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ARTICLE

Identification of Metabolites in Human Plasma and Urine after Consumption of a Polyphenol-Rich Juice Drink

William Mullen,[†] Gina Borges,[‡] Michael E. J. Lean,[§] Susan A. Roberts,[#] and Alan Crozier*,[‡]

[†]Division of Ecology and Evolutionary Biology, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom, [‡]Division of Developmental Medicine, Faculty of Medicine, University of Glasgow, Glasgow G12 8QQ, United Kingdom, [§]Unit of Human Nutrition, Division of Developmental Medicine, Queen Elizabeth Building, Royal Infirmary, University of Glasgow, Glasgow G31 2ER, United Kingdom, and [#]Global Scientific and Regulatory Affairs, The Coca-Cola Company, Atlanta, Georgia 30313

A polyphenol-rich (P-R) juice drink was developed as a potential approach to increase intake of dietary polyphenols. Analysis of the beverage by HPLC with PDA, fluorescence, and MS detection facilitated the identification/partial identification of 40 flavonoids and related phenolic compounds. The main constituents were (−)-epigallocatechin and other green tea flavan-3-ols, phloretin-2′-*O*-glucoside, gallic acid, hesperetin-7-*O*-rutinoside, 5-*O*-caffeoylquinic acid, and procyanidins, with trace levels of several flavonols and purple grape juice anthocyanins also being present. Healthy human subjects (n = 10) consumed 350 mL of the P-R juice drink, after which plasma and urine samples were collected over a 0−24 h period. HPLC-MS analysis identified 13 metabolites in plasma and a further 20 in urine. Qualitatively, the profiles of the glucuronide, sulfated, and methylated metabolites were very similar to those detected in earlier investigations when the main components in the juice drink were consumed separately in feeding studies with coffee, green tea, orange juice, and apple cider.

KEYWORDS: Plasma metabolites; polyphenol-rich drink; HPLC-MS identifications; urinary metabolites

INTRODUCTION

There is strong epidemiological evidence that diets rich in fruits and vegetables are associated with reduced prevalences and incidences of chronic diseases (*1*) including reducing the risk of cardiovascular disease (*2*, *3*) and Alzheimer's disease (*4*) and possibly delaying mental decline in the elderly (*5*, *6*). Fruits and vegetables contain fiber, vitamins, and minerals. They also contain a diversity of phytochemicals that have been implicated in a number of the protective effects against chronic diseases (*7*) such as reducing platelet aggregation, LDL oxidation, and inflammation (*8*, *9*), improving endothelial function, and reducing blood pressure (*10*, *11*). The principal dietary phytochemicals involved in these effects are flavonoids and related phenolic compounds, of which several thousand exist in planta, with many occurring in high concentrations in only a few plant species as typified by isoflavones in soya and flavanones in citrus species (*12*).

The recommended daily intake of fruit and vegetables in the United Kingdom is a minimum of five 80 g portions of different varieties, one of which may be a fruit juice (*13*). The stress on variety is a guideline, in recognition of the diverse ranges of nutrients present in plant foods. The recommendation of the U.S. Surgeon General is to eat 5−10 portions daily (*14*). In practice, only 20−30% of the U.K. population and < 50% of people in the United States consume these quantities on a regular basis. Furthermore, there has been little increase in response to dietary recommendations over recent decades (*15*−*18*).

Fruit juices represent a simple way for most consumers, including the elderly and edentulous, to increase their intakes of many potentially beneficial compounds without major changes in dietary habit. Fruit juices and drinks can contain a diversity of polyphenolic compounds, but in some instances the levels can be relatively low (*19*), in which case consumption is unlikely to have a significant dietary impact. Furthermore, there are limits to the amount of juice that can be consumed within an energy-balanced diet. It was against this background that a study was initiated to investigate a low-calorie, polyphenol-rich (P-R) juice drink containing substantial amounts of a blend of flavonoids and related compounds, from a variety of sources, that cannot be obtained in a single portion of fruit. Given the complexity of the beverage, it was considered important to determine whether or not the bioavailability of its flavonoid and phenolic components is limited by potential competitive interactions for absorption, transport, or enzymatic sites for metabolism in the human gastrointestinal tract and elsewhere in the body.

This paper reports the flavonoid and phenolic compounds in the P-R juice drink and, after ingestion of a 350 mL volume by humans subjects, identifies the metabolites that subsequently

*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).

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Table 1. HPLC-MS Conditions Used To Analyze Flavonoids and Phenolics in the P-R Drink and Their Metabolites in Urine and Plasma[a]

method	samples analyzed	HPLC solvent A	HPLC gradient	MS detection	*m/z* segment 1	*m/z* segment 2
1	P-R drink	1.0% formic acid	60 min 5−30% B	full scan[b,c]		
2	plasma flavan-3-ol and metabolites	0.1% formic acid	60 min 4−25% B	CRM and two segment SIM[b]	481, 495	369, 399, 383
3	plasma flavan-3-ol, phloretin, and hesperetin metabolites	0.1% formic acid	60 min 8−32% B	CRM and two segment SIM[b]	465	449, 477
4	urine flavan-3-ols and phloretin metabolites	0.1% formic acid	66 min 10−30% B	CRM and two segment SIM[b]	465, 369, 383, 545	449, 529
5	urine flavan-3-ol and hesperetin metabolites	0.1% formic acid	66 min 10−30% B	CRM and two segment SIM[b]	399, 481, 495	477, 557
6	urine gallic acid metabolites	0.5% acetic acid	60 min, 5−27% B	CRM and one segment SIM[b]	263	
7	urine hydroxycinnamates	0.5% acetic acid	60 min, 5−16% B	CRM and two segment SIM[b]	259, 261, 273, 275	195, 250

[a] Analyses utilized a 250 × 4.6 mm (i.d.), 4 μm, Synergi RP-MAX column eluted at a flow rate of 1 mL/min with the specified gradient of acidified water (solvent A) in acetonitrile (solvent B) except method 3, which used a 3.0 μm i.d. column at a flow rate of 0.5 mL/min. [b] Negative ionization. [c] Positive ionization.

appear in plasma and urine. The metabolite profiles are then compared qualitatively to those obtained when the main components in the beverage were consumed separately in feeding studies with coffee (*20*), green tea (*21*), orange juice (*22*), and apple cider (*23*).

