JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 3933–3939 3933
DOI:10.1021/jf100315d

Bioavailability of Anthocyanins and Ellagitannins Following Consumption of Raspberries by Healthy Humans and Subjects with an Ileostomy†

Rocío González-Barrio,‡ Gina Borges,‡ William Mullen,§ and Alan Crozier*,‡

‡Graham Kerr Building, Division of Developmental Medicine, Faculty of Medicine and §Graham Kerr Building, Division of Ecology and Evolutionary Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

The fate of anthocyanins, ellagic acid, and ellagitannins was studied following the consumption of 300 g of raspberries by healthy human volunteers and subjects with an ileostomy. Postingestion plasma and urine from the former and ileal fluid and urine from the latter group were collected and analyzed by HPLC-PDA-MS^2. Plasma from the healthy volunteers did not contain detectable quantities of either the native raspberry polyphenolics or their metabolites. The three main raspberry anthocyanins were excreted in urine in both healthy and ileostomy volunteers 0−7 h after ingestion, in quantities corresponding to <0.1% of intake. This indicates a low level of absorption in the small intestine. With ileostomy volunteers 40% of anthocyanins and 23% of the ellagitannin sanguiin H-6 were recovered in ileal fluid with the main excretion period being the first 4 h after raspberry consumption. The recovery of ellagic acid in ileal fluid was 241%, indicating hydrolysis of ellagitannins in the stomach and/or the small intestine. Urinary excretion of ellagic acid and an ellagic acid-*O*-glucuronide was <1% of intake. No intact or conjugated forms of ellagitannins were detected in urine from either healthy subjects or ileostomy volunteers. However, in healthy subjects, but not the ileostomists, ellagitannins were catabolized with the appearance of urolithin A-*O*-glucuronide, two of its isomers, and urolithin B-*O*-glucuronide in urine collected 7−48 h after raspberry consumption. There was marked variation in the urolithin profile of individual volunteers, indicating differences in the colonic microflora responsible for ellagitannin degradation.

KEYWORDS: Ellagitannins; anthocyanins; urolithins; ellagic acid; bioavailability; metabolism; colonic metabolites; raspberries

INTRODUCTION

Raspberries (*Rubus idaeus* L.), in keeping with many other berries, are rich in flavonoids and related compounds as well as vitamin C (*1*). They contain a distinct spectrum of anthocyanins, the major components being cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside, and cyanidin-3-*O*-glucoside with smaller quantities of cyanidin-3-*O*-rutinoside, cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside, pelargonidin-3-*O*-sophoroside, pelargonidin-3-(2″-*O*-glucosyl)rutinoside, pelargonidin-3-*O*-glucoside, and pelargonidin-3-*O*-rutinoside. Substantial quantities of ellagitannins, namely, sanguiin H-6, sanguiin H-10, and lambertianin C, also occur in raspberries (*2*, *3*). There is growing evidence linking consumption of ellagitannins with human health benefits. This is derived principally from research with pomegranate juice containing high concentrations of ellagitannins in the form of punicalins and punicalagins (*4*−*8*).

Raspberries also contain small amounts of a wide variety of quercetin- and kaempferol-based flavonol conjugates, with the major components being quercetin-3-*O*-glucuronide and quercetin-3-*O*-glucoside, together with trace levels of ellagic acid and ellagic acid sugar conjugates (*9*). Ellagic acid has been reported to have antiviral activity and provide protection against cancers of the colon, lung, and esophagus, and the health benefits of raspberry consumption have been promoted on the basis of claims of a high ellagic acid content (*10*−*12*).

In an earlier study we investigated the bioavailability of raspberry anthocyanins and ellagitannins in rats (*13*). In the current investigation the fate of these compounds following the acute ingestion of a 300 g raspberry supplement by humans was monitored. The study was carried out with both healthy humans and subjects with an ileostomy. Plasma, urine, and ileal fluid were collected for up to 48 h after consumption of raspberries and analyzed by HPLC-MS^2 to investigate the absorption, metabolism, and excretion of anthocyanins and ellagitannins as they pass through the body. The use of ileostomists, as well as subjects with an intact functioning colon, can provide valuable information in studies on the bioavailability of dietary polyphenols. Compounds detected in plasma and excreted in urine of healthy volunteers but absent or present in substantially lower amounts in samples from ileostomists are clearly absorbed in the large rather

†Part of the Berry Health Symposium 2009.

*Author to whom correspondence should be addressed (telephone +44-141-330-4613; fax +44-141-330-5394; e-mail a.crozier@bio.gla.ac.uk).

Published on Web 03/10/2010

than the small intestine. Knowing what components are ingested and how much is recovered in ileal fluid provides an additional dimension to information on absorption gleaned from plasma pharmacokinetics and urinary excretion. The contents of ileal fluid also yield data on compounds that are not absorbed in the small intestine and which in healthy subjects would pass to the lower bowel and be subjected to the action of the colonic microflora. Ileostomists were originally used in an early study on the bioavailibility of quercetin and quercetin glycosides (*14*) and more recently have been an integral part of investigations into the absorption and metabolism of quercetin-3-*O*-rutinoside in tomato juice (*15*), apple juice polyphenolics (*16*, *17*), green tea flavan-3-ols (*18*, *19*), and coffee chlorogenic acids (*20*, *21*).

MATERIALS AND METHODS

Raspberries and Chemicals. Raspberries cv. Glen Ample were obtained from a commercial grower in Angus, U.K. Cyanidin-3-*O*-glucoside was purchased from Apin Chemicals (Abingdon, Oxford, U.K.), cyanidin-3-*O*-sambubioside-5-*O*-glucoside was supplied by Polyphenols (Sandnes, Norway), ellagic acid was from Sigma-Aldrich (Poole, Dorset, U.K.), and punicalagin was from Chromadex (Irvine, CA), whereas urolithins A and B were a generous gift from Dr. Navindra Seeram (University of Rhode Island, Kingston, RI). HPLC grade solvents were obtained from Rathburn Chemicals (Walkerburn, U.K.). Formic acid and sodium diethyldiothiocarbamate were purchased from Sigma-Aldrich, and acetic acid was from BDH (Poole, U.K.).

Study Design. The Glasgow Royal Infirmary Research Ethics Committee and the Glasgow University Ethical Committee approved the study protocol. Ten healthy humans (four female, six male) and four subjects with an ileostomy, and hence no colon (two female, two male), were recruited and gave their written consent to participate in this study. They were nonsmokers and not on any medication, aged between 25 and 48 years. The ileostomy volunteers had their operation at least 5 years prior to the study and had minimal resection of the small intestine. Subjects were required to follow a diet low in polyphenolic compounds by avoiding fruits and vegetables, nuts, high-fiber products, and beverages such as tea, coffee, and fruit juices, as well as to abstain from consuming alcohol for 60 h prior to the beginning of the study. After an overnight fast, the volunteers consumed 300 g of homogenized raspberries and after 3 h they continued the low polyphenolic diet for up to a further 45 h until the final urine and ileal fluid samples were collected.

