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ARTICLE

Profile of Plasma and Urine Metabolites after the Intake of Almond [*Prunus dulcis* (Mill.) D.A. Webb] Polyphenols in Humans

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Nut skins are considered to be a rich source of polyphenols and may be partially responsible for the numerous health effects associated with nut consumption. However, more bioavailability studies of nut skin polyphenols are needed to understand the health effects derived from nut consumption. The aim of the present study was to determine the profiles of both phase II and microbial-derived phenolic metabolites in plasma and urine samples before and after the intake of almond skin polyphenols by healthy human subjects (n = 2). Glucuronide, *O*-methyl glucuronide, sulfate, and *O*-methyl sulfate derivatives of (epi)catechin, as well as the glucuronide conjugates of naringenin and glucuronide and sulfate conjugates of isorhamnetin, were detected in plasma and urine samples after consumption of almond skin polyphenols. The main microbial-derived metabolites of flavanols, such as 5-(dihydroxyphenyl)-γ-valerolactone and 5-(hydroxymethoxyphenyl)-γ-valerolactone, were also detected in their glucuronide and sulfate forms. In addition, numerous metabolites derived from further microbial degradation of hydroxyphenylvalerolactones, including hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids, registered major changes in urine after the consumption of almond skin polyphenols. The urinary excretion of these microbial metabolites was estimated to account for a larger proportion of the total polyphenol ingested than phase II metabolites of (epi)catechin, indicating the important role of intestinal bacteria in the metabolism of highly polymerized almond skin polyphenols. To the authors' knowledge this study constitutes the most complete report of the absorption of almond skin polyphenols in humans.

KEYWORDS: Almond; polyphenols; metabolites; microflora; hydroxyphenylvalerolactone

INTRODUCTION

Polyphenols are among the most abundant bioactive compounds of the Mediterranean diet and may play a key role in the prevention of cardiovascular and neurodegenerative diseases and cancer. Among the foods traditionally associated with the Mediterranean diet, the consumption of almonds [*Prunus dulcis* (Mill.) D.A. Webb] has been associated with beneficial protection against cardiovascular and obesity-related diseases (*1*). Moreover, Jenkins et al. (*2*, *3*) have also demonstrated that consumption of whole almonds decreases postprandial glycemia, insulinemia, and oxidative protein damage in humans. In fact, results from in vitro studies have revealed that almond skin polyphenols act in synergy with vitamins C and E to protect low-density lipoprotein (LDL) from oxidation (*4*, *5*) and enhance the antioxidant defense in other physiological environments (*6*). Besides their role in the prevention of cardiovascular and obesity-related diseases, results from in vitro studies have revealed that ground almonds significantly increased the populations of bifidobacteria and *Eubacterium rectale*, resulting in a higher prebiotic index (4.43) than for commercial prebiotic fructooligosaccharides (4.08) (*7*), indicating a potential prebiotic effect of almond seeds.

Almond skins are considered to be a very rich source of polyphenols. Non-flavonoid compounds include protocatechuic aldehyde and protocatechuic, vanillic, *p*-hydroxybenzoic, *trans*-*p*-coumaric, and chlorogenic acids (*8*−*10*). Flavonoid compounds identified in almond skins include flavanols, flavonols, dihydroflavonols, and flavanones. The monomers (+)-catechin and (−)-epicatechin, as well as procyanidins B1, B2, B3, and B4, have been identified in almond skins (*11*, *12*). Although it was originally thought that almond skin proanthocyanidins were exclusively constituted of (+)-catechin and (−)-epicatechin units linked by B-type bonds (*11*, *13*), later studies revealed the presence of delphinidin after *n*-butanol−HCl hydrolysis of almond seed

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crude extracts, indicating the additional occurrence of prodelphinidins in almonds (*12*). More recently, Monagas et al. (*10*) have confirmed the presence of A-type procyanidins and both A- and B-type propelargonidin and prodelphinidin polymers up to decamers for the first time in almond skins. Concerning flavonols, the -3-*O*-glucosides, -3-*O*-galactosides, and -3-*O*-rutinosides of quercetin, kaempferol, and isorhamnetin, as well as their corresponding aglycones, have been identified in almond skins (*8*−*10*, *14*−*16*). Recently, morin (*16*), dihydrokaempferol (*9*), and dihydroquercetin (taxifolin) (*10*) have also been reported. Finally, the flavanones identified in almond skins include naringenin-7-*O*-glucoside, eriodictyol-7-*O*-glucoside, eriodictyol-7-*O*-galactoside, and their corresponding aglycones (*8*−*10*).

The in vivo physiological importance derived from the consumption of almond polyphenols would depend on their bioavailability. However, studies concerning the bioavailability of almond polyphenols are scarce. Polyphenols bioavailability is strongly influenced by their chemical structure (*17*, *18*). Nonglycosylated polyphenols contained in almond skins, such as monomeric flavanols and dimeric procyanidins (to a lower extent), would be directly absorbed in the small intestine, where they would be first conjugated and later metabolized in the liver into methyl, glucuronide, and sulfate derivatives by phase II enzymes. However, oligomers presenting a mean degree of polymerization (mDP) of ≥3 and polymeric flavanols (proanthocyanidins) are not absorbed and reach the colon, where they are metabolized by the intestinal microbiota into hydroxyphenylvalerolactones and various phenolic acids, including phenylpropionic, phenylacetic, and benzoic acid derivatives, that can be further absorbed and then conjugated in the liver (*19*, *20*). Conversely, glycosylated polyphenols such as flavonol glycosides must be first hydrolyzed by intestinal β-glucosidases before they can be absorbed, although the type of sugar that is attached to the flavonoid limits the absorption in the small intestine (*18*). Almond skin flavonols containing rhamnose, such as rutinosides, would be poorly absorbed and transferred to the large intestine, where they would be degraded by the rhamnosidases of the intestinal microbiota (*21*). In this sense, a large amount of polyphenols contained in almond skins would be exclusively metabolized by the intestinal microbiota.

To our knowledge, only Blumberg and his colleagues have carried out bioavailability studies in plasma after the ingestion of almond polyphenols in hamsters (*4*) and in humans (*22*). In both cases, plasma samples were subjected to hydrolysis prior to LC analysis, and only total levels of flavonoid aglycones were quantified. No phenolic metabolites in their actual conjugate forms, or phenolic metabolites derived from the microbiota activity, have been reported so far in human fluids after the intake of almond polyphenols. Taking these facts into consideration, as well as the previously described potential prebiotic effect derived from almond consumption, a pilot study was conducted with healthy human volunteers to determine the profile of both phase II and microbial-derived phenolic metabolites in plasma and urine samples after the intake of almond skin polyphenols.

