JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Microbial Metabolomic Fingerprinting in Urine after Regular Dealcoholized Red Wine Consumption in Humans

María Boto-Ordóñez,[†,‡] Mireia Urpi-Sarda,*[,†,¶] María Isabel Queipo-Ortuño,[§,∥] Dolores Corella,[⊥,∥] Francisco J. Tinahones,[§,∥] Ramon Estruch,[#,∥] and Cristina Andres-Lacueva[†,¶]

[†]Biomarkers and Nutritional & Food Metabolomics Research Group, Nutrition and Food Science Department, XaRTA, INSA, Pharmacy Faculty, University of Barcelona, Avenida Joan XXIII s/n, 08028 Barcelona, Spain

[‡]Fundació Clínic per a la Recerca Biomèdica, C/Rosselló 149-153, 08036 Barcelona, Spain

[§]Servicio Endocrinología y Nutrición del Hospital Universitario Virgen de la Victoria, Campus Universitario de Teatinos, 29010 Málaga, Spain

[∥]CIBER Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III, Madrid, Spain

[⊥]Department of Preventive Medicine, University of Valencia, Avenida Blasco Ibañez 15, 46010 Valencia, Spain

[#]Department of Internal Medicine, Hospital Clinic, Institut d'Investigació Biomèdica August Pi i Sunyer (IDIBAPS), University of Barcelona, Villarroel 170, 08036 Barcelona, Spain

[¶]Ingenio-CONSOLIDER Program, FUN-C-FOOD, CSD2007-063, Barcelona, Spain

Supporting Information

ABSTRACT: The regular consumption of dealcoholized red wine (DRW) has demonstrated benefits in cardiovascular risk factors. The analysis of phenolic metabolites formed in the organism, especially those that could come from microbiota metabolism, would help to understand these benefits. The aim of this study was to determine the widest urinary metabolomic fingerprinting of phenolics and microbial-derived phenolic acids (n = 61) after regular intake of DRW in men at high cardiovascular risk by UPLC-MS/MS using a targeted approach. Up to 49 metabolites, including phase II and microbial phenolic metabolites, increased after DRW consumption compared to baseline ($P < 0.05$). The highest percentage of increase was found for microbial metabolites from anthocyanin degradation such as syringic, p-coumaric, gallic acids and pyrogallol and from flavan-3-ols degradation such as hydroxyphenylvalerolactones and (epi)catechins. These findings provide the most complete metabolic fingerprinting after wine consumption, amplifying the spectrum of microbial derived metabolites and their potential bioactivity related with health benefits.

KEYWORDS: *dealcoholized red wine, microbiota, human urine, UPLC-MS/MS, food metabolome, biomarkers, phenolic acids*

INTRODUCTION

Red wine consumption has been associated with the prevention of several diseases, mainly cardiovascular diseases.[1,2] These effects were not only explained by its alcoholic content[3] but also by its phenolic composition.[4,5] Moreover, there is an increasing interest in developing new products derived from red wine due to its reported beneficial effects. These newly developed products from red wine have a polyphenolic content similar to red wine but without alcohol (<1.2%, v/v), which could make them suitable to be considered a functional food after complying with regulations.[6] But before making nutritional claims, bioavailability studies are necessary in order to ensure that sufficient amounts of the compound are available at target tissues after consumption of a reasonable dose.

The regular consumption of dealcoholized red wine (DRW) used in this study has been associated with benefits for blood pressure[5] and inflammatory parameters[4] in patients at high cardiovascular risk. The health benefits of polyphenols have been classically related to those originally present in foods.[7,8] However, in the past few years, there has been an increased interest in metabolites formed in the organism, particularly those formed by the intestinal microbiota.[9,10] Moreover, biological activity of these compounds produced in the gastrointestinal tract has been proved in some cases to be more active than their parent compounds.[11] One of the critical points for phenolic transformation is the interaction between polyphenols and microbiota. This interaction has been shown in two senses. First, polyphenols that arrive at the intestine can exert a prebiotic effect, stimulating the growth or inhibition of certain bacteria.[12,13] Additionally, microbial enzymes may produce new molecules from those originally present in the food, as has been established in in vivo and in vitro studies.[14] These structures are phenolic acids formed by gut bacteria through reactions of hydrolysis, ring-cleavage, decarboxylation, demethylation, reduction, and dehydroxylation.[15] In some cases, these reactions have been linked to specific bacteria such as *Enterococcus casseliflavus*, *Butyrivibrio sp C3*, *Clostridium orbiscindensor*, and *Eubacterium ramulus* associated with deglycosylation and ring fission.[16] DRW composition com-

Received: May 31, 2013
Revised: August 21, 2013
Accepted: August 28, 2013
Published: August 28, 2013

prises a wide range of compounds, from simple compounds such as phenolic acids or simple flavonoids to more complex ones such as proanthocyanidins.[17] Simple components may be absorbed in the upper part of the gastrointestinal tract and pass to the bloodstream, being exposed to metabolism in the intestine, liver, and tissues. The nonabsorbed polyphenols, such as proanthocyanidins, flavan-3-ols, or anthocyanins, can be metabolized by gut microbiota, releasing an extensive number of metabolites prior to its absorption and phase II metabolism.[11] Therefore, a complete understanding of phenolic metabolism, taking into account chemical structure, bioavailability, food matrix, background diet, and individual factors, is essential for associating its effects with its consumption.[18] To our knowledge, there is only a small number of human studies in which phenolic metabolism is studied after wine intervention, and they are mainly focused on a single component and its derived metabolites such as catechin and resveratrol.[19−23] However, foods are complex systems where several phenolic classes are present, and the number of possible metabolites found in biofluids derived from all these combinations is high[24] and thus there are more metabolites that can exert their biological activity in vivo. The importance of broadening the phenolic study to metabolites formed in the organism, especially from microbiota, would help to understand the benefits derived from consumption, bearing in mind that in some cases metabolites have been proved to be more biologically active than their parent compounds.[11] In the present study, a long-term feeding trial was performed to determine changes in the urinary excretion of microbial phenolic metabolites after DRW consumption, taking into account all the phenolic classes present in wine composition and obtaining the widest phenolic metabolic profile after DRW intake in humans.

MATERIALS AND METHODS

Standards and Reagents. The following compounds (% purity when available) were used: 2,4-dihydroxybenzoic acid (≥97%), 2,6-dihydroxybenzoic acid (98%), 2,5-dihydroxybenzoic acid (98%), 3,5-dihydroxybenzoic acid (97%), 4-hydroxybenzoic acid (≥98%), 3-hydroxybenzoic acid (≥98%), gallic acid (≥98.5%), syringic acid (≥95%), phenylacetic acid (≥98%), 3-hydroxyphenylacetic acid (≥97%), 2-hydroxyphenylacetic acid (99%), 3,4-dihydroxyphenylacetic acid (98%), 3-(4-hydroxyphenyl)propionic acid (≥98%), 3-(3,4-dihydroxyphenyl)propionic acid or dihydrocaffeic acid (98%), *p*-coumaric acid (≥98%), *o*-coumaric acid (97%), caffeic acid (≥95%), ferulic acid (≥98%), protocatechuic acid (>97%), sinapic acid (≥98%), enterolactone (95%), pyrogallol (≥98%), ethylgallate (≥96%), (−)-epicatechin (≥98%), (+)-catechin (≥98%), and *β*-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma−Aldrich (St. Louis, MO, USA). 4-Hydroxyhippuric acid (>99%) was purchased from PhytoLab GmbH & Co. KG (Vestenbergsgreuth, Germany). 3-(3-Hydroxyphenyl)propionic acid was purchased from Apin Chemicals Limited (Abingdon, UK). Vanillic acid, 4-*O*-methylgallic acid, *m*-coumaric acid, and taxifolin (>90%) were purchased from Extrasynthèse (Genay, France). Standard of epicatechin-5-*O*-glucuronide was chemically synthesized and characterized as previously published.[25] Liquid chromatography grade solvents methanol, acetonitrile, glacial acetic and formic acids were purchased from Scharlau Chemie, SA (Sentmenat, Spain). Hydrochloric acid was purchased from Panreac Química, SAU (Castellar del Vallès, Spain). Ultrapure water (Milli-Q) was obtained from Millipore (Bedford, MA, USA). Synthetic urine was prepared as previously described.[26]

Subjects and Study Design. In this study, the urine of 36 men (mean age of 61 ± 9) at baseline and after one month of DRW consumption was obtained from a previous clinical trial.[4] Baseline characteristics of the participants were included in Supporting Information Table 1. Subjects were first asked to follow a 2-week run-in period in which they were requested to exclude all grape-derived products and alcoholic beverages. After that, the subjects consumed 272 mL of DRW (0.42% alcohol) daily for 4 weeks during the meals. The Institutional Review Board of the hospital approved the study protocol, and all participants gave written consent before participating in the study. Urine samples (24 h) were collected at baseline and after the intervention period with DRW and immediately were stored at −80 °C until analysis. This trial has been registered in the Current Controlled Trials in London, International Standard Randomized Controlled Trial Number (ISRCTN88720134).

DRW was elaborated with the Merlot grape variety, from the Penedès appellation (Catalonia, Spain). The phenolic composition of DRW (Table 1) was analyzed throughout the study period (*n* = 5).[27−29] Total phenolic composition was measured by Folin−Ciocalteu.[30] Individual phenolic compounds were quantified as previously reported by Ibern-Gomez et al.[27] and Romero-Perez et al.[28] The five time points analyzed along the study period did not show significant differences in the phenolic composition (data not shown).

Table 1. Phenolic Composition (Mean ± SD) of the Dealcoholized Red Wine[a]

phenolic compound (mg/L)	DRW[b]
gallic acid[c]	73.17 ± 7.01
protocatechuic acid[c]	5.85 ± 0.51
tyrosol[c]	47.81 ± 3.90
catechin[c]	126.45 ± 13.35
epicatechin[c]	70.57 ± 8.22
procyanidins[d]	187.84 ± 15.10
trans-caftaric acid[c]	19.21 ± 1.62
trans-caffeic acid[c]	12.18 ± 0.92
trans-coutaric acid[c]	5.62 ± 0.52
2-*S*-glutathionylcaftaric[c]	10.76 ± 1.26
quercetin-3-glucuronide[c]	11.25 ± 1.42
quercetin[c]	23.82 ± 2.37
isorhamnetin[c]	2.96 ± 0.14
delphinidin-3-glucoside[c]	14.71 ± 1.62
petunidin-3-glucoside[c]	12.04 ± 1.15
peonidin-3-glucoside[c]	6.68 ± 0.57
malvidin-3-glucoside[c]	49.86 ± 4.27
malvidin-(6-acetyl)-3-glucoside[c]	10.41 ± 1.20
malvidin-(6-coumaroyl)-3-glucoside[c]	3.54 ± 0.33
trans-resveratrol[e]	2.73 ± 0.23
cis-resveratrol[e]	2.75 ± 0.15
trans-piceid[e]	10.53 ± 0.96
cis-piceid[e]	7.08 ± 0.87
total phenol (meq gallic acid/L)[f]	2694.92 ± 86.79

[a]Analyses were performed at five time points along the study in duplicate. [b]DRW, dealcoholized red wine. [c]Determined as previously described by Ibern-Gomez et al.[27] [d]Determined as previously described by Queipo-Ortuño et al.[12] [e]Analyzed following the work by Romero-Perez et al.[28] [f]Analyzed by Folin−Ciocalteu methodology.[30]

Extraction of Phenolic Acid Metabolites from Urine. Solid-phase extraction was performed using Oasis MCX 96-well plates (Waters, Milford, Massachusetts) as previously described.[26] Briefly, 1 mL of urine was subjected to enzymatic hydrolysis using *β*-glucuronidase/sulfatase from *Helix pomatia* at 37 °C for 45 min after being acidified with 50 μL of 0.58 mol/L acetic acid. Immediately afterward, samples were acidified to pH 2 with 6 mol/L HCl. The plate was conditioned with methanol and 2% formic acid in water. The hydrolyzed samples were then loaded onto the plate, washed with 2% formic acid in water, and analytes were then eluted with methanol.

dx.doi.org/10.1021/jf402394c | J. Agric. Food Chem. 2013, 61, 9166−9175

Table 2. Multiple Reaction Monitoring (MRM) Transitions, Declustering Potential (DP), and Collision Energy (CE) for Each Microbial and Conjugate Metabolite Identified in This Study

analyte	MRM transitions	identified by	DP	CE	quantified as
hydroxybenzoic acids					
2,4-dihydroxybenzoic acid	153/109	STD[a]	−50	−20	STD
2,6-dihydroxybenzoic acid	153/109	STD	−50	−20	STD
2,5-dihydroxybenzoic acid	153/109	STD	−50	−20	STD
3,5-dihydroxybenzoic acid	153/109	STD	−50	−20	STD
protocatechuic acid	153/109	STD	−50	−20	STD
vanillic acid	167/152	STD	−50	−20	STD
syringic acid	197/121	STD	−50	−25	STD
4-hydroxybenzoic acid	137/93	STD	−50	−16	STD
3-hydroxybenzoic acid	137/93	STD	−50	−16	STD
4-hydroxyhippuric acid	194/100	STD	−50	−20	STD
3-hydroxyhippuric acid	194/150	PIS[b]	−50	−20	4-hydroxyhippuric acid
gallic acid metabolites					
gallic acid	169/125	STD	−40	−20	STD
4-*O*-methylgallic acid	167/108	STD	−50	−26	STD
methylgallic acid	167/108	PIS	−50	−26	4-*O*-methylgallic acid
methylgallic sulfate	263/183	PIS	−50	−25	gallic acid
ethylgallate metabolites					
ethylgallate	197/169	STD	−50	−25	gallic acid
ethylgallate sulfate	277/197	PIS	−50	−25	gallic acid
ethylgallate glucuronide 1,2	373/197	PIS	−50	−25	epicatechin-5-*O*-glucuronide
hydroxyphenylacetic acids					
phenylacetic acid	135/91	STD	−30	−12	STD
3-hydroxyphenylacetic acid	151/107	STD	−50	−12	STD
2-hydroxyphenylacetic acid	151/107	STD	−50	−12	STD
3,4-dihydroxyphenylacetic acid	167/123	STD	−50	−12	STD
homovanillic acid	181/137	STD	−40	−10	vanillic acid
hydroxycinnamic acids					
m-coumaric acid	163/119	STD	−50	−30	STD
o-coumaric acid	163/119	STD	−50	−30	STD
p-coumaric acid	163/119	STD	−50	−30	STD
caffeic acid	179/135	STD	−50	−21	STD
ferulic acid	193/134	STD	−50	−25	STD
sinapic acid	223/164	STD	−50	−25	STD
hydroxyphenylpropionic acids					
3-(4-hydroxyphenyl)propionic acid	165/121	STD	−30	−16	STD
3-(3-hydroxyphenyl)propionic acid	165/121	STD	−30	−16	STD
dihydrocaffeic acid	181/137	STD	−40	−10	STD
flavan-3-ols					
(epi)catechin glucuronide 1,2,3,4	465/289	PIS	−50	−25	epicatechin-5-*O*-glucuronide
(epi)catechin sulfate 1,2,3	369/289	PIS	−50	−25	(epi)catechin
methyl(epi)catechin glucuronide 1,2,3	479/303	PIS	−50	−30	epicatechin-5-*O*-glucuronide
methyl(epi)catechin sulfate 1,2,3	383/303	PIS	−50	−25	(epi)catechin
glycinates					
vanilloylglycine	224/180	PIS	−50	−25	4-hydroxyhippuric acid
feruloylglycine	250/100	PIS	−50	−25	4-hydroxyhippuric acid
hydroxyphenylvalerolactones					
DHPV 1	207/163	PIS	−50	−25	(epi)catechin
DHPV 2	207/163	PIS	−50	−25	(epi)catechin
DHPV glucuronide 1,2	383/207	PIS	−50	−25	epicatechin-5-*O*-glucuronide
DHPV sulfate 1,2	287/207	PIS	−50	−25	(epi)catechin
MHPV 1	221/162	PIS	−50	−25	(epi)catechin
MHPV glucuronide 1	397/221	PIS	−50	−25	epicatechin-5-*O*-glucuronide
MHPV sulfate 1,2	301/221	PIS	−50	−25	(epi)catechin
other polyphenols					
enterolactone	297/253	STD	−50	−25	STD
pyrogallol	125/69	STD	−50	−25	STD

[a]STD, standard available. [b]PIS, product ion scan.