MATERIALS AND METHODS

Chemicals. 5-*O*-Caffeoylquinic acid, procyanidin B2, (+)-catechin, (−)-epicatechin, and L-ascorbate oxidase (EC 1.10.3.3; 4 U/mL) were purchased from Sigma-Aldrich (Poole, U.K.). Quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, phloretin-2′-*O*-glucoside, hesperetin-7-*O*-rutinoside, naringenin-7-*O*-rutinoside, ferulic acid, caffeic acid, and *p*-coumaric acid were obtained from AASC Ltd. (Southampton, U.K.). Peonidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, malvidin-3-*O*-glucoside, and 3-*O*-methylgallic acid were purchased from Extrasynthese (Genay, France), and methanol and acetonitrile were obtained from Rathburn Chemicals (Walkerburn, Scotland). Formic acid and acetic acid were obtained from Fisher Scientific (Loughborough, U.K.). Benzyl mercaptan was purchased from Lancaster Synthesis (Morecombe, U.K.). Dihydroferulic acid was obtained from Alfa Aesar (Heysham, U.K.). Phloretin-2′-*O*-glucuronide was prepared as described by Kahle et al. (*24*). Feruloylglycine was synthesized by Professor Takao Yokota (Teikyo University, Utsunomiya, Japan) as described by Stalmach et al. (*20*). Professor Junji Terao and Dr. Yoshichika Kawai (University of Tokushima, Japan) supplied a sample of (−)-epicatechin-7-*O*-glucuronide. Dr. Yukihiko Hara (Mitsui Norin Co. Ltd., Tokyo, Japan) donated standards of 3′- and 4′-*O*-methyl-(−)-epicatechin. Hesperetin-7-*O*-glucuronide was a gift from Dr. Hikaru Matsumoto (National Institute of Fruit Tree Science, Shizuoka, Japan). A polyphenol-rich (P-R) drink was supplied by The Coca Cola Co. (Atlanta, GA). Quercetin-3-*O*-glucuronide was isolated from French beans.

Juice Drink Design. The beverage was designed to include polyphenolic compounds from various sources with known or potential health benefits. In the first instance, this involved performing a supplier survey to identify potential sources of high-polyphenol-containing extracts, followed by testing potential ingredients to determine the quantity and type of phenolic compounds actually present. The quality of these products in terms of polyphenol content varied greatly, but on the basis of HPLC-MS analyses and an ex vivo screening for impact on endothelial function (*25*), sources of ingredients were selected that enabled a low-calorie, 28% juice P-R beverage to be prepared containing green tea flavan-3-ols, grape seed and pomace procyanidins, apple dihydrochalcones, procyanidins, chlorogenic acids, lemon flavanones, and grape anthocyanins, a combination of potentially protective compounds that cannot be found in any individual fruit or single-component juice. The drink also contained 168 mg of vitamin C, 13 g of carbohydrate, and 51 calories per 350 mL.

Total Phenol Content of the P-R Juice Drink. The total phenol content of the drink was determined in triplicate in gallic acid equivalents (GAE) using the Folin−Ciocalteu method (*26*).

FRAP Antioxidant Capacity of the P-R Juice Drink. The FRAP assay was used to estimate the antioxidant capacity of the drink (*27*). Samples were analyzed before and after treatment with L-ascorbate oxidase to ascertain the contribution of vitamin C to the total antioxidant capacity of the beverage. The results are expressed as millimoles per liter of Fe^{3+} reduced.

Study Design. The Glasgow Royal Infirmary Research Ethics Committee and the Glasgow University Ethics Committee approved the study protocol. Six male and four female volunteers (19−51 years of age; mean body mass index = 24.3, range = 17.4−34.7), who were nonsmokers and not on any medication, gave their written consent and participated in the study. They followed a diet low in flavonoids, which excluded fruits and vegetables, high-fiber products, and beverages such as tea, coffee, fruit juices, and wine, for 2 days before the study. After an overnight fast, volunteers consumed 350 mL of the P-R drink. Volunteers ate ham or turkey with white bread rolls 4 h after drinking the beverage and thereafter remained on a low-flavonoid diet for a further 20 h until the final blood and urine samples were collected. Blood was collected in heparinized tubes from all volunteers at different time points for 24 h postingestion and plasma separated by centrifugation at 4000*g* for 10 min at 4 °C. Two 1 mL aliquots of plasma were acidified to pH 3 with 30 μL of 50% aqueous formic acid and 100 μL of 10 mmol/L ascorbic acid, frozen in liquid nitrogen, and stored at −80 °C prior to analysis. Urine was collected prior to supplementation and over four time periods 24 h after the ingestion of the drink. The total volume for each period was recorded. After collection, urine samples were divided into aliquots and stored at −80 °C prior to analysis by HPLC with photodiode array (PDA), fluorescence (FL), and mass spectrometric (MS) detection.

Extraction of the Beverage. A 500 μL aliquot of the P-R drink was added to 500 μL of methanol and shaken for 3 min. The mixture was then centrifuged at 13000*g* at 4 °C for 5 min and the supernatant stored at −80 °C prior to analysis by HPLC-PDA-FL-MS.

Extraction of Plasma. Plasma samples were extracted using a method developed by Day et al. (*28*). Each sample was spiked with 20 μL of 10% aqueous ascorbic acid containing 0.5 mmol/L EDTA and 1 μg of ethyl gallate as an internal standard for plasma extraction efficiency. Aliquots of plasma extracts were analyzed by HPLC-PDA-MS.

Processing of Urine. Aliquots of urine were defrosted and centrifuged at 13000*g* for 10 min prior to analysis by HPLC-PDA-MS.

HPLC-PDA-FL-MS. Analysis was carried out on a Surveyor HPLC system comprising an autosampler with sampler cooler maintained at 4 °C, a photodiode array detector (Thermo Fisher Scientific, San Jose, CA) scanning from 200 to 600 nm, and a fluorometer (Jasco Corporation, Tokyo, Japan) with excitation at 285 nm and emission at 315 nm. Samples were analyzed in a 250 × 4.6 mm, 4 μm, Synergi RP-Max held at 40 °C and eluted at a flow rate of 1 mL/min, all maintained at 40 °C in a column oven, using various mobile phases and gradients (see **Table 1**). After passing through the flow cells of the PDA and FL detectors, the eluate was split and 200 μL directed to a LCQ Advantage ion trap mass spectrometer fitted with an electrospray interface (Thermo Fisher Scientific). Capillary temperature was 300 °C, sheath gas and auxiliary gas were 60 and 20 units, respectively, and the source voltage was 4 kV.

Beverage samples were analyzed using full scan MS in both positive and negative ionization modes, the scan range was from *m/z* 150 to 2000 for negative ion and from *m/z* 190 to 1000 for positive ion. Identifications are based on cochromatography with authentic standards, when available. Absorbance spectra and mass spectra, using MS^2, were used to confirm the identity of compounds previously reported in the literature (*19*). The final conditions and selection of metabolites analyzed in the plasma and urine were determined after a detailed analysis of potential metabolites identified by full scan MS^2, consecutive reaction monitoring (CRM), and/or selected reaction monitoring (SRM).

