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Human urine: Epicatechin metabolites and antioxidant activity after cocoa beverage intake

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Abstract
Associations between cocoa consumption in humans, excreted metabolites and total antioxidant capacity (TAC) have been scarcely investigated. The aims of the study were to investigate the epicatechin ((−)-Ec) metabolites excreted in urine samples after an intake of 40 g of cocoa powder along with the TAC of these urine samples and the relation between both the analyses. Each of the 21 volunteers received two interventions, one with a polyphenol-rich food (PRF) and one with a polyphenol-free food (PFF) in a randomized cross-over study. Urine samples were taken before and during 24 h at 0–6, 6–12 and 12–24 h periods after test intake. The excreted (−)-Ec metabolites and the TAC were determined in urine samples by LC-MS/MS and TEAC assay, respectively. The maximum excretion of (−)-Ec metabolites and the maximum TAC value were observed in urine samples excreted between 6 and 12 h after PRF consumption. Significance of TAC increase was found in urine samples excreted during 0–6 and 6–12 h (66.6 and 72.67%, respectively, with respect to the 0 h).

Keywords: *(+)-Epicatechin metabolites, cocoa powder, flavonoids, TEAC, antioxidant activity, urine*

Abbreviations: *(−)-Ec, (−)-epicatechin; (−)-Ec-G, (−)-epicatechin glucuronide; (−)-Ec-S, (−)-epicatechin-sulphate; TAC, total antioxidant capacity; TEAC, trolox equivalent antioxidant capacity*

Introduction

The free radicals produced in the body are toxic and, if not removed or neutralized, they react with lipids, proteins, carbohydrates and nucleic acids, causing cellular damage and excessive oxidative damage, which eventually results in cell death [1,2]. The oxidative damage accumulated during the life cycle has been proposed to play a key role in the development of age-dependent diseases such as cancer, arteriosclerosis, arthritis, neurodegenerative disorders and other conditions.

There exist some preventive and defensive systems against the attack of reactive substances in the human organisms. However, these systems cannot completely eliminate the harmful activities of such substances, particularly when their production is increased in some metabolic, physiological, pathological and other processes. The most efficient non-enzymatic antioxidants include radical scavengers (vitamin C and E), carotenoids, thiols (glutathione, thioredoxin and lipoic acid), natural flavonoids, a hormonal product of the pineal gland, melatonin, antioxidant enzyme cofactors (selenium, coenzyme Q) and other

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compounds [3,4]. An adequate intake of natural antioxidants in food is, therefore, of great importance for the protection of macromolecules against oxidative damage in cells (mainly unsaturated fatty acids in lipids, cholesterol, different functional polypeptides and proteins and nucleic acids) [5].

Polyphenols, especially flavonoids, are one of the most important groups of natural antioxidants that are consumed in the diet. Flavonoids have two aromatic end rings (an A-ring and a B-ring) at the ends bound by an oxygenated heterocycle in the middle with another ring (C-ring), an arrangement which promotes free radical scavenging. *In vitro* assays with these compounds showed a strong antioxidant activity, mainly due to the low redox potential of the molecules and their capacity to donate several electrons or hydrogen atoms [6]. Specifically, the presence of the catechol or dihydroxylated B-ring allows rapid donation of a hydrogen (electron) for the stabilization of radical species. This is considered the most important structural feature defining the "classical" antioxidant nature of flavonoids [7]. Structure-activity studies also show that flavonoids inhibit key enzymes such as NAD(P)H-oxidase (a major source of endogenous free radicals), tyrosine kinase and protein kinase based on varied hydroxylation/methylation patterns [5,8].

Phenolic compounds acting as antioxidants may also function as terminators of free radical chains and as chelators of redox-active metal ions that are capable of catalysing lipid peroxidation. They also interfere with the oxidation of lipids and other molecules by the fast donation of the hydrogen atom to the radicals. Since, the phenoxyl radical intermediates are relatively stable, they do not initiate (propagate) further radical reactions. Moreover, they act as terminators of the reaction chain by interacting with other free radicals [3].

Cocoa and cocoa-derived products have shown themselves to be a great source of flavonoids, mainly epicatechin and catechin monomers and their polymeric procyanidins. The role of chocolate flavonoids in the prevention of LDL oxidation was described for the first time in 1996 [9]. