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A New Process To Develop a Cocoa Powder with Higher Flavonoid Monomer Content and Enhanced Bioavailability in Healthy Humans

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Cocoa is a food rich in polyphenols, mainly the flavonoid procyanidins and flavan-3-ols. The improvement of the cardiovascular function in humans upon cocoa consumption has been specifically linked to the presence of flavan-3-ol derived metabolites in plasma, especially epicatechin glucuronide. In this context, a flavonoid-enriched cocoa-derived product could potentially exert stronger health benefits. The aim of the present study was to obtain a cocoa powder with a higher flavonoid content (mainly enriched in monomer compounds) and assess its flavonoid bioavailability in humans. For this purpose, an unfermented, nonroasted, and blanch-treated cocoa powder (A) was obtained. The powder contained four times more procyanidins than a conventional (B) cocoa powder. Powder A contained eight times more epicatechin and procyanidin B2 than powder B. Cocoa milk drinks were prepared with powder A (MDA) and B (MDB). The bioavailability of flavonoids in both drinks was assessed in a crossover intervention with healthy volunteers. The content of epicatechin glucuronide, the main metabolite detected in plasma, was five-fold higher upon consumption of MDA as compared with MDB. The urinary excretion of metabolites, mainly methyl epicatechin sulfate, was higher upon MDA consumption as compared with MDB, ranging from two- to 12-fold higher depending on the metabolite. These results, together with previous reports regarding the cardiovascular benefits linked to the presence of procyanidin metabolites in plasma, suggest that further clinical trials to validate the health benefits of a flavonoid-enriched cocoa powder are warranted.

KEYWORDS: ***Theobroma cacao*; processing; cocoa; procyanidins; metabolism; bioavailability; cross over; plasma; urine; human**

INTRODUCTION

Cocoa derivatives are a significant source of polyphenols, particularly procyanidins and flavan-3-ols. The polyphenol content of cocoa-derived products depends on the botanical variety as well as on genetic, agronomical, and other factors. Among these factors are those related to the processing of cocoa powder, such as postharvest handling, fermentation, drying, and roasting. Consequently, the polyphenol content of raw cocoa beans differs substantially from that in cocoa powder or chocolate. Fermentation and drying steps during cocoa bean processing result in a loss of flavonoids (*1*). In addition, high temperatures and longer roasting times reduce the content of polyphenols in cocoa. Both degradations are known to be related to enzymatic and nonenzymatic oxidations that occur during these manufacturing processes (*2*). The preservation or enhancement of cocoa procyanidins is of great importance since these compounds, despite their poor bioavailability, have been related to the health beneficial effects of cocoa, particularly in cardiovascular diseases (*3*−*5*). Flavonoid-rich cocoa products and chocolate have demonstrated relevant biological activity (*6*−*8*). The consumption of flavanol-rich cocoa has been reported to improve endothelial function (*9*), and this effect has been directly linked to the presence of procyanidin-derived metabolites in plasma (*4*). In this context, the bioavailability of food polyphenols is essential to understand their biological activity.

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Table 1. Total Polyphenol Content in Unfermented and Dried Cocoa Beans from Different Geographical Origins[a]

geographical origin	variety	total polyphenol content in dried cocoa bean sample (g/100 g)
Ivory Coast	Forastero	8.15 ± 1.82[b]
Colombia	Amazon	8.14 ± 0.37[b]
Guinea Ecuatorial	Amazon Forastero	7.24 ± 0.98[c]
Ecuador	Amazon–Trinitario–Canelo Amazon Hybrid (Clone CCN51)	8.42 ± 0.87[c]
Venezuela	Trinitario	6.43 ± 0.56[d]
Peru	Criollo	5[e]
Dominican Republic	Criollo	4[e]

[a] Results are expressed on dry basis and as gallic acid equivalents. [b] Mean of two different sample preparations. [c] Mean of three different sample preparations. [d] Mean of four different sample preparations ± standard deviations. [e] One sample preparation. Results are averages of duplicate analyses of each preparation.

Table 2. Polyphenol and Purine Composition of Cocoa Powders[a]

compound	no.	R_t (min)	cocoa powder A polyphenol-rich	cocoa powder B conventional
theobromine	1	7.8	17.77 (0.10)	22.14 (0.91)
caffeoyl aspartate	H1	9.7	0.27 (0.02)	–
B1 (dimer)	2	11.1	3.68 (0.10)	1.12 (0.01)
trimer	3	11.5	2.33 (0.02)	t
caffeoyl aspartate (isomer)	4 (H2)	11.6	3.88 (0.60)	0.37 (0.02)
tetramer	5	11.9	1.46 (0.04)	–
tetramer	6		1.30 (0.08)	–
catechin	7	12.8	6.46 (0.55)	2.02 (0.04)
caffeine	8	13.0	4.39 (0.11)	1.95 (0.05)
trimer	9	13.8	4.36 (0.46)	t
B2 (dimer)	10	14.0	24.34 (0.33)	2.62 (0.14)
p-coumaroyl aspartate	H3	14.3	1.01 (0.02)	t
tetramer	11	14.5	5.67 (0.95)	–
epicatechin	12	15.3	25.65 (0.73)	3.30 (0.29)
trimer	13	16.2	12.71 (0.84)	t
tetramer	14	16.8	10.85 (0.86)	–
pentamer	15	17.3	4.82 (0.40)	t
caffeoyl 3-hydroxy-tyrosine	H4	17.6	0.71 (0.03)	t
dimer	16	20.1	7.19 (0.91)	t
caffeoyl tyrosine	H5	20.4	0.24 (0.01)	t
quercetin 3-hexoside	F1	20.9	0.25 (0.02)	t
quercetin 3-arabinoside	F2	22.35	0.30 (0.04)	–
p-coumaroyl tyrosine	H6	22.5	t	–
quercetin	F3	23.78	0.09 (0.01)	t
oligomers + polymers	17		8.96 (1.42)	13.93 (0.60)
total procyanidins			119.78 (7.69)	22.99 (1.08)

[a] Values are mg/g cocoa powder; t, detected but not quantified; –, not detected.

It is generally accepted that polyphenol bioavailability is relatively poor (*10*), but monomeric flavan-3-ols are among those polyphenols showing higher bioavailability. The bioavailability of cocoa flavonoids has been studied in a number of surveys, and the effect of milk in the chocolate preparation has shown contradictory effects (*11*, *12*). The absorption of dimers has been previously reported (*13*), and that of oligomers has been the object of controversy although recent studies show that apple procyanidin oligomers (up to pentamers) are absorbed in the rat (*14*). Other in vitro studies showed that procyanidin oligomers (dimers and trimers) passed through the human epithelial Caco-2 cell monolayer, whereas polymers did not (*15*).

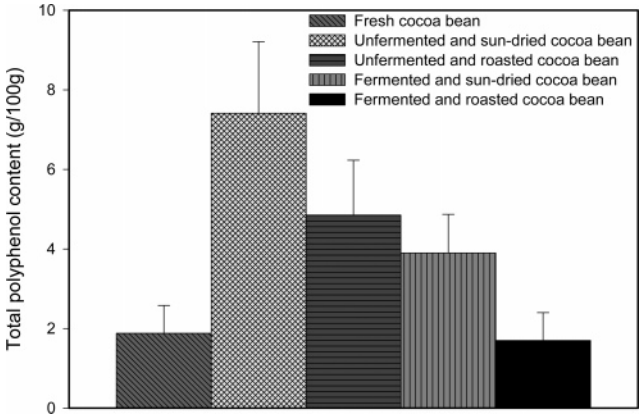

Figure 1. Total polyphenols content (g/100 g on dry basis and expressed as gallic acid equivalents) of cocoa beans obtained from different processes. Values are means of five geographical origins ± standard deviations. Means with different letters were significantly different at the 95% confidence level.