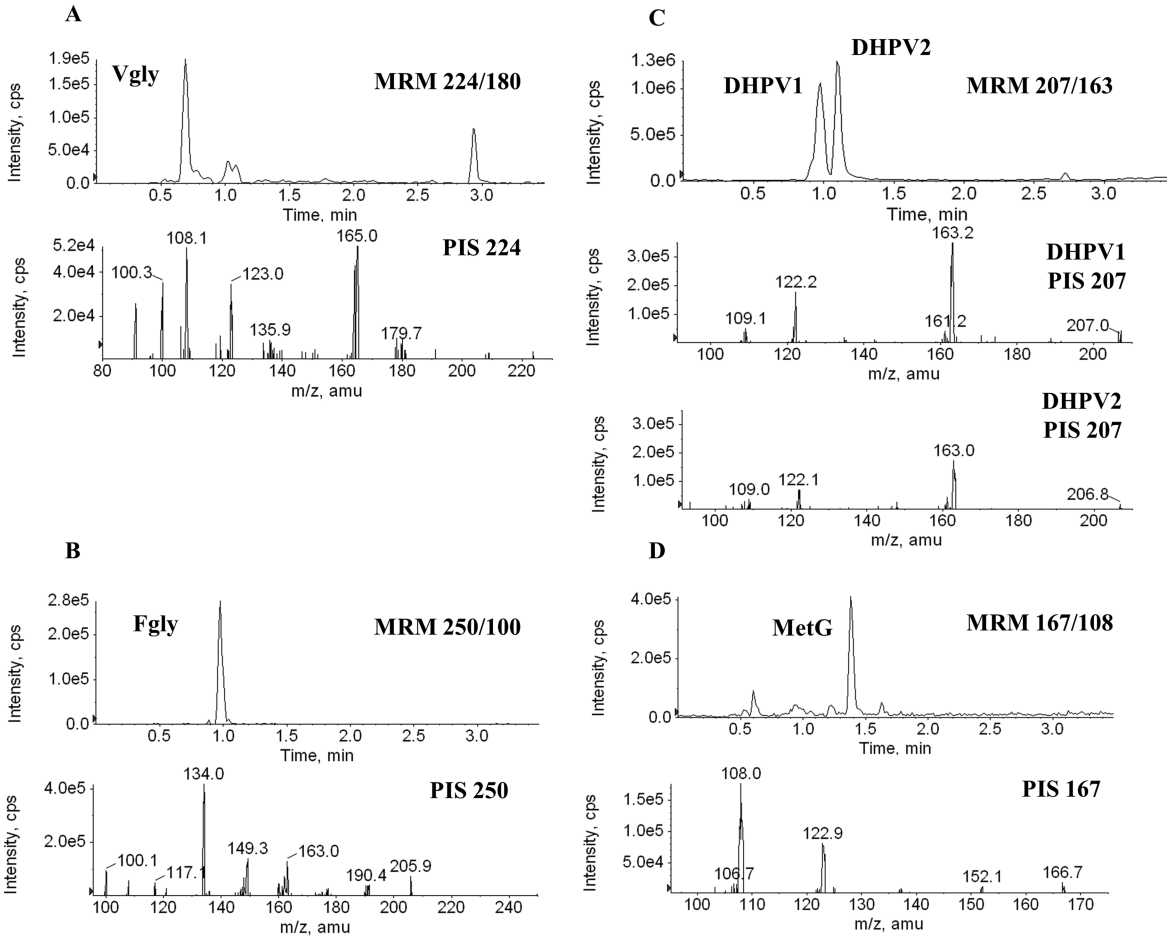

Figure 1. Multiple reaction monitoring (MRM) trace chromatograms and product ion scan (PIS) of (A) vanilloylglycine, Vgly (m/z 224), (B) feruloylglycine, Fgly (m/z 250), (C) dihydroxyphenyl-γ-valerolactone, DHPV (m/z 207), and (D) methylgallic acid, MetG (m/z 167) in hydrolyzed urine samples after DRW intake.

Eluates were evaporated to dryness and reconstituted with 100 μL of taxifolin (1.64 μmol/L) dissolved in mobile phase.

Extraction of Conjugated Phenolic Metabolites from Urine. Solid-phase extraction was performed using Oasis HLB 96-well plates (Waters, Milford, Massachusetts) as previously described.[31] Briefly, the plate was conditioned with 1 mL of methanol and 1.5 mol/L of formic acid in water. One milliliter of urine was loaded onto the cartridge plate. Then, the cartridges were washed with 1 mL of acidified water (1.5 mol/L of formic acid) and 1 mL of 5% methanol. Analytes were eluted with methanol containing 0.1% formic acid. The eluates were evaporated to dryness and reconstituted with 100 μL of taxifolin (1.64 μmol/L) dissolved in mobile phase.

UPLC-MS/MS Analysis of Conjugated and Microbial Metabolites in Urine. The analysis of metabolites in urine with or without enzymatic hydrolysis was carried out by UPLC coupled to tandem mass spectrometry (UPLC–MS/MS) adapted from a previous validated methodology.[26,31] A Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager and a refrigerated autosampler plate was used. It was coupled to an AB Sciex API 3000 triple quadrupole mass spectrometer equipped with a turbo ion spray ionizing in negative mode. The analytical column used for chromatographic separation was an Acquity UPLC BEH C18 (Milford, MA, USA) (1.7 μm, 2.1 mm × 5 mm), using a prefilter, working at 40 °C, at a flow rate of 0.5 mL/min with an injection volume of 5 μL. The linear gradient elution was carried out with 0.1% formic acid in water as phase A and 0.1% formic acid in acetonitrile as phase B at a flow rate of 500 μL/min with the following proportions (v/v) of phase A [t (min), %A]: (0,92), (2.5,50), (2.6,0), (3,0), (3.1,92), (3.5,92). The MS/MS parameters used were: collision cell exit potential (−15 V), focusing potential (−200 V), entrance potential (−10 V), nebulizer gas (10 arbitrary units), curtain gas (12 arbitrary units), collision gas (5 arbitrary units), auxiliary gas temperature (400 °C), auxiliary gas flow rate (6000 cm^3/min), and capillary voltage (−3700 V). Collision energy and declustering potential were optimized for each compound (Table 2). The identification of metabolites was done by comparing retention time with available standards or by product ion scan (PIS) when standards were not available. 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 (Table 2), using a dwell time of 10 ms. Calibration curves were constructed with available standards in synthetic urine[26] and subjected to the same procedure as the samples. Concentrations of metabolites with no available standard were estimated using the most similar compound standard curve and results were expressed as their equivalents (Table 2). Limits of detection and limits of quantification had already been published.[26]

Statistical Analysis. The MetaboAnalyst web-based platform for data analysis[32] was used for data normalization and the evaluation of mean differences of phenolic metabolites, through a t-test for paired samples. Normalization was carried out by a cube root transformation and a range scaling of the data. Statistical significance was defined as $P \leq 0.05$.

RESULTS AND DISCUSSION

Identification of Microbial-Derived and Phase II Phenolic Metabolites in Urine. A total of 37 metabolites were determined after enzymatic hydrolysis and 24 conjugated metabolites were determined without enzymatic hydrolysis.

