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**ARTICLE** *in* JOURNAL OF PROTEOME RESEARCH · NOVEMBER 2010

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# Metabolomics Study of Human Urinary Metabolome Modifications After Intake of Almond (*Prunus dulcis* (Mill.) D.A. Webb) Skin Polyphenols

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**Received June 21, 2010**

Almond, as a part of the nut family, is an important source of biological compounds, and specifically, almond skins have been considered an important source of polyphenols, including flavan-3-ols and flavonols. Polyphenol metabolism may produce several classes of metabolites that could often be more biologically active than their dietary precursor and could also become a robust new biomarker of almond polyphenol intake. In order to study urinary metabolome modifications during the 24 h after a single dose of almond skin extract, 24 volunteers ($n = 24$), who followed a polyphenol-free diet for 48 h before and during the study, ingested a dietary supplement of almond skin phenolic compounds ($n = 12$) or a placebo ($n = 12$). Urine samples were collected before ((−2)−0 h) and after (0−2 h, 2−6 h, 6−10 h, and 10−24 h) the intake and were analyzed by liquid chromatography−mass spectrometry (LC-q-TOF) and multivariate statistical analysis (principal component analysis (PCA) and orthogonal projection to latent structures (OPLS)). Putative identification of relevant biomarkers revealed a total of 34 metabolites associated with the single dose of almond extract, including host and, in particular, microbiota metabolites. As far as we know, this is the first time that conjugates of hydroxyphenylvaleric, hydroxyphenylpropionic, and hydroxyphenylacetic acids have been identified in human samples after the consumption of flavan-3-ols through a metabolomic approach. The results showed that this non-targeted approach could provide new intake biomarkers, contributing to the development of the food metabolome as an important part of the human urinary metabolome.

**Keywords:** Metabolomics • almond-polyphenols • flavan-3-ols • food metabolome • biomarkers • multivariate analysis • OPLS • S-plot

## Introduction

Regular consumption of nuts has been associated with several health-promoting properties, particularly those with important cardio-protective effects[1] and management of the metabolic syndrome.[2] Consumption of almonds, as a part of the nut family, has also been related to healthy effects. Li et al.[3] studied the effect of consumption (4 weeks) of 84 g/day of almonds on regular smokers, concluding that biomarkers of oxidative stress diminished after almond supplementation. This healthy effect was attributed to the lipid profile and to the presence of phenolic compounds in almonds, among others. Specifically, the consumption of whole almonds decreased postprandial glycemia, insulinemia, and oxidative protein damage in healthy subjects.[4] In addition, almond skin polyphenols have been found to act in synergy with vitamins C and E to protect low density lipoprotein (LDL) from oxidation.[5] In addition to these beneficial effects, *in vitro* studies have recently suggested a the potential prebiotic effect of almond seeds.[6]

Almond skin is a rich source of phenolic compounds, including flavonols (-3-*O*-glucosides and -3-*O*-rutinosides of kaempferol and isorhamnetin), flavanones (naringenin-7-*O*-glucoside), and particularly flavan-3-ols. Flavan-3-ols are composed of monomeric forms ((+)-catechin, and (−)-epicatechin) and of a series of A- and B-type proanthocyanidins (properlagonidins, procyanidins, and prodelphinidins).[7] Both epidemiological and intervention studies have reported that flavan-3-ols exhibited several beneficial effects on health by acting as antioxidant, anticarcinogenic, cardiopreventive, and neuroprotective.[8] These health-promoting properties are dependent on their bioavailability. Monomers are readily absorbed in the small intestine, dimers seem to have a very limited absorption in humans, and polymeric procyanidins are not well absorbed in their native form, reaching the colon, where they are largely metabolized by the gut microbiota.[9,10]

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10.1021/pr100639v 



Published on Web 09/21/2010

Metabolomics aims to provide a comprehensive profile of all the metabolites present in a biological sample (metabolome).[11] Metabonomics and metabolomics approaches have been applied to clinical, pharmaceutical, and toxicological applications[12] and have also recently emerged as fields of increasing interest to food and nutrition science.[13,14] Several internal and external factors could modulate the metabolome. Among these, diet is a very important external factor affecting the urinary metabolome because it produces notable changes in its composition.[15] These modifications could be related to the presence of exogenous metabolites coming from food components such as phytochemicals (food metabolome) or from microbiota metabolism.[16] Metabolomics studies of the food metabolome may lead to the discovery of new phytochemical metabolites and new markers of phytochemical intake (i.e., nutritional markers) that could allow the intake of dietary phytochemicals to be monitored, and eventually relate them to the expected biological effects.[17] In this context, nutritional biomarkers have several advantages over dietary self-reported data because they reflect a more objective assessment of nutrient intake.[17−19]

Following the hypothesis that the study of the food metabolome could open an important window in our understanding of metabolome, the aim of the present work was to apply an liquid chromatography−mass spectrometry (LC−MS)-based metabolomic strategy with multivariate analysis, in order to analyze the influence of a single intake of almond skin polyphenols on the 24 h kinetic trajectory of the human urinary metabolic profile.

## Experimental Section

**Chemicals.** (−)-Epicatechin and procyanidin B2 were purchased from Sigma-Aldrich. 4-Hydroxyhippuric acid was purchased from PhytoLab GmbH&Co.KG. Naringenin and kaempferol were purchased from Extrasynthèse. HPLC grade solvents such as methanol, acetonitrile, and formic acid were purchased from Scharlau.