MATERIALS AND METHODS

Standards and Reagents. Phenylacetic acid, 3-hydroxyphenylacetic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxy-3-methoxyphenylacetic acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylpropionic acid, *p*-coumaric acid, caffeic acid, ferulic acid, 3-hydroxy-4-methoxycinnamic acid, protocatechuic acid, 3- and 4-hydroxybenzoic acids, ethyl gallate, (−)-epicatechin, (+)-catechin, procyanidin B2, and β-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma-Aldrich (St. Louis, MO). 4-Hydroxyhippuric acid was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). Procyanidin B3 and B1, vanillic acid, *m*-coumaric acid, eriodictyol-7-*O*-glucoside, eriodictyol, kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, kaempferol-3-*O*-galactoside, dihydroquercetin, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-galactoside, isorhamnetin, naringenin-7-*O*-glucoside, naringenin, and kaempferol were purchased from Extrasynthèse (Genay, France). LC grade solvents methanol, acetonitrile, glacial acetic acid, and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Almond Extract. A commercial almond skin extract (Amanda), kindly supplied by Puleva Biotech, S.A. (Granada, Spain), was used for this study. The extract was encapsulated (350 mg of extract + 50 mg of cellulose per capsule) by the supplier and presented a total polyphenol content of 221 mg of gallic acid/g of encapsulated extract, a total proanthocyanidin content of 314.8 mg of cyanidin/g, and an antioxidant capacity (ORAC value) of 3.10 mmol of Trolox/g.

Analysis of Phenolic Compounds of the Encapsulated Almond Skin Extract by LC-DAD−Fluorescence and LC-DAD/ESI-MS. An amount of 0.025 g of the almond skin extract contained in capsules was dissolved in 50 mL of water/HCl (1000:1, v/v) and sonicated for 15 min. An aliquot (40 mL) was extracted with ethyl acetate (40 mL × 4). The organic phases were combined and dried with anhydrous Na_2SO_4 for 20 min. The extract was then taken to dryness under vacuum, dissolved in 2 mL of methanol/H_2O (50:50, v/v), and finally filtered (0.45 μm). The extractions were performed in duplicate.

A Waters (Milford, MA) liquid chromatography system, equipped with a 2695 separation module, a 2996 photodiode array detector (DAD), and a 2475 multi λ fluorescence detector coupled to the Waters Empower (version 5.0) for data acquisition and processing, was used. Separation was performed on a 150 × 4.6 mm r.d., 4 μm reversed-phase ACE 3 C_{18} (Advanced Chromatography Technologies, Aberdeen, Scotland) column at room temperature. A gradient consisting of solvent A (water/acetic acid, 98:2, v/v) and solvent B (water/acetonitrile/acetic acid, 73:25:2, v/v/v) was applied at a flow rate of 1.0 mL/min as follows (*10*): 0−80% B linear from 0 to 55 min, 80−90% B linear from 55 to 57 min, 90% B isocratic from 57 to 70 min, 90−95% B linear from 70 to 80 min, 95−100% B from 80 to 90 min, followed by washing (methanol) and re-equilibration of the column from 90 to 120 min. A 75 μL volume sample was injected into the column. The detection conditions were 210−360 nm (DAD) and 280 and 310 nm for the emission and excitation filters, respectively (fluorescence detector). Quantification was carried out by external standard calibration curves. All of the phenolic compounds were quantified at 280 nm, except flavanols, which were quantified by their fluorescence response. Due to the lack of commercial standards, oligomeric flavanols were quantified using the (−)-epicatechin calibration curve.

In addition to their UV spectra, the identification of phenolic compounds was also carried out by mass spectrometry coupled to LC. A Hewlett-Packard series 1100 (Palo Alto, CA) chromatography system, equipped with a DAD and a quadrupole mass spectrometer (Hewlett-Packard series 1100 MSD) with an electrospray interface, was used. Separation conditions were the same as described above except for the flow rate, which was set to 0.7 mL/min. The ESI source was operated in negative mode using the following parameters: drying gas (N_2) flow and temperature, 10 L/min and 350 °C, respectively; nebulizer pressure, 55 psi; and capillary voltage, 4000 V. Mass spectra were obtained using in-source collision-induced dissociation mass spectrometry, scanning negative ions from m/z 100 to 2000 using the following fragmentation program: from m/z 0 to 200 (150 V) and from m/z 200 to 2000 (300 V).

Human Experimental Design. Two healthy and non-medication-receiving volunteers (ages 41 and 29 years) were recruited for the study. Volunteers were advised to avoid alcohol and vegetable-containing foods and beverages for 48 h before the study. In the morning (9:00 a.m.), after overnight fasting, the volunteers ingested 10 capsules containing the almond skin extract (884.4 mg of total polyphenols/dose), which was ∼8 times higher than the estimated dietary intake (102−121 mg/person/day) of nut polyphenols in the Spanish diet (*23*). The volunteers did not consume any food before or with the almond skin extract. At 2:00 p.m., volunteers had a light lunch meal composed of pasta and meat, and at 8:00 p.m. they had a dinner meal composed of jam and bread. Venous blood samples were collected into vacuum tubes containing EDTA before and after ingestion (2.5 h for both volunteers and 4.5 h for volunteer 2).

After sampling, blood was placed on ice, and plasma was separated by centrifugation for 20 min at 1500*g* at 4 °C and stored at −80 °C. Urine samples were also collected at the following intervals: from 2 h before the time of ingestion (−2 to 0 h, before extract intake) and from the time of ingestion to 24 h after ingestion (0−24 h, after extract intake). Urine samples were acidified to 0.2 M HCl and were stored at −80 °C. The study protocols were approved by the Ethics Committee of Clinical Investigation of the University of Barcelona (Spain), and informed consent was obtained from all participants.

Enzymatic Hydrolysis of Urine and Plasma Samples. Urine and plasma samples were subjected to enzymatic hydrolysis as previously described (*24*) with the aim of releasing the phenolic acid/aglycone from conjugated microbial-derived phenolic metabolites and allowing their identification and total quantification in their free form. Briefly, urine samples (1 mL) were taken to pH 4.9 with 200 μL of 2 M sodium acetate, and plasma samples (1 mL) were acidified with 50 μL of 0.58 M acetic acid. Urine and plasma samples were incubated with β-glucuronidase/sulfatase at 37 °C for 45 min. Straight afterward, samples were acidified to pH 2 with 6 M HCl.