dx.doi.org/10.1021/jf402394c | J. Agric. Food Chem. 2013, 61, 9166−9175

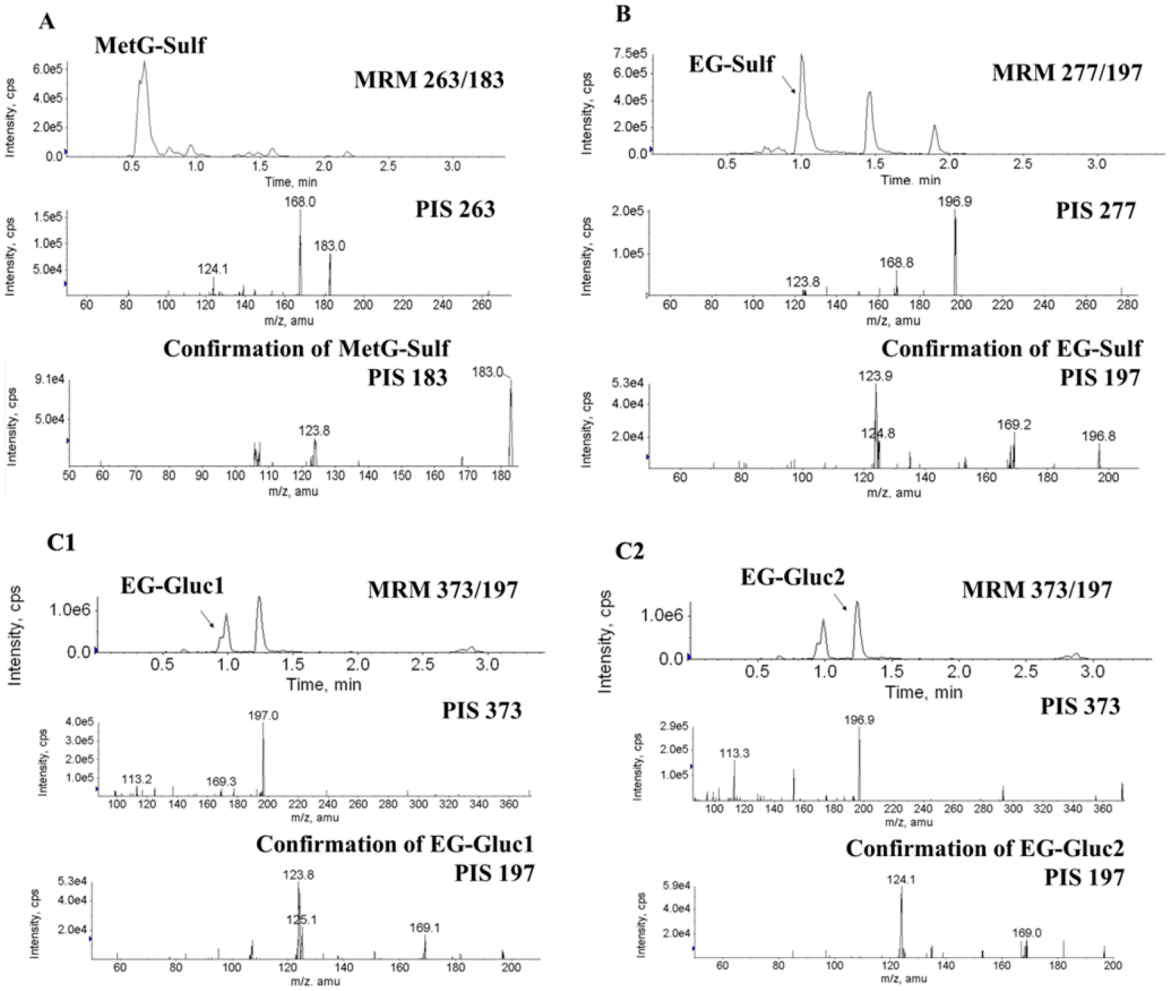

Figure 2. Multiple reaction monitoring (MRM) trace chromatograms and product ion scan (PIS) of (A) methylgallic sulfate, MetG-Sulf (*m*/*z* 263) with its confirmation through the PIS of *m*/*z* 183 in the CID-MS/MS experiments; (B) ethylgallate sulfate, EG-Sulf (*m*/*z* 277) with its confirmation through the PIS of *m*/*z* 197 in the CID-MS/MS experiments and (C1 and C2) ethylgallate glucuronide 1 and 2, EG-Gluc 1,2 (*m*/*z* 373) with its confirmation through the PIS of *m*/*z* 197 in the CID-MS/MS experiments.

From the metabolites determined after enzymatic hydrolysis, 30 were identified by comparison with the pure available standard. The other seven metabolites were tentatively identified by PIS experiments. Previously, attempts were made to identify glycinated (n = 18) metabolites of phenolic acids (results not shown) after DRW consumption but only three were positively identified based on their mass spectra. Feruloylglycine and vanilloylglycine were tentatively identified based on their published mass spectra.[33,34] The mass spectra and fragmentation pattern generated for vanilloylglycine (*m*/*z* 224) showed the ion *m*/*z* 180, loss of 44 amu (−COOH), *m*/*z* 165, loss of 59 amu (−CH_2−COOH), *m*/*z* 123, loss of 101 amu (−CO-NH-CH_2-COOH), and *m*/*z* 108, loss of 15 amu (−CH_3) and 101 amu, and *m*/*z* 100, which were coincident with the previous identifications of vanilloylglycine (Figure 1A).[33] Feruloylglycine (*m*/*z* 250) showed the fragments *m*/*z* 206, *m*/*z* 163, *m*/*z* 149, *m*/*z* 134 (loss of 101 and 15 amu), and *m*/*z* 100 reported in previous studies (Figure 1B).[34,35] 3-Hydroxyhippuric acid (*m*/*z* 194) was identified based on its mass spectra as previously published,[31,36] showing a characteristic fragment of *m*/*z* 150. In addition, two peaks of dihydroxyphenyl-γ-valerolactone (DHPV) (*m*/*z* 207) (Figure 1C) and one peak of methoxy-hydroxyphenyl-γ-valerolactone (MHPV) were identified according to the typical spectra and fragmentation pattern.[26] At MRM of 167/108, corresponding to the 4-*O*-methylgallic acid fragmentation, an additional peak was detected in samples. After studying its mass spectra, fragments coincided with those from the PIS of the 4-*O*-methylgallic acid standard (*m*/*z* 152, *m*/*z* 123, *m*/*z* 108) (Figure 1D), meaning the presence of a possible isomer, tentatively identified as 3-methylgallic acid. The concentration of some previously identified metabolites such as vanillic acid, 4-*O*-methylgallic acid, epicatechin, and MHPV were under the limit of detection of the method.

Besides the above-mentioned metabolites, conjugated metabolites derived from flavanol and microbial degradation metabolites were also investigated. Glucuronides and sulfates of (epi)catechin and methyl(epi)catechin, DHPV and MHPV, previously identified after cocoa and almond consumption,[31,37] were also found in urine. In addition, four new phenolic acid conjugates derived from methylgallic acid and ethylgallate were tentatively identified by PIS (Figure 2). The mass spectra and fragments generated by methylgallic sulfate (*m*/*z* 263) showed *m*/*z* 183, corresponding to the loss of 80 amu (−SO_3), and *m*/*z* 168, corresponding to the subsequent loss of 15 amu (−CH_3). This metabolite was confirmed through the PIS of *m*/*z* 183 in the CID-MS/MS experiments,[29] showing the fragmentation of methylgallic *m*/*z* 124, loss of 59 amu,

previously reported (Figure 2A).[38] In addition, three ethylgallate conjugates were tentatively identified. The peak at m/z 277 was identified as ethylgallate sulfate obtaining a product ion at m/z 197 (loss of 80 amu) and two peaks at m/z 373 as ethylgallate glucuronides which also showed the typical fragment of the glucuronide moiety (m/z 113 and 175) (Figure 2, C1 and C2). These conjugates were confirmed through the CID-MS/MS experiments of the product compound, ethylgallate (m/z 197), which showed its typical fragments at m/z 169 and m/z 124.[39] Additionally, more than 20 conjugated phenolic acids derived from hydroxybenzoic, hydroxyphenylacetic, hydroxycinnamic, and hydroxypropionic acids were investigated, but identification by mass spectra was not conclusive (data not shown).

Changes in Microbial-Derived and Phase II Phenolic Metabolites in Urine after DRW Consumption. The concentration of phase II and microbial-derived metabolites in urine at baseline and after consumption of DRW is presented in Tables 3 and 4. In this study, 21 phase II metabolites (Table 3) and 28 microbial metabolites (Table 4) significantly increased in urine after DRW compared to baseline.

