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Targeted metabolic profiling of phenolics in urine and plasma after regular consumption of cocoa by liquid chromatography–tandem mass spectrometry

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ABSTRACT

The biological properties of cocoa (*Theobroma cacao* L.) polyphenols are strictly dependent on their bioavailability. A long-term cocoa feeding trial was performed with subjects at high risk for cardiovascular disease. Subjects ($n = 42$) received two sachets of 20 g of cocoa powder/day with 250 mL of skimmed milk each, or only 500 mL/day of skimmed milk, both for two 4-week periods. The phenolic metabolic profile including phase II conjugated metabolites and phenolic acids derived from the intestinal microbiota was determined by LC-MS/MS in both 24-h urine and fasting plasma. The analysis of 24-h urine revealed significant increases of phase II metabolites, including glucuronides and sulfate conjugates of (−)-epicatechin, *O*-methyl-epicatechin, 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and 5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone, after regular cocoa intake. In the case of plasma, only glucuronide conjugates of dihydroxyphenylvalerolactones increased. Regular consumption of cocoa also resulted in a significant increase in the urinary excretion of colonic microbial-derived phenolic metabolites, including vanillic, 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic acids, and particularly 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone, whereas only the two latter metabolites showed a significant increase in fasting plasma. The results found herein indicate that 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone and hydroxyphenylacetic acids could be good biomarkers of the regular consumption of cocoa and therefore, of flavanol-rich foods.

1. Introduction

Cocoa (*Theobroma cacao* L.) and its derived products represent a very rich source of dietary flavonoids, containing higher amounts of flavonoids per serving than tea or red wine [1]. Spain has the largest consumption of cocoa powder products per person (1668 g/person/year), representing approximately 28% of the total cocoa consumption in this country, followed by Norway (1647 g/person/year) and Sweden (1288 g/person/year) [reports of ACNielsen, Euromonitor International]. In Spain, cocoa powder is daily consumed with milk, mainly during breakfast and as an afternoon snack. Among the Spanish population, children between 7 and 14 years old are the largest consumers, accounting for about 54% of their daily flavonoid intake (Family Food Panel, Spain 2005–2006, Taylor Nelson Sofres). Flavanols are the most abundant flavonoids in cocoa, occurring as monomers [(+)-catechin and (−)-epicatechin], and as oligomeric and polymeric forms [procyanidins]. (−)-Epicatechin has been reported as the major monomeric flavanol in cocoa, representing about 35% of the total phenolic content [2]. In contrast to most fruits which contain the (+)-catechin enantiomer, as a consequence of processing cocoa-derived products mainly contain the (−)-catechin enantiomer, which is less bioavailable [3]. Cocoa procyanidins consist primarily of (−)-epicatechin up to polymers (DP > 10) [4]. Oligomers (procyanidins B1, B2, B5 and C1) and polymers account for ≥90% of total polyphenols, and monomers for 5–10% [4].

Numerous cocoa feeding trials with humans have been performed in recent years [5,6]. Biomarkers significantly affected in these trials are related to: (i) *antioxidant effects* (decrease in LDL oxidation and oxidative stress; increase in antioxidant status) [7];

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Table 1
Nutritional and phenolic composition of the cocoa powder used in the study.

Parameter	Value
Carbohydrates (%)	65.7
Starch (%)	16.1
Sucrose (%)	<1.0
Fiber (%)	19.1
Fat (%)	5.3
Protein (%)	17.1
Moisture (%)	5.73
Ash (%)	6.17
Polyphenols (mg/g)	**Mean ± SD**
(+)-Catechin	0.26 ± 0.007
(−)-Epicatechin	1.15 ± 0.003
Procyanidin B2	0.91 ± 0.02
Vanillin	0.94 ± 0.12
Isoquercitrin	0.056 ± 0.001
Quercetin	0.005 ± <0.0001
Quercetin-3-arabinoside	0.02 ± <0.0001
Quercetin-3-glucuronide	0.003 ± <0.0001
Total polyphenols (mg catechin/g cocoa)[a]	12.38 ± 0.69

[a] Quantified by Folin–Ciocalteu method.

(ii) *antiplatelet effects* (inhibition of platelet activation and function; improvement in insulin sensitivity) [7]; (iii) *effects on lipid metabolism* (increase in HDL concentration) [8]; (iv) *effects on vascular function* (increase in NO bioactivity; lower systolic and diastolic blood pressure: improvement in endothelial function) [9,10].

Health effects derived from cocoa polyphenols depend on their bioavailability. Considering flavanols, bioavailability is influenced by their degree of polymerization [11]. Monomers are readily absorbed in the small intestine. As the result of phase II enzymes, (−)-epicatechin is converted into glucuronidated and sulfated metabolites as well as into methylated metabolites which in turn could also be glucuronidated and sulfated. However, the absorption of dimeric procyanidins in humans seems to be very limited [12,13] whereas polymeric procyanidins are not well absorbed in their native form. These polyphenols reach the colon, where they are biotransformed by the intestinal microbiota into hydroxyphenylvalerolactones and a series of organic acids [14–18] that could be further absorbed and exert several biological activities [18–23].

Accurate estimation of polyphenol intake or exposure is of high importance in order to determine the metabolic fate and to be able to calculate the polyphenol doses that could be related to certain health effects in epidemiological studies. Tandem mass spectrometry coupled to liquid chromatography has been demonstrated to be highly suitable for the analysis of phenolic metabolites enabling rapid detection and identification in a single analytical run, with adequate selectivity and sensitivity even for trace compounds [13–16,24].

Most studies related to the bioavailability of cocoa polyphenols have only considered the acute consumption of cocoa [12,16,25,26]. However, studies considering moderate and regular consumption of cocoa are required in order to evaluate the influence on polyphenol bioavailability over time, as well as the health effects derived from cocoa consumption. In the present study, a long-term cocoa feeding trial was performed with subjects at high risk for cardiovascular heart disease (CHD) who received cocoa powder/day with skimmed milk or only skimmed milk for a 4-week period, each. Urine and plasma samples from volunteers were submitted to an off-line solid-phase extraction (SPE) in 96-well plates in combination with short-run time and highly sensitive LC-MS/MS methods [13,25] in order to perform a targeted analysis of conjugated and non-conjugated metabolites derived from phase II enzymes as well as from the intestinal microbiota.