**Almond Extract Composition.** The encapsulated almond skin extract (Amanda) (total polyphenols: 221 mg of gallic acid/g; total proanthocyanidins: 314.8 mg of cyanidin/g; antioxidant capacity, ORAC: 3.10 mmol Trolox/g) was kindly supplied by Puleva Biotech, S.A. (Granada, Spain). The phenolic composition of the extract (3.5 g) included nonflavonoid compounds (protocatechuic acid, 0.47 mg; ellagic acid, 0.03 mg; vanillic acid, 1.30 mg) and flavonoid compounds included flavonols (kaempferol-3-*O*-galactoside, 0.08 mg; kaempferol-3-*O*-rutinoside, 0.82 mg; kaempferol-3-*O*-glucoside, 0.11 mg; isorhamnetin-3-*O*-galactoside, 1.01 mg; isorhamnetin-3-*O*-rutinoside, 4.39 mg; isorhamnetin-3-*O*-glucoside, 0.65 mg; kaempferol, 0.01 mg; isorhamnetin 0.08 mg), flavanones (naringenin-7-*O*-glucoside, 0.19 mg; eriodictyol-7-*O*-glucoside, 0.02 mg, naringenin, 0.19 mg; eriodictyol, 0.03 mg), dihydroflavonols (dihydroquercetin, 0.16 mg), and flavanols ((+)-catechin, 2.39 mg; (−)-epicatechin, 1.02 mg; B2 procyanidin dimer, 1.24 mg).[20]

**Subjects and Study Design.** Twenty-four healthy and non-medication-receiving volunteers with an average age of 32 ± 6.5 years (24−43 y) and with an average BMI of 22.99 ± 3.7 kg/m$^2$ (18.9−32.6 kg/m$^2$) were recruited for the study. None of them reported any history of heart disease, homeostatic disorders, or other medical conditions. The study was an open, prospective, blind, placebo-controlled, and randomized clinical trial.

Volunteers were advised to avoid alcohol and vegetable-containing foods and beverages for 48 h before the study. After overnight fasting, the volunteers were randomly distributed into a dosed group and a placebo group. The dosed group ($n = 12$) ingested 10 capsules containing the almond skin extract (thereafter termed AP-d) (3.5 g of almond extract and 0.5 g of microcrystalline cellulose), and the placebo group ($n = 12$) ingested 10 capsules containing a placebo (4 g of microcrystalline cellulose) (thereafter termed PL-d).

Urine samples were collected in the following time periods: from 2 h before to the time of intake (hereafter 0 h)]; from time of intake to 2 h after intake (hereafter 2 h); from 2 to 6 h after intake (hereafter 6 h); from 6 to 10 h after intake (hereafter 10 h); and from 10 to 24 h after intake (hereafter 24 h). The samples were stored at −80 °C. The study protocols were approved by the Ethics Committee of Clinical Investigation of the University of Barcelona and informed consent was obtained from all participants.

**Preparation of Samples.** The urine samples were thawed before analysis and centrifuged for 5 min at 12000 *g*. A 50 μL aliquot of the supernatant was diluted with 50 μL of Milli-Q water and vortex mixed; the resulting solution was transferred to a 96-well plate for LC-q-TOF analysis.

**LC-q-TOF Mass Spectrometry.** The chromatography was performed on an LC Agilent series 1200 using an RP 18 Luna 5 μm, 50 × 2.0 mm (Phenomenex, Torrance, CA). The mobile phase consisted of (A) 0.1% HCOOH and (B) acetonitrile 0.1% HCOOH. The flow rate was 600 μL/min, and the injection volume was 15 μL for both urine samples and quality controls (QCs). A linear gradient with the following proportions (v/v) of phase B (*t*, %*B*) was used: (0, 1), (4, 20), (6, 95), (7.5, 95), (8, 1), (12, 1). The LC system was coupled with a hybrid quadrupole time-of-flight (TOF) QSTAR Elite (Applied Biosystems/MDS Sciex). The MS acquisition was performed in negative ionization and full-scan (70−700 Da) mode. Spray parameters were as follows: IonSpray voltage, −3000 V; declustering potential, −80 V; focusing potential, −380 V, declustering potential 2, −10 V; ion release decay, 6 μs; ion release width, 5 μs; temperature, 400 °C; $N_2$ as curtain (50 arbitrary units) and nebulizer (60 arbitrary units) gases. The TOF was calibrated with taurocholic acid (1 pmol/μL) using the ions at $m/z$ 79.9568 and $m/z$ 514.2844. LC−MS data were acquired in two successive analysis batches. To avoid possible bias, the sequences of the injections were randomized. The MS/MS analyses were carried out using the same equipment and with the same parameters described above. The MS/MS experiments were performed with a collision energy (CE) of −30 V.

To evaluate the quality of data in the acquisition step, a procedure was used.[21] Three classes of QC samples were used for each batch:

QC1: Milli-Q water samples. These samples were injected four times in each batch.

QC2: Standard mixture solution (1 ppm) consisting of 4-hydroxyhippuric acid, (−)-epicatechin, procyanidin B2, kaempferol, and naringenin. These samples were injected four times in each batch.

QC3: Reinjection of urine samples. In order to evaluate the effect of the beginning vs final, final vs beginning and the middle part of the batch sequence, seven urine samples (four in the first batch and three in the second batch) were randomly selected from the whole list of samples and reinjected.

**Data Processing.** LC-MS data were analyzed using MarkerViewTM 1.2 software (Applied Biosystems, MDS Sciex, Toronto,

Ontario, Canada), which performs feature extraction by peak finding for each sample and alignment using mass and retention time windows for the peaks. Peak detection was performed using a minimum peak width of 1 ppm, a noise threshold of 5, and a subtraction multiple factor of 1.5. Alignment used 0.04 Da and 0.1 min tolerance windows.

**Multivariate Analysis.** Principal component analysis (PCA) and orthogonal projection to latent structures (OPLS)[22] were used (SIMCA-P+ 11.5; Umetrics, Umea, Sweden). PCA is an unsupervised analytical tool involving the calculation of linear combinations of the original descriptors, the PCs. Each PC is a linear combination of the original variables, whereby each successive PC explains the maximum amount of variance possible in the data set. This method was used for the evaluation of the analytical variability of QCs across the data acquisition. The OPLS is a modification of PLS that separates the systematic variation in $X$ into two parts, one that is linearly related to $Y$ and one that is orthogonal to $Y$. This separation facilitates the model interpretation as the variation between classes can be interpreted from the predictive component, and the variation within classes can be interpreted from the orthogonal components.[23] Here, the variable $X$ was the LC-MS data set, and $Y$ was the binary vector with the value 0 for the PL-d diet and 1 for the AP-d diet. To better visualize the contribution of ions in the separation of the classes, the S-plot was used. The S-plot combines the contribution/covariance ($p$) and reliability/correlation ($p$(corr)) from the OPLS model and helps to identify differential markers between classes.[19] A $p$(corr) of 0.5 was adopted as an arbitrary cutoff value to select the variables.