With healthy subjects, 10 mL samples of venous blood were collected at 0, 1.0, 1.5, 2.0, 3.0, 5.0, 6.5, 7.5, and 24 h postingestion, and the plasma obtained was stored as described by Stalmach et al. (*22*). In addition, all urine excreted 24 h before and over five time periods, 0−4, 4−7, 7−24, 24−32, and 32−48 h, after raspberry consumption was collected. With ileostomists, urine and ileal fluid were collected 24 h before and 0−4, 4−7, 7−24, and 24−48 h after raspberry intake. After the volumes of urine and ileal fluid collected had been recorded, aliquots were acidified to pH 3 with 50% formic acid before being stored at −80 °C.

Extraction of Raspberries. Ten grams of raspberry was homogenized with 20 mL of methanol/water/formic acid (79.9:20:0.1, v/v/v) using an Ultra-Turrax homogenizer (T25 basic, IKA Werke KG, Staufen, Germany) for 1 min at 24000 rpm, prior to being centrifuged for 10 min at 4000*g*. The pellet was re-extracted with 10 mL of the same solvent as described above, after which it was centrifuged. The two supernatants were combined and analyzed by HPLC-PDA-MS^2.

Extraction of Plasma. Plasma was extracted and processed as described by Stalmach et al. (*22*) with [2-^{14}C]quercetin-4′-glucoside as an internal standard.

Processing of Urine and Ileal Fluid. Urine samples were defrosted, vortexed, and centrifuged at 16110*g* for 10 min at 4 °C, prior to the triplicate analysis of 200 μL aliquots by HPLC-PDA-MS^2. Ileal fluid was defrosted and 2 μg of cyanidin-3-*O*-sambubioside-5-*O*-glucoside added as an internal standard to triplicate 0.5 g samples, which were homogenized in 3 mL of methanol/water/formic acid (95:4:1, v/v/v) containing 20 mM sodium diethyldiothiocarbamate for 1 min at 24000 rpm using an Ultra-Turrax homogenizer, after which they were centrifuged for 20 min at 4000*g*. The pellets were re-extracted twice, and supernatants were pooled before being reduced to dryness in vacuo. The residues were resuspended in 250 μL of acidified methanol and 4750 μL of 1% formic acid, and aliquots were analyzed by HPLC-PDA-MS^2.

Qualitative and Quantitative Analysis by HPLC-PDA-MS^2. Samples were analyzed on a Surveyor HPLC system composed of an HPLC pump, PDA detector (scanning from 200 to 700 nm), and an autosampler cooled to 4 °C (Thermo Finnigan, San Jose, CA). Separations of ellagic acid derivatives, ellagitannins, and anthocyanins from raspberry were performed using a Synergi 4 μm RP-Polar 80 Å 250 × 4.6 mm i.d. reverse phase column (Phenomenex, Macclesfield, U.K.) maintained at 40 °C. The mobile phase, pumped at a flow rate of 1 mL/min, was 1% aqueous formic acid (A) and 99% methanol containing 1% formic acid (B), establishing a linear gradient from 10 to 40% B over 60 min. Chromatograms were recorded at 280, 325, 365, and 520 nm. The same instrumentation was adopted for urolithin detection but with the use of a Synergi 4 μm RP-Max 80 Å 250 × 4.6 mm i.d. reverse phase column (Phenomenex) and a 30 min gradient of 10−50% methanol in 0.25% acetic acid. After passing through the flow cell of the diode array detector, the column eluate was split and 0.3 mL/min was directed to an LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface (ESI) operating in either positive ionization mode for anthocyanins or negative ionization mode for ellagic acid and ellagitannins. An atmospheric chemical ionization interface (APCI) in negative ion mode was used for urolithin detection as this provided the best limits of detection. Analyses were carried out using full scan, data-dependent MS^2 scanning from *m*/*z* 150 to 2000. With the ESI in positive ionization mode, capillary temperature was 300 °C, sheath gas was 50 units, auxiliary gas was 35 units, and source voltage was 2 kV. For negative ionization, capillary temperature was 325 °C, sheath gas and auxiliary gas were both 40 units, and source voltage was 4 kV. For the APCI interface, the capillary temperature was 225 °C, vaporizer temperature was 350 °C, sheath gas and auxiliary gas were both 10 units, and source voltage was 4.5 kV for negative ionization.

Anthocyanins were quantified from their chromatographic peak areas recorded at 520 nm and expressed as cyanidin-3-*O*-glucoside equivalents, ellagitannins detected at 280 nm were expressed as punicalagin equivalents, and ellagic acid and ellagic acid conjugates monitored at 365 nm were quantified in ellagic acid equivalents. Urolithin A-*O*-glucuronide and its isomers and urolithin B-*O*-glucuronide detected at 305 nm were quantified, respectively, in urolithin A and urolithin B equivalents.

Statistical Analyses. Each sample was analyzed in triplicate for each volunteer, and data are presented as mean value ± standard error ($n = 10$ for healthy volunteers and $n = 4$ for ileostomists). 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).

RESULTS

Analysis of Raspberries. The HPLC-PDA-MS^2 analysis of the raspberry extract resulted in the identification and quantification of nine anthocyanins, three ellagitannins, and conjugated and free ellagic acid. In addition to these compounds, other minor peaks were identified, in the raspberry extract, including flavonol conjugates and hydroxycinamate derivatives that appeared in the chromatograms recorded at 365 and 320 nm, respectively (*3*). However, these compounds were present in very low concentrations and were not quantified. Identifications were based on published MS^2 fragmentation data on raspberry flavonoids and polyphenolics (*2*, *3*, *13*).

The three main anthocyanins detected at 520 nm were cyanidin-3-*O*-sophoroside (*m*/*z* 611 → 287), cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside (*m*/*z* 757 → 611, 287), and cyanidin-3-*O*-glucoside (*m*/*z* 449 → 287). Other minor anthocyanins identified were pelagonidin-3-*O*-sophoroside (*m*/*z* 595 → 271), cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside (*m*/*z* 727 → 581, 287), cyanidin-3-*O*-rutinoside (*m*/*z* 595 → 287), pelargonidin-3-*O*-(2″-*O*-glucosyl)rutinoside (*m*/*z* 741 → 595, 271), pelargonidin-3-*O*-glucoside (*m*/*z* 433 → 271), and pelargonidin-3-*O*-rutinoside (*m*/*z* 579 → 271).