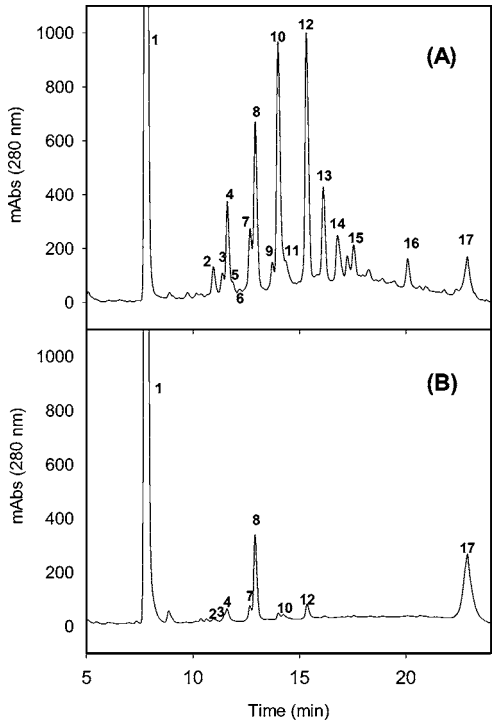

Figure 2. HPLC profile (280 nm) of flavonoid-enriched cocoa powder (**A**) and conventional cocoa powder (**B**). See **Table 2** for peak identification.

The present study was designed to prepare flavonoid-enriched cocoa powder through control of the processing steps, to characterize the flavonoid profile, and to assess its bioavailability in healthy humans.

MATERIALS AND METHODS

Solvents and Phenolic Standards. Standards of (+)-catechin, (−)-epicatechin, theobromine, and caffeine (Sigma, St. Louis, MO) were used for the quantitative determination. Organic solvents of high-performance liquid chromatography (HPLC)-grade were from Scharlab (Barcelona, Spain) and Merck (Darmstadt, Germany).

Raw Material. Unfermented and dried cocoa beans from the Ivory Cost (Forastero variety), Colombia (Amazon variety), Ecuatorial Guinea (Amazon Forastero variety), Ecuador (Amazon−Trinitario−Canelo Amazon hybrid), Venezuela (Trinitario variety), Peru (Criollo variety), and the Dominican Republic (Criollo variety) were evaluated (**Table 1**). Different types of beans were used to select the cocoa bean with

the highest total polyphenol as a raw material for the preparation of a polyphenol-enriched cocoa powder (**Figure 1**). Pods and unfermented sun-dried and fermented sun-dried cocoa beans were obtained from Guinea, the Ivory Coast, the Dominican Republic, Venezuela, and Peru. The pod samples were frozen until further analysis. The fresh beans were removed from the pods, and the polyphenol content was determined. The unfermented and fermented sun-dried cocoa beans were roasted in an oven at 150 °C for 30 min in the laboratory. The pods and unfermented sun-dried, unfermented roasted, fermented sun-dried, and fermented roasted cocoa bean samples from Guinea, the Ivory Cost, the Dominican Republic, Venezuela, and Peru were analyzed in triplicate.

Polyphenol Oxidase (PPO) Inactivation and Evaluation of Browning Degree. The PPO inactivation in fresh cocoa beans by applying heat treatment such as a blanching with water was approached. This previous assay aimed to evaluate the best conditions of time and temperature to inactivate PPO and hence to avoid the polyphenols oxidation. Different temperature (75, 85, and 95 °C) and time (5, 10, and 15 min) combinations were assayed to evaluate the impact on melanosis (browning degree). To interpret the browning degree visually, a color scale with values from 0 (absence of melanosis), 2 (slight, melanosis noticeable on some cocoa beans), 4 (slight, melanosis noticeable on most cocoa beans), 6 (moderate, melanosis noticeable on most cocoa beans), and 8 (heavy, melanosis noticeable on most cocoa beans) up to 10 (heavy, totally unacceptable) was used (*16*). To speed up the browning reaction, a few drops of catechol 1% (a PPO substrate) were added using a Pasteur pipet. Control samples were run for each heat treatment condition.

Flavonoid-Enriched Cocoa Powder Preparation. The cocoa powder was prepared as follows. A blanching treatment with water was applied to fresh cocoa beans from harvested and ripe cocoa pods that were obtained. The fresh cocoa beans were extracted manually from the cocoa pods, and the pulp was removed before the beans were thermally treated by blanching with water at an internal temperature of 95 °C for 5 min (to inactivate oxidative enzymes such as PPO). Afterward, the beans were dried in an oven at 40 °C until a moisture content of 5% was obtained. The dried beans were deshelled, milled, and partially defatted by Soxhlet extraction with petroleum ether, and the remaining powder was vacuum-dried and sieved.

Conventional Cocoa Powder. The powder with 10−12% of fat content (Granada brand) was a commercial cocoa powder produced and supplied by Natra Cacao S.L. (Valencia, Spain). This cocoa powder was produced from fermented and dried cocoa beans (Forastero variety from East Africa). The beans were roasted during the manufacturing process.

Total Polyphenols Determination. The milled cocoa samples (1 g) were extracted with 100 mL of acetone:water (70:30; v:v) under reflux at 60 °C for 2 h. Acetone was removed under vacuum at 30 °C. The total polyphenol content was determined by the spectrophotometric method of Folin−Ciocalteu (*17*). The results were expressed as gallic acid equivalents on a dry weight basis.

Polyphenol Extraction from Cocoa Powder and Cocoa Milk Drinks. Cocoa powder samples (1 g) were homogenized with a MS1 minishaker (IKA, Staufen, Germany) for 1 min with 20 mL of extraction solution (methanol:HCl; 99.9:0.1; v:v). Homogenates were sonicated in a bath type ultrasonic cleaner at 37 °C for 10 min and centrifuged at 4000*g* for 15 min in a Centromix centrifuge (Selecta, Barcelona, Spain). This procedure was repeated three times, and the supernatants were combined and concentrated under reduced pressure (35 °C) to remove methanol. The aqueous fraction was dissolved in 10 mL and passed through a Millex HV13 0.45 μm filter (Millipore, Bedford, MA). For milk drinks, the same procedure was used and 5 mL of milk drink was extracted with 5 mL of extraction solvent.

