Accepted Manuscript

Almond (*Prunus dulcis* (Mill.) D.A. Webb) polyphenols: from chemical characterization to targeted analysis of phenolic metabolites in humans

Begoña Bartolomé, María Monagas, Ignacio Garrido, Carmen Gómez-Cordovés, Pedro J. Martín-Álvarez, Rosa Lebrón-Aguilar, Mireia Urpí-Sardà, Rafael Llorach, Cristina Andrés-Lacueva

PII: S0003-9861(10)00118-9
DOI: 10.1016/j.abb.2010.03.020
Reference: YABBI 5627

To appear in: *Archives of Biochemistry and Biophysics*

Received Date: 29 January 2010
Revised Date: 17 March 2010

Please cite this article as: B. Bartolomé, M. Monagas, I. Garrido, C. Gómez-Cordovés, P.J. Martín-Álvarez, R. Lebrón-Aguilar, M. Urpí-Sardà, R. Llorach, C. Andrés-Lacueva, Almond (*Prunus dulcis* (Mill.) D.A. Webb) polyphenols: from chemical characterization to targeted analysis of phenolic metabolites in humans, *Archives of Biochemistry and Biophysics* (2010), doi: 10.1016/j.abb.2010.03.020

Almond (*Prunus dulcis* (Mill.) D.A. Webb) polyphenols: from chemical characterization to targeted analysis of phenolic metabolites in humans

Begoña Bartolomé[1*], María Monagas[1], Ignacio Garrido[1], Carmen Gómez-Cordovés[1], Pedro J. Martín-Álvarez[1], Rosa Lebrón-Aguilar[2], Mireia Urpí-Sardà[3], Rafael Llorach[3], Cristina Andrés-Lacueva[3]

[1]Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

[2]Instituto de Química-Física "Rocasolano" (CSIC), Serrano 119, 28006 Madrid, Spain

[3]Nutrition and Food Science Department, XaRTA. INSA. Pharmacy Faculty, University of Barcelona, 08028 Barcelona, Spain.

* Corresponding author: B. Bartolome (Tel: 34 91562900; Fax: 34 915644853; E-mail: bartolome@ifi.csic.es)

ABSTRACT

In this paper, a survey of our studies on almond polyphenols including their chemical characterization and further bioavailability in humans is reported. Combination of analytical techniques (LC-DAD/fluorescence, LC/ESI-MS and MALDI-TOF) allowed us, for the first time, the identification of A- and B-type procyanidin, propelargonidin and prodelphinidin polymers in almond skins. Glucuronide, *O*-methyl glucuronide, sulfate and *O*-methyl sulfate derivatives of (epi)catechin, as well as the glucuronide conjugates of naringenin and isorhamnetin, and sulfate conjugates of isorhamnetin, together with conjugates of hydroxyphenylvalerolactones were detected in plasma and urine samples after the intake of almond skin polyphenols. In addition, numerous microbial derived metabolites, including hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic and hydroxyhippuric acids were also identified. Depending of the type of metabolite, maximum urinary excretion was attained at different time in comparison to the control group in the course of the 24-h period of urine excretion, allowing us to establish the onset of microbial metabolism.

Key words. Almond skins; flavan-3-ols; conjugated phenolic metabolites; microbial-derived phenolic metabolites; hydroxyphenylvalerolactones; targeted analysis

1. INTRODUCTION

Among others foods, nuts have been traditionally associated with the Mediterranean diet. Regular nut consumption, in moderate doses, has been proved to reduce the blood levels of total and LDL cholesterol, parameters which are associated with a lower incidence of cardiovascular and obesity related diseases [1]. These healthy effects are attributed, in addition to their lipid profile and other components, to the presence of antioxidant phenolic compounds [2]. In particular, the consumption of whole almonds decreases postprandial glycemia, insulinemia and oxidative protein damage in healthy subjects [3]. Also, almond skin polyphenols have been found to act in synergy with vitamins C and E to protect LDL from oxidation and to enhance antioxidant defense [4,5]. A reduction in the risk of cancer and cardiovascular disease has also been described [6].

Almonds (*Prunus dulcis* (Mill.) D.A. Webb) are eaten raw, roasted and fried; but can also be used as ingredients in different products such as sauces, snacks and traditional Spanish products such as marzipan and "turron". Also, almonds are processed to make nutritional products such as almond milk used as a substitute cow's milk [7]. Industrial processing of almonds implies the sequential removal of the external coatings (mesocarp and brown hull) to obtain the whole almond with its skin. In further steps, whole almonds can be subjected to roasting and part of the skin falls apart, or alternatively they can be blanched to get peeled almonds [8]. The skin represents ~4% of the total almond weight and contains 70–100% of total phenols present in the nut [9].

The phenolic composition of almond skin comprises both low molecular-mass compounds and high molecular-mass polymers. Different phenolic structures corresponding to hydroxybenzoic acids and aldehydes, hydroxycinnamic acids, flavan-3-ols, flavonol glycosides and aglycones, flavanone glycosides and aglycones, and dihydroflavonol

aglycones, have been identified in almond skins [10]. In relation to the high molecular-mass phenolics, A- and B-type procyanidins and propelargonidins up to heptamers, and A- and B-type prodelphinidins up to hexamers also have been detected [8,10].

Any hypothesis about the contribution of almond skin polyphenols to the health benefits associated with almond consumption has necessary to prove that these almond polyphenols are bioavailable in human. The first bioavailability studies of almond skins polyphenols were performed in rats [4] and humans [11], flavonoid aglycones being only quantified in pre-hydrolysed plasma samples. Also, the bioaccessibility of phytochemicals from almond skins during gastrointestinal digestion and the role played by cell walls have been described using a dynamic gastric model of digestion [12, 13].

In this paper, we have summarized our recent studies covering from the previous characterization of almond skin polyphenols to their bioavailability in humans. A screening of both low and high molecular-mass of almond skins was first carried out using LC-DAD/fluorescence, LC/ESI-MS and MALDI-TOF. In order to determine the influence of the industrial processing on the phenolic composition of almond skins, samples submitted to different processes including blanching, blanching+drying and roasting were compared. Our next goal was to assess the phenolic metabolite profile in plasma and urine after an intake of almond polyphenols in humans through a pilot study. After that, a comparative placebo-controlled study was carried out in order to perform a targeted analysis of phenolic metabolites in urine samples, and finally to determine changes in the urinary metabolome after the intake of almond polyphenols.

2. MATERIALS AND METHODS

2.1. Standards and reagents. Phenolic standards were obtained from Extrasynthèse (Genay, France), Sigma/Aldrich (St. Louis, MO, USA), Fluka (Buchs, Switzerland) and PhytoLab GmbH&Co.KG (Vestenbergsgreuth, Germany). β-Glucuronidase/sulfatase (from *Helix pomatia*; ≥ 85,000 units/mL of glucuronidase) was purchased from Sigma-Aldrich (St. Louis, MO). The solvents used (methanol, acetonitrile, acetic acid and formic acid) were of LC grade.

2.2. Almond skin samples. Almond skins from mixtures of Spanish (harvest 2004) and American varieties of almonds (harvest 2004 and 2006) were kindly supplied by La Morella Nuts (Tarragona, Spain). Whole almonds were subjected to two different industrial processes: *(a)* blanching (95 ºC, 3 min) followed by peeling to remove the skins, and *(b)* roasting (145 ºC) in a continuous-working oven where the skins (roasted samples) were separated from the roasted kernels. The wet skins obtained from the blanching process were then freeze-dried in the laboratory [blanched (freeze-dried) samples] or dried in an industrial hot-air oven at 60 ºC (blanched+dried samples) until constant moisture content of skins was reached.