Ellagitannins detected at 280 nm, in accordance with previous reports (*2*, *3*, *13*), were sanguiin H-10 (*m*/*z* 1565 → 1265, 1103,

Table 1. Compounds Identified and Quantified in Raspberries by HPLC-PDA-MS[2a]

compound	μmol/300 g
cyanidin-3-*O*-sophoroside	113
cyanidin-3-(2″-*O*-glucosyl)rutinoside	45
cyanidin-3-*O*-glucoside	27
pelargonidin-3-*O*-sophoroside	3.4
cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside	1.4
cyanidin-3-*O*-rutinoside	11
pelargonidin-3-*O*-(2″-*O*-glucosylrutinoside)	2.3
pelargonidin-3-*O*-glucoside	0.8
pelargonidin-3-*O*-rutinoside	0.5
total anthocyanins	**204**
sanguiin H-10	9.5
sanguiin H-6	100
lambertianin C	13
total ellagitannins	**123**
ellagic acid-*O*-pentoside	9.1
ellagic acid	7.9
total ellagic acid	**17**

[a] Data expressed as mean values (n = 3).

933, 631), sanguiin H-6 (m/z 1869 →1567, 1265, 933, 631), and lambertianin C, which had a doubly charged molecular ion (m/z $[1401]^2$ → 1869, 1567, 1265, 933, 631). Ellagic acid (m/z 301 → 257, 229) and an ellagic acid-*O*-pentoside (m/z 433 → 301) were monitored at 365 nm.

The quantites of anthocyanins, ellagitannin, and ellagic acid derivatives in the 300 g serving of raspberries consumed by the volunteers are presented in **Table 1**. Each serving contained a total of 204 μmol of anthocyanins and 123 μmol of ellagitannins, of which 82% was sanguiin H-6. The total ellagic acid (free and conjugated) content was 17 μmol.

Analysis of Plasma. No raspberry-derived compounds were detected in the plasma of the healthy subjects. The volumes analyzed and the limits of detection were such that the concentration of the main raspberry anthocyanin, cyanidin-3-*O*-sophoroside, would have been ca. < 5 nmol/L and that of ellagic acid derivatives ca. < 10 nmol/L. Plasma was not collected from volunteers with an ileostomy.

Analysis of Ileal Fluid. Analysis of extracts of ileal fluid collected over 48 h after raspberry supplementation revealed the presence of eight of the nine raspberry anthocyanins, the exception being pelargonidin-3-*O*-rutinoside, a trace component. No anthocyanin sulfate or glucuronide metabolites were detected (**Figure 1**; **Table 2**). Most of the anthocyanins appeared in the initial 4 h after ingestion. Of the 204 μmol intake, 81 μmol of anthocyanins appeared in the 0−48 h ileal fluid, which represents a 40% recovery. With most of the anthocyanins, including the main components cyanidin-3-*O*-sophoroside and cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside, there was a 40−75% recovery. However, of the 27 μmol intake of cyanidin-3-*O*-glucoside only 1.6 μmol appeared in ileal fluid, a 5.9% recovery. At the other extreme there was a 93% recovery of the 1.4 μmol of cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside in the raspberry supplement (**Table 2**).

Of the three ellagitannins in raspberries, only the main constituent, sanguiin H-6, was detected in the 0−48 h ileal fluid with a recovery corresponding to 23% of intake. Ellagic acid, but no ellagic acid-*O*-pentoside, was also detected in ileal fluid. The 19 μmol of ellagic acid that were recovered over the 48 h period corresponds to 241% of the 7.9 μmol intake (**Table 3**).

Analysis of Urine. Quantitative estimates of anthocyanin excretion by healthy volunteers, which occurred mainly 0−4 h after consumption of raspberries, are summarized in **Table 4**. Due

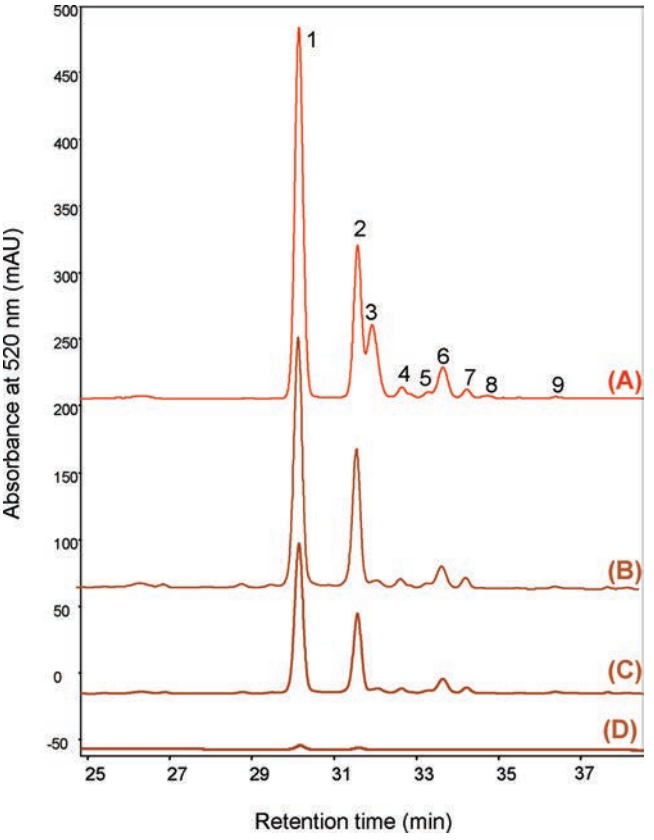

Figure 1. Gradient reversed phase HPLC profiles at 520 nm showing (**A**) the anthocyanin content of raspberries and ileal fluid collected (**B**) 0−4 h, (**C**) 4−7 h, and (**D**) 7−24 h after the ingestion of 300 g of raspberries by volunteers with an ileostomy. Peaks: (1) cyanidin-3-*O*-sophoroside, (2) cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside, (3) cyanidin-3-*O*-glucoside, (4) pelagonidin-3-*O*-sophoroside, (5) cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside, (6) cyanidin-3-*O*-rutinoside, (7) pelargonidin-3-*O*-(2″-*O*-glucosyl)rutinoside, (8) pelargonidin-3-*O*-glucoside, (9) pelargonidin-3-*O*-rutinoside.

to the low level of excretion, only the three principal raspberry anthocyanins, cyanidin-3-*O*-sophoroside, cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside, and cyanidin-3-*O*-glucoside, were present in detectable amounts. Of the 204 μmol anthocyanin intake, only 78 nmol was detected in the 0−48 h urine, a recovery of 0.04%. The 0−48 h urinary excretion of anthocyanins by ileostomy volunteers was marginally higher, at 134 nmol, equivalent a 0.07% of the total amount consumed (**Table 4**). This albeit low but slightly higher urinary excretion of anthocyanins by the ileostomy volunteers was not significantly different from that of the healthy subjects with an intact functioning colon.