2. Material and methods

2.1. Subjects

From a total of 47 volunteers recruited presenting high risk of CHD, 5 declined to participate. Therefore, 42 volunteers (19 men and 23 women, mean age of 69.7 ± 11.5 years) were included in the study. Subjects were required to present 3 or more of the following cardiovascular risk factors: tobacco smoker, diabetes mellitus (glycemic ≥ 126 mg/dL), hypertension (BP ≥ 140/90 mmHg), LDL-cholesterol ≥ 160 mg/dL, HDL-cholesterol ≤ 35 mg/dL, obesity (body mass index ≥ 25), family history of premature CHD. Exclusion criteria included: presence of previous cardiovascular event; allergic reactions to any cocoa components; gastrointestinal, neurological, psychiatric, endocrine or tumoral diseases; human immunodeficiency virus infection; chronic alcoholism or drug addiction. The institutional review board of the Hospital Clínic of Barcelona approved the study protocol and all participants gave written consent before participation in the study. This trial has been registered in the Current Controlled Trials at London, International Standard Randomized Controlled Trial Number [ISRCTN75176807: Bioavailability and effects of soluble phenols of cocoa on inflammatory biomarkers related to atherosclerosis (http://controlled-trials.com/ISRCTN75176807/)].

2.2. Study design

The study was a 4-week randomized, controlled, crossover clinical trial. After a 2-week lead-in diet, subjects received 2 sachets of 20 g/day of cocoa powder (one for breakfast and another one for dinner) with 250 mL skimmed milk each (total/day: 500 mL) (cocoa intervention) or only 500 mL/day of skimmed milk (control intervention) for 4 weeks in a random order. At baseline and after each intervention period, fasting blood samples (extracted in the morning after one night fasting) and a 24-h urine specimen were collected. Fasting blood was centrifuged immediately (1500 × *g*, 10 min) to obtain the plasma. Samples were stored at −80 °C until analysis.

The phenolic and nutritional composition of the cocoa powder (defatted and sugar-free) used in the study is presented in Table 1. Total phenolic determination was performed with the Folin–Ciocalteu method [27], and individualized phenolic compounds were determined by HPLC after extraction [28]. The mean degree of flavanol polymerization (mDP) was 8 as estimated by thiolysis [29].

2.3. Chemicals and reagents

The following compounds (% purity when available) were used. Phenylacetic acid (≥98%), 3-hydroxyphenylacetic acid (≥97%), 3,4-dihydroxyphenylacetic acid (98%), 3-methoxy-4-hydroxyphenylacetic acid (99%), 3-hydroxyphenylpropionic acid (≥98%), 3,4-dihydroxyphenylpropionic acid (≥98%), *p*-coumaric acid (≥98%), caffeic acid (≥95%), ferulic acid (≥98%), protocatechuic acid (>97%), 4-hydroxybenzoic acid (≥98%), 3-hydroxybenzoic acid (≥98%), ethyl gallate (≥96%), (−)-epicatechin (≥98%), (+)-catechin (≥98%), procyanidin B2 (≥90%), and β-glucuronidase/sulfatase (from *Helix pomatia*) were purchased from Sigma–Aldrich (St. Louis, MO). 4-Hydroxyhippuric acid (>99%) was purchased from PhytoLab GmbH&Co.KG (Vestenbergsgreuth, Germany). Vanillic acid, *m*-coumaric acid and taxifolin (>90%) were purchased from Extrasynthèse (Genay, France). HPLC grade solvents methanol, acetonitrile, glacial acetic acid and formic acid were purchased from Scharlau (Barcelona, Spain). Hydrochloric acid was purchased from Panreac (Barcelona, Spain).

Table 2
Concentrations (mean ± SEM), of phase II metabolites of epicatechin and hydroxyphenylvalerolactones in urine and plasma samples after each intervention period.

Peak	Phase II metabolite	MRM transition	Urine samples (μmol, 24 h)				Plasma samples (nmol/L)			
			Skimmed milk	Cocoa with skimmed milk			Skimmed milk	Cocoa with skimmed milk		
			Mean ± SEM	Mean ± SEM	p[a]	Δ (%)	Mean ± SEM	Mean ± SEM	p[a]	Δ (%)
Flavanols										
1	Epicatechin-*O*-glucuronide	465/289	0.10 ± 0.05	0.04 ± 0.006	0.049	−60	n.d.		n.d.	
2	Epicatechin-*O*-glucuronide	465/289	0.08 ± 0.04	0.04 ± 0.007	0.256	−50	n.d.		n.d.	
3	Epicatechin-*O*-glucuronide	465/289	0.14 ± 0.07	0.35 ± 0.07	<0.001	150	n.d.		n.d.	
4	Epicatechin-*O*-glucuronide	465/289	0.11 ± 0.04	2.26 ± 0.51	<0.001	1955	n.d.		n.d.	
5	Epicatechin-*O*-sulfate	369/289	0.12 ± 0.07	0.33 ± 0.11	0.017	175	n.d.		n.d.	
6	Epicatechin-*O*-sulfate	369/289	0.29 ± 0.06	0.85 ± 0.20	0.015	193	n.d.		n.d.	
7	Epicatechin-*O*-sulfate	369/289	0.27 ± 0.12	0.24 ± 0.05	0.308	−11	n.d.		n.d.	
8	*O*-methyl-epicatechin-*O*-glucuronide	479/303	0.13 ± 0.03	0.19 ± 0.04	0.048	46	n.d.		n.d.	
9	*O*-methyl-epicatechin-*O*-glucuronide	479/303	0.13 ± 0.04	0.20 ± 0.03	0.023	54	n.d.		n.d.	
10	*O*-methyl-epicatechin-*O*-glucuronide	479/303	0.02 ± 0.01	0.09 ± 0.03	<0.001	350	n.d.		n.d.	
11	*O*-methyl-epicatechin-*O*-sulfate	383/303	0.53 ± 0.23	4.08 ± 1.65	<0.001	104	n.d.		n.d.	
12	*O*-methyl-epicatechin-*O*-sulfate	383/303	1.11 ± 0.29	7.97 ± 1.17	<0.001	618	n.d.		n.d.	
13	*O*-methyl-epicatechin-*O*-sulfate	383/303	0.26 ± 0.08	1.74 ± 0.39	<0.001	569	n.d.		n.d.	
	Hydroxyphenylvalerolactones									
14	DHPV *O*-glucuronide	383/207	3.04 ± 0.79	8.73 ± 2.20	<0.001	187	8.29 ± 2.97	39.12 ± 14.08	0.004	372
15	DHPV *O*-glucuronide	383/207	7.27 ± 2.09	21.04 ± 4.84	<0.001	189	21.56 ± 7.31	113.93 ± 34.45	0.007	428
16	DHPV *O*-sulfate	287/207	36.44 ± 20.12	190.22 ± 122.95	0.451	422	n.d.		n.d.	
17	DHPV *O*-sulfate	287/207	452.44 ± 124.72	1003.39 ± 191.00	<0.001	122	n.d.		n.d.	
18	MHPV *O*-glucuronide	397/221	0.64 ± 0.21	1.89 ± 0.85	0.013	195	25.73 ± 3.04	36.77 ± 6.12	0.163	43
19	MHPV *O*-sulfate	301/221	3.15 ± 1.03	5.58 ± 1.35	0.021	77	n.d.		n.d.	
20	MHPV *O*-sulfate	301/221	6.64 ± 3.80	20.69 ± 6.81	<0.001	212	n.d.		n.d.	