Data were log-transformed, mean-centered, and Pareto-scaled (each variable was weighted according to $1/\sqrt{\text{SD}}$) before PCA analysis, and log-transformed and mean-centered before OPLS (SIMCA-P+ 11.5; Umetrics, Umea, Sweden). The quality of the models was evaluated by the goodness-of-fit parameter ($R^2X$), the proportion of the variance of the response variable that is explained by the model ($R^2Y$) and the predictive ability parameter ($Q^2$), which was calculated by a seven-round internal cross-validation of the data using a default option of the SIMCA-P+ 11.5 software. Validation of the models was determined by randomly permuting ($n = 100$) the order of $Y$ and fitting separate models to the permuted $Y$'s, extracting the same number of components as the original model following the procedure proposed by Kang et al[24] and Pasikanti et al.[25] The correlation coefficient between the original $Y$ and the permuted $Y$ is plotted against the cumulative $R^2$ and $Q^2$ and a regression line is calculated. Usually, $Q^2$-intercept limits for a valid model should be less than 0.05.[24] Despite this validation, the predictive ability of the OPLS-DA models was also evaluated. In this context, six samples of each model (three for each diet, prediction set) were randomly removed from the whole data set (training set). This procedure was repeated three times for each OPLS model. These training sets were used to evaluate the ability of OPLS models to classify blindly the prediction sets.[24,26]

**Metabolite Identification.** Markers contributing to the discrimination ($p$(corr) $\geq 0.5$) were identified on the basis of their exact mass, which was compared to those registered in the Human Metabolome Database (HMDB; www.hmdb.ca) and the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/) ($\Delta$mDa $\leq$ 5 mDa) using an in-house R script for R environment.[27] The database querying was improved by adding an "in-house" DB focused on those expected (theoretical or bibliographic) metabolites related to polyphenol metabolites.[20,21,28−30] Identification of cluster ions originating from the same metabolites was carried out as described previously.[21] The LC−MS/MS behavior was also compared with that proposed by the Massbank database (http://www.massbank.jp) and HMDB. Postulate identifications were achieved after a combination of LC−MS data, database information, and MS/MS experiments (Supplementary Table 2, Supporting Information).

## Results and Discussion

**Quality Parameters of Data Acquisition.** Possible artifacts could occur during the acquisition step, including possible trends caused by the order of acquisition, carry-over, sensitivity changes, or ion suppression.[31,32] A way to analyze possible trends caused by these artifacts is to use unsupervised multivariate analysis such as PCA. The hypothesis is based on the ability of PCA analysis to cluster samples in an unsupervised way, bearing in mind that if some issue arose, the artifactual trends would place the QC1, QC2, and reinjections (QC3) in a different position in the scores plot. The PC1/PC2 score plots of Supplementary Figure 1 A,B (Supporting Information) did not reveal any apparent cluster according to the batch injections or any artifactual trends in urine samples and QC3. These results suggest an acceptable overall performance. QC2 samples, corresponding to the mixture of standards, were injected in order to evaluate retention time and mass precision shifts. The retention time shift ranged from 0.01 to 0.08 min, and the mass accuracy deviation ranged from 0.5 mDa to 2.4 mDa. These data are in accordance with data reported elsewhere.[33,34] The coefficient of variation of peak area, which ranged from 2.5% to 7.5% with a mean value of 5.8%, is in line with that recently reported for metabolomic studies.[33,34] According to the obtained data, the quality of data was accepted as well as the data following further steps of the metabolomic workflow.

**Multivariate Analysis of Urinary Fingerprint Trajectory.** As previously described, the circadian rhythm causes important effects on the 24 h kinetic evolution of the urine metabolome.[21,35−38] To avoid possible influences of these effects on the urinary metabolome trajectory after almond polyphenol intake, four independent (one for each time) OPLS models comparing the PL-d vs AP-d were carried out. All of the OPLS models obtained high-quality parameters (Table 1), with an overall value of $R^2X$, $R^2Y$, and $Q^2$ of 0.28, 0.94, and 0.78, respectively. These results showed that a differentiation between both diets was obtained at all of the assayed times.

Model validation was assayed by a permutation test ($n = 100$). The $Q^2$-intercept values for all of the models were lower than 0.05 (Supplementary Figure 2, Supporting Information), indicating that the achieved separations were not related to the overfitting. Another way to validate the OPLS models is to remove some data and rebuild the model with the remaining samples.[24] For this purpose, six samples were randomly removed from each model. This procedure was repeated three times for each OPLS model using different samples each time. The obtained OPLS models yielded similar statistical parameters to those obtained by cross-validation using the entire data set (Supplementary Table 1, Supporting Information). In addition, these models were used to predict the training samples.