In the present study, 11 (epi)catechin phase II metabolites (glucuronides, sulfates, and methyl conjugates) significantly increased after DRW ($P < 0.05$) with fold changes (FC) from 1.67 to 11.43 (Table 3). According to red wine composition, procyanidins are the most abundant polyphenols, followed by flavanols and anthocyanins.[17] Procyanidins are polymeric molecules that could arrive intact to the lower gastrointestinal tract and be hydrolyzed by microbiota into more simple components before absorption.[36,40,41] Procyanidins may suffer an interflavan cleavage from microbiota activity which results in catechin and epicatechin.[11] Then, (epi)catechins which were formed from procyanidins metabolism or which were present in the original food, could be subjected to C-ring-opening, giving rise to diphenylpropan-2-ol, later converted into 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone.[11] This step has been described for *Eggerthella lenta* and *Flavonifractor plautii*.[42] In this study, two glucuronide and two sulfate conjugates of DHPV and one glucuronide and one sulfate of MHPV increased after DRW intake compared to baseline ($P < 0.05$) (Table 3). Nevertheless, the origin of valerolactones is not exclusively from (epi)catechins but also from epicatechin gallate and epigallocatechin, which could release DHPV and also trihydroxyphenylvalerolactones.[11,15] This last compound was expected to be in low concentration in the urine of the participants of this study because epigallocatechins are present in low concentrations in wine,[17] thus it could not be identified in the present study. At intestinal level, valerolactone ring may suffer a break resulting in valeric acids and a possible interconversion between both forms was described but largely displaced to the former.[11] The hydroxyphenylpropionic acids analyzed in this study significantly increased after DRW intake (Table 4). They have been described from several routes of polyphenols: (i) β-oxidation of valeric acids, (ii) ring fission of the flavonol, (iii) breakdown of naringenin, and (iv) double-bond reduction of caffeic acid.[11,43] Hydroxyphenylpropionic acids could also be transformed into hydroxycinnamic acids after microbial hydrogenation and methylation in the liver.[11,40] Almost all the hydroxycinnamic acids reported in this study increased significantly after DRW intake in a fold change of 1.29−2.31, being the highest increment for *p*-coumaric, an anthocyanin microbial metabolite[44] and also derived of coutaric acid hydrolysis.[40] The metabolic origin of hydroxybenzoic acids may come from several routes of polyphenol metabolism: (i) by β-oxidation of hydroxyphenylpropionic acids and gallates which could be further glycinated into hydroxyhippuric acids,[11,36] (ii) microbial metabolism of anthocyanins,[16,44] and (iii) from quercetin metabolism.[45] In our study, nearly all the hydroxybenzoic acids increased in a significant way after DRW intake, although no significant increase was observed for protocatechuic acid. The highest increase was observed for syringic acid (2.78-fold change), while the other hydroxybenzoic acid metabolites ranged from 1.33 to 1.93 fold changes (Table 4). This high increase was due to the fact that syringic acid is the main microbial metabolite of malvidin-3-glucoside,[16,44,46] the most prevalent anthocyanin in wine.[17] The further metabolism of syringic acid such as enzymatic

Table 3. Concentrations (Mean ± SEM) of Phase II Metabolites of (Epi)catechin, Hydroxyphenylvalerolactones, and Hydroxybenzoic Acids in 24 h Urine Samples in 36 Subjects at Baseline and after DRW Intake

	urine samples (μmol, 24 h)		
metabolites	baseline	DRW	fold change
hydroxybenzoic acids			
gallic acid metabolites			
methylgallic sulfate	2.97 ± 0.74	19.94 ± 3.08*[a]	6.71
ethylgallate metabolites			
ethylgallate sulfate	2.16 ± 0.76	15.81 ± 1.64*	7.32
ethylgallate glucuronide 1	36.73 ± 6.01	114.52 ± 10.73*	3.12
ethylgallate glucuronide 2	101.74 ± 22.40	240.98 ± 24.23*	2.37
flavan-3-ols			
(epi)catechin glucuronide 1	0.46 ± 0.14	5.26 ± 2.66*	11.43
(epi)catechin glucuronide 2	0.26 ± 0.19	2.24 ± 0.95*	8.61
(epi)catechin glucuronide 3	5.35 ± 1.09	8.92 ± 1.68*	1.67
(epi)catechin glucuronide 4	3.36 ± 0.65	10.19 ± 1.93*	3.03
(epi)catechin sulfate 1	1.38 ± 0.24	5.22 ± 0.93*	3.78
(epi)catechin sulfate 2	0.98 ± 0.26	4.13 ± 0.73*	4.21
(epi)catechin sulfate 3	0.67 ± 0.18	1.34 ± 0.35	
methyl(epi)catechin glucuronide 1	2.04 ± 0.53	8.55 ± 1.92*	4.19
methyl(epi)catechin glucuronide 2	0.96 ± 0.36	3.92 ± 0.91*	4.08
methyl(epi)catechin glucuronide 3	0.75 ± 0.23	1.38 ± 0.46	
methyl(epi)catechin sulfate 1	2.37 ± 0.33	5.62 ± 0.76*	2.37
methyl(epi)catechin sulfate 2	8.37 ± 1.67	19.42 ± 2.68*	2.32
methyl(epi)catechin sulfate 3	0.13 ± 0.05	0.60 ± 0.15*	4.61
hydroxyphenylvalerolactones			
DHPV glucuronide 1	8.22 ± 1.82	31.73 ± 5.30*	3.86
DHPV glucuronide 2	62.40 ± 13.16	145.76 ± 20.32*	2.34
DHPV sulfate 1	14.70 ± 7.84	23.69 ± 6.32*	1.61
DHPV sulfate 2	512.44 ± 52.44	889.74 ± 110.51*	1.74
MHPV glucuronide 1	23.81 ± 4.43	38.43 ± 7.08*	1.61
MHPV sulfate 1	8.38 ± 1.68	12.65 ± 2.60*	1.51
MHPV sulfate 2	23.90 ± 4.89	30.49 ± 4.42	

[a]The asterisk indicates that the mean value is significantly different from the baseline concentration ($P < 0.05$).

dx.doi.org/10.1021/jf402394c | J. Agric. Food Chem. 2013, 61, 9166−9175

Table 4. Concentrations (Mean ± SEM) of Microbial Phenolic Acids Metabolites in 24 h Urine Samples in 36 Subjects at Baseline and after DRW Intake

	urine samples (μmol, 24 h)		
metabolites	baseline	DRW	fold change
hydroxybenzoic acids			
2,4-dihydroxybenzoic acid	1.57 ± 0.17	2.67 ± 0.37*[a]	1.70
2,6-dihydroxybenzoic acid	6.19 ± 0.60	8.74 ± 0.88*	1.41
2,5-dihydroxybenzoic acid	16.23 ± 1.65	27.29 ± 2.90*	1.68
3,5-dihydroxybenzoic acid	3.93 ± 0.66	7.57 ± 1.26*	1.93
protocatechuic acid	12.10 ± 1.15	14.45 ± 1.66	
syringic acid	0.73 ± 0.15	2.03 ± 0.32*	2.78
4-hydroxybenzoic acid	25.79 ± 2.21	34.30 ± 2.81*	1.33
3-hydroxybenzoic acid	3.77 ± 1.27	5.67 ± 1.57*	1.50
4-hydroxyhippuric acid	54.05 ± 5.42	72.13 ± 9.02*	1.33
3-hydroxyhippuric acid	192.30 ± 39.81	237.58 ± 54.21	
gallic acid metabolites			
gallic acid	0.85 ± 0.18	4.76 ± 0.53*	5.60
methylgallic acid	2.97 ± 0.42	4.76 ± 0.68*	1.60
ethylgallate metabolites			
ethylgallate	1.06 ± 0.37	4.97 ± 0.73*	4.69
hydroxyphenylacetic acids			
phenylacetic acid	22.15 ± 2.21	27.66 ± 3.00*	1.25
3-hydroxyphenylacetic acid	24.72 ± 3.50	56.57 ± 6.90*	2.29
2-hydroxyphenylacetic acid	5.89 ± 0.40	7.41 ± 0.54*	1.26
3,4-dihydroxyphenylacetic acid	1.61 ± 0.17	2.37 ± 0.24*	1.47
homovanillic acid	164.35 ± 13.99	215.13 ± 25.55	
hydroxycinnamic acids			
m-coumaric acid	0.54 ± 0.09	0.83 ± 0.20*	1.54
o-coumaric acid	0.07 ± 0.02	0.10 ± 0.03	
p-coumaric acid	0.64 ± 0.07	1.48 ± 0.15*	2.31
caffeic acid	5.42 ± 0.34	7.05 ± 0.55*	1.30
ferulic acid	11.80 ± 0.98	15.25 ± 0.94*	1.29
sinapic acid	0.99 ± 0.18	1.43 ± 0.20*	1.44
hydroxyphenylpropionic acids			
3-(4-hydroxyphenyl) propionic acid	287.44 ± 27.16	389.20 ± 39.36*	1.35
3-(3-hydroxyphenyl) propionic acid	6.22 ± 1.09	10.07 ± 2.05*	1.62
dihydrocaffeic acid	14.09 ± 1.39	17.29 ± 1.50*	1.23
glycinates			
vanilloylglycine	0.80 ± 0.09	1.31 ± 0.16*	1.64
feruloylglycine	9.23 ± 1.05	11.24 ± 1.31	
hydroxyphenylvalerolactones			
DHPV 1	6.73 ± 1.21	13.61 ± 2.68*	2.02
DHPV 2	18.50 ± 3.67	37.04 ± 4.31*	2.00
other polyphenols			
enterolactone	8.73 ± 1.10	14.81 ± 3.40*	1.70
pyrogallol	1.96 ± 0.43	8.08 ± 1.78*	4.12