Nonhydrolyzed samples were also studied in plasma and urine samples to allow identification of phenolic metabolites in their actual conjugate forms.

Extraction of Phenolic Metabolites from Urine and Plasma Samples. Extraction of phenolic metabolites from hydrolyzed samples was performed using solid-phase extraction (SPE) Oasis MCX 96-well plates (Waters), a vacuum manifold, and a vacuum source as described by Urpi-Sarda et al. (*24*). Briefly, the plate was conditioned with 1 mL of methanol followed by 1 mL of 2% formic acid. The hydrolyzed plasma/urine samples were then loaded onto the plate with 100 μL of 2 μM ethyl gallate used as internal standard (IS). The plate was washed with 1 mL of 2% formic acid. Analytes were then eluted with methanol (0.5 mL × 3), and the eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100 μL of mobile phase.

The nonhydrolyzed samples were also fractionated by SPE, but using Oasis HBL 96-well plates (30 mg) (Waters). The plate was conditioned with 1 mL of methanol followed by 1 mL of 1.5 M formic acid. One milliliter of the nonhydrolyzed plasma/urine samples was then loaded onto the plate with 100 μL of 2 μM ethyl gallate used as IS. The plate was washed with 1 mL of 1.5 M formic acid and 1 mL of 5% methanol. The plates were thoroughly dried by vacuum. Analytes were then eluted with 2 mL of methanol containing 0.1% formic acid and 2 mL of methanol containing NH_3 (pH 5), and the eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100 μL of mobile phase.

Analysis of Phenolic Metabolites in Urine and Plasma Samples by LC-ESI-MS/MS. The analyses of phenolic metabolites in urine and plasma samples were carried out by LC-ESI-MS/MS on an Agilent 1200 system equipped with a quaternary pump and a refrigerated autosampler plate (Waldbronn, Germany) and coupled to an Applied Biosystems API 3000 triple-quadrupole mass spectrometer (PE Sciex, Concord, ON, Canada) equipped with a turbo ion spray ionizing in negative mode. A Phenomenex Luna C18 analytical column [50 × 2.0 mm i.d., 5 μm] (Torrance, CA) with mobile phases A (100% water and 0.1% formic acid) and B (100% acetonitrile and 0.1% formic acid) was used. For the nonhydrolyzed samples, the gradient was linear of 4−100% of mobile phase B from 0 to 8 min, followed by washing and re-equilibration of the column for 2 min, at a flow rate of 500 μL/min. For the hydrolyzed samples, the gradient was linear of 8−100% of mobile phase B from 0 to 7 min, followed by washing and re-equilibration of the column for 3 min, at a flow rate of 400 μL/min. The volume injected was 15 μL. MS/MS parameters used were as follows: capillary voltage, −3700 V; focusing potential, −200 V; entrance potential, −10 V; declustering potential, −50 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 6000 cm^3/min. For quantification purposes data were collected in the multiple reaction monitoring (MRM) mode, tracking the transition of parent and product ions specific for each compound with a dwell time of 100 ms. Phenolic standard curves were constructed with standard solutions subjected to the same SPE procedure as the samples. Conjugated metabolites of (epi)catechin, narigenin, and isorhamnetin were quantified using the calibration curves of (−)-epicatechin, naringenin, and isorhamnetin, respectively. Hydroxyphenylvalerolactones and their conjugated metabolites were quantified as (−)-epicatechin. The mean recovery of analytes ranged from 87 to 109% (*24*, *25*). Accuracy ranged from 87.5 to 113.8%, and precision met acceptance criteria (< 15% RSD). The LODs varied between 0.050 and 44.4 μg/L (*24*, *25*).

Table 1. Concentration of Individual Phenolic Compounds in the Encapsulated Almond Skin Extract

	mean ± SD[a] (μg/g)
non-flavonoids	
protocatechuic acid	134.86 ± 20.77
vanillic acid	372.82 ± 41.16
ellagic acid	9.00 ± 1.34
flavonoids	
kaempferol-3-*O*-galactoside	22.24 ± 1.31
kaempferol-3-*O*-rutinoside	235.63 ± 23.50
kaempferol-3-*O*-glucoside	32.45 ± 3.86
isorhamnetin-3-*O*-galactoside	289.51 ± 32.34
isorhamnetin-3-*O*-rutinoside	1253.06 ± 130.76
isorhamnetin-3-*O*-glucoside	185.85 ± 20.11
kaempferol	4.28 ± 0.34
isorhamnetin	24.21 ± 3.53
naringenin-7-*O*-glucoside	55.19 ±6.31
eriodictyol-7-*O*-glucoside	5.17 ±0.69
naringenin	54.87 ± 8.72
eriodictyol	7.82 ± 1.67
dihydroquercetin	46.99 ± 8.13
(+)-catechin	682.83 ± 55.46
(−)-epicatechin	292.48 ± 34.46
procyanidin dimers B3 + B1	269.45 ± 41.69
procyanidin dimer B2	355.34 ± 7.18
procyanidin dimer B7	112.63 ± 8.29
unknown A-type procyanidin dimer (A-1)	99.37 ± 7.14
unknown A-type procyanidin dimer (A-2)	33.54 ± 2.97
unknown A-type procyanidin dimer (A-3)	69.03 ± 7.49
unknown B-type propelargonidin dimer	117.91 ± 15.76
unknown A-type propelargonidin dimer	50.44 ± 0.44
procyanidin trimer C1	173.97 ± 10.27

[a] Mean (n = 2) ± standard deviation (SD).

Determination of Total Polyphenols in Urine Samples. The analysis of total polyphenols in urine samples was performed using the Folin−Ciocalteu (F-C) reagent after SPE of phenolic compounds using Oasis MAX 96-well plates (Waters) (*26*). Urine samples were thawed on an ice bed for 3 h and then centrifuged for 10 min at 4 °C. One milliliter of supernatant was diluted with 1 mL of Milli-Q water. The MAX 96-well plate was conditioned with 1 mL of methanol followed by 1 mL of 50 mM sodium acetate (pH 7). The sample was then loaded onto the plate and washed with 50 mM sodium acetate (pH 7) and 5% (v/v) methanol. Polyphenols were then eluted with 2% formic acid in methanol (1.8 mL).