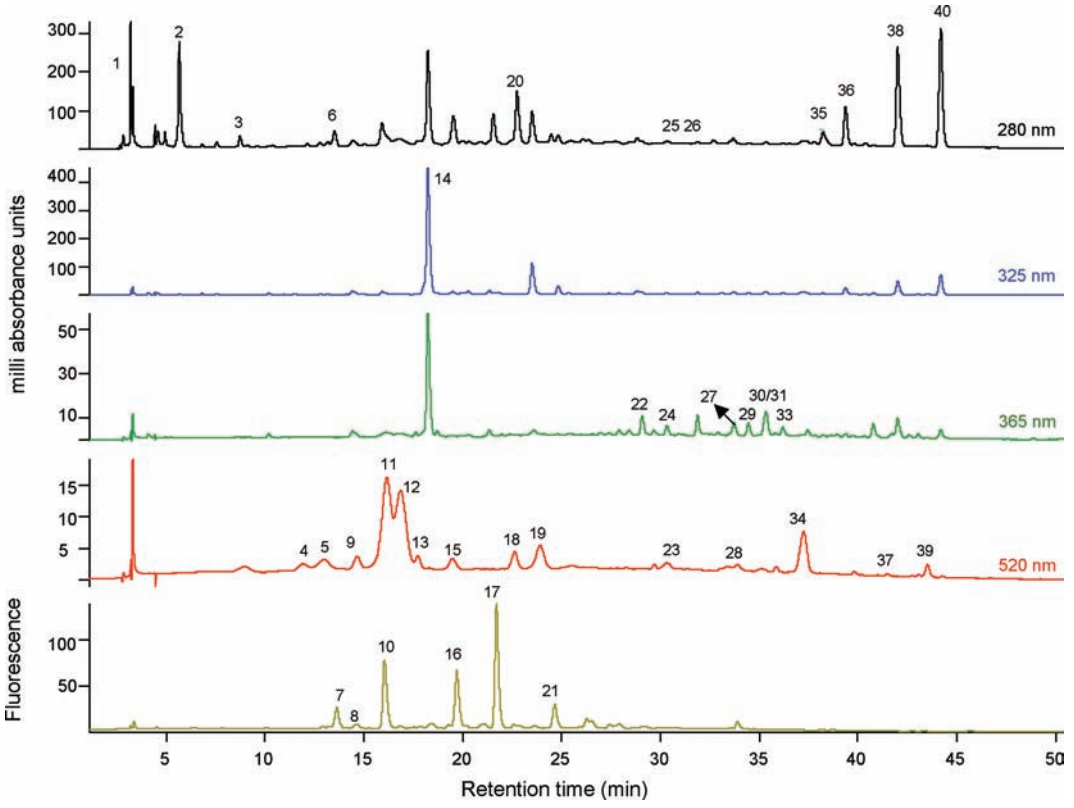

Figure 1. Gradient, reversed phase HPLC analysis of the P-R drink with PDA detection at 280, 325, 365, and 520 nm and fluorescence detection at excitation 285 nm/emission 315 nm. For identification of numbered peaks see **Table 3**.

Table 2. FRAP Antioxidant Capacity and Folin Total Phenolics Content of the P-R Drink[a]

FRAP (mmol/L Fe^{3+})	FRAP minus vitamin C (mmol/L Fe^{3+})	contribution of vitamin C (%)	total phenolics (mmol/L GAE)
17.5 ± 0.1	16.1 ± 0.1	8.5	10.6 ± 0.1

[a] Data expressed as mean values ± standard error (n = 3). GAE, gallic acid equivalents.

Analysis of Procyanidin. Thiolytic degradation was carried out on freeze-dried aliquots of the drink, which were reacted with 400 μL of benzyl mercaptan (5% in methanol, v/v) in 200 μL of acidified methanol (3.3% HCl, v/v) at 40 °C for 30 min, with samples being vortexed every 10 min (*29*). The HPLC-PDA-FL-MS analysis consisted of a 60 min gradient of 3−55% acetonitrile in 1% aqueous formic acid. The flow rate was 1 mL/min, and a fluorometric detector was also used with excitation at 280 nm and emission at 310 nm.

RESULTS

Antioxidant Capacity and Total Phenolics Content of the P-R Drink. The FRAP antioxidant capacity of the beverage was 17.5 ± 0.1 mmol/L Fe^{3+}, of which 8.5% was contributed by vitamin C, whereas the concentration of total phenolics level was 10.6 ± 0.1 mmol/L GAE (**Table 2**). Both of these values, especially the antioxidant capacity, are higher than those of the commercial juices and fruit drinks analyzed by Mullen et al. (*19*).

Analysis of the P-R Drink. HPLC-PDA-FL traces obtained when analyzing the P-R drink are illustrated in **Figure 1**. In total, 40 compounds were identified or partially identified on the basis of their mass spectra, cochromatography with reference compounds, when available, and by reference to the literature. This information is summarized in **Table 3**, and the bases of the identifications are the same as those detailed in earlier publications (*19*, *30*, *31*).

Quantitative analysis of the beverage revealed that the 350 mL consumed by the volunteers contained 101 mg of grape seed and apple procyanidins, with an average degree of polymerization of 3.9, as well as the compounds listed in **Table 3**. Of the 40 flavonoids and related phenolic compounds identified in the beverage, 26 were present in quantifiable amounts (**Table 4**). The predominant ingredients in the drink, structures of which are illustrated in **Figure 2**, were green tea flavan-3-ols, with the principal component being 187 μmol of (−)-epigallocatechin. Also quantified were 5-*O*-caffeoylquinic acid and dihydrochalcones from apples, flavanones from citrus, gallic acid, which is also a green tea-derived compound, and trace amounts of six flavonols and a similar number of purple grape anthocyanins. The beverage, because of the selected ingredients, had a much more diverse composition of polyphenolic compounds than that found in any single-component fruit juice or drink (*19*).

Qualitative Analysis of Plasma. The complexity of the metabolites meant that it was not possible to carry out analysis of all compounds in a single run (see **Table 1**). A total of 13 plasma metabolites were detected and identified as described below and summarized in **Table 5**. All of the metabolites identified in plasma were also present in urine. The peak numbers assigned to plasma metabolites match those in urine.

It should be noted that analysis of flavan-3-ols and their metabolites is somewhat more subtle than is generally appreciated. For instance, without reference compounds that can be separated by reversed phase HPLC, MS is unable to distinguish between (−)-epicatechin and (+)-catechin metabolites or (−)-epigallocatechin and (+)-gallocatechin metabolites. There is also evidence of both green tea processing and postconsumption converting (+)-flavan-3-ols to their (−)-stereoisomers, which, together with their associated metabolites, cannot be discriminated by reversed phase HPLC-MS. We therefore refer to flavan-3-ol metabolites as (epi)catechins or (epi)gallocatechins.