[a]The asterisk indicates that the mean value is significantly different from the baseline concentration ($P < 0.05$).

demethylation of the B-ring could degenerate into gallic acid (5.60-fold change, Table 4), and the subsequent decarboxylation[44] could release pyrogallol (4.12-fold change, Table 4). In vitro studies have shown that the incubation of other minor wine anthocyanins such as cyanidin and peonidin with microbiota[16] released protocatechuic and vanillic acids. Delphinidin and petunidin derivatives, present in wine composition in lower concentrations than malvidin derivatives, could also suffer microbial degradation. These compounds could produce gallic and 3-methylgallic acids, respectively.[46−48] In our study, gallic and methylgallic acids showed a 5.60- and 1.60-fold significantly increase, respectively, after DRW intake (Table 4). Gallic acid metabolites have been clearly associated with wine consumption as they are present in wine composition and could also be released from anthocyanins, gallates, through the cleveage of gallic ester moiety, and syringic acid.[44,49−52] In addition, ethylgallate, which was originally present in wine,[53] also increased after DRW, along with its glucuronide and sulfate metabolites. The formation of hydroxyphenylacetic acids could come from three described routes: (i) α-oxidation of hydroxyphenylpropionic acids,[11] (ii) through the cleveage of the upper unit of dimeric procyanidins, or (iii) quercetin degradation via ring fission.[11,41,43] Participants of this study significantly increased the concentrations of mono-, dihydrolyphenylacetic, and phenylacetic acids, except for homovanillic acid, after one-month of DRW intake.

However, not only flavanols but flavonols and anthocyanins are present in DRW composition. Other minority components such as tyrosol and hydroxytyrosol can be metabolized to homovanillic alcohol, homovanillic acid, 3,4-dihydroxyphenylacetaldehyde and 3,4-dihydroxyphenylacetic acid,[54] or sinapic, which could also be transformed into syringic acid.[49] In addition, in this study, the concentration of enterolactone metabolite increased after DRW intake (Table 4). This has been described as a metabolite of lignans, which have been in wines[55] and was formed by selected strains of *Bifidobacterium* genus and *Lactonifactor longoviformis*.[16,56] Therefore, the increases of phenolic acids in urine after DRW consumption would be represented by the proportion of the phenolic compounds ingested through red wine and by the proportion of microbial transformation of different classes of wine polyphenols.

The targeted metabolism of phenolic acids after wine products intake has not been deeply studied. Intervention studies with wine and derived products studied the phase II metabolism of individual classes of polyphenols such as catechins or resveratrol.[22,29] A few other studies have implied the microbiota metabolism of wine phenolics. Cacceta et al.[23] determined 4-*O*-methylgallic acid, caffeic acid, and protocatechuic acid in plasma after intake of RW and DRW. While the two first metabolites increased significantly after wine consumption, no significant differences were observed for protocatechuic acid.[23] These results in plasma are in accordance with our study with urine samples. In addition, the similar content of urinary protocatechuic acid as well as other nonsignificant phenolic acids between baseline and after DRW intake could imply its origin from the habitual dietary pattern of participants which was maintained during the study with no differences in nutrient intake, daily intake of antioxidants, and fat intake.[4] Recently, two human intervention studies reported the gut microbial derived degradation products after the intake of extracts of grape juice during four days[57] or four weeks[58] by GC-MS. In their studies, authors found the strongest urinary markers for syringic acid, 3- and 4-hydroxyhippuric acid, pyrogallol, 3-hydroxyphenylacetic acid, 3-hydroxyphenylpropionic, and 4-hydroxymandelic acid.[57,58] These results are also in accordance to our study, except for 3-hydroxyhippuric acid, which no significant differences were observed between baseline and DRW intake period, and for 4-

hydroxymandelic acid that was not determined in the present study.

The potential prebiotic effect of rich phenolic sources such as DRW has already been reported,[12] but the role of these metabolites at intestinal level remains unknown. Some of the metabolites formed in the organism, such as hydroxyphenylpropionic or hydroxybenzoic acids, have been proved to have the ability to inhibit the growth of pathogenic bacteria and nonpathogenic bacteria[59] in in vitro studies and proposed as being responsible for phenolic health benefits in the organism.[11] However, more studies are needed to clarify this point because huge interindividual variability is described for polyphenol, probably to the high variability of bacterial species,[60] and thus beneficial effects.[18] Changes in the bacterial population may modify the metabolites that have formed, so the approach of urinary metabolism could be the key to understanding what is happening at intestinal level and linking to its biological effects.

To our knowledge, this study constitutes the most complete report of gut and microbial metabolites derived from wine consumption in humans. The numerous metabolites described to come from microbial degradation highlight the important role of intestinal bacteria in polyphenol degradation, modulating bioavailability and possible effects in the organism.

■ ASSOCIATED CONTENT

Supporting Information

Baseline characteristics of the participants. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: 00-34-934034844. E-mail: murpi@ub.edu.

Funding

This work has been supported by CICIT AGL2006-14228-C03-02, AGL2009-13906-C02-01, and the Ingenio-CONSOLIDER program, FUN-C-FOOD (CSD2007−063) from the Spanish Ministerio de Economía y Competitividad (MINECO). M.B.-O. thanks the FPU predoctoral program from the Spanish Ministry of Education, Culture and Sport. M.U.-S. thanks the "Ramon y Cajal" program from the MINECO and Fondo Social Europeo.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Torres S.A. for providing the dealcoholized red wine used in this study.

■ REFERENCES

(1) Renaud, S.; de Lorgeril, M. Wine, alcohol, platelets, and the French paradox for coronary heart disease. *Lancet* **1992**, *339*, 1523−1526.

(2) Arranz, S.; Chiva-Blanch, G.; Valderas-Martinez, P.; Medina-Remon, A.; Lamuela-Raventos, R. M.; Estruch, R. Wine, beer, alcohol and polyphenols on cardiovascular disease and cancer. *Nutrients* **2012**, *4*, 759−781.

(3) Chiva-Blanch, G.; Urpi-Sarda, M.; Ros, E.; Valderas-Martinez, P.; Casas, R.; Arranz, S.; Guillen, M.; Lamuela-Raventos, R. M.; Llorach, R.; Andres-Lacueva, C.; Estruch, R. Effects of red wine polyphenols and alcohol on glucose metabolism and the lipid profile: a randomized clinical trial. *Clin. Nutr.* **2013**, *32*, 200−206.

(4) Chiva-Blanch, G.; Urpi-Sarda, M.; Llorach, R.; Rotches-Ribalta, M.; Guillen, M.; Casas, R.; Arranz, S.; Valderas-Martinez, P.; Portoles, O.; Corella, D.; Tinahones, F.; Lamuela-Raventos, R. M.; Andres-Lacueva, C.; Estruch, R. Differential effects of polyphenols and alcohol of red wine on the expression of adhesion molecules and inflammatory cytokines related to atherosclerosis: a randomized clinical trial. *Am. J. Clin. Nutr.* **2012**, *95*, 326−334.

(5) Chiva-Blanch, G.; Urpi-Sarda, M.; Ros, E.; Arranz, S.; Valderas-Martinez, P.; Casas, R.; Sacanella, E.; Llorach, R.; Lamuela-Raventos, R. M.; Andres-Lacueva, C.; Estruch, R. Dealcoholized red wine decreases systolic and diastolic blood pressure and increases plasma nitric oxide: short communication. *Circ. Res.* **2012**, *111*, 1065−1068.

(6) Regulation (EC) No 1924/2006 of the European Parliament and of the Council of the European Union of 20 December 2006 on nutrition and health claims made on foods. *Official J. Eur. Union* **2006**, *L 404*, 30/12/2006.

(7) Baur, J. A.; Sinclair, D. A. Therapeutic potential of resveratrol: the in vivo evidence. *Nature Rev. Drug Discovery.* **2006**, *5*, 493−506.