Likewise, only trace levels of ellagic acid and ellagic acid-*O*-glucuronide were detected in urine from healthy subjects and ileostomists (**Table 5**). The recovery levels were low compared to the intake of the parent compounds and in reality are probably even lower when it is taken into account that both compounds could be derived from breakdown of the ellagitannins by the colonic microflora. Ellagic acid formed in this way is an intermediate in the production of urolithins, which are glucuronidated during passage through the wall of the large intestine en route to the circulatory system prior to urinary excretion (*23*−*25*). In the current study, no urolithins were detected in urine from ileostomists, but they were present in the urine of healthy subjects with an intact colon following raspberry consumption, confirming that they are formed in the large intestine.

Employing procedures originally utilized by Espín et al. (*26*), we identified different urolithins in the urine from healthy

Table 2. Recovery of Anthocyanins in Ileal Fluid Collected 0−4, 4−7, and 7−24 h after the Consumption of 300 g of Raspberries[a]

anthocyanin	0−4 h	4−7 h	7−24 h	total
cyanidin-3-*O*-sophoroside	41 ± 1	3.3 ± 1.0	0.4 ± 0.2	45 ± 2 (*40%*)
cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside	22 ± 1	1.5 ± 0.4	0.2 ± 0.1	24 ± 1 (*53%*)
cyanidin-3-*O*-glucoside	1.5 ± 0.2	0.1 ± 0.0	nd	1.6 ± 0.2 (*5.9%*)
pelargonidin-3-*O*-sophoroside	1.9 ± 0.2	0.1 ± 0.0	nd	2.0 ± 0.2 (*59%*)
cyanidin-3-*O*-(2″-*O*-xylosyl)rutinoside	1.2 ± 0.1	0.1 ± 0.4	nd	1.3 ± 0.9 (*93%*)
cyanidin-3-*O*-rutinoside	5.6 ± 0.2	0.2 ± 0.1	0.1 ± 0.1	5.9 ± 0.4 (*54%*)
pelargonidin-3-*O*-(2″-*O*-glucosyl)rutinoside	1.1 ± 0.1	0.2 ± 0.1	nd	1.3 ± 0.2 (*57%*)
pelargonidin-3-*O*-glucoside	0.6 ± 0.0	nd	nd	0.6 ± 0.0 (*75%*)
total	**75 ± 3**	**5.5 ± 1.4**	**0.7 ± 0.5**	**81 ± 4 (*40%*)**

[a] Data presented as mean values in μmol ± standard error ($n = 4$). Italicized figures in parentheses represent the amounts recovered as a percentage of the quantity ingested. nd, not detected. Anthocyanins were not detected in ileal fluid samples collected >24 h after raspberry intake.

Table 3. Recovery of Ellagic Acid, Ellagic Acid-*O*-pentoside, Sanguiin H-6, Lambertianin C, and Sanguiin H-10 from Ileal Fluid Collected 0−4, 4−7, and 7−24 h after Consumption of 300 g of Raspberries[a]

compound	0−4 h	4−7 h	7−24 h	total
ellagic acid	13 ± 3	4.3 ± 0.2	1.8 ± 0.5	19 ± 3 (*241%*)
ellagic acid-*O*-pentoside	nd	nd	nd	nd
sanguiin H-6	16 ± 0	5.1 ± 0.7	2.1 ± 0.2	23 ± 1 (*23%*)
lambertianin C	nd	nd	nd	nd
sanguiin H-10	nd	nd	nd	nd

[a] Data presented as mean values in μmol ± standard error ($n = 4$) and italicized within parentheses as a percentage of the total amounts ingested. nd, not detected. Ellagic acid, ellagic acid-*O*-pentoside, sanguiin H-6, lambertianin C, and sanguiin H-10 were not detected in urine collected >24 h after raspberry intake.

Table 4. Quantities of Anthocyanins Excreted in Urine by Humans with an Intact Colon and Subjects with an Ileostomy 0−4 and 4−7 h after Consumption of 300 g of Raspberries[a]

volunteers	anthocyanin	0−4 h	4−7 h	total
with a colon	cyanidin-3-*O*-sophoroside	34 ± 6	8.5 ± 2.3	43 ± 8 (*0.04%*)
	cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside	19 ± 3	6.8 ± 2.0	26 ± 5 (*0.06%*)
	cyanidin-3-*O*-glucoside	7.3 ± 1.5	1.4 ± 0.6	8.7 ± 1.9 (*0.03%*)
	total	**61 ± 10**	**17 ± 5**	**78 ± 14 (*0.04%*)**
without a colon	cyanidin-3-*O*-sophoroside	69 ± 7	13 ± 2	82 ± 7 (*0.07%*)
	cyanidin-3-*O*-(2″-*O*-glucosyl)rutinoside	33 ± 3	8.3 ± 1.5	41 ± 4 (*0.09%*)
	cyanidin-3-*O*-glucoside	10 ± 2	0.4 ± 0.3	10 ± 3 (*0.04%*)
	total	**112 ± 12**	**22 ± 4**	**134 ± 14 (*0.07%*)**

[a] Data presented as mean values in nmol ± standard error ($n = 10$ for healthy volunteers and $n = 4$ for ileostomy volunteers). Italicized figures in parentheses represent the amount excreted as a percentage of the amounts of anthocyanins ingested. Anthocyanins were not detected in urine samples collected >7 h after raspberry intake.