The determination of total polyphenols was performed by mixing 15 μL of the eluted fraction with 170 μL of Milli-Q water in the thermo microtiter 96-well plate (NuncTM, Roskilde, Denmark), followed by the addition of 12 μL of F-C reagent and 30 μL of sodium carbonate (200 g/L). The mixture was incubated for 1 h at room temperature in the dark. After the reaction period, 73 μL of Milli-Q water was added with the multichannel pipet. Absorbance was measured at 765 nm in UV−vis Thermo Multiskan Spectrum spectrophotometers (Vantaa, Finland), and the results were expressed as micrograms of gallic acid equivalent per milliliter of urine.

RESULTS AND DISCUSSION

Phenolic Composition of the Encapsulated Almond Skin Extract. The analysis of the encapsulated almond skin extract by LC-DAD−fluorescence and LC-DAD-ESI-MS revealed the presence of both non-flavonoid and flavonoid phenolic compounds (**Table 1**). Non-flavonoid compounds mainly include protocatechuic acid, ellagic acid, and vanillic acid, the latter present in the largest concentration. Flavonoid compounds include flavanols

Table 2. Concentrations of Conjugated Phenolic Metabolites Detected in Nonhydrolyzed Human Plasma and Urine before and after Consumption of Almond Skin Extract

			plasma[a] (nM)				urine[b] (nM)			
			before		after		before		after	
metabolite	MRM transition	compound	mean	SD	mean	SD	mean	SD	mean	SD
flavan-3-ols										
(epi)catechin-*O*-glucuronide 1	465→289	**1**	nd[c]		nd		0.55	0.78	6.95	3.29
(epi)catechin-*O*-glucuronide 2	465→289	**2**	nd		nd		2.25	0.05	6.39	0.89
(epi)catechin-*O*-glucuronide 3	465→289	**3**	nd		19.51	3.99	3.08	0.17	21.35	3.91
O-methyl (epi)catechin-*O*-glucuronide 1	479→303	**4**	nd		8.54	3.47	1.93	0.70	25.30	9.74
O-methyl (epi)catechin-*O*-glucuronide 2	479→303	**5**	nd		7.94	3.98	2.28	0.62	46.92	7.28
(epi)catechin sulfate 1	369→289	**6**	nd		5.59[d]		5.33	1.77	972.70	344.64
(epi)catechin sulfate 2	369→289	**7**	nd		14.71	9.02	10.62	5.79	200.40	28.55
O-methyl (epi)catechin sulfate 1	383→303	**8**	nd		41.82	±17.17	8.24	4.04	586.31	±90.34
O-methyl (epi)catechin sulfate 2	383→303	**9**	nd		17.83[d]		7.14	1.83	537.34	132.70
O-methyl (epi)catechin sulfate 3	383→303	**10**	nd		nd		2.90	0.57	87.52	10.37
hydroxyphenylvalerolactones										
5-(dihydroxyphenyl)-γ-valerolactone glucuronide derivative 1	383→207	**11**	nd		nd		18.03	3.95	2939.65	501.22
5-(dihydroxyphenyl)-γ-valerolactone glucuronide derivative 2	383→207	**12**	nd		nd		35.28	±17.30	7682.78	1942.14
5-(dihydroxyphenyl)-γ-valerolactone sulfate derivative	287→207	**13**	278.81	160.36	368.23	224.37	3969.68	283.23	449558.92	146817.62
5-(hydroxymethoxyphenyl)-γ-valerolactone glucuronide derivative 1	397→221	**14**	7.62	6.11	5.94	4.84	362.61	97.5	832.89	291.5
5-(hydroxymethoxyphenyl)-γ-valerolactone glucuronide derivative 2	397→221	**15**	nd		nd		6.18	1.68	117.89	41.19
5-(hydroxymethoxyphenyl)-γ-valerolactone sulfate derivative 1	301→221	**16**	nd		nd		57.26	50.50	1495.68	828.12
5-(hydroxymethoxyphenyl)-γ-valerolactone sulfate derivative 2	301→221	**17**	nd		nd		14.36	7.15	704.80	266.53
flavanones										
naringenin-*O*-glucuronide 1	447→271	**18**	0.84	0.15	3.35	0.17	2.81	1.44	14.97	6.41
naringenin-*O*-glucuronide 2	447→271	**19**	0.61	0.05	1.27	0.17	8.15	8.35	15.43	5.61
flavonols										
isorhamnetin-*O*- glucuronide 1	491→315	**20**	1.68	0.03	2.40	1.46	5.08	2.74	6.71	0.55
isorhamnetin-*O*-glucuronide 2	491→315	**21**	1.14	0.81	1.49	0.54	4.54	1.38	4.89	1.72
isorhamnetin sulfate	395→315	**22**	nd		nd		37.39	4.54	62.75	29.13

[a] Mean ($n = 2$) ± standard deviation (SD). Before (at 0 h); after (at 2.5 h). [b] Mean ($n = 2$) ± standard deviation (SD). Before (from −2 to 0 h); after (from 0 to 24 h). [c] nd, not detected. [d] Detected in only one volunteer.

(50.4% of total flavonoids), flavonols (45.8%), flavanones (2.8%), and dihydroflavonols (1.1%). Isorhamnetin glycosides were more abundant than the corresponding glycosides of kaempferol, and naringenin glucoside was more abundant than the corresponding eriodictyol glucoside. However, the rutinoside derivatives were present in the largest concentration independent of the flavanol aglycone. Among flavanols, (+)-catechin was the predominant monomeric form, whereas procyanidin B2 was the most abundant oligomeric form. In addition, several mass signals were detected at *m*/*z* 591 corresponding to A-type prodelphinidin dimers, but UV peaks were not well resolved for quantification purposes (data not shown). These results are in agreement with previous reports describing the phenolic composition of almond skins (*10*).