(8) Cos, P.; De Bruyne, T.; Hermans, N.; Apers, S.; Berghe, D. V.; Vlietinck, A. J. Proanthocyanidins in health care: current and new trends. *Curr. Med. Chem.* **2004**, *11*, 1345−1359.

(9) Crozier, A.; Jaganath, I. B.; Clifford, M. N. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat. Prod. Rep.* **2009**, *26*, 1001−1043.

(10) Del Rio, D.; Costa, L. G.; Lean, M. E.; Crozier, A. Polyphenols and health: what compounds are involved? *Nutr. Metab. Cardiovasc. Dis.* **2010**, *20*, 1−6.

(11) Monagas, M.; Urpi-Sarda, M.; Sanchez-Patan, F.; Llorach, R.; Garrido, I.; Gomez-Cordoves, C.; Andres-Lacueva, C.; Bartolome, B. Insights into the metabolism and microbial biotransformation of dietary flavan-3-ols and the bioactivity of their metabolites. *Food Funct.* **2010**, *1*, 233−253.

(12) Queipo-Ortuno, M. I.; Boto-Ordonez, M.; Murri, M.; Gomez-Zumaquero, J. M.; Clemente-Postigo, M.; Estruch, R.; Cardona Diaz, F.; Andres-Lacueva, C.; Tinahones, F. J. Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *Am. J. Clin. Nutr.* **2012**, *95*, 1323−1334.

(13) Tabasco, R.; Sanchez-Patan, F.; Monagas, M.; Bartolome, B.; Victoria Moreno-Arribas, M.; Pelaez, C.; Requena, T. Effect of grape polyphenols on lactic acid bacteria and bifidobacteria growth: resistance and metabolism. *Food Microbiol.* **2011**, *28*, 1345−1352.

(14) Sanchez-Patan, F.; Cueva, C.; Monagas, M.; Walton, G. E.; Gibson, G. R.; Quintanilla-Lopez, J. E.; Lebron-Aguilar, R.; Martin-Alvarez, P. J.; Moreno-Arribas, M. V.; Bartolome, B. In vitro fermentation of a red wine extract by human gut microbiota: changes in microbial groups and formation of phenolic metabolites. *J. Agric. Food Chem.* **2012**, *60*, 2136−2147.

(15) Selma, M. V.; Espin, J. C.; Tomas-Barberan, F. A. Interaction between phenolics and gut microbiota: role in human health. *J. Agric. Food Chem.* **2009**, *57*, 6485−6501.

(16) Aura, A.-M. Microbial metabolism of dietary phenolic compounds in the colon. *Phytochem. Rev.* **2008**, *7*, 407−429.

(17) Neveu, V.; Perez-Jimenez, J.; Vos, F.; Crespy, V.; du Chaffaut, L.; Mennen, L.; Knox, C.; Eisner, R.; Cruz, J.; Wishart, D.; Scalbert, A. Phenol-Explorer: an online comprehensive database on polyphenol contents in foods. *Database (Oxford)* **2010**, bap024.

(18) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S−242S.

(19) Urpi-Sarda, M.; Zamora-Ros, R.; Lamuela-Raventos, R.; Cherubini, A.; Jauregui, O.; De la Torre, R.; Covas, M. I.; Estruch, R.; Jaeger, W.; Andres-Lacueva, C. HPLC-tandem mass spectrometric method to characterize resveratrol metabolism in humans. *Clin. Chem.* **2007**, *53*, 292−299.

(20) Vitaglione, P.; Sforza, S.; Galaverna, G.; Ghidini, C.; Caporaso, N.; Vescovi, P. P.; Fogliano, V.; Marchelli, R. Bioavailability of *trans*-resveratrol from red wine in humans. *Mol. Nutr. Food Res.* **2005**, *49*, 495−504.