For the determination of total phenolic content, blanched, blanched+dried and roasted skins were milled in a Janke & Kunkel mill (Ika Labortechnik, Wilmington, North Carolina, USA). Ground skins (0.05 g) were extracted with 10 mL of methanol/HCl (1000:1, v/v) by sonication for 5 min followed by an extra 15 min resting period. The mixture was then centrifuged (3024 g, 5 min, 5 ºC) and filtered (0.45 μm) for the determination of total phenolic content by the method of Singleton and Rossie [14]. Gallic acid was used as standard to prepare the calibration curve. The results were expressed as mg of gallic acid equivalents per gram of almond skin. Analysis was performed in triplicate.

For the human studies, a commercial almond skin extract (Amanda®) made from the Spanish Marcona variety, was kindly supplied by Puleva Biotech, S.A (Granada, Spain). The almond skin extract consisted of water extraction at 60 °C followed by purification by adsorption chromatography using water-alcohol mixtures. The extract was encapsulated (350 mg extract + 50 mg cellulose per capsule) by the supplier. The phenolic composition of the encapsulated almond skin extract was determined by LC-DAD-fluorescence and LC-DAD-ESI-MS [15, 16]. Total non-flavonoid phenolic compounds (protocatechuic acid, ellagic acid and vanillic acid) accounted for 517 ± 63 μg/g of the encapsulated extract. The total concentration of the different groups of flavonoid phenolic compounds was: 2047 ± 215 μg/g for *flavonols* (aglycones and -3-*O*-galactosides, -3-*O*-rutinosides and -3-*O*-glucosides of kaempferol and isorhamnetin); 123 ± 17 μg/g for *flavanones* (aglycones and -7-*O*-glucosides of naringenin and eriodictyol); 47.0 ± 8.1 μg/g for *dihydroflavonols* (dihydroquercetin); 975 ± 90 μg/g for *monomeric flavan-3-ols* [(+)-catechin and (-)-epicatechin], and 1282 ± 101 μg/g for *olygomeric flavan-3-ols* (A- and B-type properlagonidins and procyanidins). The encapsulated almond skin extract presented a total polyphenol content of 221 ± 6 mg gallic acid/g, a total proanthocyanidin content of 315 ± 14 mg cyanidin/g, and an antioxidant capacity (ORAC value) of 3.10 ± 0.20 mg mmol Trolox equivalents/g [15].

2.3. Determination of the phenolic composition of almond skins by LC-DAD-fluorescence and MALDI-TOF MS

For phenolic extraction, 2.10 g of ground skins were mixed with 30 mL of methanol/HCl (1000/1, v/v) and sonicated for 15 min followed by an extra 15 min of resting period. This procedure was performed twice. The mixture was then centrifuged (3024 *g*, 10 min, 5 ºC). The supernatant was separated and the pellet was submitted to two further extractions. The

supernatants were combined and the mixture was dried under vacuum. The residue was dissolved in 40 mL of distilled water and extracted with ethyl acetate (40 mL x 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) for HPLC analysis. The extractions were performed in duplicate.

For the LC analysis, a Waters (Milford, MA, USA) liquid chromatography system equipped with a 600-MS controller, a 717Plus autosampler, a 996 photodiode-array detector (DAD), and a fluorescence detector coupled to the Waters Empower (version 5.0) for data acquisition and processing was used. Separation was performed on a 250 x 4.6 mm r.d., 4µm reversed-phase Nova-Pak C_{18} (Waters, Milford, MA, USA) 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 90-120 min. A 75 µL volume sample was injected into the column. The detection conditions were: 210-360 nm (DAD); 280 nm 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 flavan-3-ols that were quantified by their fluorescence response. Due to the lack of commercial standards, oligomeric flavan-3-ols were quantified using the (-)-epicatechin calibration curve.

For the MALDI-TOF analysis, the ethyl-acetate-purified extracts (1µL) were mixed with 4 µL of the matrix consisting of 2,5-dihydroxybenzoic acid (gentisic acid) at a concentration of 20 mg/mL in water [10]. Then, 1µL of this solution was spotted onto a flat stainless-steel sample

plate and dried in air. MALDI-TOF measurements were performed using a Voyager DE-PRO mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with a pulsed nitrogen laser (λ=337 nm, 10 ns pulse width, and 20 Hz frequency) and a delayed extraction ion source. Ions generated by the laser desorption were introduced into the flight tube (1.3 m flight path) with an acceleration voltage of 25 kV, 94% grid voltage, 0.05% ion guide wire voltage, and a delay time of 100 ns in the linear positive ion mode. All mass spectra were collected by averaging the signals of at least 3000 laser shots over the *m/z* range 300-6000. Angiotensin I and bovine insulin were used for external calibration.

2.4. Pilot study of acute intake of almond polyphenols in humans. Two healthy and non-medication-receiving volunteers were recruited. They were advised to avoid alcohol and vegetable- and fruit-containing foods and beverages for 48 h before the study. In the morning (9.00), after overnight fasting, the volunteers ingested 10 capsules containing the almond skin extract (884 mg total polyphenols/dose), which was ~8 times higher than the estimated dietary intake (102 to 121 mg/person/day) of nut polyphenols in the Spanish diet [17]. The volunteers did not consume any food before or with the almond skin extract. At 14.00, volunteers had a light lunch meal composed of pasta and meat, and at 20.00 they had a dinner meal composed of ham 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 ºC and stored at -80 ºC. Urine samples were also collected at the following intervals: from 2 h before the time of ingestion **(-2–0 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 ºC. 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.

202

203 **2.5. Placebo-controlled study of acute intake of almond polyphenols in humans.** Six-teen
204 healthy and non-receiving medication volunteers [n = 12 for the **intake group**, and n= 4 for
205 **the placebo (control) group**] aged 29.8 ± 5.2 years were recruited for the study. Volunteers
206 followed the same diet protocol before and during the day of the intake as in the pilot study.
207 The intake group ingested the same dosis of almond skin polyphenol extract (10 capsules)
208 whereas the control group ingested 10 capsules containing 450 mg of microcrystalline
209 cellulose. But for this study, urine samples were collected in the following time periods: from
210 2 h before to the time of intake **[(-2)-0 h]**; from time of intake (0 h) to 2 h after intake **(0-2 h)**;
211 from 2 h to 6 h after intake **(2-6 h)**; from 6 h to 10 h after intake **(6-10 h)**, and from 10 h to 24
212 h after intake **(10-24 h)**. Urine samples were acidified to 0.2 M HCl, and were stored at -80
213 ºC. The study protocols were approved by the Ethics Committee of Clinical Investigation of
214 the University of Barcelona (Spain) and informed consent was obtained from all participants.

215

216 **2.6. Enzymatic hydrolysis of plasma and urine samples.**

217 Samples were subjected to enzymatic hydrolysis as previously described Urpi-Sarda et al.
218 [18]. Briefly, urine samples (1 mL) were taken to pH 4.9 with 200 µL of 2 M sodium acetate,
219 and subsequently incubated with β-glucuronidase/sulfatase (9444 units/mL of glucuronidase)
220 at 37ºC for 45 min. Straight afterwards, samples were acidified to pH 2 with 6 M HCl.

221

222 **2.7. Extraction of phenolic metabolites from plasma and urine samples.**

223 To perform the targeted analysis of microbial-derived metabolites, extraction from
224 hydrolyzed samples was performed using solid-phase extraction (SPE) Oasis® MCX 96-well
225 plates (Waters, Mildford, Massachusetts), a vacuum manifold and a vacuum source as
226 described by Urpi-Sarda et al [18]. Briefly, the plate was conditioned with 1mL of methanol
227 followed by 1 mL of 2% formic acid. The hydrolyzed urine samples were then loaded onto

the plate with 100 µL of 2 µM ethyl gallate as internal standard (IS). The plate was washed with 1 mL of 2% formic acid. Analytes were then eluted with methanol (0.5 mL x 3) and the eluates were evaporated to dryness under a stream of nitrogen. Residues were reconstituted with 100 µL of mobile phase.