Table 5. Recovery of Ellagic Acid and Metabolites from Urine Collected from Volunteers with an Intact Colon and Subjects with an Ileostomy 0−4 h and 4−7 h after Consumption of 300 g of Raspberries[a]

volunteers	compound	0−4 h	4−7 h	total
with an intact colon	ellagic acid	6.7 ± 1.6	1.2 ± 0.4	7.9 ± 1.4 (*0.05%*)
	ellagic acid-*O*-glucuronide	5.2 ± 1.1	nd	5.2 ± 1.1 (*0.03%*)
	total	**12 ± 2**	**1.2 ± 0.4**	**13 ± 0 (*0.08%*)**
with an ileostomy	ellagic acid	25 ± 5	0.9 ± 0.2	26 ± 5 (*0.15%*)
	ellagic acid-*O*-glucuronide	7.8 ± 3.4	nd	7.8 ± 3.4 (*0.15%*)
	total	**33 ± 8**	**0.9 ± 0.2**	**34 ± 8 (*0.20%*)**

[a] Data presented as mean values in nmol ± standard error ($n = 10$ for healthy subjects and $n = 4$ for ileostomy volunteers) and italicized within parentheses as a percentage of the total amounts of ellagic acid and ellagic acid-*O*-pentoside ingested. nd, not detected. Ellagic acid and ellagic acid-*O*-glucuronide were not detected in urine collected >7 h after raspberry intake.

volunteers, on the basis of their absorbance spectrum and MS^2 fragmentation pattern. These metabolites are summarized in **Table 6**. The main urolithin excreted by most of the healthy volunteers with an intact colon was, in the absence of a standard, tentatively identified as urolithin A-3-*O*-glucuronide (**Figures 2** and **3**) on the basis of its relative HPLC retention and a negatively charged molecular ion ($[M - H]^-$) at m/z 403 that with a loss of 176 amu (cleavage of a glucuronyl group) produced an MS^2 daughter ion at m/z 227 (urolithin A aglycone). Urolithin B-3-*O*-glucuronide (**Figures 2** and **3**) was likewise tentatively identified in the urine of one of the volunteers. It had a $[M - H]^-$ ion at m/z 387, which, with a 176 amu loss, yielded a MS^2 fragment ion at m/z 211 (urolithin B aglycone). In addition, HPLC-PDA-MS^2 analysis detected two possible urolithin A-*O*-glucuronide isomers. Both yielded the same MS^2 spectrum as urolithin A-*O*-glucuronide but had a different absorbance spectrum and HPLC retention time (**Figure 2**). These different properties are probably indicative of different substitution positions of the hydroxyl groups on the benzopyranone nucleus which, based on known urolithin structures (*26*), could potentially be at C4 or C9 (see **Figure 3**). This is the first report of the possible occurrence of isomers of urolithin A-*O*-glucuronide in human urine following consumption of ellagitannins, although two urolithin C-*O*-glucuronide isomers had been detected in the jejunum of Iberian pigs fed ellagitannin-rich acorns (*26*).

The putative urolithin A-*O*-glucuronide isomers were excreted only by volunteer 10 and were the main urolithins, along with lesser amounts of urolithin A-*O*-glucuronide, in urine collected 7−48 h after raspberry consumption (**Table 6**). Volunteer 5 was the sole producer of urolithin B-*O*-glucuronide, which was excreted along with urolithin A-*O*-glucuronide 24−48 h after raspberry intake. With the exception of volunteer 1, who produced no urolithins, the other volunteers excreted only urolithin A-*O*-glucuronide. In most instances urinary excretion of urolithin-*O*-glucuronides extended to the 32−48 h collection period after ingestion of raspberries. Among the nine urolithin producers, total excretion greatly varied, ranging from 1.3 to 44 μmol, which on a mole-for-mole basis is equivalent to 0.3−8.6% of ellagitannin/ellagic acid intake (**Table 6**). However, this may well be an overestimate of conversion of ellagitannins to urolithins as in

Table 6. Quantities of Urolithins Excreted in Urine by Humans with an Intact Colon 7−24, 24−32, and 32−48 h after Consumption of 300 g of Raspberries[a]

volunteer	collection period (h)	UroA-GlcA	UroA-GlcA (isomer 1)	UroA-GlcA (isomer 2)	UroB-GlcA	total urolithins
1	7−24	nd	nd	nd	nd	nd
	24−32	nd	nd	nd	nd	nd
	32−48	nd	nd	nd	nd	nd
					total	**nd (*0.0%*)**
2	7−24	nd	nd	nd	nd	nd
	24−32	0.4 ± 0.0	nd	nd	nd	0.4 ± 0.0
	32−48	0.9 ± 0.0	nd	nd	nd	0.9 ± 0.0
					total	**1.3 ± 0.0 (*0.3%*)**
3	7−24	nd	nd	nd	nd	nd
	24−32	0.7 ± 0.1	nd	nd	nd	0.7 ± 0.1
	32−48	2.5 ± 0.0	nd	nd	nd	2.5 ± 0.0
					total	**3.2 ± 0.1 (*0.6%*)**
4	7−24	nd	nd	nd	nd	nd
	24−32	5.1 ± 0.1	nd	nd	nd	5.1 ± 0.1
	32−48	3.1 ± 0.0	nd	nd	nd	3.1 ± 0.0
					total	**8.2 ± 0.1 (*1.6%*)**
5	7−24	nd	nd	nd	nd	nd
	24−32	4.3 ± 0.2	nd	nd	3.9 ± 0.0	8.3 ± 0.2
	32−48	3.3 ± 0.1	nd	nd	4.0 ± 0.0	7.3 ± 0.1
					total	**16 ± 0 (*3.1%*)**
6	7−24	3.0 ± 0.1	nd	nd	nd	3.0 ± 0.1
	24−32	1.6 ± 0.0	nd	nd	nd	1.6 ± 0.0
	32−48	1.7 ± 0.0	nd	nd	nd	1.7 ± 0.0
					total	**6.3 ± 0.0 (*1.2%*)**
7	7−24	8.1 ± 0.0	nd	nd	nd	8.1 ± 0.0
	24−32	3.6 ± 0.0	nd	nd	nd	3.6 ± 0.0
	32−48	1.6 ± 0.4	nd	nd	nd	1.6 ± 0.4
					total	**13.3 ± 0.3 (*2.6%*)**
8	7−24	9.8 ± 0.1	nd	nd	nd	9.8 ± 0.1
	24−32	4.1 ± 0.1	nd	nd	nd	4.1 ± 0.1
	32−48	1.3 ± 0.1	nd	nd	nd	1.3 ± 0.1
					total	**15.2 ± 0.2 (*3.0%*)**
9	7−24	27 ± 0	nd	nd	nd	27 ± 0
	24−32	10 ± 0	nd	nd	nd	10 ± 0
	32−48	6.0 ± 0.2	nd	nd	nd	6.0 ± 0.2
					total	**44 ± 0 (*8.6%*)**
10	7−24	1.2 ± 0.0	2.0 ± 0.1	7.1 ± 0.0	nd	10 ± 0
	24−32	0.9 ± 0.0	1.9 ± 0.0	6.1 ± 0.1	nd	8.9 ± 0.1
	32−48	nd	1.0 ± 0.0	3.0 ± 0.0	nd	4.0 ± 0.1
					total	**23 ± 0 (*4.6%*)**

[a] Data expressed as μmol ± standard error (n = 3). Italicized figures within parentheses represent the 0−48 h urolithin excretion as a percentage total amount of ellagitannins and ellagic acid ingested. Urolithins were not detected in urine collected <7 h after raspberry intake. nd, not detected; UroA-GlcA, urolithin A-*O*-glucuronide; UroB-GlcA, urolithin B-*O*-glucuronide.

theory 1 mol of sanguiin H-6 can produce 4 mol of ellagic acid, each of which can be metabolized to urolithins.