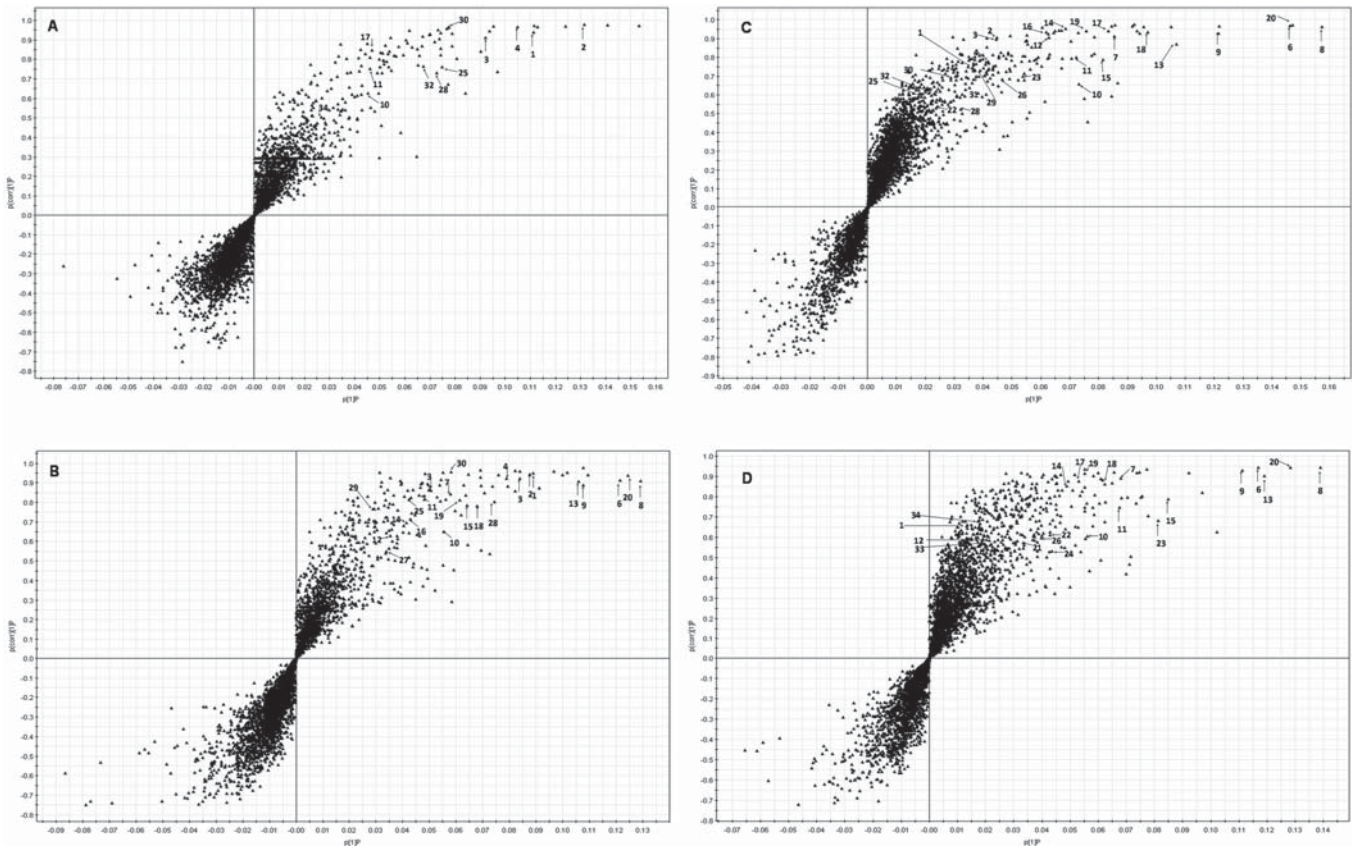


**Figure 1.** S-plots associated with the OPLS score plots obtained for data derived from LC−MS of urine obtained at 2 h (A), 6 h (B), 10 (C), and 24 h (D) after AP-d and PL-d intake. The numbers for the metabolites used (quasi-molar ions) are as given in Table 2.

**Table 1.** Summary of Parameters for Assessing OPLS Modeling Quality

| MODELS | no.[a] | $R^2X_{cum}$[b] | $R^2Y_{cum}$[b] | $Q^2_{cum}$[b] |
|---|---|---|---|---|
| 2 h | 1P[c] + 1O[d] | 0.34 | 0.95 | 0.70 |
| 6 h | 1P + 1O | 0.31 | 0.94 | 0.83 |
| 10 h | 1P + 1O | 0.33 | 0.96 | 0.83 |
| 24 h | 1P + 0O | 0.12 | 0.92 | 0.75 |

[a] No., number of components. [b] $R^2X_{cum}$ and $R^2Y_{cum}$ are the cumulative modeled variations in the $X$ and $Y$ matrix, respectively, and $Q^2Y_{cum}$ is the cumulative predicted variation in the $Y$ matrix. [c] Predictive component. [d] Orthogonal component.

The results showed that all of the samples were correctly classified (Supplementary Figure 3, Supporting Information).

**Detection and Identification of Biomarkers Related to Almond Polyphenol Intake.** The S-plots[19] associated with the scores plots were generated to reveal the markers responsible for the AP-d diet (Figure 1). The markers with higher $p$ and $p$(corr) values were the more relevant ions. The upper-right quadrant of each S-plot in Figure 1 displays the most important ions in the urine samples related to AP-d intake. The ions in the middle of the figure did not show any relevance in the model.

A list of markers detected in the four S-plots ($p$(corr) ≥ 0.5) was submitted for metabolite identification procedure. The results are summarized in Table 2, which provides compound information including retention time, detected, calculated and theoretical mass, statistical parameters, and putative identifications. The Supplementary Table 2, Supporting Information, provides the MS/MS data.

A total of 34 metabolites (Table 2, Figure 1) were putatively identified in the different urine fractions. Eleven metabolites were present as cluster ions composed of a group of ions (i.e., molecular or parent ions and daughter ions arising from the possible in-source fragmentation under ESI). Metabolites were classified into different groups of compounds, namely, flavonoid conjugates, hydroxyphenylvalerolactone conjugates, 4-hydroxy-5-(phenyl)-valeric acid conjugates, hydroxyphenylpropionic acid conjugates, hydroxyphenylacetic acid conjugates, and other phenolic acid conjugates.

Among flavonoid conjugates, (epi)catechin sulfate, two *O*-methyl-(epi)catechin sulfates, and narigenin-*O*-glucuronide (metabolites 1−4) (Table 2) were identified as metabolites significantly contributing to changes in the urine metabolome after the intake of almond skin extract (Figure 1A,B). These results are in accordance with our previous studies which reported significant increases in the urine concentration of these compounds after the intake of almond skin extract.[20] Phenylvalerolactones constituted a major group of metabolites (Table 2, metabolites 5−16) and were presented as mono-, di-, and trisubstituted phenyl structures in the form of glucuronic acid and sulfate conjugates, or as a combination of methoxy-glucuronide and methoxy-sulfate derivatives (Table 2). These compounds could undergo in-source fragmentation resulting in signals corresponding to the loss of the glucuronide (−176 Da) or sulfate (−80 Da) moieties, which also showed statistical relevance in the case of metabolites 6, 8, and 10, and metabolites 13, 14, and 15, respectively. Besides the fragments corresponding to the loss of different conjugate groups (i.e., $m/z$ 207 for dihydroxyphenylvalerolactones or $m/z$ 221 for methoxyhydroxyphenylvalerolactones), the MS/MS spectra of phenylvalerolactone conjugates were characterized by the product ion at $m/z$ 163 resulting from the decarboxylation (−44 Da) of