Identification of Phase II and Microbial-Derived Phenolic Metabolites in Human Plasma and Urine. The concentration of phenolic metabolites detected in nonhydrolyzed and hydrolyzed samples before and after consumption of the almond skin extract is presented in **Tables 2** and **3**, respectively. The presence of metabolites derived from the main phenolic compounds (i.e., flavanols, flavonols, and flavanones) identified in the almond skin extract (**Table 1**) was investigated in both fluids. With regard to flavanols, three mass signals were detected at *m*/*z* 465/289 and two at *m*/*z* 369/289, indicating the possible presence of glucuronide (+176 amu) and sulfate (+80 amu) derivatives of (epi)catechin (**Figure 1**; **Table 2**). In addition, two signals were detected at *m*/*z* 479/303 and three at *m*/*z* 383/303, suggesting they were possible glucuronide and sulfate derivatives of *O*-methyl(epi)catechin, respectively (**Figure 1**; **Table 2**). These conjugated metabolites of (epi)catechin were detected in plasma and urine samples, with some exceptions in the plasma samples (**Table 2**). Different signals detected at the same *m*/*z* may belong to different regioisomers resulting from conjugation at different OH groups of the flavanol molecule. In humans, methylation of (−)-epicatechin has been reported to occur at C-3′ and at C-4′ and glucuronidation at C-3′, C-5, and C-7 (*27*). However, the active positions for flavanol sulfation have not been clearly identified. These metabolites may have been formed in the small intestine and/or further in the liver by phase II enzymes after absorption of (epi)catechin in the small intestine (*18*).

Conjugated metabolites, derived from the microbial degradation of flavanols, were also investigated. In particular, hydroxyphenylvalerolactones, originating from the C-ring-opening of flavanols by the colonic microflora followed by further lactonization, are considered to be main microbial metabolites derived from the biotransformation of flavanols (*28*−*31*). Bearing in mind that procyanidins are the most abundant type of proanthocyanidins in the almond extract (**Table 1**), conjugated metabolites of 5-(dihydroxyphenyl)-γ-valerolactone [i.e., 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone] were targeted (**Figure 2**; **Table 2**). Two mass signals were detected at *m*/*z* 383/207, indicating the possible occurrence of two different glucuronide derivatives of a 5-(dihydroxyphenyl)-γ-valerolactone molecule, probably originating from the esterification of glucuronic acid at the two different OH groups of the phenyl ring of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone. One mass signal detected at *m*/*z* 287/207 was tentatively assigned to the corresponding sulfate conjugate (**Figure 2**). At *m*/*z* 397/221 two signals were detected, suggesting the presence of glucuronide derivatives of 5-(hydroxymethoxyphenyl)-γ-valerolactone. Similarly, two signals detected at *m*/*z* 301/221 were

Table 3. Concentration of Microbial-Derived Phenolic Metabolites Detected in Hydrolyzed Human Plasma and Urine before and after Consumption of Almond Skin Extract

		plasma[a] (nM)				urine[b] (nM)			
		before		after		before		after	
metabolite	MRM transition	mean	SD	mean	SD	mean	SD	mean	SD
hydroxyphenylvalerolactones									
5-(dihydroxyphenyl)-γ-valerolactone	207→163	nd[c]		nd		121.09	±11.70	23553.03	±1230.73
5-(hydroxyphenyl)-γ-valerolactone	191→147	nd		nd		48.81	10.12	229.21	54.93
hydroxyphenylpropionic acids									
3,4-dihydroxyphenylpropionic acid	181→137	2880.36	365.83	2787.25	14.53	2805.14	415.97	3618.37	787.73
3-hydroxyphenylpropionic acid	165→121	2038.17	367.60	1780.23	335.28	8.16[d]		51.61[d]	
hydroxyphenylacetic acids									
3,4-dihydroxyphenylacetic acid	167→123	406.28	112.62	385.83	30.04	1759.11	559.81	3122.01	161.09
3-hydroxyphenylacetic acid	151→107	986.95	143.93	939.50	52.28	2421.92	2366.71	7012.71	4401.08
4-hydroxy-3-methoxyphenylacetic acid	181→137	755.81	189.16	571.49	162.27	6327.50	2186.86	25212.61	10960.16
phenylacetic acid	135→91	8481.61	2343.50	8733.33	2384.11	5577.11	116.15	6716.28	921.82
hydroxycinnamic acids									
m-coumaric acid	163→119	17.94	9.58	22.93	4.98	15.54	8.91	29.35	17.15
p-coumaric acid	163→119	29.29	0.46	19.14	2.66	14.86	17.72	20.65	12.90
caffeic acid	179→135	509.99	95.05	484.26	24.04	809.06	318.26	1200.01	43.82
ferulic acid	193→134	46.50	2.68	25.38	12.68	111.13	17.70	365.45	258.27
3-hydroxy-4-methoxycinnamic acid	193→134	71.52	26.28	66.74	5.61	3034.48	128.10	4541.98	1705.02
hydroxybenzoic acids									
3-hydroxybenzoic acid	137→93	nd		nd		206.26	140.96	315.91	±10.28
4-hydroxybenzoic acid	137→93	6177.80	824.05	6527.09	±234.47	5529.69	1759.09	7034.03	240.27
protocatechuic acid	153→109	4553.05	792.78	4222.13	265.67	9012.44	1414.96	9451.31	1943.47
vanillic acid	167→152	2821.25	478.51	3087.02	273.06	218.39	111.27	1676.85	1183.74
hydroxyhippuric acids									
4-hydroxyhippuric acid	194→100	14.56	1.47	11.46	0.11	15475.48	13393.70	17909.39	12733.87

[a] Mean ($n = 2$) ± standard deviation (SD). Before (at 0 h); after (at 2.5 h). [b] Mean ($n = 2$) ± standard deviation (SD). Before (from −2 to 0 h); after (from 0 to 24 h). [c] nd, not detected. [d] Quantified in only one volunteer.

tentatively assigned to the corresponding sulfate derivative of the same molecule. These metabolites were detected in both fluids, although with some exceptions in the plasma samples (**Table 2**), and have been previously reported in human urine after the consumption of tea catechins (*32*, *33*). The identification of these compounds was aided by the disappearance of the peaks after enzymatic hydrolysis with β-glucuronidase/sulfatase and by the appearance of peaks of corresponding aglycones, as will be described below.

With regard to other flavonoids, conjugated metabolites from main flavanones (naringenin) and flavonols (isorhamnetin) were studied (**Figure 3**; **Table 2**). At *m*/*z* 447/271 two signals were detected in both plasma and urine samples tentatively assigned to glucuronide derivatives of naringenin (*m*/*z* 447/271) originating from the metabolism of almond skin naringenin-7-*O*-glucoside and naringenin. In addition, two signals were detected at *m*/*z* 491/315 and one at *m*/*z* 395/315, indicating that they were possible glucuronide and sulfate derivatives of isorhamnetin (**Figure 3**; **Table 2**). These latter metabolites may have originated from the metabolism of isorhamnetin-3-*O*-galactoside and isorhamnetin-3-*O*-glucoside present in the almond skin extract (**Table 1**) because isorhamnetin-3-*O*-rutinoside needs to reach the colon to be metabolized by the microbiota (*21*).