[a] Wilcoxon non-parametric test for 2 related samples; n.d.: not detected; SEM: standard error of the mean; DHPV: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; MHPV: 5-(3′-methoxy,4′-hydroxyphenyl)-γ-valerolactone.

Table 3
Concentrations (mean ± SEM) of flavanols and colonic microbial metabolites in urine and plasma samples after each intervention period.

No.	Colonic Microbial Metabolite	MRM transition	Urine samples (μmol, 24 h)				Plasma samples (μmol/L)			
			Skimmed milk	Cocoa with skimmed milk			Skimmed milk	Cocoa with skimmed milk		
			Mean ± SEM	Mean ± SEM	p[a]	Δ (%)	Mean ± SEM	Mean ± SEM	p[a]	Δ (%)
	Flavanols									
21	(−)-Epicatechin	289/245	0.007 ± 0.003	0.24 ± 0.06	<0.001	3328	n.d.		n.d.	
	Hydroxyphenylvalerolactones									
22	DHPV	207/163	7.27 ± 1.69	34.13 ± 7.95	<0.001	369	0.11 ± 0.04	0.48 ± 0.09	0.004	336
23	MHPV	221/162	n.d.		n.d.		n.d.		n.d.	
	Hydroxyphenylpropionic acids									
24	3,4-Dihydroxyphenylpropionic acid	181/137	4.27 ± 0.75	2.92 ± 0.56	0.092	−32	0.19 ± 0.04	0.20 ± 0.04	0.751	5
25	3-Hydroxyphenylpropionic acid	165/121	6.34 ± 2.13	4.74 ± 2.22	0.235	−25	0.16 ± 0.04	0.23 ± 0.08	0.159	44
	Hydroxyphenylacetic acids									
26	3,4-Dihydroxyphenylacetic acid	167/123	0.82 ± 0.10	1.44 ± 0.27	0.012	76	0.10 ± 0.02	0.11 ± 0.02	0.808	10
27	3-Methoxy-4-hydroxyphenylacetic acid	181/137	35.00 ± 6.75	34.82 ± 6.99	0.694	−1	n.d.		n.d.	
28	3-Hydroxyphenylacetic acid	151/107	14.93 ± 3.53	31.23 ± 7.29	0.002	109	0.06 ± 0.02	0.12 ± 0.02	0.005	100
	Phenylacetic acid	135/91	42.77 ± 5.51	49.30 ± 7.57	0.589	15	19.21 ± 1.28	20.32 ± 1.20	0.732	6
	Hydroxycinnamic acids									
29	*m*-Coumaric acid	163/119	0.60 ± 0.14	0.71 ± 0.40	0.706	18	n.d.		n.d.	
30	*p*-Coumaric acid	163/119	0.32 ± 0.08	0.31 ± 0.06	0.945	−3	0.03 ± 0.004	0.03 ± 0.005	0.316	0
31	Caffeic acid	179/135	0.19 ± 0.07	0.07 ± 0.02	0.212	−63	0.09 ± 0.01	0.08 ± 0.01	0.909	−11
32	Ferulic acid	193/134	5.39 ± 1.17	4.28 ± 0.99	0.326	−21	0.22 ± 0.02	0.21 ± 0.02	0.849	−5
	Hydroxybenzoic acids									
33	Protocatechuic acid	153/109	29.70 ± 4.29	34.47 ± 6.48	0.641	16	10.09 ± 1.57	10.52 ± 1.61	0.889	4
34	Vanillic acid	167/152	21.11 ± 4.24	52.44 ± 10.06	<0.001	148	2.80 ± 0.34	2.71 ± 0.31	0.713	−3
35	4-Hydroxybenzoic acid	137/93	35.69 ± 5.59	37.34 ± 5.57	0.922	5	9.37 ± 0.46	9.73 ± 0.50	0.751	4
36	3-Hydroxybenzoic acid	137/93	1.58 ± 0.43	2.29 ± 0.94	0.454	45	n.d.		n.d.	
37	4-Hydroxyhippuric acid	194/100	57.30 ± 8.83	57.80 ± 8.68	0.974	1	0.09 ± 0.01	0.11 ± 0.02	0.469	22
38	3-Hydroxyhippuric acid	194/150	176.76 ± 29.96	136.69 ± 24.50	0.067	−23	0.30 ± 0.10	0.48 ± 0.18	0.209	60

n.d.: not detected; SEM: standard error of the mean; DHPV: 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone; MHPV: 5-(3′-methoxy,4′-hydroxyphenyl)-γ-valerolactone.

[a] Wilcoxon non-parametric test for 2 related samples.

2.4. Sample preparation

2.4.1. Determination of conjugated metabolites

Solid-phase extraction was performed using Oasis® HLB 96-well plates (Waters, Milford, Massachusetts) as previously described [25]. Briefly, the plate was conditioned with 1 mL of methanol and acidified water (1.5 mol/L formic acid). One milliliter of plasma with 20 μL of *O*-phosphoric acid or 3 mL of urine samples, both with ethyl gallate as internal standard (IS), were loaded onto the cartridge plate. Then, the cartridges were washed and analytes were eluted with methanol containing formic acid (1.5 mol/L). The eluates were evaporated to dryness and reconstituted with taxifolin dissolved in mobile phase A as an additional external standard.