**Table 2.** Identification of Metabolites after a Single Intake of the Almond Skin Extract by Human Volunteers

| metabolite | retention time (min) | detected mass | *p*(contribution) 2 h | 6 h | 10 h | 24 h | *p*(corr)[b] (confidence) 2 h | 6 h | 10 h | 24 h | metabolite putative identification | assignation | theoretical mass [M − H]⁻ | mass difference (mDa)[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Flavonoid conjugates | | | | | | | |
| 1 | 5.53 | 369.0245 | 0.11 | 0.09 | 0.04 | 0.01 | 0.94 | 0.95 | 0.74 | 0.66 | (epi)catechin sulfate | $[M-H]^-$ | 369.0286 | 4.06 |
| 2 | 6.10 | 383.0414 | 0.13 | 0.09 | 0.04 | 0.02 | 0.98 | 0.94 | 0.91 | 0.79 | *O*-methyl (epi)catechin sulfate | $[M-H]^-$ | 383.0442 | 2.81 |
| 3 | 6.25 | 383.0415 | 0.09 | 0.08 | 0.04 | −[b] | 0.91 | 0.92 | 0.90 | − | *O*-methyl (epi)catechin sulfate | $[M-H]^-$ | 383.0442 | 2.71 |
| 4 | 6.25 | 447.0900 | 0.10 | 0.08 | 0.04 | − | 0.97 | 0.92 | 0.78 | − | naringenin -*O*-glucuronide | $[M-H]^-$ | 447.0933 | 3.27 |
| | | | | | | | Hydroxyphenyl−valerolactone conjugates[c] | | | | | | | |
| 5 | 4.53 | 399.0959 | − | − | − | 0.02 | − | − | − | 0.83 | 5-(trihydroxyphenyl)-$\gamma$-valerolactone glucuronide | $[M-H]^-$ | 399.0933 | −2.63 |
| 6 | 4.62 | 383.0964 | − | 0.12 | 0.15 | 0.12 | − | 0.91 | 0.97 | 0.94 | 5-(dihydroxyphenyl)-$\gamma$-valerolactone glucuronide | $[M-H]^-$ | 383.0984 | 1.95 |
| | | *207.0644* | − | 0.04 | 0.07 | 0.04 | − | 0.75 | 0.94 | 0.84 | | *[M − H-glucuronide]⁻* | *207.0663* | *1.87* |
| 7 | 4.70 | 367.1004 | − | 0.06 | 0.09 | 0.07 | − | 0.84 | 0.91 | 0.89 | 5-(hydroxyphenyl)-$\gamma$-valerolactone glucuronide | $[M-H]^-$ | 367.1034 | 3.04 |
| 8 | 4.82 | 383.0986 | − | 0.13 | 0.16 | 0.14 | − | 0.91 | 0.96 | 0.94 | 5-(dihydroxyphenyl)-$\gamma$-valerolactone glucuronide | $[M-H]^-$ | 383.0984 | −0.25 |
| | | *207.0647* | − | 0.07 | 0.09 | 0.07 | − | 0.88 | 0.97 | 0.92 | | *[M − H-glucuronide]⁻* | *207.0663* | *1.57* |
| 9 | 4.98 | 397.1138 | − | 0.11 | 0.12 | 0.11 | − | 0.89 | 0.93 | 0.93 | 5-(hydroxy-methoxy-phenyl)-$\gamma$-valerolactone glucuronide | $[M-H]^-$ | 397.1140 | 0.2 |
| 10 | 5.07 | 463.0547 | 0.05 | 0.06 | 0.07 | 0.06 | 0.63 | 0.65 | 0.66 | 0.60 | 5-(dihydroxyphenyl)-$\gamma$-valerolactone sulfate glucuronide | $[M-H]^-$ | 463.0552 | 0.47 |
| | | *287.0215* | − | 0.02 | 0.04 | 0.02 | − | 0.51 | 0.67 | 0.54 | | *[M − H-glucuronide]⁻* | *287.0231* | *1.58* |
| 11 | 5.12 | 367.1030 | 0.05 | 0.05 | 0.07 | 0.07 | 0.75 | 0.83 | 0.80 | 0.75 | 5-(hydroxyphenyl)-$\gamma$-valerolactone glucuronide | $[M-H]^-$ | 367.1034 | 0.44 |
| 12 | 5.38 | 397.1138 | − | 0.04 | 0.06 | 0.02 | − | 0.63 | 0.91 | 0.59 | 5-(hydroxy-methoxy-phenyl)-$\gamma$-valerolactone glucuronide | $[M-H]^-$ | 397.1140 | 0.2 |
| 13 | 6.10 | 287.0264 | − | 0.11 | 0.11 | 0.12 | − | 0.91 | 0.87 | 0.90 | 5-(dihydroxyphenyl)-$\gamma$-valerolactone sulfate | $[M-H]^-$ | 287.0231 | −3.32 |
| | | *207.0705* | − | 0.07 | 0.08 | − | − | 0.56 | 0.60 | − | | *[M − H-sulfate]⁻* | *207.0663* | *−4.23* |
| 14 | 6.22 | 301.0368 | − | 0.04 | 0.07 | 0.05 | − | 0.70 | 0.95 | 0.85 | 5-(hydroxy-methoxy-phenyl)-$\gamma$-valerolactone sulfate | $[M-H]^-$ | 301.0387 | 1.93 |
| | | *221.0802* | − | 0.03 | 0.04 | 0.02 | − | 0.59 | 0.80 | 0.69 | | *[M − H-sulfate]⁻* | *221.0819* | *1.72* |
