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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).