Crossover Experiment Intervention Design. To evaluate the effect on flavonoid bioavailability of both cocoa powder extracts, these were dissolved in semiskimmed milk and given to six healthy volunteers (five females and one male) on a randomized double-blind crossover study. The study conformed to the principles outlined in the "Declaration of Helsinki" and was approved by the Ethical Committee of the Universidad Católica, San Antonio (UCAM). The protocol was fully explained to the volunteers who gave their written consent prior to participation. Inclusion criteria were no smoking and a generally healthy status. Exclusion criteria involved a vegetarian diet, weight-reducing dietary regimen, alcoholism, consumption of vitamin supplements or nutraceuticals, diabetes, hyperlipidemia, cardiovascular disease, history of gastrointestinal disease, or any other chronic disease. The age of the volunteers ranged from 20 to 30 years. The volunteers followed a controlled diet in which catechin, epicatechin, and procyanidin-containing sources including grapes, cocoa-derived products, wine, and apples were strictly forbidden for 1 week before the intervention. The intake of the rest of polyphenol-containing sources (fruits, vegetables, tea, coffee, juices, and olive oil) was also restricted. In addition, the volunteers provided a diet history with the food and drinks consumed 1 week before and during the intervention.

Two drinks were prepared with semiskimmed milk (250 mL), one with the flavonoid-enriched cocoa powder (milk drink with powder A, MDA) and another one with the conventional cocoa powder (MDB). The identity of the drinks was unknown both to the volunteers and to the scientists in charge of this part of the study. The drink was prepared just before administration and ingested at room temperature, without applying any thermal treatment. Each drink contained 12 g of cocoa powder and 28 g of sugar according to other authors (*6*). MDA was prepared with 7 g of flavonoid-enriched powder plus 5 g of conventional cocoa powder while MDB was prepared with 12 g of conventional cocoa powder. Both powders were fully soluble in milk, and no residues remained in the drinking vessel. Volunteers ingested the drinks in the same place where the blood samples were collected. Blood samples were drawn before the intake of milk drinks (base blood) and 1, 2, and 3 h after the intake. In addition, volunteers collected 24 h urine volume before the intake of beverages (base urine), and 24 and 48 h after the intake (24 h volume urine in each case). Urine samples were collected in plastic bottles containing 1 g/L ascorbic acid and immediately stored at −70 °C until the analysis. The first group drank the MDA beverage, and the second group drank the MDB drink. Next, the crossover study was carried out after 2 weeks of washout. Then, the first group drank the MDB beverage, and the second group drank the MDA drinks (blood and urine samples were collected as described above).

Plasma Samples. Plasma samples were processed as previously reported (*18*). Briefly, plasma samples (1.5 mL) were mixed with 555 μL of a water solution containing ascorbic acid (0.2 g/L) and ethylenediaminetetraacetic acid (1 mg/mL). To break possible polyphenol−plasmatic protein linkages, 30 μL of *o*-phosphoric acid was added. Samples were homogenized and centrifuged in a Sigma 1−13 microcentrifuge (Braun Biotech. International, Melsungen, Germany) at 14000*g* at 4 °C. The supernatants were filtered over Oasis HLB 3 mL (60 mg) cartridges (Waters), and these were washed with distilled water and polyphenol metabolites eluted with methanol. The methanol fraction was taken to dryness under nitrogen flow at room temperature, and the extract was then redissolved in methanol−0.2 M HCl (50:50; v:v).

Urine Samples. Urine samples (10 mL) were directly filtered through reversed-phase C-18 Sep Pack cartridges (*19*) and washed with distilled water (30 mL), and the polyphenols were eluted with methanol (2 mL). The methanol extract was then taken to dryness under nitrogen flow at room temperature. The samples were then redissolved in 200 mM HCl in methanol (0.2 mL) and centrifuged. A sample of 100 μL of the supernatant was analyzed by HPLC−diode array detection−tandem mass spectrometry (DAD-MS/MS).

HPLC-MS/MS Analyses of Cocoa Procyanidins and Derived Physiological Metabolites. The HPLC system was equipped with an Agilent 1100 Series diode array and a mass detector in series (Agilent Technologies, Waldbronn, Germany). It consisted of a G1312A binary pump, a G1313A autosampler, a G1322A degasser, and a G1315B photodiode array detector controlled by a ChemStation software (Agilent, v. 08.03). Spectroscopic data from all peaks were accumulated in the range of 240−400 nm, and chromatograms were recorded at 280 nm. The mass detector was a G2445A ion-trap mass spectrometer equipped with an electrospray ionization (ESI) system and controlled by LCMSD software (Agilent, v. 4.1.). Nitrogen was used as the nebulizing gas at a pressure of 65 psi, and the flow was adjusted to 11 L/min for cocoa extract analyses and 25 psi and 7 L/min for plasma and urine metabolites analyses, respectively. The heated capillary was maintained at 355 °C. The full scan mass covered the range m/z 100−

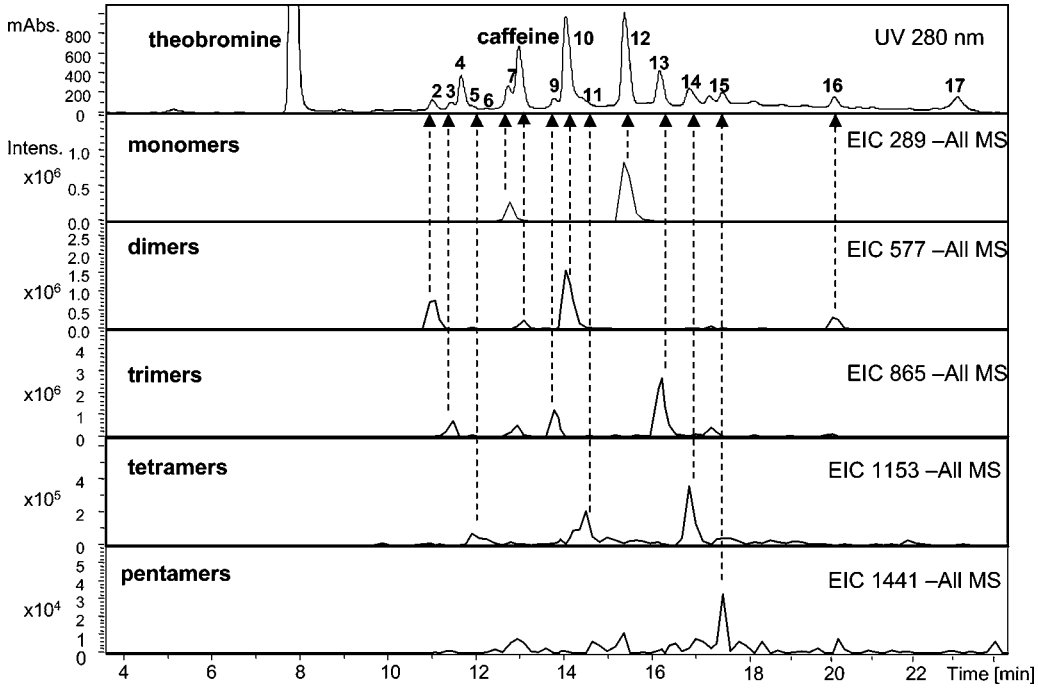

Figure 3. UV (280 nm) and extracted ion chromatogram of flavonoid-enriched cocoa powder showing flavanol-derived compounds.