Table 3. HPLC-MS^2-Based Identifications of Vitamin C and Phenolic Compounds in the P-R Drink

peak	t_R (min)	$[M - H]^-$ (m/z)[a]	MS^2 (m/z)	compound
1	3.6	175	157	vitamin C
2	5.6	169	125	gallic acid
3	8.6	305	261, 221	(+)-gallocatechin
4	11.8	611$^+$	449, 287	cyanidin-3,5-*O*-diglucoside
5	12.6	641$^+$	479, 317	petunidin-3,5-*O*-diglucoside
6	12.9	305	261, 221	(−)-epigallocatechin
7	13.0	577	425, 289	procyanidin B1 dimer
8	13.4	577	425, 289	procyanidin B3 dimer
9	14.5	465$^+$	303	delphinidin-3-*O*-glucoside
10	15.5	289	245	(+)-catechin
11	15.9	625$^+$	463, 301	peonidin-3,5-*O*-diglucoside
12	16.6	655$^+$	493, 331	malvidin-3,5-*O*-diglucoside
13	17.4	449$^+$	287	cyanidin-3-*O*-glucoside
14	17.9	353	191	5-*O*-caffeoylquinic acid
15	19.0	479	317	petunidin-3-*O*-glucoside
16	19.1	577	425, 289	procyanidin B2 dimer
17	21.0	289	245	(−)-epicatechin
18	22.1	463$^+$	317	peonidin-3-*O*-glucoside
19	23.3	493$^+$	331	malvidin-3-*O*-glucoside
20	22.3	457	331, 305	(−)-epigallocatechin gallate
21	23.9	865	577, 289	procyanidin trimer
22	28.4	479	317	myricetin-*O*-hexoside
23	29.6	773$^+$	611, 303	delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside
24	31.2	771	609, 301	quercetin-*O*-rutinosyl-*O*-glucoside
25	31.9	441	305	(−)-epicatechin gallate
26	32.9	577	577, 289	procyanidin dimer
27	33.1	609	301	quercetin-3-*O*-rutinoside
28	33.2	787$^+$	625, 317	petunidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside
29	33.7	463	301	quercetin-3-*O*-galactoside
30/31	34.5	463/477	301	quercetin-3-*O*-glucoside/quercetin-3-*O*-glucuronide
32	35.1	611$^+$	303	delphinidin-3-*O*-(6″-*O*-*p*-coumaroyl)glucoside
33	35.4	755	593, 285	kaempferol conjugate
34	36.5	801$^+$	639, 331	malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside
35	37.5	579	459, 271	naringenin-7-*O*-neohesperidoside
36	38.6	567	273	phloretin-2′-*O*-(2″-*O*-xylosyl)glucoside
37	39.1	625$^+$	317	petunidin-3-*O*-*p*-coumaroylglucoside
38	41.6	609	301	hesperetin-7-*O*-rutinoside
39	42.7	639$^+$	331	malvidin-3-*O*-*p*-coumaroylglucoside
40	43.4	435	273	phloretin-2′-*O*-glucoside

[a] $[M - H]^-$ negatively charged molecular ion; $^+$ indicates positively charged molecular ion.

Peak M1 [HPLC retention time (t_R) 10.6 min when analyzed using method 2] had a negatively charge molecular ion $[M - H]^-$ at m/z 481, which on MS^2 fragmented with a 176 amu loss, indicative of cleavage of a glucuronide unit, producing an (epi)gallocatechin-like ion at m/z 305. Further fragmentation in MS^3 of the ion at m/z 305 produced the same fragmentation pattern observed with peaks 3 and 6 in the P-R drink (**Table 3**). This metabolite is as an (epi)gallocatechin-*O*-glucuronide (*21*).

Peak M2 (t_R 15.4 min, method 2) had a $[M - H]^-$ at m/z 495, which on MS^2 also fragmented with a 176 amu loss, yielding a methyl-*O*-(epi)gallocatechin ion at m/z 319. Upon MS^3 this ion produced a series of ions, including one at m/z 137 characteristic of methylation at the 4′-position (*32*). This peak was therefore identified as a 4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide (*21*).

Table 4. Flavonoid and Phenolic Composition of the P-R Juice Drink[a]

compound	μmol/350 mL
gallic acid	52
total phenolic acids	*52*
(−)-epicatechin	77
(+)-catechin	50
(−)-epigallocatechin	187
(+)-gallocatechin	48
(−)-epicatechin-3-*O*-gallate	6.2
(−)-epigallocatechin-3-*O*-gallate	65
procyanidin B1 dimer	15
procyanidin B2 dimer	39
total flavan-3-ols	*487*
peonidin-3,5-*O*-diglucoside	1.8
malvidin-3,5-*O*-diglucoside	3.4
peonidin-3-*O*-glucoside	0.6
malvidin-3-*O*-glucoside	0.7
malvidin-3-*O*-(6″-*O*-*p*-coumaroyl)-5-*O*-diglucoside	0.6
malvidin-3-*O*-*p*-(6″-*O*-*p*-coumaroyl)glucoside	0.7
total anthocyanins	*7.8*
5-*O*-caffeoylquinic acid	46
total chlorogenic acids	*46*
hesperetin-7′-*O*-rutinoside	45
naringenin-7-*O*-neohesperidoside	5.9
total flavanones	*51*
phloretin-2′-*O*-glucoside	68
phloretin-2′-*O*-(2″-xylosyl)glucoside	15
total dihydrochalcones	*83*
quercetin-3-*O*-rutinoside	1.3
quercetin-3-*O*-galactoside	1.6
quercetin-3-*O*-glucoside	1.3
quercetin-*O*-glucuronide	2.3
myricetin-*O*-hexoside	2.7
quercetin-*O*-rutinosylglucoside	1.0
total flavonols	*10*
total flavonoids and phenolic compounds	*737*

[a] Identification and quantification were based on HPLC with PDA and fluorescence detection and full scan data dependent tandem MS. Data are expressed as mean values (n = 3). The P-R juice drink also contained 101 mg/350 mL of procyanidins.

Peak M4 (t_R 17.6 min, method 2) produced a $[M - H]^-$ at m/z 465, which upon MS^2 gave rise to a fragment at m/z 289. MS^3 of this ion produced ions at m/z 245, 205, and 179, which matched the mass spectrum of an (epi)catechin. This peak did not cochromatograph with (−)-epicatechin-7-*O*-glucuronide and may be the 3′-*O*-glucuronide, which has been identified in human urine after ingestion of (−)-epicatechin (*33*). However, in the absence of a reference compound, this peak is designated as an incompletely characterized (epi)catechin-*O*-glucuronide (*21*).

Peaks M7 and M10 (t_R 26.7 and 32.1 min, method 2) both produced a $[M - H]^-$ at m/z 369, which, with an 80 amu loss indicating cleavage of a sulfate unit, yielded an (epi)catechin MS^2 ion at m/z 289, which further fragmented producing a spectrum of ions matching that of (epi)catechin. These two peaks are, therefore, (epi)catechin-*O*-sulfates (*21*).

Peak M8 (t_R 30.2 min, method 2) had a $[M - H]^-$ at m/z 399, which on MS^2 gave rise to an ion at m/z 319. Like M7 and M10, the 80 amu loss indicates cleavage of a sulfate moiety. MS^3 fragmentation of the aglycone ion at m/z 319 produced a number of ions including, like M2, a fragment at m/z 137. This mass spectrum, therefore, is that of a 4′-*O*-methyl-(epi)gallocatechin-*O*-sulfate (*21*, *32*).