2.4.2. Determination of colonic microbial metabolites

Solid-phase extraction was performed using Oasis® MCX 96-well plates (Waters, Milford, Massachusetts) as previously described [13]. Briefly, 1 mL of urine or 1 mL of plasma samples were spiked with ethyl gallate as IS and subjected to enzymatic hydrolysis as previously described [13]. The plate was conditioned with methanol and acidified water (2% formic acid). The hydrolyzed samples were then loaded onto the plate, washed with acidified water (2% formic acid) and analytes were then eluted with methanol and evaporated to dryness. Residues were reconstituted with taxifolin dissolved in mobile phase A.

2.5. LC-MS/MS

The analyses of hydrolyzed and non-hydrolyzed samples were carried out by liquid chromatography–tandem mass spectrometry (LC-MS/MS). LC analyses were performed using an Agilent 1200 system equipped with a quaternary pump and a refrigerated autosampler plate (Waldbronn, Germany). An Applied Biosystems API 3000 Triple Quadrupole mass spectrometer (PE Sciex, Concord, Ontario, Canada), equipped with a Turbo Ion Spray ionizing in negative mode, was used. A Phenomenex Luna C_{18} analytical column [50 mm × 2.0 mm i.d., 5 μm] (Torrance, CA) with mobile phase A (0.1% formic acid in water) and B (0.1% formic acid in acetonitrile) was used. The linear gradient for the determination of phase II metabolites at a flow rate of 400 μL/min was (% mobile phase B, time (min)): (9, 0), (80, 6), (100, 6.5), (100, 8). And the linear gradient for the determination of colonic microbial metabolites at a flow rate of 400 μL/min was (% mobile phase B, time (min)): (8, 0), (50, 4), (100, 5.2) and (100, 7) [13]. In each case, the column was re-equilibrated for 6 min and the sample volume injected was 15 μL. MS/MS parameters used were as follows: capillary voltage, −3700 V; focusing potential, −200 V; entrance potential, −10 V; declustering potential, −50 V; nebulizer gas, 10 (arbitrary units); curtain gas, 12 (arbitrary units); collision gas, 5 (arbitrary units); auxiliary gas temperature, 400 °C; auxiliary gas flow rate, 6000 cm^3/min. The collision energy was −25 V for phase II metabolites of epicatechin. The collision energy for phenolic acids was optimized for each compound as previously published [13]. 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. MRM transitions for phase II metabolites and ring-fission metabolites of epicatechin are shown in Table 2. The following transitions were also screened: epicatechin sulfoglucuronide (545/289); *O*-methyl epicatechin sulfoglucuronide (559/303); ethyl gallate (197/169); taxifolin (303/285). A dwell time of 80 ms was used in this experiment.

MRM transitions for colonic microbial metabolites are shown in Table 3. In addition, the following transitions were also screened: 5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (MHPV) (221/162); procyanidin dimer (577/289); ethyl gallate (197/169); taxifolin (303/285). A dwell time of 65 ms was used in this experiment.

When no commercial standards were available, the concentration was expressed as (−)-epicatechin equivalents [24,25]. 3-Hydroxyhippuric acid was expressed as 4-hydroxyhippuric acid equivalents.

2.6. Statistical analysis

SPSS Statistical Analysis System, Version 14.0 (SPSS) was used to perform the statistical analysis. Descriptive statistics were used to obtain the mean and the standard error of the mean (SEM) of the different variables after each intervention period. Because the data were non-parametric (Kolmogorov–Smirnov test) and presented non-homogeneous variances (Levene's test), the Wilcoxon test for related samples was used to compare changes in outcome variables in response to the intervention trial. Statistical tests were two-tailed, and the significance level was 0.05.

3. Results and discussion

3.1. Targeted analysis of conjugated metabolites

In contrast to procyanidins, monomeric flavanols are directly absorbed in the small intestine, where they are first conjugated, and subsequently metabolized in the liver into methyl, glucuronide and sulfate derivatives [11]. These forms pass into the bile through enterohepatic circulation and may reach the colon, together with the procyanidins which escape small intestine absorption, to be further degraded by the intestinal microbiota. In order to study the metabolic profile of the conjugation of epicatechin and its ring-fission metabolites, non-hydrolyzed urine and plasma samples collected after cocoa consumption were screened. The analysis of urine revealed the presence of signals corresponding to four epicatechin-*O*-glucuronides (MRM 465/289), three *O*-methyl-epicatechin-*O*-glucuronides (MRM 479/303), three epicatechin-*O*-sulfates (MRM 369/289), and three *O*-methyl-epicatechin-*O*-sulfates (MRM 383/303) (Fig. 1A). However, these metabolites were absent from fasting plasma samples as expected from their pharmacokinetic parameters ($T_{max} \sim 2$ h) [12,26]. With regards to epicatechin-*O*-glucuronides (MRM 465/289), three of the signals (peaks 1–3) have already been confirmed in rat samples after cocoa intake [30], whereas the fourth signal (peak 4) was confirmed by a product ion scan (PIS) of the deprotonated molecule (Fig. 1B). The PIS of peak 4 showed the deprotonated molecule (*m*/*z* 465), the ion at *m*/*z* 327 and *m*/*z* 289 corresponding to epicatechin aglycone, as reported by Sang et al. [31] after tea consumption, and finally, the ions at *m*/*z* 175 and *m*/*z* 113 corresponding to the glucuronic acid moiety (Fig. 1B). Previously, Natsume et al. observed epicatechin-3′-*O*-glucuronide in human urine and epicatechin-7-*O*-glucuronide in rat urine, both after epicatechin intake [32]. The different chromatographic retention times of these compounds could help us to tentatively indicate that peak 3 could correspond to epicatechin-7-*O*-glucuronide and peak 4 to epicatechin-3′-*O*-glucuronide. The three signals (peaks 8–10) corresponding to *O*-methyl-epicatechin-*O*-glucuronides (MRM 479/303) (Fig. 1A) were previously confirmed in rat samples after cocoa intake [30]. Similarly, the three epicatechin-*O*-sulfates (peaks 5–7; MRM 369/289) (Fig. 1A) were also confirmed in a previous human study after acute intake of cocoa [25]. With regards to *O*-methyl-epicatechin-*O*-sulfates (MRM 383/303), the three signals (peaks 11–13) were identified (Fig. 1A) by a PIS of the deprotonated molecule (*m*/*z* 383) (Fig. 1C). The PIS of these peaks showed the deprotonated molecule (*m*/*z* 383) and the ions at *m*/*z* 303 corresponding to methyl-epicatechin, *m*/*z* 137 corresponding to the