For the targeted analysis of conjugated metabolites, non-hydrolyzed samples were fractionated by SPE, using Oasis HBL 96-well plates (30 mg) (Waters, Mildford, Massachusetts). The plate was conditioned with 1mL of methanol followed by 1 mL of 1.5 M formic acid. One milliliter of the non-hydrolyzed 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 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.

2.8. Analysis of phenolic metabolites in urine and plasma samples by LC-ESI-MS/MS.

The analysis of phenolic metabolites in urine and plasma samples were carried out by LC-ESI-MS/MS on a 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, Ontario, Canada) equipped with a Turbo IonSpray ionizing in negative mode. A Phenomenex Luna C18 analytical column [50 x 2.0 mm i.d., 5µm] (Torrance, CA) with mobile phase A (100% water and 0.1% formic acid) and B (100% acetonitrile and 0.1% formic acid) was used as described by Urpi-Sarda et al [15]. For the non-hydrolyzed 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, 400ºC; 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. For the conjugated metabolites of flavan-3-ols, the following transitions were targeted: (epi)catechin-*O*-glucuronide (465/289), *O*-methyl (epi)catechin-*O*-glucuronide (479/303), (epi)catechin sulfate (369/289), and *O*-methyl (epi)catechin sulfate (383/303). For conjugated metabolites of hydroxyphenylvalerolactones, the following transitions were targeted: 5-(dihydroxyphenyl)-γ-valerolactone glucuronide (383/207); 5-(dihydroxyphenyl)-γ-valerolactone sulfate (287/207); 5-(hydroxy-methoxy-phenyl)-γ-valerolactone glucuronide (397/221); 5-(hydroxy-methoxy-phenyl)-γ-valerolactone sulfate (301/221). For the conjugated metabolites of flavonols, the following transitions were targeted: naringenin-*O*-glucuronide (447/271); isorhamnetin-*O*-glucuronide (491/315), and isorhamnetin sulfate (395/315). For microbial-derived phenolic metabolites, the following transitions were targeted: 5-(dihydroxyphenyl)-γ-valerolactone (207/163); 5-(hydroxyphenyl)-γ-valerolactone (191/147); 3,4-dihydroxyphenylpropionic acid (181/137); 3-hydroxyphenylpropionic acid (165/121); 3,4-dihydroxyphenylacetic acid (167/123); 3-hydroxyphenylacetic acid (151/107); 4-hydroxy-3-methoxy-phenylacetic acid (181/137); phenylacetic acid (135/91); *m*-coumaric acid (163/119); *p*-coumaric acid (163/119); caffeic acid (179/135); ferulic acid (193/134); 3-hydroxy-4-methoxy-cinnamic acid (193/134); 3-hydroxybenzoic acid (137/93); 4-hydroxybenzoic acid (137/93); protocatechuic acid (153/109); vanillic acid (167/152), and 4-hydroxyhippuric acid (194/100).

Phenolic standard curves were constructed with standard solutions subjected to the same SPE procedure than the samples. Conjugated metabolites of (epi)catechin, naringenin and isorhamnetin were quantified using the calibration curves of (-)-epicatechin, naringenin and isorhamnetin, respectively. Hydroxyphenylvalerolactones and their conjugated metabolites were quantified as (-)-epicatechin.

2.9. Statistical methods. The statistical methods used for data analysis were: *a)* Repeated Measures Analysis of Variance (ANOVA) to test jointly the effects of the two factors: time as within-subjects factor, with 5 levels: (-2)-0 h, (0-2 h), (2-6 h), (6-10 h) and (10-24 h), and the groups of consumption of almond skin extracts (intake and control), as a categorical factor; *b)* and Principal Component Analysis (PCA), from standardized variables, to examine the relationship between all targeted metabolites at all time periods. The STATISTICA program for Windows, version 7.1 was used for data processing (StatSoft, Inc., 2005, www.statsoft.com).

3. RESULTS

3.1. Characterization of phenolic compounds from almond skins

A total of 31 low molecular-mass phenolic compounds were identified and quantified (Table 1) in almond skins by LC-DAD/fluorescence and LC/ESI-MS using their retention time and UV and mass spectra [10]. These compounds corresponded to different flavonoids and non-flavonoids: hydroxybenzoic acids (*p*-hydroxybenzoic acid, vanillic acid, and protocatechuic acid) and aldehydes (protocatechuic aldehyde), hydroxycinnamic acids (*trans-p*-coumaric acid and 3-*O*-caffeoylquinic acid), flavan-3-ols ((+)-catechin, (–)-epicatechin, procyanidins B3, B1, B2, B7, B5, C1, three unknown A-type procyanidin dimers and an unknown A-type procyanidin trimer), flavonol glycosides (kaempferol-3-*O*-rutinoside, kaempferol-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isorhamnetin-3-*O*-glucoside, and quercetin-3-*O*-

306 glucoside), flavanone glycosides (naringenin-7-*O*-glucoside and eriodictyol-7-*O*-glucoside),
307 flavonol aglycones (kaempferol, quercetin, and isorhamnetin), dihydroflavonol aglycones
308 (dihydroquercetin) and flavanone aglycones (naringenin and eriodictyol).
309
310 Concerning high-molecular mass flavan-3-ol polymers, Table 2 describes the $[M+Na]^+$
311 signals detected by MALDI-TOF-MS in the almond skin extracts [10]. They corresponded to
312 a series of A-type (containing one A-type linkage) and B-type (oligomers and polymers
313 composed of: *i)* homopolymers of (epi)catechin (procyanidins), *ii)* heteropolymers composed
314 of one (epi)afzelechin unit and the rest of (epi)catechin units (propelargonidins), and *iii)*
315 heteropolymers containing one unit of (epi)gallocatechin unit and the rest of (epi)catechin
316 units (prodelphinidins) (Table 2). A- and B-type procyanidins and propelargonidins were
317 detected up to heptamers, and A- and B-type prodelphinidins up to hexamers (Table 2).
318
319 **3.2. Influence of industrial processing**
320 The total polyphenol content of skins from mixtures of Spanish and American almond
321 varieties ranged from 9.10 to 32.1 mg of gallic acid equivalents per gram of almond skin.
322 Both roasted skins and blanched+dried skins showed a higher phenolic content than blanched
323 skins (1.9-2.8-fold and 1.2-1.6-fold increase, respectively) [8].
324 Figure 1 illustrates the influence of industrial processing on the content of individual phenolic
325 by group category: Σ hydroxybenzoic acid and aldehydes, Σ hydroxycinnamic acids, Σ flavan-
326 3-ols, Σ flavonol glycosides, Σ flavanone glycosides, Σ flavonol aglycones, Σ dihydroflavonol
327 aglycones, and Σ flavanone aglycones. Comparing roasted skins (R) with blanched skins (B) ,
328 the phenolic content of the different phenolic groups was approximately 2-fold higher in R
329 samples, except for Σ flavonol aglycones. Differences in phenolic content were more relevant
330 for Σ flavan-3-ols and Σ flavonol glycosides which are the main phenolic groups in almond
331 skins (Figure 1).

Similarly, industrial drying of blanched almond skins (BD) produced an increase in the content of the different phenolic groups (Figure 1). Again, these differences were more relevant for Σflavan-3-ols and Σflavonol glycosides. An increase in the content of Σ hydroxybenzoic acids and aldehydes, Σ hydroxycinnamic acids and Σflavanone glycosides, was also observed but not for all varieties. However, Σ flavonols and Σ flavanone aglycones showed a decrease of phenolic content in BD samples (Figure 1).

