoic acid, and m-hydroxyphenylacetic acid. Data were log transformed before statistical analysis. Bars with different letters are significantly different, P < 0.05 (Tukey’s test). ND, not detected.

diHPAc, VA, and mHBA increased significantly during the first day after the chocolate meal, and mHPP, FE, and mHPAc increased significantly during the second day. Among these 6 aromatic acids, only VA excretion returned to the basal excretion level during the second day; the excretion of the other 5 aromatic acids during the second day was either significantly higher than before chocolate consumption or was even higher than that during the first day after the test meal (mHPAc).

The kinetics of the 6 aromatic acids that showed variation in urinary excretion were examined in more detail. VA clearly differed from the other aromatic acids because it showed a peak excretion shortly after chocolate consumption (0–3 h) (Figure 3). The excretion of the other 5 compounds increased in the 6–48 h after chocolate intake.

DISCUSSION

The present results show that the excretion of phenolic acids is influenced by the consumption of chocolate. Several microbial metabolites of various polyphenols were identified previously in
reach the inner tissues in their intact form. They are not depolymerized in the stomach and reach the small intestine (24). Because of their high molecular weight, they are hardly absorbed in the small intestine (15). Attempts to detect proanthocyanidin dimers in plasma found either none (14, 44) or only minute amounts (18, 45), showing that they are poorly absorbed in the small intestine.

The biological effects of proanthocyanidins or proanthocyanidin-rich foods may therefore be at least partially explained by the formation of more easily absorbed phenolic acids. These phenolic acids may contribute to the prevention of oxidative stress in inner tissues observed after chocolate consumption (5, 21, 46). The antioxidant capacity [azo-bis(amidinopropane) assay] and plasma thiobarbituric acid–reactive substances observed 6 h after chocolate consumption were higher and lower, respectively, than were those observed after only 2 h (47), whereas the maximal epicatechin concentration was reached 2 h after chocolate intake (43). The plasma concentrations of some phenolic acids formed in the colon exceed those of the parent flavonoids after regular consumption of a flavonoid-rich diet (48). This stresses the likely implication of microbial metabolites in antioxidant protection and their possible role in the health effects of cocoa polyphenols.

LYR contributed to the study design, data collection and analysis, and the writing of the manuscript. CR and GW contributed to the study design and the writing of the manuscript. AS contributed to the study design, data analysis, and the writing of the manuscript. MG, IM, and SAL contributed to data collection and analysis; and CL contributed to data analysis. None of the authors had any conflict of interest.

REFERENCES
6. Adamson GE, Lazarus SA, Mitchell AE, et al. HPLC method for the determination of phenolic acids before consumption of the test meal (Figure 3).
7. The previous identification of nonphenolic aromatic acids such as PAc and phenylpropionic acid as metabolites of catechins and proanthocyanidins was not observed in the present study. Their yields in human subjects may be too low to significantly affect their urinary excretion.
8. The total amounts of phenolic acids derived from flavanols and excreted during the 2 d after chocolate intake can be calculated from the data shown in Figure 3. The consumption of 80 g chocolate led to a urinary excretion of 202 μmol flavanol-derived aromatic acids (mHPP + diHPAc + mHPAc + mHBA). These phenolic acids, which are reducing agents, also contribute to antioxidant protection (41, 42). This total amount of phenolic acids excreted in urine is similar to that of catechin monomers excreted after consumption of an equivalent amount of chocolate. The consumption of 96 g chocolate resulted in an excretion of 215 μmol total epicatechin (methylated and nonmethylated forms) (43).
9. The reported health effects of polyphenols could be explained by direct effects of the polyphenols in their ingested form or by the effects of metabolites formed in the body. Proanthocyanidins, the most abundant polyphenols in chocolate, are probably unable to reach the inner tissues in their intact form. They are not depolymerized in the stomach and reach the small intestine intact (24).