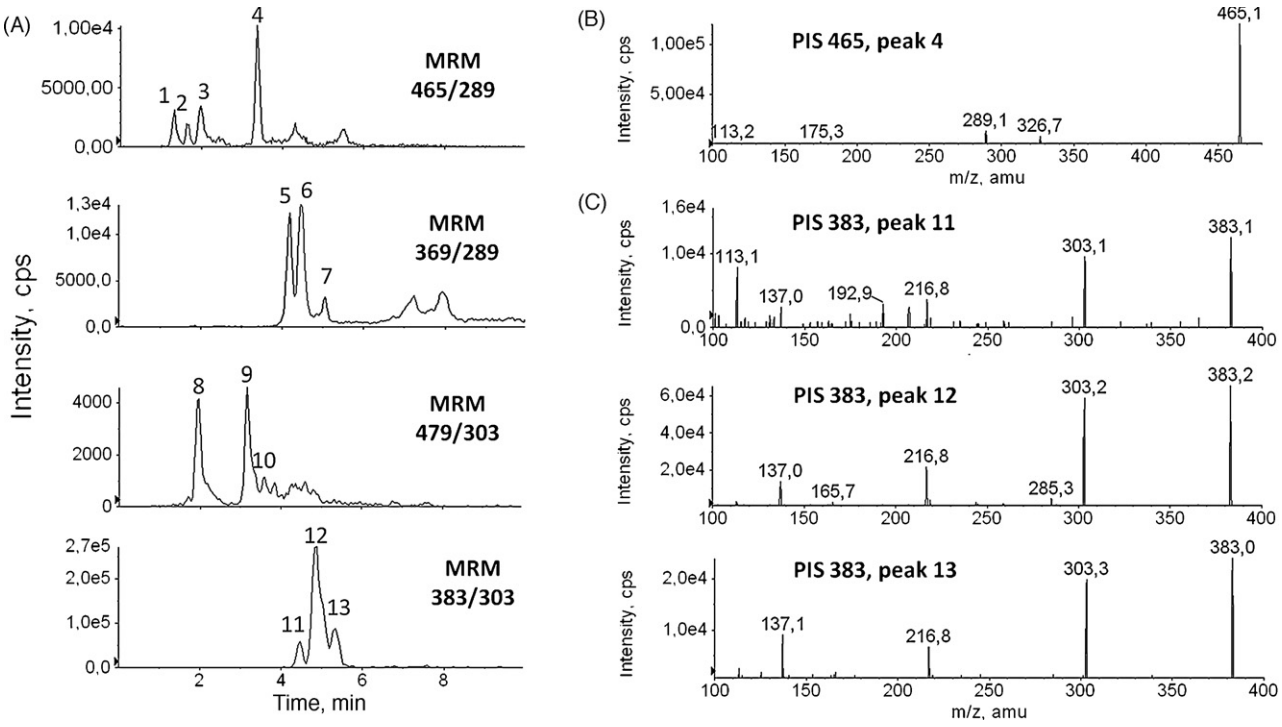

Fig. 1. (A) MRM trace chromatogram of phase II metabolites of epicatechin: epicatechin-*O*-glucuronides (465/289), epicatechin-*O*-sulfates (369/289), *O*-methyl-epicatechin-*O*-glucuronides (479/289), *O*-methyl-epicatechin-*O*-sulfates (383/289). (B) Product ion scan (PIS) of epicatechin-*O*-glucuronide (*m*/*z* 465). (C) Product ion scan (PIS) of *O*-methyl-epicatechin-*O*-sulfates (*m*/*z* 383).

classic epicatechin-related A-ring fragment, and *m*/*z* 217 suggesting that the sulfatation occurred on the A-ring at position 5 or 7 (Fig. 1C), as confirmed by Sang et al. [31] after tea consumption.

Conjugated metabolites derived from the ring-fission of epicatechin/procyanidins by the colonic microbiota were also studied. These metabolites were identified based on their PIS and compared with those described by Sang et al. after tea consumption in humans [31]. Two glucuronide and sulfate conjugates of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (DHPV) were identified based on their PIS (Fig. 2A and B). The PIS of the glucuronide conjugates (peaks 14 and 15) and sulfate conjugates (peaks 16 and 17) showed the deprotonated molecule (*m*/*z* 383 and *m*/*z* 287, respectively) and the ions at *m*/*z* 207 corresponding to DHPV, *m*/*z* 163 corresponding to the loss of 44 amu (CO_2), and *m*/*z* 175 and *m*/*z* 113 corresponding to the glucuronic acid moiety in the case of glucuronidated conjugates (Fig. 2A). Glucuronidated and sulfated metabolites of MHPV were also identified (Fig. 2C and D). The PIS of glucuronide (peak 18) and sulfate (peaks 19 and 20) conjugates showed the deprotonated molecule (*m*/*z* 397 and *m*/*z* 301, respectively), the ions at *m*/*z* 221 corresponding to MHPV, *m*/*z* 206 corresponding to the loss of 15 amu (CH_3) from MHPV, and *m*/*z* 175 and *m*/*z* 113 corresponding to the glucuronic acid moiety in the case of glucuronidated conjugates (Fig. 2C).