Peaks M11, M13, M14, and M15 (t_R 33.6, 37.6, 41.1, and 43.9 min, respectively, method 2) all had a $[M - H]^-$ at m/z 383, which on MS^2 fragmented with an 80 amu loss to yield an ion at m/z 303.

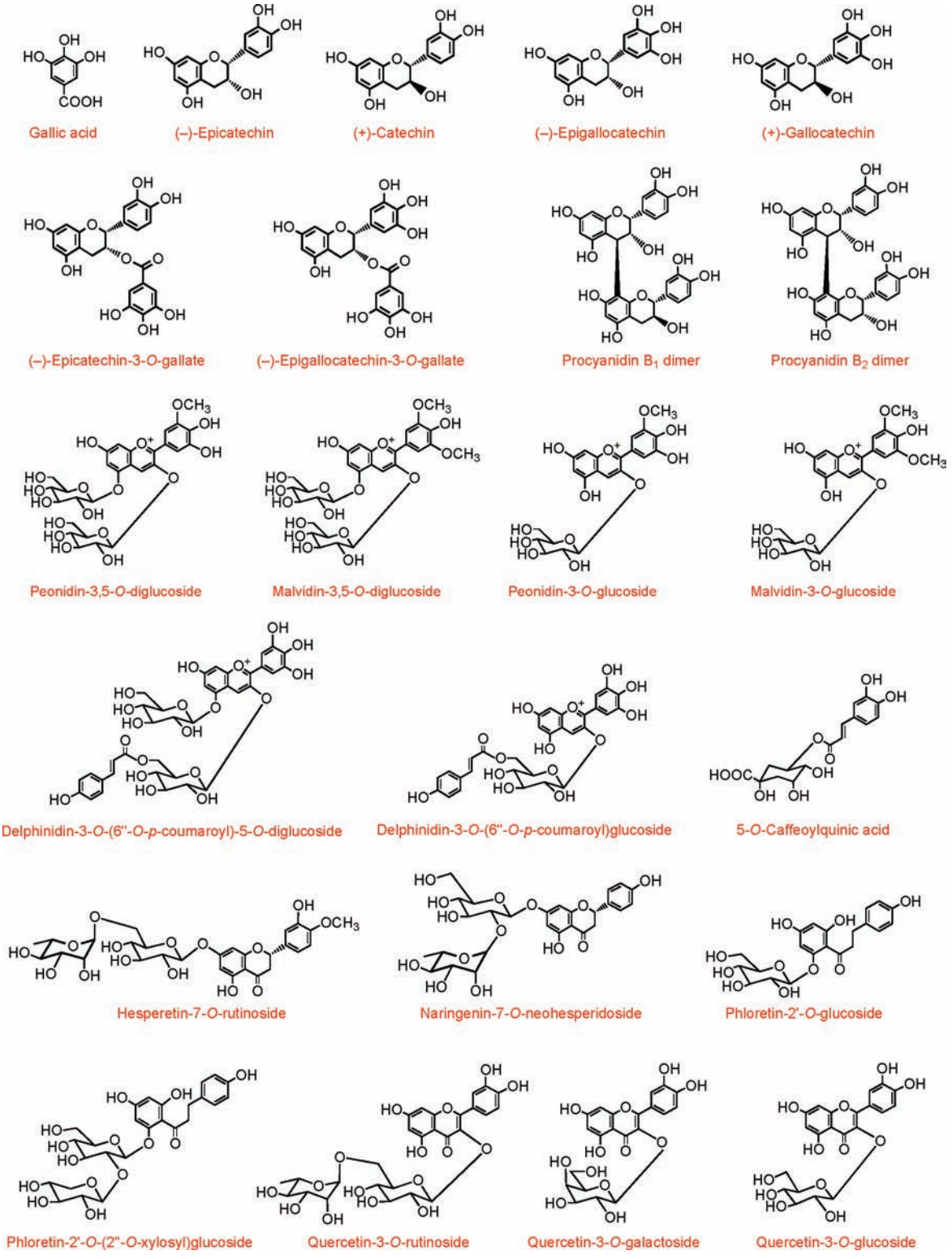

Figure 2. Structures of the main flavonoids and related compounds in the P-R drink.

MS^3 of the *m*/*z* 303 fragment revealed a series of ions typical of a methyl-*O*-(epi)catechin. Only peak M13 had an ion at *m*/*z* 137, indicating that it was a 4′-*O*-methyl-(epi)catechin-*O*-sulfate. All other peaks were 3′-*O*-methyl-(epi)catechin-*O*-sulfates (*21*, *32*).

Peak M16 (t_R 40.9 min, method 3) yielded a $[M - H]^-$ at *m*/*z* 449, which on MS^2 gave rise to an ion at *m*/*z* 273, demonstrating cleavage of a glucuronide moiety. Cochromatography and the mass spectrum of a standard identified this compound as phloretin-2′-*O*-glucuronide (*23*).

Peaks M18 and M21 (t_R 42.9 and 45.7 min, respectively, method 3) both had a $[M - H]^-$ at *m*/*z* 477, which produces a MS^2 fragment ion at *m*/*z* 301. Cochromatography and the mass spectrum of a standard demonstrated that M18 was hesperetin-7-*O*-glucuronide, whereas M21 is an undesignated hesperetin-*O*-glucuronide (*22*, *34*).

Qualitative Analysis of Urine. A total of 33 different metabolites were identified in urine; because of this and the complexity of the urinary matrix, a total of four different analytical procedures

Table 5. HPLC-MS^3 Identification of Metabolites in Human Plasma and Urine Collected 0−24 h after the Ingestion of 350 mL of the P-R Drink[a]