Metabolites derived from the microbial degradation of almond skin flavanols were studied after enzymatic hydrolysis of plasma and urine samples. The targeted metabolites were classified into different groups according to their chemical structure (**Table 3**): hydroxyphenylvalerolactones and hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids. Among hydroxyphenylvalerolactones, besides 5-(dihydroxyphenyl)-γ-valerolactone (*m*/*z* 207/163), the transition corresponding to 5-(hydroxyphenyl)-γ-valerolactone (*m*/*z* 191/147) was additionally studied in hydrolyzed samples. In plasma, no hydroxyphenylvalerolactone derivatives were detected, whereas in urine the two forms (i.e., mono- and dihydroxyphenyl-γ-valerolactones) were detected. The mass spectra of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (*m*/*z* 207) reported in a previous work (*24*) allowed the identification of dihydroxyphenyl-γ-valerolactone. The monohydroxylated form 5-(hydroxyphenyl)-γ-valerolactone could originate directly from (epi)afzlechin units of properlagonidins or more likely from dehydroxylation of (epi)catechin (i.e., procyanidins), which occur in larger concentrations than the former compounds in the almond skin extract (**Table 1**). Subsequent fission of the valerolactone ring leads to hydroxyphenylvaleric acids (*34*−*37*), which are further degraded to a series of phenolic acids. The targeted-studied phenolic acids (hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic, and hydroxyhippuric acids) were identified in both plasma and urine samples, with the exception of 3-hydroxybenzoic acid in plasma (**Table 3**). Hydroxyphenylpropionic and hydroxybenzoic acids originate from the β-oxidation of hydroxyphenylvaleric acids (*37*). α-Oxidation of hydroxyphenylpropionic acids may also occur, leading to hydroxyphenylacetic acids (*38*). Phenolic acids hydroxylated at C-3 (i.e., 3-hydroxyphenylpropionic and 3-hydroxybenzoic acids) are likely to be produced via the selective dehydroxylation of the OH group of dihydroxylated compounds at C-4 (*37*), whereas C4-hydroxylated phenolic acids (i.e., 4-hydroxybenzoic acid) are the result of the dehydroxylation at C-3, although it seems to be characteristic of the degradation only of procyanidins and not of monomeric flavanols (*19*, *20*, *35*, *36*). Other microbial metabolites targeted in the present study are derived from further metabolism in the liver and kidney, including 4-hydroxyhippuric acid, from conjugation of 4-hydroxybenzoic acid with glycine (*39*); *m*- and *p*-coumaric acids, from dehydrogenation of 3- and 4-hydroxypropionic acids, respectively; ferulic acid by further parahydroxylation

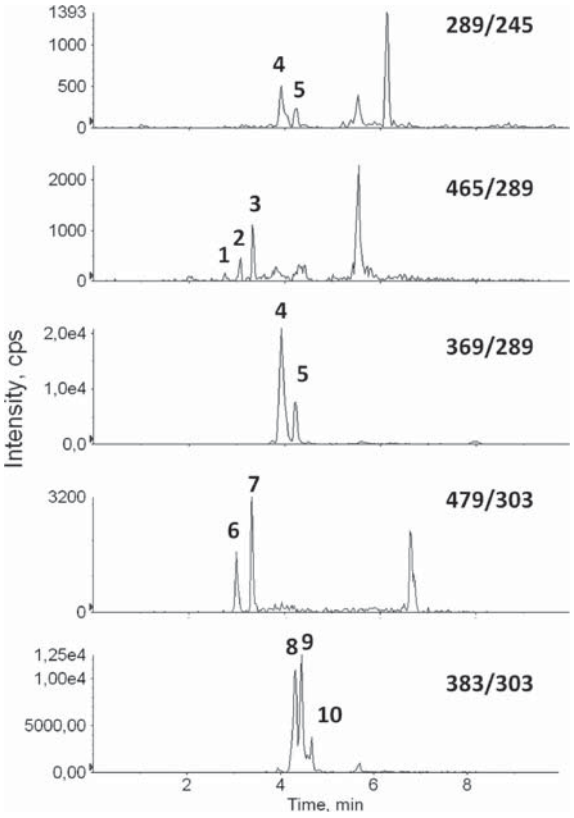

Figure 1. MRM trace chromatograms of (epi)catechin-*O*-glucuronides (465/289), (epi)catechin sulfates (369/289), *O*-methyl (epi)catechin-*O*-glucuronides (479/303), and *O*-methyl (epi)catechin sulfates (383/303) detected in urine samples after intake of the almond extract. The MRM trace chromatogram corresponding to (epi)catechin (289/245) is also shown to confirm metabolites.