3.2. Quantitative determination of conjugated metabolites

A significant increase in the urinary levels of different conjugates of epicatechin and DHPV was found in non-hydrolyzed 24-h urine samples after the consumption of cocoa in relation to the milk consumption (Table 2). Both epicatechin and hydroxyphenylvalerolactone derivatives have been quantified as (−)-epicatechin in order to estimate the changes in phenolic metabolite content after cocoa consumption. Among phase II metabolites of epicatechin, epicatechin-*O*-glucuronide (peak 4) and *O*-methyl-epicatechin-*O*-sulfates (peaks 12 and 13) presented the largest increment in urine as a consequence of cocoa intake. However, other metabolites such as epicatechin-*O*-glucuronide (peak 2) and epicatechin-*O*-sulfate (peak 7) did not show significant changes (Table 2). Regular consumption of 40 g of cocoa powder per day resulted in a urinary excretion of 18.38 μmol of phase II metabolites of epicatechin in 24 h, which represents an overall increment of 458% in comparison to the intake of milk. Our results are in agreement with those of Natsume et al. [32], who reported that 3′-*O*-glucuronide was the main glucuronide in human urine. Also, our epicatechin metabolic profile seems in accordance with that of Tomas-Barberan et al. [26] who found methyl-epicatechin sulfates as major urinary metabolites, followed by glucuronide, sulfate and methyl-glucuronide conjugates, after the intake by humans of a single dose of cocoa powder. Roura et al. [33] also found that sulfated metabolites of epicatechin were the predominant metabolites in urine. Finally, also in agreement with our findings, major amounts of *O*-methyl-(epi)catechin-*O*-sulfates, followed by sulfates and glucuronide conjugates, have been reported after the intake of a single dose of tea extracts [34,35]. Differences in the metabolic profile of epicatechin may be attributed to the different flavan-3-ol composition of the matrix, cocoa or tea.

A significant increase in the urinary levels of hydroxyphenylvalerolactone conjugates was also registered after cocoa intake, with the exception of DHPV *O*-sulfate (peak 16) which did not show a significant change due to large interindividual variations (Table 2). After the cocoa intake period, an overall increment of 145% was shown for this group of metabolites when compared to the milk intake. In the case of fasting plasma, two glucuronide derivatives of DHPV (peaks 14 and 15) and one glucuronide derivative of MHPV (peak 18) were detected in non-hydrolyzed samples. In particular, the two former conjugates of DHPV were found in significant amounts after cocoa consumption (Table 2).

There are very few studies concerning the metabolic fate of monomeric flavanols and procyanidins in humans by the intestinal microbiota. Consumption of tea catechins by humans resulted in the formation of hydroxyphenylvalerolactones in plasma and urine [14,15]. Similarly, the occurrence of these metabolites was

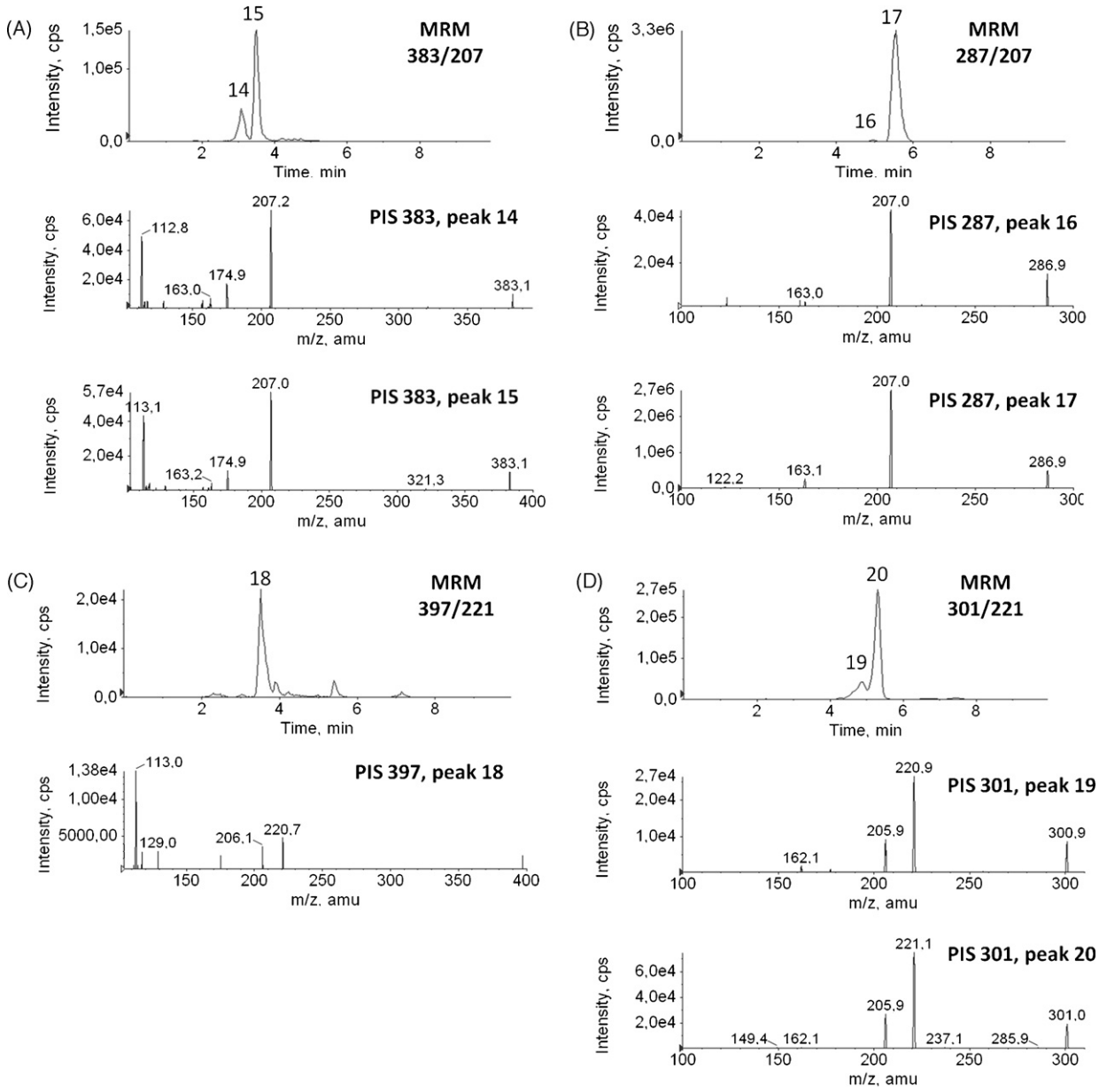

Fig. 2. MRM trace chromatograms and product ion spectra (PIS) of 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (DHPV) *O*-glucuronides (A), 5-(3′,4′-dihydroxyphenyl)-γ-valerolactone (DHPV) *O*-sulfates (B), 5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (MHPV) *O*-glucuronide (C), and 5-(3′-methoxy-4′-hydroxyphenyl)-γ-valerolactone (MHPV) *O*-sulfates (D).