peak	t_R (min)	$[M-H]^-$ (*m*/*z*)	MS^2 (*m*/*z*)	MS^3 (*m*/*z*)	compound	location[b]
M1	10.6	481	305	261, 221, 219	(epi)gallocatechin-*O*-glucuronide	P, U
M2	15.4	495	319	305, 235, 137	4′-*O*-methyl-(epi)gallocatechin-*O*-glucuronide	P, U
M3	11.1	545	465, 369, 289	245, 205, 179	(epi)catechin-*O*-glucuronide-O-sulfate	U
M4	17.6	465	289	245, 205, 179	(epi)catechin-*O*-glucuronide	P, U
M5	17.4	369	289	245, 205, 179	(epi)catechin-*O*-sulfate	U
M6	17.6	545	465, 369, 289	245, 205, 179	(epi)catechin-*O*-glucuronide-*O*-sulfate	U
M7	26.7	369	289	245, 205, 179	(epi)catechin-*O*-sulfate	P, U
M8	30.2	399	319	305, 235, 137	4′-*O*-methyl-(epi)gallocatechin-*O*-sulfate	P, U
M9	22.2	399	319	305, 235, 137	4′-*O*-methyl-(epi)gallocatechin-*O*-sulfate	U
M10	32.1	369	289	245, 205, 179	(epi)catechin-*O*-sulfate	P, U
M11	33.6	383	303	285, 259, 219	3′-*O*-methyl-(epi)catechin-*O*-sulfate	P, U
M12	28.1	383	303	285, 259, 219	3′-*O*-methyl-(epi)catechin-*O*-sulfate	U
M13	37.6	383	303	285, 259, 137	4′-*O*-methyl-(epi)catechin-*O*-sulfate	P, U
M14	41.1	383	303	285, 259, 219	3′-*O*-methyl-(epi)catechin-*O*-sulfate	P, U
M15	43.9	383	303	285, 259, 219	3′-*O*-methyl-(epi)catechin-*O*-sulfate	P, U
M16	40.9	449	273		phloretin-2′-*O*-glucuronide	P, U
M17	46.6	449	273		phloretin-*O*-glucuronide	U
M18	42.9	477	301		hesperetin-7-*O*-glucuronide	P, U
M19	47.9	557	477, 381, 301		hesperetin-*O*-glucuronide-*O*-sulfate	U
M20	49.6	529	353, 449, 273		phloretin-*O*-glucuronide-*O*-sulfate	U
M21	45.7	477	301		hesperetin-*O*-glucuronide	P, U
M22	50.4	477	301		hesperetin-*O*-glucuronide	U
M23	51.3	529	353, 449, 273		phloretin-*O*-glucuronide-*O*-sulfate	U
M24	52.4	529	353, 449, 273		phloretin-*O*-glucuronide-*O*-sulfate	U
M25	8.9	261	181	137, 119	dihydrocaffeic acid-3-*O*-sulfate	U
M26	10.2	275	195	177, 151, 123	dihydroferulic acid-3-*O*-sulfate	U
M27	11.1	259	179	135	caffeic acid-3-*O*-sulfate	U
M28	12.0	273	193	178, 149, 133	ferulic acid-4-*O*-sulfate	U
M29	14.9	263	183	169, 139, 123	*O*-methyl gallic acid-*O*-sulfate	U
M30	19.5	250	206, 191, 177		feruloylglycine	U
M31	20.5	263	183	169, 139, 123	*O*-methyl gallic acid-*O*-sulfate	U
M32	22.6	195	177,151,123		dihydroferulic acid	U
M33	26.4	195	177,151,123		dihydro(iso)ferulic acid	U

[a] See **Table 1** and the text for HPLC conditions employed. [b] P, plasma; U, urine.

had to be employed. Peaks M1−24 were analyzed using methods 4 and 5 and peaks M25−33 with methods 6 and 7 (see **Table 1**). As a consequence, because of the use of different HPLC conditions, there were differences in retention times when the same metabolite was detected in both urine and plasma. The HPLC and mass spectral properties of the identified urinary metabolites are summarized in **Table 5**. The identification of metabolites M1, M2, M4, M7, M8, M10, M11, M13−15, M16, M21, and M22, which were detected in both plasma and urine, was as outlined in the previous section. The criteria for identification of the additional urinary metabolites is summarized below with typical HPLC-SIM traces illustrated in **Figures 3** and **4**.

Peaks M3 and M6 (t_R 11.1 and 17.6 min, respectively, method 4) had a $[M-H]^-$ at *m*/*z* 545, which upon MS^2 fragmented to produce daughter ions at *m*/*z* 465, 369, and 289. This is typical of a compound O-conjugated at two positions on the flavonoid skeleton with the neutral losses of 80, 176, and 256 amu, indicating cleavage of both a sulfate and a glucuronide moiety. MS^3 fragmentation of the *m*/*z* 289 aglycone ion produced a typical (epi)catechin mass spectrum. These compounds were therefore tentatively identified as (epi)catechin-*O*-glucuronide-*O*-sulfates (*21*).

Peaks M5 (t_R 17.4 min, method 4) had the same mass spectrum as peaks M7 and M10 and is, therefore, an additional (epi)catechin-*O*-sulfate (*21*).

Peak M9 (t_R 22.2 min, method 5) had the same mass spectrum as peak M8 and is, thus, a second 4′-*O*-methyl-(epi)gallocatechin-*O*-sulfate (*21*, *32*).

Peak M12 (t_R 28.1 min, method 4) with a $[M-H]^-$ at *m*/*z* 383 had the same mass spectrum as peaks M11, M14, and M15 and is, therefore, a 3′-*O*-methyl-(epi)catechin-*O*-sulfate (*21*, *32*).

Peak M17 (t_R 46.6 min, method 4) had a mass spectrum similar to that of M16, phloretin-2′-*O*-glucuronide, from which it was chromatographically distinct, and was identified as an uncharacterized phloretin-*O*-glucuronide (*23*).

Peak M19 (t_R 47.9 min, method 5) had a $[M-H]^-$ at *m*/*z* 557, which yielded MS^2 fragment ions at *m*/*z* 477, 381, and 301. This demonstrates cleavage of glucuronide and sulfate units, whereas MS^3 fragmentation of *m*/*z* 301 produced a mass spectrum characteristic of the flavanone hesperetin. Peak M19 was identified as a hesperetin-*O*-glucuronide-*O*-sulfate (*22*).

Peaks M20, M23, and M24 (t_R 49.6, 51.3, and 52.4 min, respectively, method 4) all had a $[M-H]^-$ at *m*/*z* 529, and MS^2 produced sequential losses of 80, 176, and 256 amu, indicating the presence of a glucuronide/sulfate conjugate. The aglycone ion at *m*/*z* 273 was phloretin. These three metabolites are, therefore, phloretin-*O*-glucuronide-*O*-sulfates (*23*).

Peak M22 (t_R 50.4 min, method 5) had a different HPLC retention time but the same mass spectrum as M18 and M21 and is identified as an additional hesperetin-*O*-glucuronide.

Peak M25 (t_R 8.9 min, method 7) produced a $[M-H]^-$ at *m*/*z* 261, which with an 80 amu loss yielded a MS^2 fragment at *m*/*z* 181, which on further fragmentation gave rise to predominant ions at *m*/*z* 137 and 119, characteristic of dihydrocaffeic acid. The retention time and MS^3 characteristics identified this peak as dihydrocaffeic acid-3-*O*-sulfate, previously detected in urine after the ingestion of coffee by human subjects (*20*).