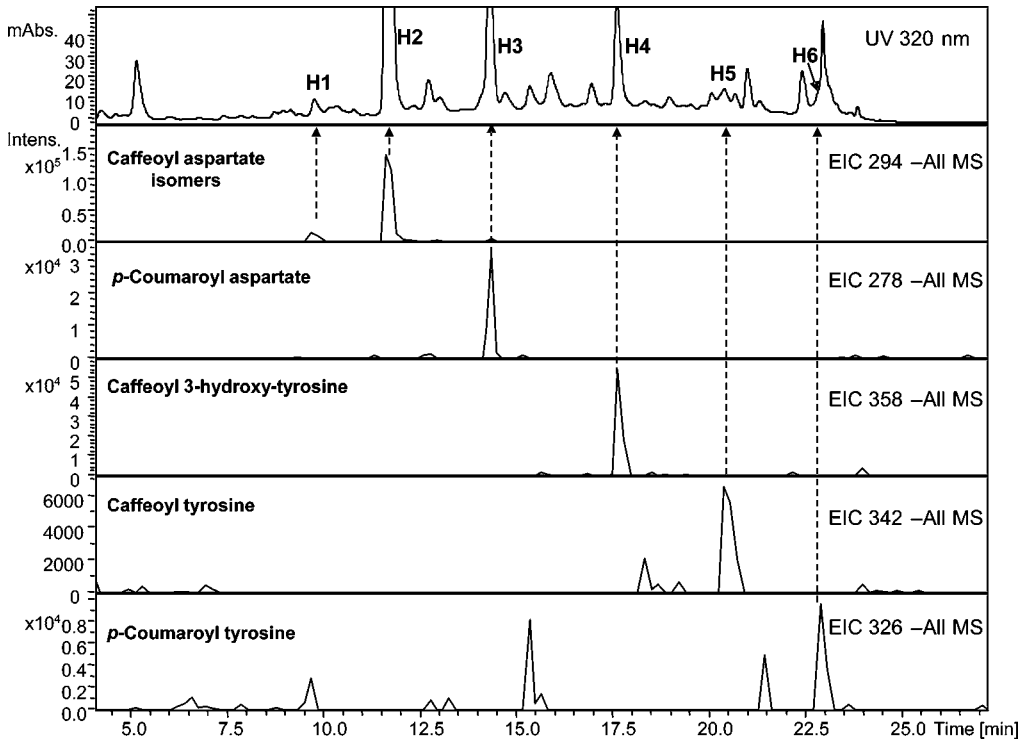

Figure 4. UV (320 nm) and extracted ion chromatogram of flavonoid-enriched cocoa powder showing caffeic acid derivatives. See **Table 2** for details.

1500. Collision-induced fragmentation experiments were performed in the ion trap using helium as the collision gas, with voltage ramping cycles from 0.3 up to 2 V. MS data were acquired in the negative ionization mode.

Cocoa Extract. Prior to the qualitative analysis of cocoa extract, epicatechin and catechin were characterized and the conditions were optimized for maximum sensitivity by LC-ESI-MS/MS ion trap mass spectrometry. Epicatechin and catechin showed in the negative mode the same deprotonated molecular ion $[M - H]^-$ at *m*/*z* 289. The ideal conditions for epicatechin analysis were obtained by direct infusion to the electrospray source of 20 μM epicatechin (600 μL/h) in conjunction with 1 mL/min 20% acetontrile/80% water−formic acid (95:5, v:v) isocratic flow (flow injection analysis, FIA). Then, the polyphenol extracts from cocoa powder and chocolate milk drinks were analyzed on the HPLC-DAD-MS/MS system using water−formic acid (95:5; v:v) (solvent A) and acetonitrile (solvent B). Elution was performed with a linear gradient of B in A starting from 6% B to reach 25% B after 18 min and 60% B at 20 min, maintaining the system isocratically in 60% B until 25 min, and then increasing up to 90% B at 26 min, remaining at 90% B until 32 min when the initial conditions were recovered. The system was left for 10 min under the initial conditions before a new injection for stabilization. A 250 mm × 4 mm i.d. Mediterranean Sea 5 μm, C-18 column (Tecknochroma, Barcelona, Spain) was used, and elution was with a solvent flow rate of 1 mL/min. Identification of polyphenols was achieved using their UV spectra, retention times, ion mass (MS), and MS/MS daughter fragments using

Table 3. HPLC-MS/MS Analysis of Hydroxycinnamic Acid Amides in Cocoa Powder

structure	no.	MS $[M-H]^-$	MS^{2-}	R_t (min)
N-[3′,4′-dihydroxy-(*Z*)-cinnamoyl]-L-aspartic acid	H1	294	276, 179, 132	9.7
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid	H2	294	276, 179, 132	11.6
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-aspartic acid	H3	278	235, 163, 132	14.3
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-3-hydroxy-L-tyrosine	H4	358	313, 222, 178, 161	17.6
N-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-tyrosine	H5	342	222, 206, 178, 163, 135, 119	20.4
N-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine	H6	326	282, 206, 188, 163, 147	22.9

external standards (when available). Flavan-3-ols and procyanidins were quantified as catechin at 280 nm, and flavonols and hydroxycinnamic acid derivatives were quantified as rutin and chlorogenic acid, respectively, at 320 nm. Theobromine and caffeine were quantified at 275 nm using the corresponding commercial standards.

Plasma and Urine Analyses. Chromatographic separations were carried out on the same 250 mm × 4 mm i.d. Mediterranean Sea 5 μm, C-18 column (Tecknokroma) protected with a 4 mm × 4 mm Mediterranean Sea guard column, with 1% formic acid (A) and acetonitrile (B) as solvents, starting with 4% B and using a gradient to obtain 25% B at 18 min, 60% at 20, 60% at 25 min, and 90% B at 26 min. The flow rate was 0.5 mL/min, and the injection volumes varied between 10 and 90 μL.

As described for the cocoa extract, physiological polyphenol metabolites (epicatechin glucuronide and epicatechin sulfate) were optimized for maximum detection by LC-ESI-MS/MS using FIA of the urine methanolic extract from a volunteer who ingested MDA. FIA of the urine extract allowed us to optimize the detection of procyanidin metabolites despite the lack of commercially available standards. The different ions were selected using FIA, and different analytical conditions were applied to optimize the detection. The total chromatogram time was divided in segments to set the ideal conditions for each compound (segment 0−12 min for epicatechin, 12−17 min for catechin, 17−22.5 min for epicatechin glucuronide, and 22.5−40 min for epicatechin sulfate). The quantitation of the peaks was carried out by the selected monitoring ion mode. The final LC-MS/MS analyses were performed the same day using the autosampler to avoid fluctuation in the ionization of the equipment.

Statistics. Data obtained from **Figure 1** were analyzed using the Statgraphics plus software version 5.1. Statistical significance was assessed by analysis of variance (ANOVA), and significant differences between means were confirmed using Duncan's multiple range test at the 95% confidence level. Data from **Figures 7** and **9** were analyzed using one-way ANOVA followed by Dunnett's multiple comparisons post test. Data were deemed significant at $p < 0.005$.

RESULTS AND DISCUSSION

Preparation of Flavonoid-Enriched Cocoa Powder. The polyphenol contents of seven samples of cocoa beans from different geographical origins and belonging to six different botanical varieties were evaluated (**Table 1**). The CCN51 clone from Ecuador had the highest total polyphenol content while the Criollo variety from the Dominican Republic had the lowest. As indicated by its high total polyphenol content, the hybrid clone CCN51 from pods harvested from the Quevedo region in Ecuador was selected for the preparation of flavonoid-enriched cocoa powder. This triple hybrid came from a cross of the Amazonic IMC67 clone with the Trinitary ICS95 clone, which at the same time has been crossed with the genotype Canelo Amazonic, from the Quevedo area.