confirmed by *in vitro* fermentation studies performed with (−)-epicatechin-3-*O*-gallate, (−)-epicatechin and (+)-catechin [36], or with procyanidin dimer B3 [37]. According to Meselhy et al. [36], renal excretion of DHPV occurred within the first 24 h after the incubation of (−)-epicatechin-3-*O*-gallate with human intestinal bacteria, or after the oral administration of (−)-epicatechin [38]. In humans, maximum concentration of DHPV has been reported to occur at 3–6 h for pure (−)-epicatechin or at 8–24 h after green tea consumption [14,15]. In agreement with these studies, DHPV presented a significant increase in 24-h urine and plasma samples after consumption of cocoa flavanols, in comparison to the intake of milk. Our results are also consistent with previous studies that reported that DHPV mainly existed in sulfate and glucuronide conjugated forms in the urine of rats after administration of epicatechin, the level of conjugation being higher than for (−)-epicatechin [38]. Peak plasma level of DHPV has been reported to be around 13 h after tea intake in humans [14,15]. Therefore, detection of DHPV in fasting plasma in the present study may be due to the intake of the second cocoa dose (dinner dose) or to their lengthy presence in the blood through enterohepatic circulation. To our knowledge this is the first report of hydroxyphenylvalerolactones and their conjugate metabolites in human urine and plasma after regular intake of cocoa polyphenols.

3.3. Targeted analysis and quantitative determination of microbial metabolites

The microbial degradation pathway of monomeric flavanols and procyanidins, which has been partially elucidated by the results of *in vitro* and *in vivo* animal studies [31,39,40], is illustrated in Fig. 3. It involves the following reactions: (a) reductive cleavage of the heterocyclic C-ring resulting in the formation of diphenylpropan-2-ols; (b) lactonization of diphenylpropan-2-ols into hydroxyphenylvalerolactones; (c) fission of the valerolac-

tone ring leading to hydroxyphenylvaleric acids [36,37]; (d) β-oxidation of the side chain of hydroxyphenylvaleric acids resulting in hydroxyphenylpropionic and hydroxybenzoic acids [36]; (e) α-oxidation of hydroxyphenylpropionic acid leading to phenylacetic acids [40], although a recent study suggests that they may exclusively arise from the degradation of procyanidins [39]; (f) 3- and 4-dehydroxylation reactions resulting in 4- and 3-hydroxylated phenolic acids, respectively [36,37,40–42]. After absorption microbial-derived phenolic metabolites could be conjugated into their glucuronide and sulfate esters in the liver and kidney before excretion in the urine. Other reactions occurring in the liver and kidney include: conjugation glycine [41], dehydrogenation, hydroxylation and methylation [40].

In order to do a targeted study of phenolic metabolites derived from the colonic microbiota, hydrolyzed urine and plasma samples were screened as previously described [13]. A total of 19 metabolites, including flavanol [(−)-epicatechin] and microbial-derived metabolites, were identified and quantified by LC/MS-MS (Table 3).

As expected from the changes observed in their corresponding conjugated forms, (−)-epicatechin and DHPV showed the largest increment in 24-h urine after cocoa consumption, followed by vanillic acid, 3-hydroxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid (Table 3). The urinary levels of the remaining metabolites kept virtually constant after cocoa consumption. Regular consumption of 40 g of cocoa powder with skimmed milk per day resulted in a urinary excretion of 485.2 μmol of microbial-derived phenolic acids in 24 h, which represents an overall increment of 10% in comparison with the intake of milk. Hydroxyphenylacetic acids (3-methoxy-4-hydroxyphenylacetic acid; 3-hydroxyphenylacetic acid; phenylacetic acid) and hydroxybenzoic acids (protocatechuic acid, vanillic acid, 4-hydroxybenzoic acid, and 3- and 4-hydroxyhippuric acids) were among the predominant urinary metabolites after cocoa consumption. It is important to highlight that microbial-derived phenolic metabolites represented the largest proportion of total phenolic metabolites in urine, whereas (−)-epicatechin represented only a minor proportion (0.09% of total metabolite excretion). This is consistent with reports from other feeding trials performed with wine [41] or sorghum bran [43] that as cocoa contain a high fraction of polymerized polyphenols, whereas the