DISCUSSION

After acute consumption of a 300 g serving of raspberries, containing principally anthocyanins and ellagitannins along with smaller amounts of ellagic acid and an ellagic acid-*O*-pentoside (**Table 1**), by healthy human subjects, neither the parent compounds nor any of their metabolites appeared in detectable quantities in the plasma samples collected over the ensuing 24 h period. This is in keeping with other bioavailability studies in which anthocyanins have only rarely been detected in plasma (*27*). Other investigators have, however, detected ellagic acid in plasma, albeit with somewhat different pharmacokinetic profiles, after the ingestion of a pomegranate juice (*28*) and a pomegranate extract (*29*) containing ellagic acid and ellagitannins.

Analysis of ileal fluid collected 0−48 h after ingestion of raspberries by subjects with an ileostomy revealed the presence of 81 μmol of the 204 μmol intake of anthocyanins, a recovery of 40% (**Table 2**). This is the first report of the presence of anthocyanins in ileal fluid and indicates that despite the impact of pH on the stability of anthocynanins in the small intestine, in subjects with an intact colon, unexpectedly large amounts will

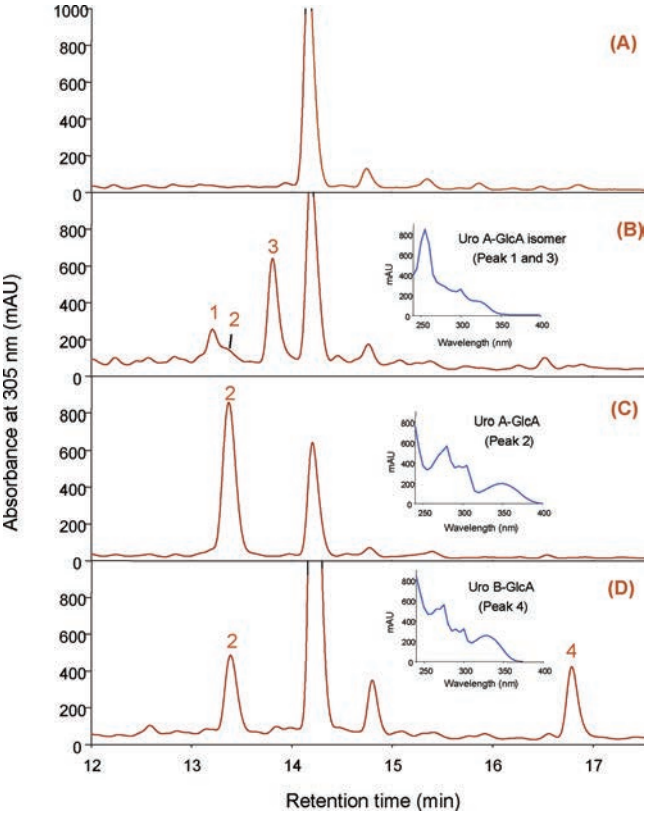

Figure 2. Gradient reversed phase HPLC profiles at 305 nm showing the urolithin content of urine collected from individual healthy volunteers before and after the ingestion of 300 g of raspberries: urine from (**A**) volunteer 9, 0−24 h before raspberry intake; (**B**) volunteer 10, 7−24 h after ingestion of raspberries; (**C**) volunteer 9, 7−24 h after ingestion of raspberries; and (**D**) volunteer 5, 24−32 h after ingestion of raspberries. Peaks: (1) urolithin A-*O*-glucuronide isomer 1, (2) urolithin A-*O*-glucuronide, (3) urolithin A-*O*-glucuronide isomer 2, (4) urolithin B-*O*-glucuronide.

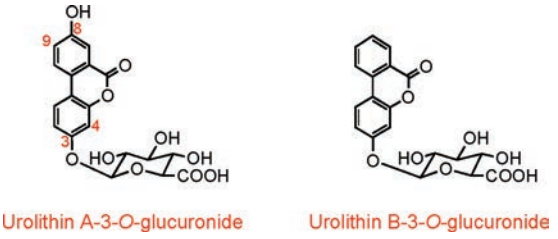

Figure 3. Structures of urolithin A-*O*-glucuronide and urolithin B-*O*-glucuronide.

pass from the small to the large intestine where the cyanidin-based anthocyanins are likely to be degraded into low molecular weight phenolic acids by the colonic microflora (*30*).

Ellagic acid-*O*-pentoside was not detected in ileal fluid, which did contain 19 μmol of ellagic acid, which corresponds to 241% of intake. This 11 μmol increase on the amount ingested is probably attributable to ellagic acid released by breakdown of ellagitannins. Although structurally too large to be absorbed in the small intestine, neither of the minor ellagitannins, sanguiin H-10 and lambertianin C, was excreted in ileal fluid, and there was only a 23% recovery of sanguiin H-6 (**Table 3**). This ca. 100 μmol loss of ellagitannins was not accompanied by the appearance of substantive amounts of ellagic acid in either plasma or urine (**Table 5**). Ellagic acid has limited solubility and may be lost under certain circumstances as it has been recovered as a precipitate in the abdominal cavity after its intraperitoneal administration to rats (*31*). Other factors affecting the recovery of ellagic acid could be its ionization at physiological pH to form poorly soluble complexes with calcium and magnesium ions and its ability to bind to intestinal epithelial cells (*32*, *33*). In addition, the possibility of irreversible binding of ellagitannins to proteins (*34*) in the gastrointestinal tract could contribute to their seemingly low recovery in ileal fluid.

The timing and quantity of urinary excretion of anthocyanins, ellagic acid, and ellagic acid-*O*-glucuronide indicated low-level absorption in the small intestine and, in keeping with this, there was an absence of major differences in the amounts excreted by healthy subjects and ileostomists (**Tables 4** and **5**).

Urolithins, which are formed from ellagic acid produced in the colon by microflora-mediated breakdown of ellagitannins, were absent in the urine of ileostomy volunteers but present in urine excreted by healthy subjects with a functioning large intestine. There was large person-to-person variation in the timing, quantity, and types of urolithins excreted in urine (**Table 6**). One subject (volunteer 1) produced no urolithins, whereas the other nine excreted quantities ranging from 1.3 to 44 μmol. With four of the volunteers (2−5) they were excreted 24−32 and 32−48 h after raspberry ingestion, whereas in five instances excretion also occurred in the 7−24 h collection period (volunteers 6−10). The spectrum of urolithins also varied. Seven subjects excreted only urolithin A-*O*-glucuronide (volunteers 2−4 and 6−9), and volunteer 5 produced *O*-glucuronides of both urolithins A and B, whereas the urine from volunteer 10 contained urolithin A-*O*-glucuronide and, uniquely, two isomers of urolithin A-*O*-glucuronide (**Table 6**).