The astringency of cocoa beans, due to their high procyanidin content, has been previously reported as a potential negative sensorial factor. In this context, the decrease of the astringency of cocoa beans by adding external PPO has been previously proposed (*20*). However, because of the increasing interest to preserve the content of health beneficial compounds, our aim was to maintain the flavonoid content as high as possible. The enzyme PPO is the key enzyme in the melanogenesis pathway in fruits and vegetables giving rise to the often undesired so-called "enzymatic browning" (*21*). A major role of PPO to promote browning in cocoa beans cannot be ruled out since catechins, epicatechins, and anthocyanins are PPO substrates. For this purpose, different temperature−time combinations were assessed to evaluate the impact on browning degree and hence select the best conditions to decrease the oxidation reactions and enzymatic polymerization. Whereas long treatments at 95 °C promoted nonenzymatic browning, a blanching treatment and an internal temperature of 95 °C for 5 min were the optimal conditions where the lowest enzymatic browning of the fresh beans occurred. The high temperature needed could be justified by the relatively high thermal stability previously reported for isolated cocoa bean PPO (*22*).

Table 4. Polyphenol and Purine Content of the Prepared Cocoa Milk Drinks

peak	compound	MDA (mg/250 mL)	MDB (mg/250 mL)
1	theobromine	182.89 (29.20)	230.61 (0.87)
2	B1	16.40 (2.65)	t
4 (H2)	caffeoyl aspartate	17.64 (0.44)	2.26 (0.03)
7	catechin	14.67 (1.31)	t
8	caffeine	28.43 (4.60)	21.71 (0.32)
9	trimer	24.20 (1.14)	
10	B2	68.79 (1.56)	15.25 (0.25)
11	tetramer	17.62 (0.19)	
12	epicatechin	138.15 (2.64)	27.85 (0.10)
13	trimer	54.62 (1.74)	t
14	tetramer	45.52 (5.97)	t
15	tetramer	15.25 (0.52)	t
16	dimer + trimer + tetramer	25.29 (1.46)	
17	oligomers + polymers	106.86 (11.21)	164.45 (1.24)
	total procyanidins	527.37	207.55

Data for total polyphenol content from different types of cocoa beans are represented in **Figure 1**. According to the results, significant differences in total polyphenol content were observed between unfermented sun-dried cocoa beans and fermented sun-dried cocoa beans. In addition, there was a significant difference in total polyphenol content between unfermented sun-dried cocoa beans and unfermented roasted cocoa bean and between fermented sun-dried cocoa bean and fermented roasted cocoa bean. Consequently, the fermentation and roasting processes resulted in a significant decrease of total polyphenol content. Therefore, the nonfermented cocoa bean without roasting was selected as a raw material for the preparation of the flavonoid-enriched cocoa powder.

Characterization of the Obtained Flavonoid-Enriched Cocoa Powder. Phenolic compounds were characterized using HPLC-DAD-MS/MS methods. A quick survey of the UV spectra of the different phenolics in the extracts showed that three different groups of compounds were present in significant amounts: procyanidins, with UV maxima (λ_{max}) around 280 nm, hydroxycinnamates at around 320 nm, and flavonols at 350 nm. In addition, theobromine and caffeine were observed with

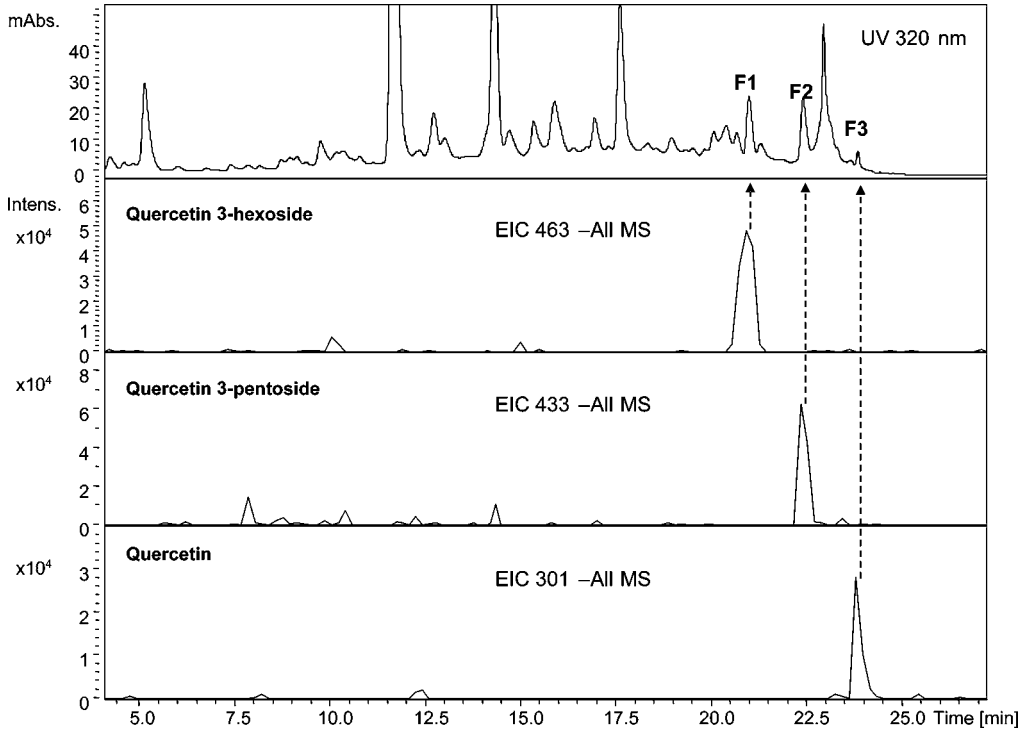

Figure 5. UV (320 nm) and extracted ion chromatogram of flavonoid-enriched cocoa powder showing flavonol derivatives. See **Table 2** for details.

UV λ_{max} around 275 nm. Thus, an HPLC UV chromatogram at 280 nm reflected mainly the procyanidin and xanthic base profile while that at 320 nm showed hydroxycinnamates and flavonols. In the extracts, theobromine (**1**) and caffeine (**8**) were easily identified by their UV spectra (λ_{max} 274 nm) and by HPLC-MS/MS in the positive mode (theobromine $[M + H]^+$ *m*/*z* 181 and caffeine $[M + H]^+$ *m*/*z* 195).

Catechin (**7**), epicatechin (**12**) as flavan-3-ol monomers, four dimers [procyanidin B1 (**2**) and B2 (**10**) and two unidentified dimers (**8** coeluting with caffeine and **16**)], three trimers (**3**, **9**, and **13**), four tetramers (**5**, **6**, **11**, and **14**) and one pentamer (**15**; other minor pentamers were detected but not quantified) were detected and quantified (**Table 2** and **Figure 2**). The HPLC-MS/MS analyses with the extracted ion chromatogram for each parent ion showed which peak in the HPLC chromatogram corresponded to every type of compound (monomers, dimer, trimers, etc.) (**Figure 3**). The fragments obtained after the MS/MS analyses confirmed the nature of these compounds in agreement with previous reports (*22*−*24*). Thus, dimers showed $[M - H]^-$ ions at *m*/*z* 577, consistent with catechin and/or epicatechin-based dimers, and all four compounds of this type detected gave the same fragment ions under the MS^2 experiments (559, 451, 425, 407, 289, and 287). Trimers showed $[M - H]^-$ ions at *m*/*z* 865 (three molecules of catechin and/or epicatechin), and all of them gave similar fragment ions under the MS^2 experiment (847, 739, 713, 695, 577, 451, 425, 407, 287, and 243). In the same way, all tetramers ($[M - H]^-$ at *m*/*z* 1153) showed similar fragment ions (*m*/*z* 1136, 1027, 1001, 983, 908, 866, 865, 813, 739, 695, 575, and 425). All of these fragments confirmed the nature of the main procyanidins present in cocoa.