Peak M26 (t_R 10.2 min, method 7) had a $[M-H]^-$ at *m*/*z* 275, which produced a MS^2 fragment ion, with a loss of 80 amu, at *m*/*z* 195, the molecular ion of dihydroferulic acid. MS^3 fragmentation produced a number of identifier ions at *m*/*z* 177, 151, 136, 123,

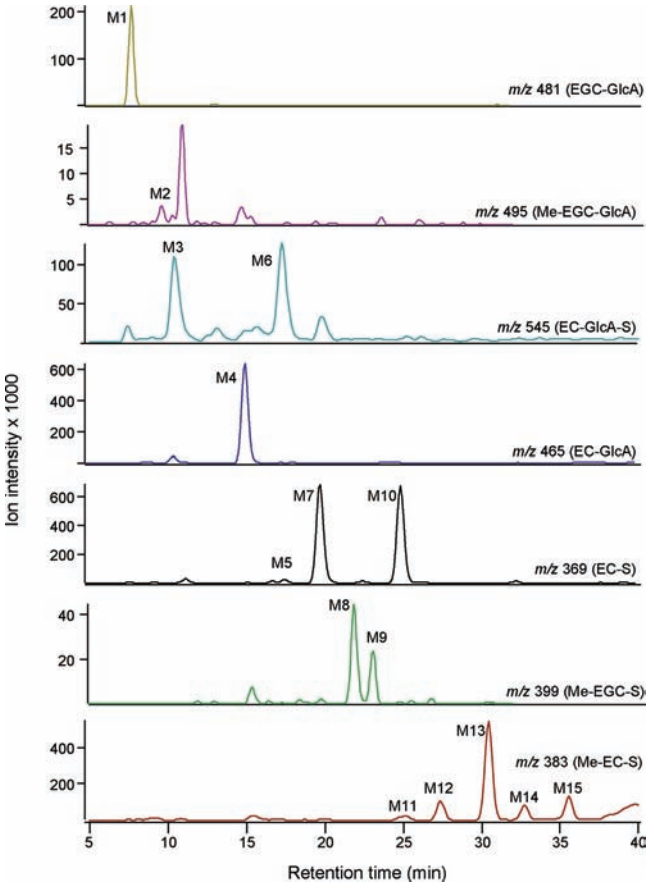

Figure 3. HPLC-SIM traces of (epi)catechin and (epi)gallocatechin metabolites in urine with detection at *m/z* 481, 495, 545, 465, 369, 399, and 383. EC-GlcA, (epi)catechin-*O*-glucuronide; EC-S, (epi)catechin-*O*-sulfate; Me-EC-S, *O*-methyl-(epi)catechin-*O*-sulfate; EC-GlcA-S, (epi)catechin-*O*-glucuronide-sulfate; EGCGlcA, (epi)gallocatechin-*O*-glucuronide; Me-EC-GlcA, *O*-methyl-(epi)gallocatechin-*O*-glucuronide; Me-EC-S, *O*-methyl-(epi)gallocatechin-*O*-sulfate. For identification of peaks M1−15, see **Table 5**. Note, in the qualitative SIM traces illustrated in this figure only the peaks designated M1−15 yielded the appropriate ions when subjected to further fragmentation. Thus, for instance, the larger unlabeled peak in the *m/z* 495 trace is not an *O*-methyl-(epi)gallocatechin-*O*-glucuronide as, unlike peak M2, the *m/z* 495 ion did not produce a MS^2 daughter ion at *m/z* 319.

and 119. This fragmentation pattern and matching retention time with a previous study (*20*) identified this metabolite as dihydroferulic acid-4-*O*-sulfate.

Peak M27 (t_R 11.1 min, method 7) produced a $[M - H]^-$ at *m/z* 259, which fragmented on MS^2 to *m/z* 179, the molecular ion of caffeic acid. MS^3 of this ion produced a single fragment at *m/z* 135. In keeping with an earlier investigation, this metabolite is identified as caffeic acid-3-*O*-sulfate (*20*).

Peak M28 (t_R 12.0 min, method 7) had $[M - H]^-$ molecular ion and MS^2 spectrum 14 amu higher than that of M27. The MS^2 *m/z* 193 fragment had an MS^3 spectrum characteristic of ferulic acid with ions at *m/z* 178, 149, and 134. This mass spectrum and the elution profile identified this compound as ferulic acid-4-*O*-sulfate (*20*).

Peaks M29 and M31 (t_R 14.9 and 20.5 min, respectively, method 6) both had a $[M - H]^-$ at *m/z* 263, which fragmented with a loss of 80 amu to *m/z* 183, the $[M - H]^-$ of methyl gallic acid. MS^3 fragmentation of *m/z* 183 produced ions at *m/z* 169, 139, and 123. This matched the spectrum of the standard of 3-*O*-methyl gallic acid. These two peaks are, therefore, *O*-methyl-gallic acid-*O*-sulfates.

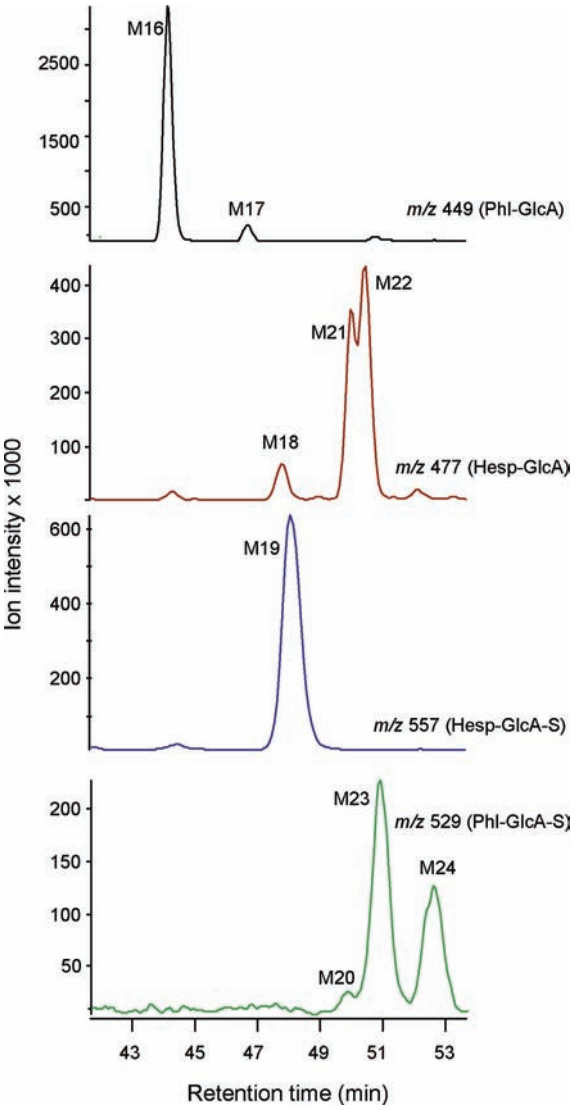

Figure 4. HPLC-SIM traces of flavanone and dihydroxychalcone metabolites in urine with detection at *m/z* 449, 477, 557, and 529. Phl-GlcA, phloretin-*O*-glucuronides; Phl-GlcA-S, phloretin-*O*-glucuronide-*O*-sulfates; Hesp-GlcA, hesperetin-*O*-glucuronides; Hesp-GlcA-S, hesperetin-*O*-glucuronide-*O*-sulfate. For identification of peaks M16−24, see **Table 5**.

Peak M30 (t_R 19.5 min, method 7) was characterized by a $[M - H]^-$ at *m/z* 250, which fragmented yielding ions at *m/z* 206, 191, 177, 149, and 100. Cochromatography with a reference compound and the resultant mass spectrum identified this compound as feruloylglycine (*20*).

Peaks M32 and M33 (t_R 22.6 and 26.4 min, respectively, method 7) both had a $[M - H]^-$ at *m/z* 195, which fragmented to produce ions at *m/z* 177, 151, and 123. The mass spectrum, relative HPLC retention times, and previous identifications with authentic standards are all in keeping with M32 being dihydroferulic acid and M33 being dihydro(iso)ferulic acid (*20*).

DISCUSSION

The analyses undertaken showed that the P-R juice drink was rich in antioxidant potential and phenolic compounds (**Table 2**) and contained a complex mixture of 40 flavonoids and related compounds (**Table 3**) of which 26, together with procyanidins, were present in quantifiable amounts (**Table 4**). The main components in the beverage were derived from green tea, apples, citrus, and grapes. Following ingestion of 350 mL of the beverage

by human subjects, 13 metabolites were identified in plasma. These compounds were also detected in urine along with an additional 20 metabolites (**Table 5**). Despite the complexity of the juice drink, the plasma and urine metabolites were qualitatively very similar to those detected in individual feeding studies we have undertaken, which also utilized HPLC-MS-based analysis of metabolites without recourse to cleavage of conjugating moieties with enzyme preparations containing sulfatase and glucuronidase activity (*20*−*23*).

Following ingestion of the P-R juice drink, hesperetin-7-*O*-rutinoside yielded hesperetin-7-*O*-glucuronide and an uncharacterized hesperetin-*O*-glucuronide in plasma, whereas urine contained an additional hesperetin-*O*-glucuronide and a hesperetin-*O*-glucuronide-sulfate (**Table 5**). This spectrum of metabolites is the same as that observed after the consumption of orange juice (*22*). Likewise, the phloretin metabolites in plasma and urine (**Table 5**) were similar to those detected after the consumption of apple cider with ca. 50% of the phloretin-*O*-glycoside content of the P-R drink (*23*). The profile of hydroxycinnamates observed in urine, dihydroferulic acid-3-*O*-sulfate, caffeic acid-3-*O*-sulfate, ferulic acid-4-*O*-sulfate and feruloylglycine, dihydroferulic acid, and dihydro(iso)ferulic acid (**Table 5**), was very similar to the main urinary hydroxycinnamate metabolites detected in a study in which volunteers drank coffee. In contrast to the investigation with coffee, no plasma hydroxycinnamates were detected in the present study and several of the minor urinary hydroxycinnamates were absent. This presumably is a dose effect as the ingested coffee contained 412 μmol of chlorogenic acids (*20*), compared to the 46 μmol intake of 5-*O*-caffeoylquinic acid with the P-R juice drink (**Table 4**).

No anthocyanins were detected in either plasma or urine after consumption of the P-R drink. This is not surprising as the beverage contained only low levels of the main anthocyanins peonidin-3,5-*O*-diglucoside and malvidin-3,5-*O*-diglucoside (**Figure 1**; **Table 4**), despite its red color. Also, with the exception of pelargonidin-3-*O*-glucoside, anthocyanins are poorly absorbed and are rarely detected in plasma and, typically, are excreted in quantities corresponding to $<0.05\%$ of intake (*35*).

The drink also contained a number of flavonol glycosides (**Table 3**). However, they were minor components (**Table 4**), and neither quercetin nor myricetin metabolites accumulated in either plasma or urine in consistently detectable quantities.

The spectrum of plasma and urinary glucuronide, sulfate, and methylated (epi)catechin metabolites detected after consumption of the P-R drink was very similar to that found after consumption of cocoa containing low levels of flavan-3-ols (*36*), whereas the (epi)catechin and (epi)gallocatechin metabolites closely resembled those observed after acute ingestion of green tea (*21*).

In a recent cocoa study the flavan-3-ol metabolites detected in plasma were an (epi)catechin-*O*-sulfate and an *O*-methyl-(epi)catechin-*O*-sulfate (*36*), whereas, with a higher (epi)catechin dose, when green tea is ingested glucuronide metabolites are also detected in plasma (*21*). This is in agreement with two reports on (−)-epicatechin bioavailability, which also found sulfate conjugates to be the major metabolites in plasma (*37*, *38*). All four investigations used liquid−liquid extraction procedures to precipitate proteins and purify plasma prior to analysis. In contrast, there are two publications, both dealing with cocoa intake, in which (epi)catechin-*O*-glucuronides rather than sulfates are reported to be the principal metabolites in plasma (*39*, *40*). In both instances, plasma was purified by solid phase extraction. There is, however, concurrence that sulfates are the predominant (epi)catechin metabolites in urine, from studies in which samples were analyzed directly without prior purification by solid phase extraction (*21*, *36*−*40*). Interestingly, in a study in which (−)-epicatechin was fed to humans and rats and urine was subjected to solid phase extraction, no sulfate metabolites were detected (*33*). The stability of the (epi)catechin-*O*-sulfate metabolites during purification by solid phase extraction, and other procedures, merits further investigation.

Although the P-R drink also contained a substantial quantity of procyanidins, no procyanidin dimers, trimers, or pentamers, or their glucuronide metabolites, were detected in any of the plasma or urine samples. Trace levels of procyanidins, but not (−)-catechin and (+)-epicatechin, inhibit platelet aggregation in vitro and suppress the synthesis of the vasoconstriction peptide, endothelin-1, by cultured endothelial cells, and it has been proposed that with only minimal absorption dietary procyanidins may exert similar effects in vivo (*41*). Although it is widely believed that dietary procyanidins are not absorbed into the circulatory system to any extent (see ref *7*), one investigation in which individual procyandins were fed to rats has detected dimers through pentamers in plasma that was extracted with 8 mol/L urea. It was suggested that the use of urea prevented irreversible binding of procyanidins to plasma proteins, which occurs when the more traditional methanol/acetonitrile is used for extraction (*42*). However, in this study, the procyanidins were administered by gavage at an extremely high dose, 1 g/kg of body weight, and it remains to be determined if procyanidins can be similarly detected in urea-extracted plasma after the ingestion of more nutritionally relevant quantities.

The P-R juice drink contained 52 μmol/350 mL of gallic acid, and in vivo there would be the potential for more gallic acid to be produced by cleavage of (−)-epicatechin-3-*O*-gallate and (−)-epigallocatechin-3-*O*-gallate. Urine was found to contain two *O*-methyl-gallic acid-*O*-sulfates (**Table 5**). Although methylated gallic acid has been detected in urine (*43*, *44*), this is the first report of the occurrence of sulfated derivatives.

In conclusion, the data obtained in this study demonstrate that following consumption of the P-R drink the spectrum of plasma and urinary metabolites detected by HPLC-MS is very similar to what would be expected by combining the metabolite profiles obtained in previous studies, with a more limited spectrum of individual flavonoids and related compounds, in feeds with green tea, cocoa, apple cider, orange juice, and coffee (*20*−*23*, *36*). Accordingly, there is no evidence to suggest saturation or inhibition of either absorption or the pathways associated with the metabolism of the ingested polyphenols.

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