These interindividual variations in urinary excretion of urolithins are almost certainly a consequence of the dependency of ellagitannin degradation on the composition of the colonic microflora. Similar variability was also observed in earlier feeding studies with raspberries and pomegranate juice (*28*, *35*, *36*). The nature of the interindividual differences and the specific bacteria involved in the metabolic processes that produce urolithins are, as yet, unknown.

It is of interest that phenolic compounds detected in ileal fluid collected after raspberry consumption were exclusively native to raspberries (**Tables 2** and **3**). This is in marked contrast to ileal fluid collected after ingestion of coffee, which contains not just chlorogenic acids but their sulfate and glucuronide metabolites (*21*). Likewise, after green tea consumption, ileal fluid contains parent green tea flavan-3-ols as well as their methyl, sulfate, and glucuronide metabolites (*19*). This indicates that sulfotransferases, uridine-5′-diphosphate glucuronosyltransferases and catechol-*O*-methyltransferases, in the wall of the small intestine, which catalyze the formation of these metabolites, possess a high degree of substrate specificity. Alternatively, it could also reflect various degrees of enterohepatic circulation, returning metabolites from the circulatory system to the small intestine via the bile.

In conclusion, the data obtained in the present study indicate low-level absorption and excretion of anthocyanins and ellagic acid after the ingestion of raspberries by humans. The ca. 40% recovery of anthocyanins and 23% recovery of sanguiin H-6 in ileal fluid show that in healthy subjects with an intact colon substantial quantities of these compounds will pass from the small to the large intestine, where they will be subjected to the action of the colonic microflora. This is evident from the excretion of ellagitannin-derived urolithin-*O*-glucuronides in the urine of healthy subjects but not volunteers with an ileostomy. There were marked qualitative and quantitative variations in the urolithin profile of individial volunteers, indicating differences in the colonic microflora responsible for ellagitannin degradation.

ACKNOWLEDGMENT

We are especially grateful to the volunteers who participated in the study.

LITERATURE CITED

(1) Borges, G.; Degeneve, A.; Mullen, W.; Crozier, A. Identification of flavonoid and phenolic antioxidants in black currants, blueberries, raspberries, red currants, and cranberries. *J. Agric. Food Chem.* **2010**, *58*, DOI: 10.1021/jf902263n.

(2) Mullen, W.; McGinn, J.; Lean, M. E. J.; Gardner, P.; Duthie, G. G.; Yokota, T.; Crozier, A. Ellagitannins, flavonoids, and other phenolics in red raspberries and their contribution to antioxidant capacity and vasorelaxation properties. *J. Agric. Food Chem.* **2002**, *50*, 5191–5196.

(3) Mullen, W.; Yokota, T.; Lean, M. E. J.; Crozier, A. Analysis of ellagitannins and conjugates of ellagic acid and quercetin in raspberry fruits by LC-MS^n. *Phytochemistry* **2003**, *64*, 617–624.

(4) Aviram, M.; Dornfeld, L.; Rosenblat, M.; Volkova, N.; Kaplan, M.; Colemann, R.; Hayek, T.; Presser, D.; Fuhman, B. Pomegranate juice consumption reduces oxidative stress, atherogenic modifications to LDL, and platelet aggregation: studies in humans and in atherosclerotic apolipoprotein E-deficient mice. *Am. J. Clin. Nutr.* **2000**, *71*, 1062–1076.

(5) Aviram, M.; Dornfeld, L. Pomegranate juice consumption inhibits serum angiotensin converting enzyme activity and reduces systolic blood pressure. *Atherosclerosis* **2001**, *158*, 195–198.

(6) Pantuck, A. J.; Lepper, J. T.; Zomorodian, N.; Aronson, W.; Hong, J.; Barnard, R. J.; Seeram, N.; Liker, H.; Wang, H.; Elashoff, R.; Heber, D.; Aviram, M.; Ignarro, L.; Belldegrum, A. Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer. *Clin. Cancer Res.* **2006**, *2*, 4018–4026.

(7) Rosenblat, M.; Hayek, T.; Aviram, M. Anti-oxidative effects of pomegranate juice consumption by diabetic pacients on serum and on macrophages. *Atherosclerosis* **2006**, *187*, 363–371.

(8) Belal, S. K .M.; Abdel-Rahman, A. H.; Mohamed, D. S.; Osman, H. E. H.; Hassan, N. A. Protective effect of pomegranate fruit juice against aeromonas hydrophila-induced intestinal histopathological changes in mice. *World Appl. Sci. J.* **2009**, *7*, 245–254.

(9) Zafrilla, P.; Ferreres, F.; Tomás-Barberán, F. A. Effect of processing and storage on the antioxidant ellagic acid derivatives and flavonoids of red raspberries (*Rubus idaeus*) jams. *J. Agric. Food Chem.* **2001**, *49*, 3651–3655.

(10) Mandal, S.; Stoner, G .D. Inhibition of *N*-nitrosobenzylmethylamine-induced oesophageal tumorigenesis in rats by ellagic acid. *Carcinogenesis* **1990**, *11*, 55–61.

(11) Rao, C. V.; Tokumo, K.; Rigotty, J.; Zang, E.; Kelloff, G.; Reddy, B. S. Chemoprevention of colon carcinogenesis by dietary administration of piroxicam, α-difluoromethylornithine, 16-α-fluoro-5-androsten-17-one, and ellagic acid individually and in combination. *Cancer Res.* **1991**, *51*, 4528–4534.