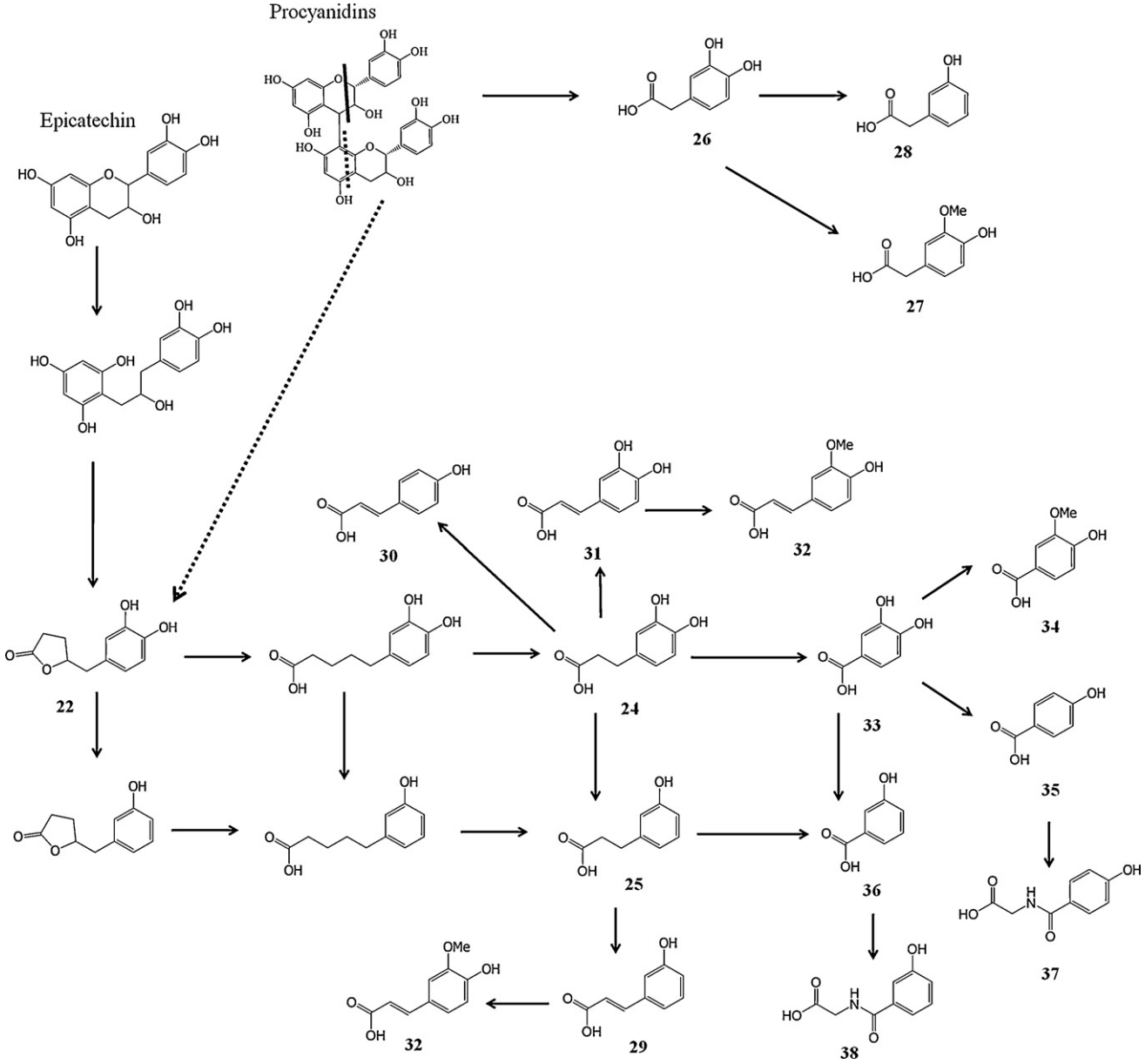

Fig. 3. Metabolites originated from the microbial degradation and further liver metabolism of catechins and procyanidins. Figure numbers are referred to Table 3.

contrary has been observed for a diet rich in monomeric forms [40].

Many of the above-mentioned metabolites were also detected in fasting plasma samples, with the exception of (−)-epicatechin, 3-methoxy-4-hydroxyphenylacetic acid, *m*-coumaric acid and 3-hydroxybenzoic acid (Table 3). In comparison to urine, only DHPV and 3-hydroxyphenylacetic acid presented a significant increase after cocoa consumption. The predominant metabolites in fasting plasma after the consumption of cocoa were phenylacetic acid, protocatechuic acid, 4-hydroxybenzoic acid and vanillic acid. Although fewer metabolites were detected in plasma, changes observed in the metabolic profile seem to be consistent with those observed in urine. To our knowledge, this is the first report related to the fasting plasmatic levels of microbial-derived phenolic metabolites in human subjects after regular cocoa intake.

Recently, it has been suggested that 3,4-dihydroxyphenylacetic acid seems to be a characteristic and major metabolite derived exclusively from the microbial degradation of procyanidins but not from monomers [39]. Gonthier et al. reported that both 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid increased in urine after administration of a procyanidin dimer B3 to rats [40]. Other studies have reported an increased urinary excretion of 3-hydroxypropionic acid and 3-hydrophenylacetic acid after human consumption of grape seed polyphenols [17]. Rios et al. reported a significant increase in the urinary excretion of 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 3-hydroxyphenylacetic acid, ferulic acid, vanillic acid, and 3-hydroxybenzoic acid in healthy humans after acute consumption of flavanol-rich chocolate (80 g) [16]. According to these authors, the increase in the levels of vanillic acid observed after cocoa consumption, which is also in agreement with the results found in the present study, most likely originated from the oxidation of vanillin added to the product as vanilla flavour (Table 1). With the exception of this metabolite, which showed a maximum excretion during the first 3 h after chocolate intake, the excretion of the remaining microbial metabolite increased between 6 and 48 h after consumption and registered maximum levels between 24 and 48 h [16]. This could explain the fact that no additional significant compositional changes were observed in 24-h urine after cocoa consumption in the present study. Another aspect to be considered is that the mean age of volunteers included in this study was 69.7 ± 11.5 years, and that age, together with other factors such as stress, disease or diet, affects the human microbiota and therefore produces changes in the microbial metabolome, leading to large interindividual variation in metabolite concentrations [44]. In addition, it is important to highlight that phenolic compounds themselves are able to modulate the microbiota [45]. A change in the population of bacteria towards tannin-resistant Gram-negative species (i.e. *Enterobacteriaceae* and *Bacteriodes*) have been reported after the 3-week administration of condensed tannins to rats [46]. Therefore, the long-term ingestion of cocoa phenolics may have produced changes in the bacterial population of volunteers, which may help to explain the differences in the urine metabolic profile in comparison to the study of Rios et al. [16]. In this context, Gu et al. [43] reported that urinary excretion of 3-hydroxyphenylpropionic acid was high in rats fed a high amount of sorghum bran (20–40% of the diet) over 50 days, whereas the concentration of hydroxyphenylacetic acid was high when a low amount of sorghum bran was administered, but also decreased when higher amounts were administered.

4. Conclusions

In conclusion, the LC/MS-MS analysis of 24-urine and fasting plasma after regular consumption of cocoa revealed that monomeric flavanols in cocoa were mainly absorbed and metabolized as glucuronide, sulfate, *O*-methylglucuronide and *O*-methylsulfate derivatives of (−)-epicatechin, whereas the occurrence of glucuronide and sulfate derivatives of DHPV and its methyl ester (MHPV) could be indicative of the absorption of both monomeric flavanols and procyanidins present in cocoa. However, microbial-derived phenolic acids were the most abundant metabolites in both urine and fasting plasma. Some of these phenolic acids have been recently proved to inhibit the secretion of proinflammatory cytokine involved in the early stages of atherosclerosis from LPS-induced human PBMC [20]. As a consequence of the intake of cocoa, changes were observed in some microbial-derived metabolites, in particular for DHPV, 3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylacetic acid. This is the first report of DHPV after regular long-term feeding of cocoa polyphenols to human subjects. The results found herein indicate that these compounds could be good biomarkers of cocoa consumption. The fasting plasma gives sufficient information about dietary exposure and individual response to diet. One aspect that deserves further consideration is the effect of long-term feeding of food containing highly polymerized proanthocyanidins on the human colonic microbiota population, posterior metabolism and its final implication for the gut and overall health.

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