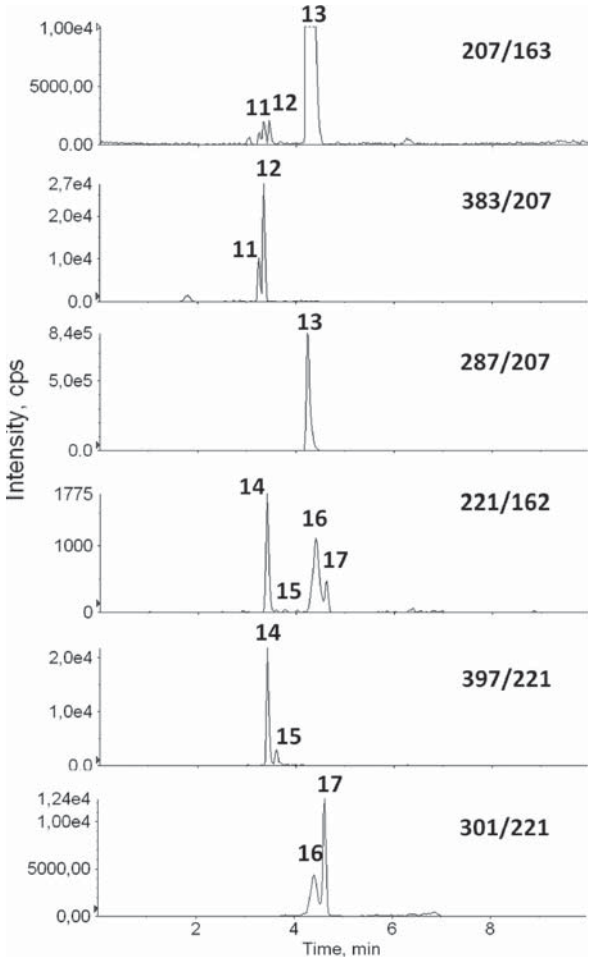

Figure 2. MRM trace chromatograms of 5-(dihydroxyphenyl)-γ-valerolactone glucuronide derivatives (383/207), 5-(dihydroxyphenyl)-γ-valerolactone sulfate derivatives (287/207), 5-(hydroxymethoxyphenyl)-γ-valerolactone glucuronide derivatives (397/221), and 5-(hydroxymethoxyphenyl)-γ-valerolactone sulfate derivatives (301/221). MRM trace chromatograms of aglycones, 5-(dihydroxyphenyl)-γ-valerolactone (207/163), and 5-(hydroxymethoxyphenyl)-γ-valerolactone (221/162) are also shown to confirm metabolites.

and methylation of *m*-coumaric acid (*20*, *38*); and vanillic and 4-hydroxy-3-methoxyphenylacetic acid by methylation of protocatechuic acid and 3,4-dihydroxyphenylacetic acid, respectively (*20*).

Microbial metabolites derived from flavanones and flavonols were also identified. Naringenin-7-*O*-rutinoside was degraded into 4-hydroxyphenylpropionic acid and phenylpropionic acid by human fecal microbiota in vitro (*40*). In the case of isorhamnetin glycosides, there is no previous report of microbial metabolites, but 4-hydroxy-3-methoxyphenylpropionic and phenylacetic acids, as well as vanillic acid, are expected to be formed from the degradation pathway characteristic of other flavonols. Among the possible metabolites derived from the microbial degradation of naringenin and isorhamnetin, only vanillic acid and 4-hydroxy-3-methoxyphenylacetic acid were targeted in the present study.

Changes in Conjugated Phenolic Metabolites in Human Plasma and Urine after the Consumption of the Almond Skin Extract. The concentration of conjugated phenolic metabolites (phase II and microbial-derived) detected in nonhydrolyzed human plasma and urine before and after consumption of almond skin extract is presented in **Table 2**. Among targeted compounds, conjugated metabolites of epicatechin [*O*-glucuronide (3), *O*-methyl glucuronides (1 and 2), sulfates (1 and 2), and *O*-methyl sulfates (1 and 2)] were detected in only plasma after the intake of the almond skin extract, indicating that they arise from the metabolism of almond skin polyphenols. Other metabolites, including some conjugated metabolites of hydroxyphenylvalerolactones [5-(dihydroxyphenyl)-γ-valerolactone sulfate], as well as glucuronides of naringenin and isorhamnetin, were detected in plasma before consumption of almond skin extract, but their levels increased in all of them at 2.5 h after intake. Both (epi)catechin and hydroxyphenylvalerolactone derivatives have been quantified as (−)-epicatechin to estimate the changes in phenolic metabolite content after consumption of the almond skin extract. Taking this approach into account, the consumption of 3.5 g of almond skin extract resulted in a 57% increase in the total concentration of plasma conjugated metabolites at 2.5 h after intake. These findings seem to be in accordance with the pharmacokinetics of (epi)catechin in plasma ($t_{max} \approx 2$ h), which indicates absorption in the small intestine (*41*). For naringenin, a $t_{max} \approx 5$ h in plasma has been reported after the consumption of orange juice (*42*), although it could appear earlier. In the case of hydroxyphenylvalerolactones, pharmacokinetic data of these compounds in plasma are very limited. According to Li et al. (*28*), t_{max} of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone occurred at 5−12 h after green tea consumption in humans. Meng et al. (*29*) reported the appearance of this compound in human plasma at approximately 5 h after the intake of green tea, achieving the maximum concentration at 12 h. Also in agreement with these results, Kohri et al. (*30*) reported that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone

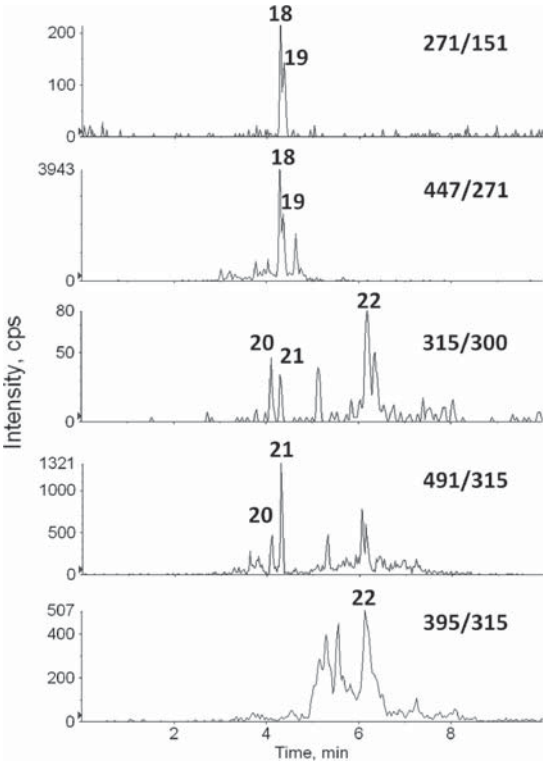

Figure 3. MRM trace chromatograms of naringenin-*O*-glucuronides (447/271), isorhamnetin-*O*-glucuronides (491/315), and isorhamnetin sulfate (395/315). MRM trace chromatograms of aglycones, naringenin (271/151), and isorhamnetin (315/300) are also shown to confirm metabolites.

appeared in plasma at 6 h after the administration of epicatechin gallate to rats, although in this case t_{max} was achieved at 24 h. These findings help to explain why only a small amount of hydroxyvalerolactone conjugated metabolites could be detected in the 2.5 h plasma sample of the present study. However, these metabolites increased at 4.5 h after the intake of the almond skin extract, and other new metabolites such as 5-(dihydroxyphenyl)-γ-valerolactone glucuronide derivatives (1 and 2) not detected at 2.5 h were also detected at 4.5 h after intake (results not shown), indicating that a longer time is required for the microbial biotransformation of almond polyphenols in the colon.