| 15 | 6.38 | 271.0283 | − | 0.06 | 0.08 | 0.08 | − | 0.78 | 0.79 | 0.79 | 5-(hydroxyphenyl)-$\gamma$-valerolactone sulfate | $[M-H]^-$ | 271.0282 | −0.13 |
| | | *191.0691* | − | − | 0.03 | 0.03 | − | − | 0.59 | 0.55 | | *[M − H-sulfate]⁻* | *191.0714* | *2.26* |
| 16 | 6.43 | 301.0359 | − | 0.04 | 0.06 | 0.04 | − | 0.72 | 0.92 | 0.81 | 5-(hydroxy-methoxy-phenyl)-$\gamma$-valerolactone sulfate | | | |
| | | | | | | | 4-Hydroxy-5-(phenyl)-valeric acid conjugates[d] | | | | | | | |
| 17 | 4.02 | 401.1049 | 0.05 | 0.07 | 0.08 | 0.05 | 0.85 | 0.88 | 0.94 | 0.88 | 4-hydroxy-5-(dihydroxyphenyl)-valeric acid glucuronide[d] | $[M-H]^-$ | 401.1089 | 4.01 |
| 18 | 4.18 | 401.1074 | − | 0.07 | 0.10 | 0.06 | − | 0.78 | 0.94 | 0.86 | 4-hydroxy-5-(dihydroxyphenyl)-valeric acid glucuronide | $[M-H]^-$ | 401.1089 | 1.51 |
| 19 | 4.30 | 415.1233 | − | 0.06 | 0.08 | 0.06 | − | 0.81 | 0.94 | 0.91 | 4-hydroxy-5-(hydroxy-methoxy-phenyl)-valeric acid glucuronide | $[M-H]^-$ | 415.1246 | 1.26 |
| 20 | 5.12 | 305.0351 | 0.03 | 0.12 | 0.15 | 0.13 | − | 0.94 | 0.97 | 0.94 | 4-hydroxy-5-(dihydroxyphenyl)-valeric acid sulfate | $[M-H]^-$ | 305.0337 | −1.45 |
| | | *225.0765* | − | 0.06 | 0.07 | 0.05 | − | 0.76 | 0.89 | 0.67 | | *[M − H-sulfate]⁻* | *225.0768* | *0.33* |
| | | *207.0667* | − | − | 0.03 | − | − | − | 0.79 | − | | *[M − H-sulfate-$H_2O$]⁻* | *207.0663* | *−0.43* |
| | | *163.0771* | − | − | 0.02 | 0.02 | − | − | 0.63 | 0.62 | | *[M − H-sulfate-$H_2O-CO_2$]⁻* | *163.0758* | *−1.26* |
| | | *101.0249* | − | 0.01 | 0.03 | 0.01 | − | 0.51 | 0.91 | 0.59 | | *[M−H-$C_7H_7O_2$]⁻* | *101.0238* | −1.07 |
| 21 | 5.92 | 385.1149 | − | − | − | 0.03 | − | − | − | 0.57 | 4-hydroxy-5-(hydroxyphenyl)-valeric acid glucuronide | $[M-H]^-$ | 385.1140 | −0.9 |
| 22 | 6.00 | 399.1273 | − | − | 0.02 | 0.04 | − | − | 0.54 | 0.61 | 4-hydroxy-5-(methoxyphenyl)-valeric acid glucuronide | $[M-H]^-$ | 399.1297 | 2.35 |
| 23 | 6.93 | 289.0404 | − | − | 0.05 | 0.08 | − | − | 0.70 | 0.68 | 4-hydroxy-5-(hydroxyphenyl)-valeric acid sulfate | $[M-H]^-$ | 289.0387 | −1.67 |
| | | *209.0810* | − | − | 0.02 | 0.04 | − | − | 0.57 | 0.58 | | *[M − H-sulfate]⁻* | *209.0819* | *0.92* |
| 24 | 7.00 | 273.0426 | − | − | − | 0.04 | − | − | − | 0.53 | 4-hydroxy-5-(phenyl)-valeric acid sulfate | $[M-H]^-$ | 273.0438 | 1.22 |
| | | | | | | | Hydroxyhenylacetic acid conjugates | | | | | | | |
| 25 | 3.63 | 343.0678 | 0.07 | 0.04 | 0.02 | − | 0.76 | 0.82 | 0.59 | − | 2-(dihydroxyphenyl)-acetic acid glucuronide | $[M-H]^-$ | 343.0671 | −0.75 |
| 26 | 4.27 | 246.9916 | − | − | 0.05 | 0.04 | − | − | 0.68 | 0.60 | 2-(dihydroxyphenyl)-acetic acid sulfate | $[M-H]^-$ | 246.9918 | 0.19 |
| | | *203.0009* | − | − | 0.04 | 0.03 | − | − | 0.74 | 0.74 | | *[M − H−$CO_2$]⁻* | *203.0014* | *0.46* |
| | | *123.0454* | − | − | 0.04 | 0.04 | − | − | 0.74 | 0.62 | | *[M − H−$CO_2$- sulfate]⁻* | *123.0446* | *−0.84* |
| 27 | 4.53 | 357.0833 | − | 0.03 | − | − | − | 0.55 | − | − | 2-(hydroxy-methoxy-phenyl)-acetic acid glucuronide | $[M-H]^-$ | 357.0827 | −0.6 |
| 28 | 5.03 | 423.0194 | 0.07 | 0.07 | 0.03 | − | 0.73 | 0.80 | 0.52 | − | 2-(dihydroxyphenyl)-acetic acid sulfate glucuronide | $[M-H]^-$ | 423.0239 | 4.47 |
| 29 | 5.28 | 230.9957 | − | 0.03 | 0.04 | − | − | 0.77 | 0.73 | − | 2-(hydroxyphenyl)-acetic acid sulfate | $[M-H]^-$ | 230.9969 | 1.17 |
| | | *151.0397* | − | 0.04 | − | − | − | 0.57 | − | − | | *[M − H-sulfate]⁻* | *151.0401* | *0.36* |