3.3. Identification of phenolic metabolites in human plasma and urine

To determine the main phenolic metabolites originated from the ingestion of almond polyphenols, a pilot study with two healthy volunteers was first carried out. Plasma (0 h and 2.5 h after the intake) and urine samples (2-0 h before the intake and 0-24 h after the intake) were subjected to hydrolysis for the determination of microbial-derived phenolic acids. Non-hydrolyzed samples were used for the determination of the phenolic conjugated forms.

A total of 22 conjugates derived from phase II metabolism of flavanols, flavonols, flavanones, and hydroxyphenylvalerolactones (a microbial metabolite of flavan-3-ols) [19-21] were identified in non-hydrolyzed samples (Table 3). For all these metabolites, an increase in concentration was observed after the intake of the almond skin extract (Table 3). The majority of the metabolites were detected in both plasma and urine samples, except for the conjugates of hydroxyphenylvalerolactones and isorhamnetin that were mainly detected in urine (0-24 h). This could be attributed to the fact that their formation from the microbial degradation from flavan-3-ols and isorhamnetin-3-*O*-rutinoside, respectively, may require a longer period of time [15].

The following microbial-derived phenolic acids were detected in the hydrolyzed samples: 3,4-dihydroxyphenylpropionic, 3-hydroxyphenylpropionic, 3,4-dihydroxyphenylacetic, 3-

358 hydroxyphenylacetic, 4-hydroxy-3-methoxy-phenylacetic, phenylacetic, *m*- and *p*-coumaric,
359 caffeic, ferulic, 3-hydroxy-4-methoxy-cinnamic,, 3-hydroxybenzoic, 4-hydroxybenzoic,
360 protocatechuic, vanillic, and 4-hydroxyhippuric (Table 3). All targeted metabolites were
361 identified in plasma and urine samples with the exception of 3-hydroxybenzoic acid in
362 plasma. In the case of urine samples, an increase in the concentration of all metabolites was
363 observed 24 h after the intake of the almond skin extract. However, no changes were observed
364 in plasma after 2.5 h from the intake, which was expected since this was a short time for the
365 formation of these metabolites by the microbiota.
366
367 **3.4. Targeted analysis of the phenolic metabolites in urine samples**
368 A further placebo-controlled study was carried out with sixteen healthy and non-receiving
369 medication volunteers *(n* = 12 for the intake group, and *n* = 4 for the control group) targeting
370 the metabolites above identified in urine after the intake of the almond skin extract. As
371 representative results, Figure 2 illustrates the changes registered in the urinary levels of
372 (epi)catechin sulfate #1, 5-(hydroxymethoxyphenyl)-γ-valerolactone sulfate #1, naringenin-*O*-
373 glucuronide #1, isorhamnetin sulfate, 3,4-dihydroxyphenylpropionic acid, 4-hydroxy-3-
374 methoxy-phenylacetic acid, caffeic acid, and protocatechuic acid.
375
376 For (epi)catechin sulfate #1 and naringenin-*O*-glucuronide #1 significant changes ($p < 0.05$)
377 were observed for the time period of urine collection, and between the intake and control
378 groups (Figure 2A and 2C). The urinary excretion of these metabolites increased from (-2)-0
379 h to 0-2 h after the intake, reaching the maximum level at 2-6 h. For isorhamnetin sulfate,
380 significant changes ($p < 0.05$) were only registered for the time period of urine collection,
381 although the urinary excretion of last fraction (10-24 h) was higher for the intake group
382 (Figure 2D).
383

Concerning the conjugated forms of hydroxyphenylvalerolactones, significant changes ($p < 0.05$) were registered for 5-(hydroxymethoxyphenyl)-γ-valerolactone sulfate #1 for the time period, and between the intake and control groups (Figure 2B). The excretion of this compound started at 2-6 h after the intake, and kept increasing up to 10-24 h after the intake.

Concerning microbial-derived metabolites, changes in the levels of 3,4-dihydroxyphenylpropionic acid (Figure 2E), 4-hydroxy-3-methoxy-phenylacetic acid (Figure 2F), caffeic acid (Figure 2G), and protocatechuic acid (Figure 2H) were only significant ($p < 0.05$) for the time period of sample collection. Therefore, the small differences observed in the urinary excretion levels between intake and control groups in the course of the 24-h period, suggested the need of a larger time period for these microbial-derived metabolites to be excreted.

To determine the influence of both conjugated and microbial-derived phenolic metabolites in the urinary metabolome as consequence of the intake of the almond skin extract, a PCA was performed including all targeted metabolites at all time periods. Two new components, PC1 (that explained 37.7% of the total variance of the data) and PC2 (that explained 20.2% of the total variance of the data) resulted from the analysis (Figure 3). PC1 was negatively correlated with 5-(dihydroxyphenyl)-γ-valerolactone sulfate, 5-(hydroxy-methoxy-phenyl)-γ-valerolactone glucuronide #1, 5-(hydroxy-methoxy-phenyl)-γ-valerolactone sulfate #1, 4-hydroxyhippuric acid, 3- and 4-hydroxybenzoic acids, protocatechuic acid, phenylacetic acid, 3,4-dihydroxyphenyl acetic acid, 4-hydroxy-3-methoxy-phenylacetic acid, *m*-coumaric acid, caffeic acid, ferulic acid, and 3-hydroxy-4-methoxy-cinnamic acid (loadings < -0.7). This is to mean, PC1 was more related to metabolites of microbial origin. Contrary, PC2 was positively correlated with (epi)catechin-*O*-glucuronide #3, *O*-methyl (epi)catechin-*O*-glucuronide #1 and #2, (epi)catechin sulfate #1 and #2, and *O*-methyl (epi)catechin sulfate #1, #2 and #3

410 (loadings > 0.7), so PC2 was more related to conjugated metabolites formed after the intake
411 of the almond polyphenols. In addition, it is important to highlight that urine samples were
412 distributed in the plane defined by these two components according to the time period of
413 sample collection: (-2)-0 h, 0-2 h, 2-6 h, 6-10 h, and 10-24 h (Figure 3).
414
415 **4. DISCUSSION**
416
417 Our results concerning the phenolic composition of almond skins from different origins and
418 harvests, suggested that almond skin can be considered as a good source of polyphenols to be
419 used in the elaboration of dietary ingredients. The total polyphenol content of almond skin is
420 ≥ 10 mg per gram, which is higher than other well-known materials such as red grape skin,
421 but lower than grape seeds [8]. From the data of the individual phenolic compound content, it
422 can be stated that the phenolic composition of almond skins is characterized by the prevalence
423 of flavan-3-ols (represented 33-56% of the total compounds identified for the different
424 almond varieties) and flavonol glycosides (9-36%). Other minor phenolic compound found in
425 almond skins corresponded to hydroxybenzoic acids and aldehydes (6-26%), flavanol
426 aglycones (1.7-18%), flavanol glycosides (3-7.7%), flavanone aglycones (0.69-5.4%),
427 hydroxycinnamic acids (0.65-2.6%), and dihydroflavonol aglycones (0-2.8%) [8]. Also, the
428 most abundant compounds present in almond skin was (+)-catechin, representing 10-23 % of
429 the total phenolic compounds identified, and isorhamnetin-3-*O*-rutinoside accounting for 6.8-
430 17% [8].
431
432 Although almonds can eaten raw, almonds are usually subjected to industrial processing to
433 remove the skin. Our results indicate that the phenolic content of almond skins depends on the
434 industrial processing used. Subjecting the almonds to high temperatures (i.e., blanching,
435 drying, roasting) could promote degradation of polymeric compounds such as

436 proanthocyanidins [22], hydrolysis of glycosylated flavonoids [23] and the decomposition of
437 the aglycones [24], which could explain the increase observed in the content of monomeric
438 and oligomeric flavan-3-ols after drying or roasting, and the decline of the flavonols and
439 flavanones aglycones found after these treatments. Moreover, blanching could have produced
440 solubilization of many phenolic compounds present in the almond skin. The influence of nut
441 processing in the phenolic composition of the skin has also been reported for peanut [22] and
442 hazelnut skins [25].