(21) Bell, J. R. C.; Donovan, J. L.; Wong, R.; Waterhouse, A. L.; German, J. B.; Walzem, R. L.; Kasim-Karakas, S. E. (+)-Catechin in human plasma after ingestion of a single serving of reconstituted red wine. *Am. J. Clin. Nutr.* **2000**, *71*, 103−108.
(22) Donovan, J. L.; Bell, J. R.; Kasim-Karakas, S.; German, J. B.; Walzem, R. L.; Hansen, R. J.; Waterhouse, A. L. Catechin is present as metabolites in human plasma after consumption of red wine. *J. Nutr.* **1999**, *129*, 1662−1668.
(23) Caccetta, R. A. A.; Croft, K. D.; Beilin, L. J.; Puddey, I. B. Ingestion of red wine significantly increases plasma phenolic acid concentrations but does not acutely affect ex vivo lipoprotein oxidizability. *Am. J. Clin. Nutr.* **2000**, *71*, 67−74.
(24) Rothwell, J. A.; Urpi-Sarda, M.; Boto-Ordonez, M.; Knox, C.; Llorach, R.; Eisner, R.; Cruz, J.; Neveu, V.; Wishart, D.; Manach, C.; Andres-Lacueva, C.; Scalbert, A. Phenol-Explorer 2.0: a major update of the Phenol-Explorer database integrating data on polyphenol metabolism and pharmacokinetics in humans and experimental animals. *Database (Oxford)* **2012**, bas031.
(25) Gonzalez-Manzano, S.; Gonzalez-Paramas, A.; Santos-Buelga, C.; Duenas, M. Preparation and characterization of catechin sulfates, glucuronides, and methylethers with metabolic interest. *J. Agric. Food Chem.* **2009**, *57*, 1231−1238.
(26) Urpi-Sarda, M.; Monagas, M.; Khan, N.; Lamuela-Raventos, R. M.; Santos-Buelga, C.; Sacanella, E.; Castell, M.; Permanyer, J.; Andres-Lacueva, C. Epicatechin, procyanidins, and phenolic microbial metabolites after cocoa intake in humans and rats. *Anal. Bioanal. Chem.* **2009**, *394*, 1545−1556.
(27) Ibern-Gómez, M.; Andrés-Lacueva, C.; Lamuela-Raventós, R. M.; Waterhouse, A. L. Rapid HPLC analysis of phenolic compounds in red wines. *Am. J. Enol. Vitic.* **2002**, *53*, 218−221.
(28) Romero-Perez, A. I.; Ibern-Gomez, M.; Lamuela-Raventos, R. M.; de La Torre-Boronat, M. C. Piceid, the major resveratrol derivative in grape juices. *J. Agric. Food Chem.* **1999**, *47*, 1533−1536.
(29) Rotches-Ribalta, M.; Urpi-Sarda, M.; Llorach, R.; Boto-Ordonez, M.; Jauregui, O.; Chiva-Blanch, G.; Perez-Garcia, L.; Jaeger, W.; Guillen, M.; Corella, D.; Tinahones, F. J.; Estruch, R.; Andres-Lacueva, C. Gut and microbial resveratrol metabolite profiling after moderate long-term consumption of red wine versus dealcoholized red wine in humans by an optimized ultra-high-pressure liquid chromatography tandem mass spectrometry method. *J. Chromatogr., A* **2012**, *1265*, 105−113.
(30) Singleton, V. L.; Rossi, J. A. Colorimetry of total phenolics with phosphomolybdic−phosphotungstic acid reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144−158.
(31) Urpi-Sarda, M.; Monagas, M.; Khan, N.; Llorach, R.; Lamuela-Raventos, R. M.; Jauregui, O.; Estruch, R.; Izquierdo-Pulido, M.; Andres-Lacueva, C. Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography-tandem mass spectrometry. *J. Chromatogr., A* **2009**, *1216*, 7258−7267.
(32) Xia, J.; Wishart, D. S. Web-based inference of biological patterns, functions and pathways from metabolomic data using MetaboAnalyst. *Nature Protoc.* **2011**, *6*, 743−760.
(33) Cao, Y. G.; Zhang, L.; Ma, C.; Chang, B. B.; Chen, Y. C.; Tang, Y. Q.; Liu, X. D.; Liu, X. Q. Metabolism of protocatechuic acid influences fatty acid oxidation in rat heart: new anti-angina mechanism implication. *Biochem. Pharmacol.* **2009**, *77*, 1096−1104.
(34) Stalmach, A.; Mullen, W.; Barron, D.; Uchida, K.; Yokota, T.; Cavin, C.; Steiling, H.; Williamson, G.; Crozier, A. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug. Metab. Dispos.* **2009**, *37*, 1749−1758.
(35) Espín, J. C.; González-Barrio, R.; Cerdá, B.; López-Bote, C.; Rey, A. I.; Tomás-Barberán, F. A. Iberian pig as a model to clarify obscure points in the bioavailability and metabolism of ellagitannins in humans. *J. Agric. Food Chem.* **2007**, *55*, 10476−10485.
(36) Gonthier, M. P.; Donovan, J. L.; Texier, O.; Felgines, C.; Remesy, C.; Scalbert, A. Metabolism of dietary procyanidins in rats. *Free Radic. Biol. Med.* **2003**, *35*, 837−844.
(37) Urpi-Sarda, M.; Garrido, I.; Monagas, M.; Gomez-Cordoves, C.; Medina-Remon, A.; Andres-Lacueva, C.; Bartolome, B. Profile of plasma and urine metabolites after the intake of almond [*Prunus dulcis* (Mill.) D.A. Webb] polyphenols in humans. *J. Agric. Food Chem.* **2009**, *57*, 10134−10142.
(38) Ito, H.; Gonthier, M. P.; Manach, C.; Morand, C.; Mennen, L.; Remesy, C.; Scalbert, A. Polyphenol levels in human urine after intake of six different polyphenol-rich beverages. *Br. J. Nutr.* **2005**, *94*, 500−509.
(39) Sun, J.; Liang, F.; Bin, Y.; Li, P.; Duan, C. Screening non-colored phenolics in red wines using liquid chromatography/ultraviolet and mass spectrometry/mass spectrometry libraries. *Molecules* **2007**, *12*, 679−693.
(40) Gonthier, M. P.; Cheynier, V.; Donovan, J. L.; Manach, C.; Morand, C.; Mila, I.; Lapierre, C.; Remesy, C.; Scalbert, A. Microbial aromatic acid metabolites formed in the gut account for a major fraction of the polyphenols excreted in urine of rats fed red wine polyphenols. *J. Nutr.* **2003**, *133*, 461−467.
(41) Appeldoorn, M. M.; Vincken, J. P.; Aura, A. M.; Hollman, P. C.; Gruppen, H. Procyanidin dimers are metabolized by human microbiota with 2-(3,4-dihydroxyphenyl)acetic acid and 5-(3,4-dihydroxyphenyl)-gamma-valerolactone as the major metabolites. *J. Agric. Food Chem.* **2009**, *57*, 1084−1092.
(42) Kutschera, M.; Engst, W.; Blaut, M.; Braune, A. Isolation of catechin-converting human intestinal bacteria. *J. Appl. Microbiol.* **2011**, *111*, 165−175.
(43) Rechner, A. R.; Smith, M. A.; Kuhnle, G.; Gibson, G. R.; Debnam, E. S.; Srai, S. K.; Moore, K. P.; Rice-Evans, C. A. Colonic metabolism of dietary polyphenols: influence of structure on microbial fermentation products. *Free Radic. Biol. Med.* **2004**, *36*, 212−225.
(44) Hidalgo, M.; Oruna-Concha, M. J.; Kolida, S.; Walton, G. E.; Kallithraka, S.; Spencer, J. P.; de Pascual-Teresa, S. Metabolism of anthocyanins by human gut microflora and their influence on gut bacterial growth. *J. Agric. Food Chem.* **2012**, *60*, 3882−3890.
(45) Loke, W. M.; Jenner, A. M.; Proudfoot, J. M.; McKinley, A. J.; Hodgson, J. M.; Halliwell, B.; Croft, K. D. A metabolite profiling approach to identify biomarkers of flavonoid intake in humans. *J. Nutr.* **2009**, *139*, 2309−2314.
(46) Kern, M.; Fridrich, D.; Reichert, J.; Skrbek, S.; Nussher, A.; Hofem, S.; Vatter, S.; Pahlke, G.; Rüfer, C.; Marko, D. Limited stability in cell culture medium and hydrogen peroxide formation affect the growth inhibitory properties of delphinidin and its degradation product gallic acid. *Mol. Nutr. Food Res.* **2007**, *51*, 1163−1172.
(47) Forester, S. C.; Waterhouse, A. L. Identification of Cabernet Sauvignon anthocyanin gut microflora metabolites. *J. Agric. Food Chem.* **2008**, *56*, 9299−9304.
(48) Jimenez-Giron, A.; Queipo-Ortuno, M. I.; Boto-Ordonez, M.; Munoz-Gonzalez, I.; Sanchez-Patan, F.; Monagas, M.; Martin-Alvarez, P. J.; Murri, M.; Tinahones, F. J.; Andres-Lacueva, C.; Bartolome, B.; Moreno-Arribas, M. V. Comparative study of microbial-derived phenolic metabolites in human feces after intake of gin, red wine, and dealcoholized red wine. *J. Agric. Food Chem.* **2013**, *61*, 3909−3915.
(49) Nurmi, T.; Mursu, J.; Heinonen, M.; Nurmi, A.; Hiltunen, R.; Voutilainen, S. Metabolism of berry anthocyanins to phenolic acids in humans. *J. Agric. Food Chem.* **2009**, *57*, 2274−2281.
(50) Ávila, M.; Hidalgo, M.; Sánchez-Moreno, C.; Pelaez, C.; Requena, T.; Pascual-Teresa, S. Bioconversion of anthocyanin glycosides by Bifidobacteria and Lactobacillus. *Food Res. Int.* **2009**, *42*, 1453−1461.
(51) Gao, S. H.; Zhan, Q.; Li, J. X.; Yang, Q.; Li, X.; Chen, W. S.; Sun, L. N. LC-MS/MS method for the simultaneous determination of ethyl gallate and its major metabolite in rat plasma. *Biomed. Chromatogr.* **2010**, *24*, 472−478.
(52) Hodgson, J. M.; Morton, L. W.; Puddey, I. B.; Beilin, L. J.; Croft, K. D. Gallic acid metabolites are markers of black tea intake in humans. *J. Agric. Food Chem.* **2000**, *48*, 2276−2280.
(53) Monagas, M.; Gomez-Cordoves, C.; Bartolome, B.; Laureano, O.; Ricardo da Silva, J. M. Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from *Vitis vinifera* L. Cv.

Graciano, Tempranillo, and Cabernet Sauvignon. *J. Agric. Food Chem.* **2003**, *51*, 6475−6481.
(54) Tuck, K. L.; Hayball, P. J. Major phenolic compounds in olive oil: metabolism and health effects. *J. Nutr. Biochem.* **2002**, *13*, 636−644.
(55) Nurmi, T.; Heinonen, S.; Mazur, W.; Deyama, T.; Nishibe, S.; Adlercreutz, H. Lignans in selected wines. *Food Chem.* **2003**, *83*, 303−309.
(56) Roncaglia, L.; Amaretti, A.; Raimondi, S.; Leonardi, A.; Rossi, M. Role of bifidobacteria in the activation of the lignan secoisolariciresinol diglucoside. *Appl. Microbiol. Biotechnol.* **2011**, *92*, 159−168.
(57) Jacobs, D. M.; Fuhrmann, J. C.; van Dorsten, F. A.; Rein, D.; Peters, S.; van Velzen, E. J.; Hollebrands, B.; Draijer, R.; van Duynhoven, J.; Garczarek, U. Impact of short-term intake of red wine and grape polyphenol extract on the human metabolome. *J. Agric. Food Chem.* **2012**, *60*, 3078−3085.
(58) van Dorsten, F. A.; Grün, C. H.; van Velzen, E. J. J.; Jacobs, D. M.; Draijer, R.; van Duynhoven, J. P. M. The metabolic fate of red wine and grape juice polyphenols in humans assessed by metabolomics. *Mol. Nutr. Food Res.* **2010**, *54*, 897−908.
(59) Requena, T.; Monagas, M.; Pozo-Bayón, M. A.; Martín-Álvarez, P. J.; Bartolomé, B.; del Campo, R.; Ávila, M.; Martínez-Cuesta, M. C.; Peláez, C.; Moreno-Arribas, M. V. Perspectives of the potential implications of wine polyphenols on human oral and gut microbiota. *Trends Food Sci. Technol.* **2010**, *21*, 332−344.
(60) Blaut, M.; Clavel, T. Metabolic diversity of the intestinal microbiota: implications for health and disease. *J. Nutr.* **2007**, *137*, 751S−755S.