**Table 2.** Continued

| metabolite | retention time (min) | detected mass | *p*(contribution) 2 h | 6 h | 10 h | 24 h | *p*(corr)[b] (confidence) 2 h | 6 h | 10 h | 24 h | metabolite putative identification | assignation | theoretical mass $[M + H]^-$ | mass difference (mDa)[a] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | Hydroxyphenylpropionic acid conjugates | | | |
| 30 | 4.38 | 341.0880 | 0.08 | 0.06 | 0.03 | – | 0.96 | 0.96 | 0.69 | – | 3-(hydroxyphenyl)-propionic acid glucuronide | $[M - H]^-$ | 341.0878 | −0.21 |
| 31 | 5.18 | 261.0094 | – | – | 0.04 | 0.06 | – | – | 0.70 | 0.75 | 3-(dihydroxyphenyl)-propionic acid sulfate | $[M - H]^-$ | 261.0074 | −1.96 |
| | | *181.0493* | – | – | 0.04 | 0.04 | – | – | 0.64 | 0.79 | | *[M − H-sulfate]*$^-$ | *181.0506* | *1.32* |
| | | *137.0603* | – | – | 0.02 | 0.03 | – | – | 0.54 | 0.58 | | *[M − H-sulfate-CO$_2$]*$^-$ | *137.0608* | *0.49* |
| | | | | | | | | | | | Other phenolic acid conjugates | | | |
| 32 | 3.25 | 343.0685 | 0.07 | 0.05 | 0.02 | – | 0.77 | 0.86 | 0.61 | – | vanillic acid glucuronide | $[M - H]^-$ | 343.0671 | −1.45 |
| | | *167.0354* | *0.02* | *0.02* | *0.02* | – | *0.59* | *0.83* | *0.56* | – | | *[M − H-glucuronide]*$^-$ | *167.0350* | *−0.43* |
| | | *152.0116* | *0.02* | *0.02* | *0.01* | – | *0.68* | *0.81* | *0.58* | – | | *[M − H- CH$_3$]*$^-$ | *152.0109* | *−0.68* |
| 33 | 3.87 | 194.0445 | – | – | – | 0.02 | – | – | – | 0.57 | hydroxyhippuric acid | $[M - H]^-$ | 194.0459 | 1.37 |
| 34 | 4.28 | 369.0806 | 0.03 | – | – | 0.02 | 0.50 | – | – | 0.68 | ferulic acid glucuronide | $[M - H]^-$ | 369.0827 | 2.10 |

[a] Obtained as (theoretical mass-detected mass). [b] Denotes the ***p*(corr)** values lower than cutoff 0.5. [c] Structural identification agrees with Urpi-Sarda et al.;[20] Llorach et al.;[21] Fardet et al.;[29] Sang et al.[30] [d] Structural identification agrees with Khori et al., 2003 (MS/MS information Supplemental Table 2, Supporting Information).

the $\gamma$-valerolactone ring, as previously described[9,30,39] (Supplementary Table 2, Supporting Information). An alternative fragmentation of the aglycone could result in the fragment ion at $m/z$ 85 resulting from the loss of $C_4H_5O_2$.

Of particular relevance was the detection of eight metabolites corresponding to the group of 4-hydroxy-5-(phenyl)-valeric acid conjugates (Table 2, metabolites 17−24). These metabolites were presented as non-, mono-, and disubstituted phenyl-4-hydroxyvaleric acids in the form of glucuronide, sulfate, and methoxy-glucuronide conjugates. For metabolite 20, tentatively identified as 4-hydroxy-5-(dihydroxyphenyl)-valeric acid sulfate ($[M - H]^- = 305.0351$), the in-source fragmentation resulted in the formation of four product ions of statistical relevance. These product ions corresponded to the loss of the sulfate moiety ($[M - H - \text{sulfate}]^-$) resulting in the aglycone, followed by subsequent elimination of a water molecule ($[M - H - \text{sulfate} - H_2O]^-$) and decarboxylation ($[M - H - \text{sulfate} - H_2O - CO_2]^-$). The fourth in-source daughter ion corresponded to an alternative fragmentation of the aglycone ($[M - H - C_7H_7O_2]^-$). These ions were also the main product ions obtained in the MS/MS spectra of both 4-hydroxy-5-(dihydroxyphenyl)- and 4-hydroxy-5-(hydroxyphenyl)-valeric acid conjugates (Supplementary Table 2, Supporting Information). This MS/MS behavior is in line with that previously described for the MS/MS fragmentation of 4-hydroxy-hydroxyphenyl valeric acids.[40]

Metabolites belonging to the hydroxyphenylacetic acid group (Table 2, metabolites 25−29) were found in a larger number than those of the hydroxyphenylpropionic acid group (Table 2, metabolites 30 and 31). These metabolites were mainly present as glucuronide and sulfate conjugates of both di- and monosubstituted derivatives. In addition, a combined glucuronide-methoxy conjugate (metabolite 27) was also detected in the case of the phenylacetic acid group. Signals derived from in-source fragmentation corresponding to the product ion derived from the loss of the sulfate group ($[M - H - \text{sulfate}]^-$), followed or not by previous decarboxylation ($[M - H - CO_2]^-$, $[M - H - CO_2 - \text{sulfate}]^-$ or $[M - H - \text{sulfate} - CO_2]^-$), were also of statistical relevance for metabolites 26, 29, and 31. Hydroxyphenylpropionic acid conjugates (sulfates) have been recently identified in the urine of rats fed grape antioxidant dietary fiber, which contains a large proportion of polyphenolic compounds, mainly oligomeric and polymeric procyanidins.[41] As far as we know, this is the first time that conjugates of hydroxyphenylacetic acids have been identified in humans after consumption of flavan-3-ols.

Conjugates of other phenolic acids, including vanillic and ferulic acids (metabolites 32 and 34, respectively), were also identified as metabolites contributing to the discrimination between the intake and control groups (Figure 1). In the case of vanillic acid, a signal derived from in-source fragmentation corresponding to the loss of the glucuronic acid ($[M - H - \text{glucuronide}]^-$) and methyl ($[M - H - CH_3]^-$) moieties were of statistical relevance. Hydroxyhippuric acid, a conjugate of hydroxybenzoic acid with glycine, was also tentatively identified (metabolite 33). Ferulic and hydroxyhippuric acids have already been related to the consumption of procyanidins from both cocoa and almond.[9,20]

**Metabolic Interpretation of Biomarkers of Almond Polyphenol Intake.** Changes observed in the urine metabolome during the first 0−6 h after the intake of almond polyphenols were largely attributed to polyphenol host metabolites, mainly flavonoids (naringenin and (epi)catechin) and vanillic acid

conjugates (Table 2, Figure 1). Although vanillic acid could also arise from the microbial metabolism of flavonoids, its early urinary excretion is most likely related to absorption in the small intestine of the phenolic acid itself, originally present in the almond skin extract, as has been described in the case of vanillin-containing cocoa products.[9,21] The contribution of naringenin-*O*-glucuronide and vanillic acid glucuronide to the urine metabolome was observed up to 10 h after the intake, whereas for (epi)catechin conjugates it was extended up to 24 h (Table 2, Figure 1). These findings could suggest that whereas the first (epi)catechin urinary excretion (0−6 h) pattern could be related to the absorption of monomers, tissue metabolism and further excretion, the second part (6−24 h) is probably related to the microbial degradation of polymers and further absorption of monomers.