443

444 It is known that polyphenol bioavailability is strongly influenced by their chemical structure
445 [26, 27]. Monomeric flavan-3-ols and dimeric procyanidins (to a lower extent), would be
446 directly absorbed in the small intestine, where they would be first conjugated, and later in the
447 liver into methyl, glucuronide and sulfate derivatives by phase II enzymes. However,
448 oligomers presenting a mean degree of polymerization (mDP) ≥3 and polymeric flavan-3-ols
449 (proanthocyanidins) are not absorbed and reach the colon where they are metabolized by the
450 intestinal microbiota into hydroxyphenylvalerolactones and various phenolic acids, including
451 phenylpropionic, phenylacetic and benzoic acid derivatives, that can be further absorbed and
452 then conjugated in the liver [28, 29]. Conversely, glycosylated polyphenols such as flavonol
453 glycosides must be first hydrolyzed by intestinal β-glucosidases before they can be absorbed,
454 although the type of sugar that is attached to the flavonoid limits the absorption in the small
455 intestine [27]. Flavonols containing rhamnose, would be poorly absorbed and transferred to
456 the large intestine where they would be degraded by the rhamnosidases of the intestinal
457 microbiota [30]. The results obtained through the human studies reported in this paper prove
458 that the monomeric flavan-3-ols, and flavonol and flavanone glycosides coming from the
459 almond skin are absorbed and further conjugated, as they were detected as phase-II
460 metabolites (glucuronide, *O*-methyl glucuronide, sulfate and *O*-methyl sulfate derivatives of
461 (epi)catechin, as well as the glucuronide conjugates of naringenin and glucuronide, and

462 sulfate conjugates of isorhamnetin) in both plasma and urine. Besides, these compounds plus
463 the olygomeric flavan-3-ols (that would be not absorbed in the small intestine) would be
464 degraded by the intestinal microbiota and lead to hydroxyphenylvalerolactones and phenolic
465 acids (hydroxyphenylpropionic, hydroxyphenylacetic, hydroxycinnamic, hydroxybenzoic and
466 hydroxyhippuric acids) that in urine over a 0-24 h period time.

467

468 The results of the human studies also concluded that the maximum urinary excretion of the
469 different phenolic metabolites derived from the consumption of almond skins was attained at
470 different time over a 24-h period after the intake. For flavonol (i.e., (epi)catechin sulfate #1)
471 and flavanone (i.e., naringenin-*O*-glucuronide #1) conjugates, the maximum was achieved
472 from 2 to 6 hours, as expected for their early intestinal absorption. These data are in
473 agreement with previous studies that reported that the maximum urinary excretion of
474 (epi)catechin conjugated metabolites at 0-6 h [31] and at 0-2h [32] after intake of cocoa
475 powder either with milk or water, and at 0-5 h after the intake of green tea [33]. However, for
476 the conjugates of hydroxyphenylvaleractones [i.e., 5-(hydroxymethoxyphenyl)-γ-
477 valerolactone sulfate #1], the maximum was attained latter, from 6 to 24 h, as expected for
478 their colonic origin. The later urinary excretion of flavonol conjugates (i.e., isorhamnetin
479 sulphate) was related to the high content of isorhamnetin-3-*O*-rutinoside found in the almond
480 skin [10], which would suffer a later colonic transformation. The maximum urinary excretion
481 for microbial-derived phenolic acids (i.e., 3,4-dihydroxyphenylpropionic acid, 4-hydroxy-3-
482 methoxy-phenylacetic acid, caffeic acid and protocatechuic acid) seemed to occur after 24 h
483 period of urine collection used in our study. This finding agrees with the study of Rios et al.
484 [34], who reported that maximum urinary levels of 3-hydroxyphenylpropionic, 3-
485 hydroxyphenylacetic, 3-hydroxybenzoic and ferulic acid was attained between 24 and 48 h
486 after the intake of chocolate, although no control group was considered in this study, so
487 changes could only be compared to baseline values.

In conclusion, almond skins have proved to contain a wide range of phenolic compounds. Of particular importance in the occurrence of A- and B-type proanthocyanidins, comprising procyanidins, propelargonidins and prodelphinidins. Among the industrial processing used to remove almond skins, roasting is the most suitable type of to obtain skin extracts with the highest phenolic content. Our studies also demonstrate that almond skin polyphenols are bioavailable in humans as they were detected as phase-II and microbial-derived metabolites in plasma and urine samples. Depending on the type of metabolite, maximum urine excretion was attained at different time in the course of the 24-h period, allowing us to establish the onset of microbial metabolism. Further investigations of the potential of these phenolic metabolites as biomarkers of almond skin consumption are currently being carried out using metabolomic approaches.

ACKNOWLEDGMENTS

We are thankful to La Morella Nuts (Tarragona, Spain) and Puleva Biotech, S.A (Granada, Spain) for kindly supplying of the samples. This work has received financial support from the CONSOLIDER INGENIO 2010 Programme: FUN-C-FOOD CSD2007-063 from the Spanish Ministry of Science and Innovation (MICINN). I.G. was the recipient of a fellowship from the I3P Program funding by the European Social Fund. M.U-S thanks the FPI fellowship from MICINN. M.M thanks the "Ramón y Cajal" post-doctoral program. R.LL thanks the "Fondo de Investigación Sanitaria" post-doctoral program (F.I.S. CD06/00161) from MICINN.

509 **LITERATURE CITED**

510 [1] C.Y. Chen, K. Lapsley, J. Blumberg, A nutrition and health perspective on almonds, J.
511 Sci. Food Agric. 86 (2006) 2245-2250.

512 [2] S.S.K Wijeratne, M.M. Abou-Zaid, F. Shahidi, Antioxidant polyphenols in almond and its
513 coproducts, J. Agric. Food Chem. 54 (2006) 312-318.

514 [3] D.J.A. Jenkins, C.W.C. Kendall, A. Marchie, Dose response of almonds on coronary heart
515 disease risk factors: Blood lipids, oxidized low-density lipoproteins, lipoprotein(a),
516 homocysteine, and pulmonary nitric oxide: A randomized, controlled, crossover trial,
517 Circulation 106 (2002) 1327-1332.

518 [4] C.Y. Chen, P.E. Milbury, K. Lapsley, J.B. Blumberg, Flavonoids from almond skins are
519 bioavailable and act synergistically with vitamins C and E to enhance hamster and human
520 LDL resistance to oxidation, J. Nutr. 135 (2005)1366-73.

521 [5] C.Y. Chen, P.E. Milbury, S.K. Chung, J. Blumberg, Effect of almond skin polyphenolics
522 and quercetin on human LDL and apolipoprotein B-100 oxidation and conformation, J. Nutr.
523 Biochem. 18 (2007) 785-794.

524 [6] C.Y.O. Chen, J.B. Blumberg, Phytochemical composition of nuts, Asia Pac. J. Clin. Nutr.
525 17 (2008) 329-332.

526 [7] J. Aranceta, C. Perez Rodrigo, A. Naska,V. Ruiz Vadillo, A. Trichopoulou, Nut
527 consumption in Spain and other countries. Brit. J. Nutr. 96 (Suppl. 2) (2006) S3-S11.