(12) Boukharta, M.; Jalbert, G.; Castonguay, A. Biodistribution of ellagic acid and dose-related inhibition of lung tumorigenesis in A/J mice. *Nutr. Cancer Int. J.* **1992**, *18*, 181–189.
(13) Borges, G.; Roowi, S.; Rouanet, J.-M.; Duthie, G. G.; Lean, M. E.J.; Crozier, A. The bioavailability of raspberry anthocyanins and ellagitannins in rats. *Mol. Nutr. Food Res.* **2007**, *51*, 714–725.
(14) Hollman, P. C. H.; Devries, J. H. M.; Vanleeuwen, S. D.; Mengelers, M. J. B.; Katan, M. B. Absorption of dietary quercetin glycosides and quercetin in healthy ileostomy volunteers. *Am. J. Clin. Nutr.* **1995**, *62*, 1276–1282.
(15) Jaganath, I. B.; Mullen, W.; Edwards, C. A.; Crozier, A. The relative contribution of the small and large intestine to the absorption and metabolism of rutin in man. *Free Radical Res.* **2006**, *40*, 1035–1046.
(16) Kahle, K.; Huemmer, W.; Kempf, M.; Scheppach, W.; Erk, T.; Richling, E. Polyphenols are intensively metabolized in the human gastrointestinal tract after apple juice consumption. *J. Agric. Food Chem.* **2007**, *55*, 10605–10614.
(17) Marks, S. C.; Mullen, W.; Borges, G.; Crozier, A. Absorption, metabolism and excretion of cider dihydrochalcones in health humans and subjects with an ileostomy. *J. Agric. Food Chem.* **2009**, *57*, 2009–2015.
(18) Roowi, S.; Stalmach, A.; Mullen., W.; Lean, M. E. J.; Edwards, C. A.; Crozier, A. Green tea flavan-3-ols: colonic degradation and urinary excretion of catabolites by humans. *J. Agric. Food Chem.* **2010**, *58*, 1296–1304.
(19) Stalmach, A.; Mullen, W.; Steiling, H.; Williamson, G.; Crozier, A. Absorption, metabolism, efflux and excretion of green tea flavan-3-ols in humans with an ileostomy. *Mol. Nutr. Food Res.* **2010**, *54*, 323−334.
(20) Olthof, M. R.; Hollman, P. C. H.; Buijsman, M. N. C. P.; Amelsvoort, J. M. M.; Katan, M. B. Chlorogenic acid, quercetin-3-rutinoside and black tea phenols are extensively metabolized in humans. *J. Nutr.* **2003**, *133*, 1806–1814.
(21) Stalmach, A.; Steiling, H.; Williamson, G.; Crozier, A. Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Arch. Biochem. Biophys.* **2010**, in press.
(22) Stalmach, A.; Mullen, W.; Barron, D.; Uchida, K.; Yokota, T.; Cavin, C.; Steiling, H.; Williamson, G.; Crozier, A. Metabolite of hydroxycinnamates derivatives in plasma and urine after the ingestion of coffe by humans: identification of biomarkers of coffe consumption. *Drug Metab. Dispos.* **2009**, *37*, 1749–1758.
(23) Doyle, B.; Griffiths, L. A. The metabolism of ellagic acid in the rat. *Xenobiotica* **1980**, *10*, 247–256.
(24) Cerdá, B.; Periago, P. M.; Espín, J. C.; Tomás-Barberán, F. A. Identification of urolithin A as a metabolite produced by human colon microflora from ellagic acid and related compounds. *J. Agric. Food Chem.* **2005**, *53*, 5571–5576.
(25) Larrosa, M.; Tomás-Barberán, F. A.; Espín, J. C. The hydrolysable tannin punicalagin releases ellagic acid which induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J. Nutr. Biochem.* **2006**, *17*, 611–625.
(26) Espín, J. C.; González-Barrio, R.; Cerdá, B.; López-Bote, C.; Tomás-Barberán, F. A. Iberian pig as a model to clarify obscure points in the bioavailablility and metabolim of ellagitannins in humans. *J. Agric. Food Chem.* **2007**, *55*, 10476–10485.
(27) Wu, X. L; Prior, R. L. Fate of anthocyanins and antioxidant capacity in contents of the gastrointestinal tract of weanling pigs following black raspberry consumption. *J. Agric. Food Chem.* **2006**, *54*, 583–589.
(28) Seeram, N. P.; Henning, S. M.; Zhang, Y. J.; Suchard, M.; Li, Z. P.; Heber, D. Pomegranate juice ellagitannin metabolites are present in human plasma and some persist in urine for up to 48 h. *J. Nutr.* **2006**, *136*, 2481–2485.
(29) Mertens-Talcott, S. U.; Jilma-Stohlawetz, P.; Rios, J.; Hingorani, L.; Derendorf, H. Asorption, metabolism, and antioxidant effects of pomegranate (*Punica granatum* L.) polyphenols after ingestion of a standardized extract in healthy human volunteers. *J. Agric. Food Chem.* **2006**, *54*, 583–589.
(30) Aura, A. M.; Martin-López, P.; O'Leary, K. A.; Williamson, G.; Oksman-Caldentey, K. M.; Poutanen, K.; Santos-Buelga, C. In vitro metabolism of anthocyanins by human gut microflora. *Eur. J. Nutr.* **2005**, *44*, 133–42.
(31) Smart, R. C.; Huang, M.-T.; Chang, R. L.; Sayer, J. M.; Jerina, D. M.; Conney, A. H. Disposition of the naturally occurring antimutagenic plant phenol, ellagic acid, and its synthetic derivatives, 3-*O*-decylellagic acid and 3,3′-di-*O*-methylellagic acid in mice. *Carcinogenesis* **1986**, *7*, 1663–1667.
(32) Whitley, A. C.; Stoner, G. D.; Darby, M. V.; Walle, T. Intestinal epithelial cell accumulation of the cancer preventive polyphenol ellagic acid-extensive binding to protein and DNA. *Biochem. Pharmacol.* **2003**, *66*, 907–915.
(33) Seeram, N.; Lee, R.; Heber, D. Bioavailability of ellagic acid in human plasma after consumption of ellagitannins from pomegranate (*Punica granatum* L.) juice. *Clin. Chim. Acta* **2004**, *348*, 63–68.
(34) Haslam, E. *Practical Polyphenolics. From Structure to Molecular Recognition and Physiological Action*; Cambridge University Press: Cambridge, U.K., 1998.
(35) Cerdá, B.; Espín, J. C.; Parra, A.; Martínez, P.; Tomás-Barberán, F. A. The potent in vitro antioxidant ellagitannins from pomegranate juice are metabolized into bioavailable but poor antioxidant hydroxy-6*H*-dibenzopyran-6-one derivatives by the colonic microflora of healthy humans. *Eur. J. Nutr.* **2004**, *43*, 205–220.
(36) Cerdá, B.; Tomás-Barberán, F. A.; Espín, J. C. Metabolism of chemopreventive and antioxidant ellagitannins from strawberries, raspberries, walnuts and oak-aged wines in humans: Identification of biomarkers and individual variability. *J. Agric. Food Chem.* **2005**, *53*, 227–235.

Received for review January 26, 2010. Revised manuscript received February 23, 2010. Accepted March 1, 2010. The investigation was funded by a grant from the Washington Red Raspberry Commission and Fundación Séneca, Spain, which provided R.G.-B. with a postdoctoral fellowship.