The excretion profile of phenyl-$\gamma$-valerolactone conjugates (with the exception of metabolites 10 and 11) indicated that these compounds contributed significantly to changes in the urine metabolome from 2−24 h after the intake of the almond skin extract (Table 2, Figure 1). In particular, hydroxyphenylvalerolactones are considered as main metabolites exclusively arising from the first stages of the colonic microbial metabolism of flavan-3-ols, after the opening of the C-ring into diphenylpron-2-ols.[42] The excretion pattern found in this study seems to be in accordance with its colonic microbial origin and is also in line with previous studies performed with other flavan-3-ol-rich products such as tea or cocoa.[21,43,44] The highest correlation *p*(corr) (Table 2, Figure 1) for these metabolites was observed from 6−10 h for most conjugates of both mono- and disubstituted phenyl-$\gamma$-valerolactones, whereas trihydroxyphenyl-$\gamma$-valerolactone glucuronide mainly contributed to changes in the urine metabolome at 10−24 h after intake. This finding could be attributed to the fact that (epi)gallocatechin units in almond prodelphinidins may be forming part of the inner extension rather than terminal units, in such a way that they could be only accessible to the gut microbiota after extended degradation of terminal units, as has been proposed for dimeric procyanidins.[45]

4-Hydroxy-5-(phenyl)-valeric acid conjugates presented an excretion pattern similar to phenyl-$\gamma$-valerolactones, significantly contributing to changes in the urine metabolome from 2 to 24 h after the intake of the almond skin extract (Table 2, Figure 1). However, differences were observed in the excretion profile according to the hydroxylation pattern of the phenyl ring from non- to mono- and dihydroxylated forms (including methoxylated metabolites). For dihydroxylated derivatives (metabolites 17−20), the highest *p*(corr) was found 6−10 h after the intake; for monohydroxylated forms (metabolites 21−23) a contribution to changes in the urine metabolome was observed at 6−24 h, and finally, for nonhydroxylated (metabolite 24) it was found at 10−24 h after the intake. These findings suggest that dehydroxylation reactions gradually occur as colonic metabolism progresses. The occurrence of 4-hydroxy-5-(hydroxyphenyl)-valeric acid was first described after oral administration of (−)-epicatechin-3-*O*-gallate to rats.[30] Recently, this metabolite has also been identified after cocoa intake using a LC-q-TOF metabolomic approach.[21] However, to our knowledge this study constitutes the first report of non- and monohydroxyphenyl-4-hydroxyvaleric acids after human intake of a polyphenol-rich source. It has been proposed that hydroxyphenyl-4-hydroxyvaleric acids could arise from the degradation of diphenylpropan-2-ols, together with hydroxyphenyl-$\gamma$-valerolactones,[30] but this pathway still needs to be confirmed. Although it is not[30] possible to establish the correct sequence of formation from the results found herein, a concurrent formation of 4-hydroxy-5-(phenyl)-valeric acids with phenyl-$\gamma$-valerolactones or posterior formation from these metabolites could be possible.[30]

In the case of phenylacetic and phenylpropionic acids, the excretion pattern was more difficult to establish in terms of the hydroxylation profile of the phenyl ring (Table 2). Apparently, the urinary excretion of glucuronide derivates of the mono- and dihydroxylated forms of both phenylacetic and phenylpropionic acids mainly contributed (highest *p*(corr)) to the discrimination between the intake and control groups in the first hours of urine collection (0−6 h), whereas sulfate derivatives significantly contributed to later fractions (10 and 24 h). Sulfate conjugation has been reported to occur in the colon through microbial arilsulphotransferase activity,[46] although its contribution does not seem to be relevant. The discrete number of hydroxyphenylacetic and hydroxyphenylpropionic acid derivatives in comparison to hydroxyphenyl-$\gamma$-valerolactones and 4-hydroxy-5-(phenyl)-valeric acids suggests a slow formation of the latter followed by a rapid turnover into the former, as has been proposed by Appledoorn et al.[45]

## Conclusion

On the basis of the proposed identification, changes observed in the urine metabolome as a consequence of the intake of almond skin extract were attributed partly to the excretion of polyphenol host metabolites, but mainly to a large group of microbial-derived polyphenolic metabolites. It is difficult to consider these metabolites as biomarkers of almond polyphenol intake since most of them have also been identified after consumption of cocoa, tea, or other sources of flavan-3-ols. However, this fact makes these metabolites robust biomarkers of the consumption of flavan-3-ol-rich foods. In addition to this interpretation, it should be noted that as far as we know, this is the first time that conjugates of hydroxyphenyl-valeric, hydroxyphenyl-propionic, and hydroxyphenyl-acetic acids have been identified in human samples after consumption of flavan-3-ols, and obviously they could be considered as potential markers of almond polyphenol intake. It is important to notice the large number of metabolites of both phenyl-$\gamma$-valerolactones and 4-hydroxy-5-(phenyl)-valeric acids identified in the present study after the intake of almond skin extract, in comparison to other flavan-3-ol sources such as cocoa, for which metabolomic studies have revealed the contribution of only a limited number of derivatives from these metabolites.[21] In this context, these results confirm the importance of gut microbiota on the metabolism of polyphenols and, in particular, on the metabolism of flavan-3-ols. The results obtained highlight the importance of using a non-targeted metabolomic approach to obtain new biomarkers. These biomarkers will be very useful to develop the concept of the food metabolome, and also in further clinical and epidemiological studies, where it is often difficult to estimate the real intake of polyphenols.

**Acknowledgment.** This research was supported by Spanish national grants: AGL2009-13906-C02-01/ALI, as well as by the CONSOLIDER INGENIO Program, project FUNCFOOD (CSD2007-063). R.L1. and M.U.-S. would like to thank the postdoctoral programs (FIS CD06/00161 and Sara Borrell CD09/00134, respectively), I.G., the I3P Program and M.M., the Ramon y Cajal program, all from the Ministry of Science and Innovation.

**Supporting Information Available:** PCA scores plots; validation plots; table of parameters for assessing OPLS modeling quality; table of MS/MS experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR100639V