528 [8] I. Garrido, M. Monagas, C. Gómez-Cordovés, B. Bartolomé B, Polyphenols and
529 antioxidant properties of almond skins: influence of industrial processing, J. Food. Sci. 73
530 (2008) 106-115.

531 [9] P.E. Milbury, C.Y. Chen, G.G. Dolnikowski, J.B. Blumberg, Determination of flavonoids
532 and phenolics and their distribution in almonds, J. Agric. Food. Chem. 54 (2006) 5027-5033.

533 [10] M. Monagas, I. Garrido, R. Lebrón-Aguilar, B. Bartolome, C. Gómez-Cordovés, Almond
534 (Prunus dulcis (Mill.) D.A. Webb) skins as a potential source of bioactive polyphenols, J.
535 Agric. Food Chem. 55 (2007) 8498-8507.
536 [11] P.E.C. Milbury, C.Y. Chen, H.K. Kwak, J.B. Blumberg, Almond polyphenolics feeding
537 alters LDL oxidizability and glutathione metabolism without altering ORACpca in older
538 adults, Free Rad. Biol. Med. 37 (2004) S38.
539 [12] G. Mandalari, C. Nueno-Palop, G. Bisignano, M.S.J. Wickham, A. Narbad, Potential
540 prebiotic properties of almond (*Amygdalus communis* L.) seeds, Applied Environ. Microbiol.
541 74 (2008) 4264-4270.
542 [13] G. Mandalari, G. Rich, G. Bisignano, M. Parker, K. Waldron, M. Wickham, Almond
543 skins digestion using an in vitro dynamic gastric model: phytochemicals release and gut
544 health, FASEB J., 23 (2009) 563.38
545 [14] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with
546 phosphomolibdicphosphotungstic acid reagent, Am. J. Enol. Vitic 16 (1965) 144-158.
547 [15] M. Urpi-Sarda, I. Garrido, M. Monagas, C. Gómez-Cordovés, A. Medina-Remón1, C.
548 Andres-Lacueva, B. Bartolomé, Profile of plasma and urine metabolites after the intake of
549 almond (Prunus dulcis (Mill.) D.A. Webb) polyphenols in humans, J. Agric. Food Chem. 57
550 (2009) 10134-10142.
551 [16] I. Garrido, M. Urpí-Sardà, M. Monagas, C. Gómez-Cordovés, P.J. Martín-Álvarez, R.
552 Llorach, B. Bartolomé, C. Andrés-Lacueva, Targeted analysis of conjugated and microbial-
553 derived phenolic metabolites in human urine after consumption of an almond skin phenolic
554 extract, J. Nutr. Submitted (2010).
555 [17] F. Saura-Calixto, J. Serrano, I. Goñi, Intake and bioaccessibility of total polyphenols in a
556 whole diet, Food Chem. 101 (2007) 492-501.

557 [18] M. Urpi-Sarda, M. Monagas, N. Khan, Epicatechin, procyanidins, and phenolic
558 microbial metabolites after cocoa intake in humans and rats, Anal. Bioanal. Chem. 394 (2009)
559 1545-1556.
560 [19] X. Meng, S. Sang, N. Zhu, Identification and characterization of methylated and ring-
561 fission metabolites of tea catechins formed in humans, mice, and rats, Chem. Res. Toxicol. 15
562 (2002) 1042-1050.
563 [20] T. Kohri, M. Suzuki, F. Nanjo, Identification of metabolites of (-)-epicatechin gallate and
564 their metabolic fate in the rat, J. Agric. Food Chem. 51 (2003) 5561-5566.
565 [21] M.M. Appeldoorn, J.P Vincken, A.M. Aura, P.C.H. Hollman, H. Gruppen, Procyanidin
566 dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-
567 (3,4-dihydroxyphenyl)-γ- valerolactone as the major metabolites, J. Agric. Food Chem. 57
568 (2009) 1084-1092.
569 [22] J. Yu, M. Ahmedna, I. Goktepe, J. Dai, Peanut skin procyanidins: composition and
570 antioxidant activities as affected by processing, J. Food Compos. Anal. 19 (2006) 364–371.
571 [23] S. Rohn, N. Buchner, G. Driemel, M. Rauser, L.W. Kroh, Thermal degradation of onion
572 quercetin glucosides under roasting conditions, J. Agric. Food. Chem. 55 (2007) 1568-1573.
573 [24] N. Buchner, A. Krumbein, S. Rohn, L.W. Kroh, Effect of thermal processing on the
574 flavonols rutin and quercetin, Rapid. Commun. Mass. 20 (2006) 3229-3235.
575 [25] M. Locatelli, F. Travaglia, J.D. Cöisson, A. Martelli, C. Stévigny, M. Arlorio, Total
576 antioxidant activity of hazelnut skin (Nocciola Piemonte PGI): Impact of different roasting
577 conditions, Food Chem. 119 (2010) 1647-1655.
578 [26] A. Scalbert, G. Williamson, Dietary intake and bioavailability of polyphenols, J. Nutr.
579 130 (SUPPL. 8) (2000) 2073S-2085S.
580 [27] C. Manach, A. Scalbert, C. Morand, C. Rémésy, L. Jiménez, Polyphenols: Food sources
581 and bioavailability. Am. J. Clin Nutr.79 (2004) 727-747.

582 [28] M.P. Gonthier, V. Cheynier, J.L. Donovan, C. Manach, C. Morand, I. Mila, C. Lapierre,
583 C. Rémésy, A. Scalbert, Microbial aromatic acid metabolites formed in the gut account for a
584 major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. J. Nutr.
585 133 (2003) 461-467.
586 [29] M.P. Gonthier, J.L. Donovan, O. Texier, C. Felgines, C. Remesy, A. Scalbert,
587 Metabolism of dietary procyanidins in rats, Free Rad. Biol. Med. 35 (2003) 837-844.
588 [30] P.C.H. Hollman, J.M.P. Van Trijp, M.J.B. Mengelers, J.H.M. De Vries, M.B. Katan,
589 Bioavailability of the dietary antioxidant flavonol quercetin in man, Cancer Letters 114
590 (1997) 139-140.
591 [31] E. Roura, C. Andrés-Lacueva, R. Estruch, M.L.M. Bilbao, M. Izquierdo-Pulido, R.M.
592 Lamuela-Raventós, The effects of milk as a food matrix for polyphenols on the excretion
593 profile of cocoa (-)-epicatechin metabolites in healthy human subjects, Br. J. Nutr. 100 (2008)
594 846-851.
595 [32] W. Mullen, G. Borges, J.L. Donovan, Milk decreases urinary excretion but not plasma
596 pharmacokinetics of cocoa flavan-3-ol metabolites in humans, Am. J. Clin. Nutr. 89 (2009)
597 1784-1791.
598 [33] A. Stalmach, S. Troufflard, M. Serafini, A. Crozier, Absorption, metabolism and
599 excretion of Choladi green tea flavan-3-ols by humans, Mol. Nutr. Food Res 53 (2009) S44-
600 S53.
601 [34] L.Y. Rios, M.P. Gonthier, C. Remesy, Chocolate intake increases urinary excretion of
602 polyphenol-derived phenolic acids in healthy human subjects, Am. J. Clin. Nutr. 77 (2003)
603 912-918.

604 **Table 1.** Phenolic content of blanched almond skins.