The analysis of urine revealed an increase in the urinary level of all targeted conjugated metabolites as a consequence of the consumption of the almond skin extract. Among these metabolites, 5-(dihydroxyphenyl)-γ-valerolactone glucuronides (1 and 2), 5-(dihydroxyphenyl)-γ-valerolactone sulfate, and (epi)catechin sulfate (1) presented the largest increases in concentration (163-, 218-, 113-, and 182-fold increases, respectively). Consumption of green tea catechins (*28*, *29*) or cocoa (*24*) in humans has also resulted in an increase in the formation of hydroxyphenylvalerolactones. Maximum renal excretion of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone in humans has been reported to have occurred at 3−6 h for pure (−)-epicatechin or at 8−24 h after green tea consumption, although it could vary greatly between subjects (*28*, *29*). In our study, consumption of 3.5 g of almond skin extract resulted in a 102-fold increase in the total urinary concentration of conjugated metabolites 24 h after intake. It was also calculated that phase II metabolites of (epi)catechin, naringenin, and isorhamnetin accounted for 44, 5.3, and 1.4%, respectively, of the total monomeric flavanol [(+)-catechin + (−)-epicatechin], total naringenin (naringenin and its -7-*O*-glucoside derivative), and total isorhamnetin (isorhamnetin and its -3-*O*-galactoside, -3-*O*-rutinoside, and -3-*O*-glucoside derivatives), ingested in the almond skin extract (**Table 1**). Urinary excretion ranges of 2.1−55.0 and 1.1−30.2% have been reported for (epi)catechin and naringenin, respectively (*41*). However, there are no data currently available of the urinary excretion rates of isorhamnetin, although its pharmacokinetics in plasma seem to be similar to that of quercetin after the consumption of almond skins (*4*).

Changes in Microbial-Derived Phenolic Metabolites in Human Plasma and Urine after the Consumption of the Almond Skin Extract. The concentration of microbial-derived metabolites detected in hydrolyzed human plasma and urine before and after the consumption of almond skin extract is presented in **Table 3**. The plasmatic level of microbial-derived phenolic metabolites remained practically constant after consumption of almond skin extract. In fact, the plasmatic concentration of total microbial-derived phenolic metabolites accounted for 29.79 μM before consumption and for 29.66 μM after consumption of almond skin extract. According to Kohri et al. (*30*), subsequent microbial degradation of products of hydroxyvalerolactones, such as 3-hydroxyphenylpropionic acid and *m*-coumaric acid, appeared in rat plasma 6 h after consumption of (−)-epicatechin gallate, reaching maximum levels at 24 h. Therefore, the 2.5 h plasma sample may not reflect microbial metabolism because almond polyphenols need a longer time to reach the large intestine and be biotransformed.

In contrast, major changes were observed in the urinary levels of all microbial metabolites after consumption of almond skin extract. Hydroxyphenylvalerolactones, in particular 5-(dihydroxyphenyl)-γ-valerolactone, exhibited the largest increase in concentration (195-fold increase). Among the remaining compounds, the concentrations of vanillic acid and 3-hydroxyphenylpropionic acid also exhibited large changes (8- and 6-fold increases, respectively). These results seem to be consistent with those of Rios et al. (*43*), who reported a significant increase in the urinary excretion of these metabolites, among others, in healthy humans after acute consumption of a flavanol-rich chocolate. Most microbial-derived metabolites were found to increase from 6 to 48 h after consumption and registered maximum levels from 24 to 48 h (*43*). Also in line with our results, Ward et al. (*44*) reported an increased urinary excretion of 3-hydroxypropionic acid and 3-hydroxyphenylacetic acid after human consumption of grape seed polyphenols. As a consequence of the consumption of almond skin extract, the total urinary microbial metabolite concentration increased by 109%. The total polyphenol content of the 24 h urine sample (as determined by the F-C method) taken after the consumption of almond skin extract was 212.5 mg of gallic acid and accounted for 24% of the total polyphenol intake (884.4 mg of gallic acid). Microbial-derived phenolic metabolites (**Table 3**) represented 4.9% of the total polyphenol intake (884.4 mg of gallic acid), whereas (epi)catechin conjugates (**Table 2**) represented only 0.11% of this intake. These findings seem to be in line with the results reported by Gonthier et al. (*19*) for rats fed wine polyphenols, in which microbial metabolites and catechins [(+)-catechin + 3′-*O*-methylcatechin] represented 9.2 and 1.2% of the total polyphenol intake, respectively. Lower yields in the case of the almond skin extract could be due to the structural complexity (A- and B-type properlagonidins, procyanidins, and prodelphidins) and the high mDP of the almond skin extract (total proanthocyanidins, 315 mg/g) in comparison to the wine powder used in the rat study (146 mg/g) (*19*).

In conclusion, to our knowledge this study constitutes the most complete report of the bioavailability of almond skin polyphenols in humans, including both phase II and microbial metabolism. Almond skin polyphenols were absorbed as phase II metabolites,

mainly including glucuronide, *O*-methyl glucuronide, sulfate, and *O*-methyl sulfate derivatives of (epi)catechin, which arise from the metabolism of monomeric flavanols, as well as glucuronide and sulfate derivatives of isorhamnetin and glucuronide derivatives of naringenin. Of particular importance was the detection of microbial-derived metabolites of flavanols, 5-(dihydroxyphenyl)-γ-valerolactone and 5-(hydroxymethoxyphenyl)-γ-valerolactone in their glucuronide and sulfate forms, which confirmed the absorption of both monomeric and polymeric flavanols from almond skins and subsequent conjugation in the liver. Numerous metabolites derived from the further microbial degradation of hydroxyvalerolactones were also detected. The urinary excretion of these metabolites accounted for a larger proportion of the total polyphenol ingested than phase II metabolites of monomeric flavanols, indicating the important role of intestinal bacteria in the metabolism of highly polymerized almond skin polyphenols.

ABBREVIATIONS USED

LDL, low-density lipoprotein; DAD, diode array detector; mDP, mean degree of polymerization; SPE, solid-phase extraction; IS, internal standard; MRM, multiple reaction monitoring; F-C, Folin−Ciocalteu.

SAFETY

Guidelines for work with organic solvents and acids were respected. Universal precautions for the handling of chemicals and fluids were applied.

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