Several compounds with characteristic UV spectra of caffeic acid derivatives (λ_{max} 289 sh, 325 nm) were also detected in the chromatograms at 320 nm (**Figure 4**). None of these compounds coincided in retention time and MS/MS spectra with those of an authentic marker of chlorogenic acid or related hydroxycinnamoyl quinic or hydroxycinnamoyl glucose derivatives. The fragments obtained from the isolated ions after MS/MS of the main compound (**4**; **H2**) coincided with those recently reported for *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-L-aspartic acid (*25*) and allowed the identification of the rest of the hydroxycinnamoyl derivatives in the cocoa powder extract (**Figure 4**). In addition, the MS/MS analyses allowed the characterization of the rest of hydroxycinnamic derivatives as hydroxycinnamic acid amides (**Table 3**). These were combinations of caffeic and *p*-coumaric acids with aspartic acid, tyrosine, and 3-hydroxytyrosine. In the previous report (*25*), the content of these hydroxycinnamic acid derivatives in chocolate or other related food products was not reported. This is therefore the first evaluation of the content of these compounds in cocoa powder and cocoa milk drinks.

In addition, three flavonoids were identified as quercetin 3-hexoside (probably glucoside), quercetin 3-arabinoside (*26*), and quercetin aglycone (**Table 2**) and were easily observed in the HPLC chromatograms recorded at 320 nm (**Figure 5**). The extracted ion chromatograms recorded in the HPLC-MS/MS analyses at the M−H values for quercetin 3-hexoside (*m*/*z* 463), quercetin 3-pentoside (*m*/*z* 433), and quercetin aglycone (*m*/*z* 301) are shown in **Figure 5**, and the nature of the glycosides was confirmed by HPLC-MS/MS of the isolated ions that rendered the corresponding quercetin aglycone at *m*/*z* 301.

Comparison of Conventional and Flavonoid-Enriched Cocoa Powders. Cocoa powders [flavonoid-enriched (A) and conventional (B)] were extracted and analyzed by HPLC-DAD-MS/MS. The phenolic compounds described above were detected in both cocoa powders although the quantification of some compounds was not possible in the conventional cocoa powder due to the small amounts present (**Figure 2**).

The phenolic content of the conventional powder B was much smaller than that of A (**Table 2** and **Figure 2**). Regarding flavonols, only quercetin 3-hexoside and quercetin aglycone were detected in the HPLC-MS/MS analysis (extracted ion chromatogram) of B while quercetin 3-arabinoside was not detected at all. Their quantification was not possible due to the small amount present that prevented their quantification in the

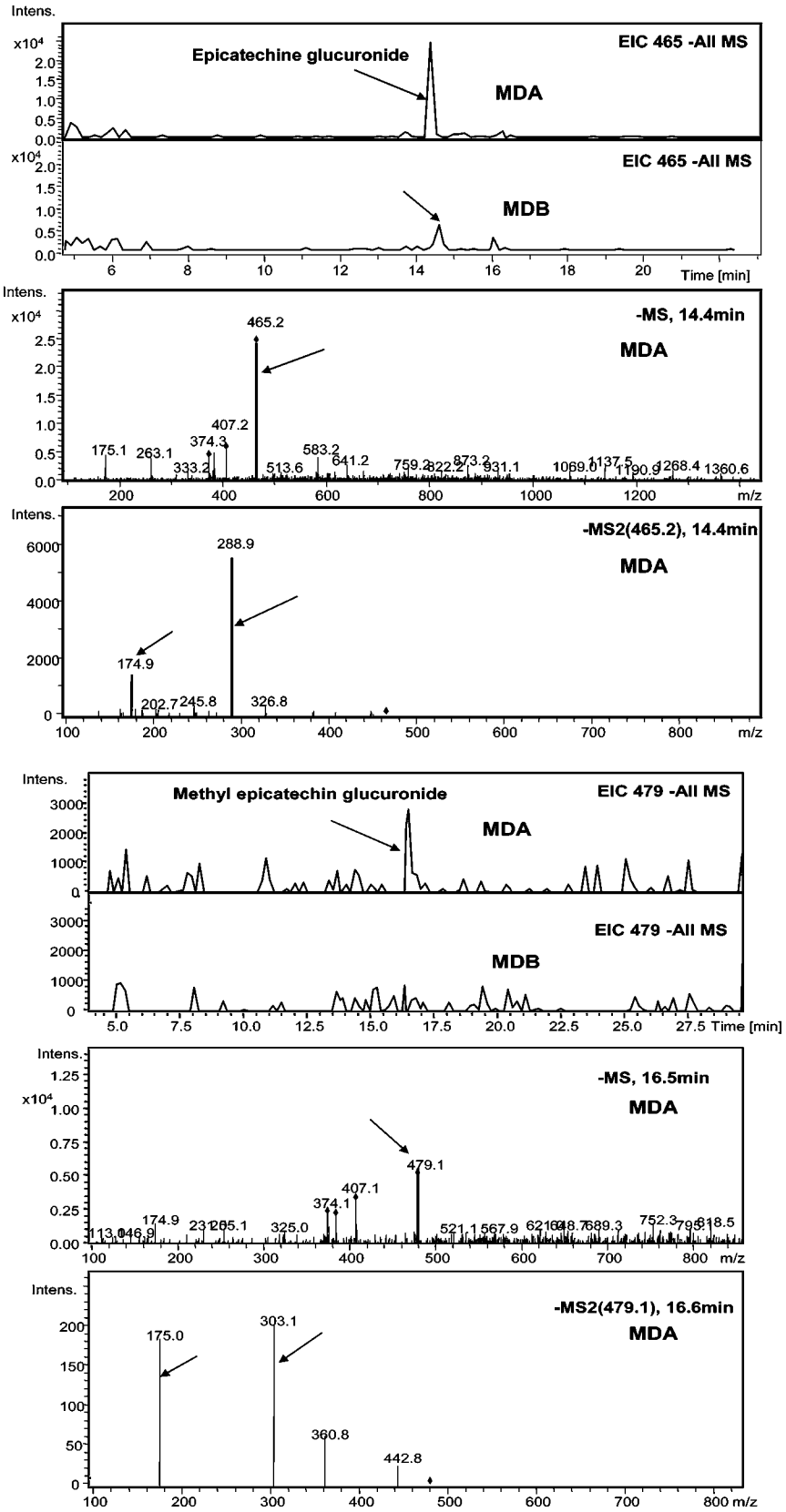

Figure 6. LC-MS/MS analysis of plasma samples from the same volunteer after drinking either MDA or MDB showing the main glucuronide derivatives (epicatechin glucuronide and methyl epicatechin glucuronide).