Compound	Content (µg/g)
Hydroxybenzoic acids and aldehydes	**30.2 - 53.9**
p-Hydroxybenzoic acid	3.08 - 7.18
Vanillic acid	11.1 - 19.2
Protocatechuic acid	6.69 - 17.2
Protocatechuic aldehyde	5.45 - 13.7
Hydroxycinnamic acids	**1.78 - 3.77**
trans p-Coumaric acid	0 - 1.06
3-*O*-Caffeoylquinic acid	**1.76 - 3.77**
Flavan-3-ols	**64.8 - 121**
(+)-Catechin	20.1 - 38.3
(-)-Epicatechin	7.17 - 26.5
B3+B1	13.0 - 19.6
B2	2.34 - 9.64
B7	3.72 - 5.57
B5	2.29 - 5.38
C1	3.69 - 5.96
Unknown dimer A [(epi)catechin→A→(epi)catechin]	2.36 - 3.51
Unknown dimer A [(epi)catechin→A→(epi)catechin]	1.16 - 4.76
Unknown dimer A [(epi)catechin→A→(epi)catechin]	3,21 - 4.89
Unknown trimer A [(epi)catechin-(epi)catechin→A→(epi)catechin]	2.47 - 4.93
Flavonol glycosides	**15.6 - 130**
Kaempferol-3-*O*-rutinoside	5.26 - 40.7
Kaempferol-3-*O*-glucoside	0 - 14.2
Isorhamnetin-3-*O*-rutinoside	5.34 - 58.0
Isorhamnetin-3-*O*-glucoside	5.02 - 15.3
Quercetin-3-*O*-glucoside	0 - 1.71
Flavanone glycosides	**4.99 - 28.5**
Naringenin-7-*O*-glucoside	2.34 - 25.9
Eriodictyol-7-*O*-glucoside	2.5 - 2.65
Flavonol aglycones	**9.69 - 29.6**
Kaempferol	2.75 - 12.1
Quercetin	1.02 - 4.89
Isorhamnetin	5.92 - 16.0
Dihydroflavonol aglycones	**0 - 10.3**
Dihydroquercetin	0 - 10.3
Flavanone aglycones	**7.25 - 19.9**
Naringenin	3.70 - 12.1
Eriodictyol	2.98 - 7.76
Total	**165 - 370**

Table 2. Proanthocyanidin signals ($[M+Na]^+$) detected by MALDI-TOF-MS in almond skins extracts.

[a]DP	[b]Type	Propelargonidins		Procyanidins		Prodelphinidins	
		$[M+Na]^+$ calculated	$[M+Na]^+$ observed	$[M+Na]^+$ calculated	$[M+Na]^+$ observed	$[M+Na]^+$ calculated	$[M+Na]^+$ observed
1				313.3	313.1		
2	A	583.1	583.2				
2	B	585.1	585.2				
2	A			599.1	599.2		
2	B			601.1	601.2		
2	A					615.1	615.2
2	B					617.1	617.2
3	B	873.2	873.2				
3	A			887.2	887.2		
3	B			889.2	889.2		
3	A					903.2	903.2
3	B					905.2	905.2
4	A	1159.2	1159.3				
4	B	1161.3	1161.2				
4	A			1175.2	1175.2		
4	B			1177.3	1177.2		
4	A					1191.2	1191.2
4	B					1193.2	1193.3
5	B	1449.3	1449.3				
5	A			1463.3	1463.3		
5	B			1465.3	1465.2		
5	A					1479.3	1479.2
5	B					1482.3	1481.2
6	B	1737.4	1737.4				
6	A			1751.4	1752.2		
6	B			1753.4	1754.0		
6	A					1767.3	1767.4
7	A	2023.4	2024.8				
7	B	2025.4					
7	A			2039.4	2039.6		
7	B			2041.4			

[a]DP: Degree of polymerization [b] A: A-type proantocyanidins containing one A-type linkage; B: B-type proanthocyanidins

Table 3. Phenolic metabolites identified in plasma and urine after the intake of the almond polyphenol extract in humans.

Non hydrolized samples				Hydrolyzed samples			
Metabolite	**MRM transition**	**Plasma (at 2.5h)**	**Urine (0-24h)**	**Metabolite**	**MRM transition**	**Plasma (at 2.5h)**	**Urine (0-24h)**
Flavan-3-ols				***Hydroxyphenylpropionic acids***			
(Epi)catechin-*O*-glucuronide #1	465/289		X[a]	3,4-Dihydroxyphenylpropionic acid	181/137	+[b]	X
(Epi)catechin-*O*-glucuronide #2	465/289	X	X	3-Hydroxyphenylpropionic acid	165/121	+	X
(Epi)catechin-*O*-glucuronide #3	465/289	X	X	***Hydroxyphenylacetic acids***			
O-methyl (epi)catechin-*O*-glucuronide #1	479/303	X	X	3,4-Dihydroxyphenylacetic acid	167/123	+	X
O-methyl (epi)catechin-*O*-glucuronide #2	479/303	X	X	3-Hydroxyphenylacetic acid	151/107	+	X
(Epi)catechin sulfate #1	369/289	X	X	4-Hydroxy-3-methoxy-phenylacetic acid	181/137	+	X
(Epi)catechin sulfate #2	369/289	X	X	Phenylacetic acid	135/91	+	X
O-methyl (epi)catechin sulfate #1	383/303	X	X	***Hydroxycinnamic acids***			
O-methyl (epi)catechin sulfate #2	383/303	X	X	m-Coumaric acid	163/119	+	X
O-methyl (epi)catechin sulfate #3	383/303		X	p-Coumaric acid	163/119	+	X
Flavanones				Caffeic acid	179/135	+	X
Naringenin-*O*-glucuronide #1	447/271	X	X	Ferulic acid	193/134	+	X
Naringenin-*O*-glucuronide #2	447/271	X	X	3-Hydroxy-4-methoxy-cinnamic acid	193/134	+	X
Flavonols				***Hydroxybenzoic acids***			
Isorhamnetin-*O*-glucuronide #1	491/315		X	3-Hydroxybenzoic acid	137/93		X
Isorhamnetin-*O*-glucuronide #2	491/315		X	4-Hydroxybenzoic acid	137/93	+	X
Isorhamnetin sulfate	395/315		X	Protocatechuic acid	153/109	+	X
Hydroxyphenylvalerolactones				Vanillic acic	167/152	+	X
5-(Dihydroxyphenyl)-γ-valerolactone glucuronide derivative #1	383/207		X	***Hydroxyhippuric acids***			
5-(Dihydroxyphenyl)-γ-valerolactone glucuronide derivative #2	383/207		X	4-Hydroxyhippuric acid	194/100	+	X
5-(Dihydroxyphenyl)-γ-valerolactone sulfate derivative	287/207	X	X				
5-(Hydroxy-methoxy-phenyl)-γ-valerolactone glucuronide derivative #1	397/221		X				
5-(Hydroxy-methoxy-phenyl)-γ-valerolactone glucuronide derivative #2	397/221		X				
5-(Hydroxy-methoxy-phenyl)-γ-valerolactone sulfate derivative #1	301/221		X				
5-(Hydroxy-methoxy-phenyl)-γ-valerolactone sulfate derivative #2	301/221		X				

X[a] The concentration of the compound increased after the intake.

+[b] The compound was detected but its concentration did not increase after the intake.

FIGURE LEGENDS

Figure 1. Influence of industrial processing (blanching [B], blanching+drying [BD] and roasting [R]) on the content of the different phenolic groups in almond skins. A) Σ Hydroxybenzoic acids and aldehydes, B) Σ Hydroxycinnamic acids, C) Σ Flavan-3-ols, D) Σ Flavonol glycosides, E) Σ Flavanone glycosides, F) Σ Flavonol aglycones, G) Σ ΣDihydroflavonol aglycones, H) Σ Flavanone aglycones, I) Σ all groups.

Figure 2. Changes registered in the urinary levels of some representative phenolic metabolites. A) (Epi)catechin sulfate #1, B) 5-(Hydroxymethoxyphenyl)-γ-valerolactone sulfate #1, C) Naringenin-*O*-glucuronide #1, D) Isorhamnetin sulfate, E) 3,4-Dihydroxyphenylpropionic acid, F) 4-Hydroxy-3-methoxy-phenylacetic acid, G) Caffeic acid, H) Protocatechuic acid.

Figure 3. Representation of the plane of the first and second principal components resulting from the application of principal components analysis (PCA) to all targeted metabolites for the intake group (n =12) at all time periods. Numbers in figure indicate the centre of each time period (1 for (-2)-0 h; 2 for 0-2 h; 3 for 2-6 h; 4 for 6-10 h, and 5 for 10-24 h), and arrows, changes in the plane in function of the time period.

Figure 1.

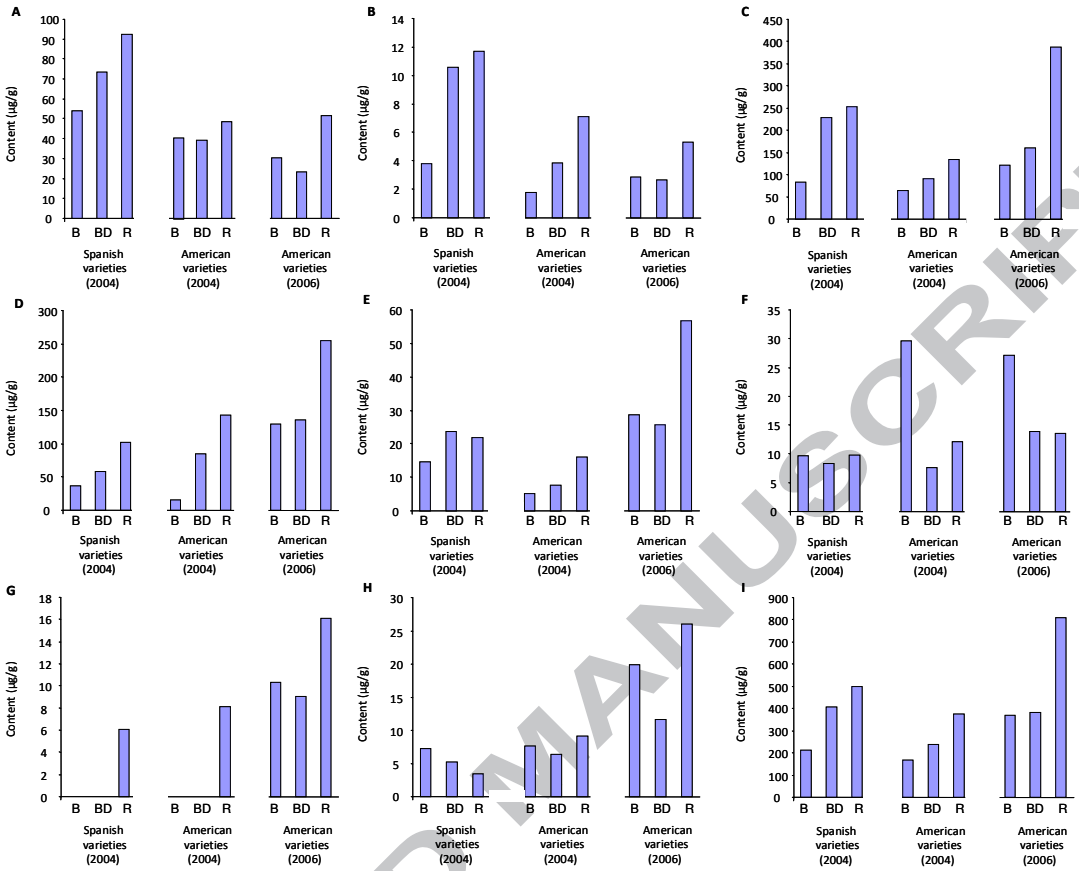

Figure 2.

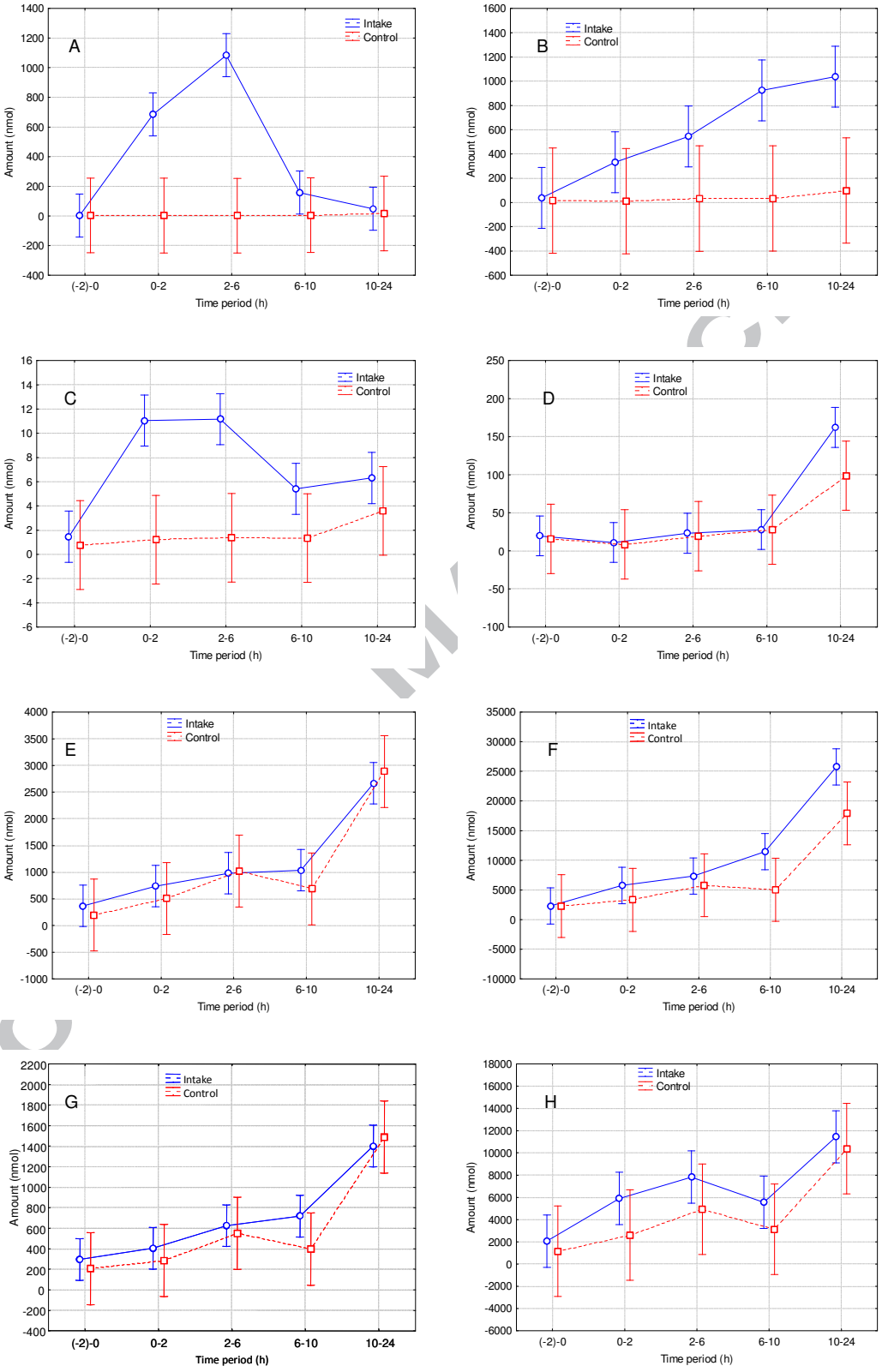

Figure 3.