UV chromatograms. The relative amounts of 3′,4′,- and 4′-hydroxy-(*E*)-cinnamoyl-L-aspartic acid in cocoa powders A and B showed that the former was relatively more degraded during the cocoa powder manufacturing in the conventional cocoa powder, while it was better preserved in A (**Table 4**).

The theobromine content of both cocoa powders was smaller in powder A, while the caffeine content was two-fold higher in the powder A. However, the most significant differences were found in the phenolic composition, particularly in the flavan-3-ol content (**Table 2**). Thus, cocoa powder A contained four

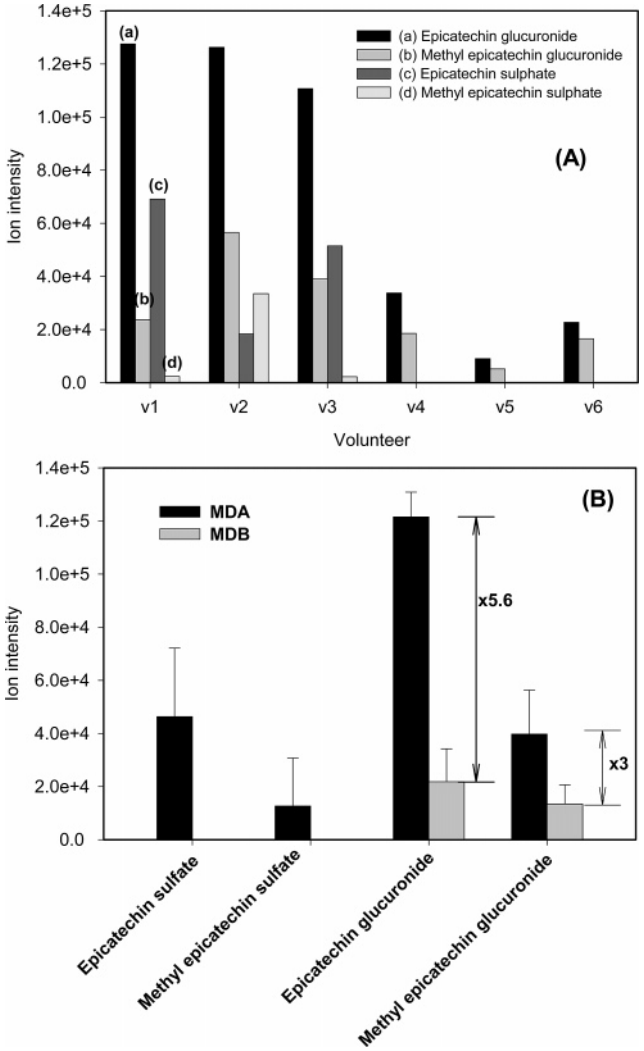

Figure 7. (**A**) Ion intensity of procyanidin metabolites (the sum of the values obtained in the three sampling times) in plasma after MDA (v1–v3) or MDB (v4–v6) consumption. (**B**) Mean ion intensity of procyanidin metabolites in plasma of volunteers upon drinking MDA or MDB. Values between brackets represent the increase (-fold) of each metabolite when comparing MDA and MDB consumption.

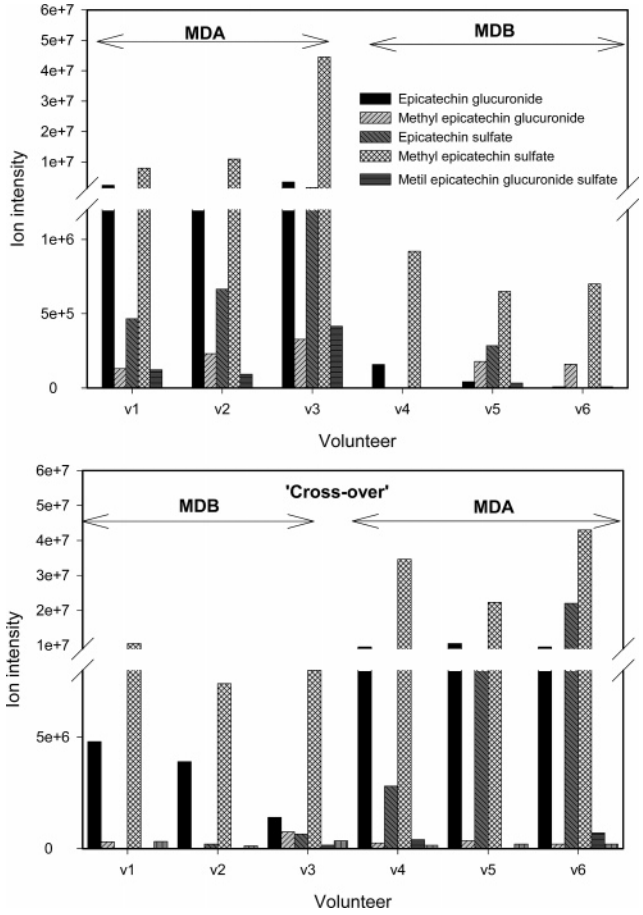

Figure 8. Individual urine excretion of procyanidin-derived metabolites (the sum of the two sampling times) upon consumption of cocoa milk drinks.

times more procyanidins than the conventional cocoa powder B and the difference was even higher when flavan-3-ol monomers were compared, as A contained eight times more epicatechin or procyanidin B2 than B. The content of theobromine was higher in the conventional cocoa than in the flavonoid-enriched powder, while caffeine was present in smaller amounts in the conventional powder. This could be explained by the higher degree of fermentation in the conventional cocoa powder, as fermentation can lead to a decrease in caffeine and an increase in theobromine, in a similar manner to what has been observed during black tea fermentation that transforms caffeine in theophylline (*27*). A similar mechanism could be envisaged for the changes occurring during the fermentation in both products as caffeine (*N*,*N*-dimethyl-xanthine) can be transformed, losing one methyl or another leading to theophylline in tea and theobromine in cocoa.

With these cocoa powders A and B, cocoa milk drinks were prepared as indicated above, and then, the polyphenols were extracted and analyzed by HPLC-MS/MS to evaluate the phenolic intake in the human intervention assay. As could be expected, both drinks had a similar phenolic profile, with approximately similar contents of theobromine and caffeine, but the cocoa milk drink (MDB) prepared with conventional cocoa powder had a smaller phenolic content (**Table 4**).

Bioavailability Study. Both MDA and MDB drinks were fully accepted by volunteers regarding their sensory properties (color, taste, etc.; results not shown). In the plasma samples, polyphenols present in the ingested drink and their conjugated metabolites were searched. Thus, *O*-methyl epicatechin (m/z^- 303), epicatechin-*O*-glucuronide (m/z^- 465), *O*-methyl epicatechin-*O*-glucuronide (m/z^-, 479), epicatechin sulfate (m/z^-, 369), *O*-methyl epicatechin sulfate (m/z^-, 383), epicatechin-*O*-glucuronide sulfate (m/z^-, 545), and *O*-methyl epicatechin-*O*-glucuronide sulfate (m/z^-, 559) were searched using the extracted ion chromatogram mode. The plasma chromatograms at 280 nm showed that the main compounds associated with the intake of the cocoa milk drinks were theobromine and caffeine. The amount of the xanthic bases (methylated purines) detected in plasma after the intake of both the conventional cocoa powder drink (MDB) and the flavonoid-enriched drink (MDA) was in the same range, confirming that the intake of cocoa was similar after the intake of both drinks and that the increase observed in the content of specific phenolic metabolites in plasma was not due to a higher intake of cocoa but to a higher intake of polyphenols in the ingredient used for the preparation of the cocoa milk drink.

The chromatogram at 280 nm (the maximum wavelength for procyanidins and their conjugated metabolites) did not show any evident peak corresponding to procyanidin metabolites (results not shown). This is not unexpected due to the poor response factor of procyanidins under UV detection. After the MS and MS/MS analyses, a peak for epicatechin-*O*-glucuronide

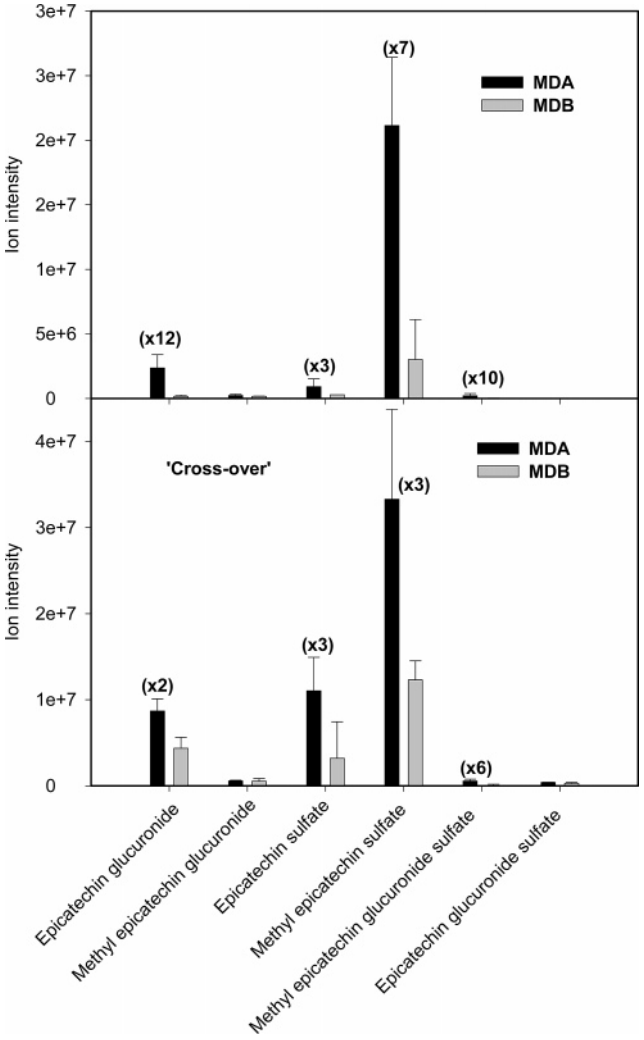

Figure 9. Mean ion intensity of procyanidin metabolites (the sum of the two sampling times) in urine of volunteers upon drinking MDA or MDB. Values between brackets represent the increase (-fold) of each metabolite when comparing MDA and MDB consumption.

was detected, and this was identified in the UV chromatogram as a small shoulder behind one peak not associated with the cocoa drink intake. This prevented the quantification of the metabolites in plasma using UV detection. The MS/MS analyses using the extracted ion chromatogram mode allowed the detection of one peak for epicatechin glucuronide (EIC, m/z^- 465) and another for methyl epicatechin glucuronide (EIC, m/z 479). After ion trapping and fragmentation, the fragments for epicatechin, methyl epicatechin, and glucuronic acid were detected (**Figure 6**) and confirmed the nature of the plasma metabolites.

The main procyanidin metabolites detected in plasma after the milk cocoa drink intake were epicatechin glucuronide, methyl epicatechin glucuronide, epicatechin sulfate, and methyl epicatechin sulfate; the glucuronides were present in larger amounts than the sulfates. Other complex metabolites, such as epicatechin glucuronide sulfate and methyl epicatechin glucuronide sulfate, were detected in samples from some volunteers that drank the flavonoid-rich drink but as trace quantities that prevented the correct integration after the extracted ion chromatogram analysis. The volunteers that ingested the cocoa milk drink containing the flavonoid-enriched ingredient showed a higher content of flavonoid metabolites in plasma than the conventional cocoa drink (**Figure 7A**). The quantification after enzymatic hydrolysis to yield the aglycones was not possible due to the poor ionization of the aglycone; therefore (due to the lack of available standards), the relative quantitation of the metabolites present in plasma after the intake of both drinks was achieved using the intensity of the different isolate ions, which showed a higher plasma concentration in those volunteers that had ingested the flavonoid-enriched cocoa drink (MDA) (**Figure 7B**). The accumulation of epicatechin glucuronide in those volunteers that ingested the flavonoid-enriched drink was approximately five-fold than that of the regular drink and three-fold in the case of methyl epicatechin glucuronide. The sulfated metabolites were not detected in volunteers that had taken the MDB cocoa drink, probably because these metabolites were present at concentrations below the detection limit.

From the pharmacokinetic point of view, the different metabolites were evaluated in plasma just before the cocoa drink intake and 1, 2, and 3 h after the intake (results not shown). This study showed that the maximum plasma concentration was reached between 1 and 2 h after the intake, depending on the individual, in accordance with previous reports (*4*). The total clearing of the metabolites was not always observed after 3 h. As could be expected, a relevant interindividual variability was observed.

When the urine samples were analyzed, a similar behavior was observed. The main metabolites observed in this case were epicatechin sulfate, methyl epicatechin sulfate, and epicatechin glucuronide (**Figure 8**). The highest excretion of these compounds occurred after 24 h of the intake (results not shown), suggesting that the enterohepatic circulation occurred and that the late metabolism in the liver produced mainly sulfate conjugates, while the early metabolism (1 and 2 h) produced mainly the glucuronide and methyl ether conjugates in the cells of the gastrointestinal tract. Our results confirm those previously reported by Roura et al. (*18*) and Baba et al. (*28*) who detected the sulfated derivatives as the most abundant metabolites in urine upon cocoa consumption. The metabolite content present in the urine of volunteers after the intake of flavonoid-enriched drink was always higher than that of the volunteers that had the conventional drink (from three- to 12-fold depending on the metabolite), and this difference was also observed in the crossover experiment (**Figure 9**), which indicated that despite the interindividual variability, the procyanidins bioavailability upon consumption of MDA was always higher.

All of these studies confirm that through improved processing, the flavonoid composition of cocoa powder can be enhanced (mainly flavanol monomers and dimers) and that this enhancement leads to an increase in the cocoa flavonoid metabolites present in plasma and in urine showing a higher bioavailability. Taking into account the poor bioavailability of oligomeric procyanidins, both the higher content in plasma and the higher urine excretion of epicatechin-derived metabolites upon consumption of MDA seems to be mainly related with the higher flavonoid monomer content of cocoa powder A. Previous reports have linked the cardiovascular beneficial effects of cocoa consumption with the accumulation of procyanidin metabolites in plasma (*4*). In this context, a higher health beneficial effect upon the intake of a cocoa-derived product based on a process that preserves flavonoid content cannot be ruled out.

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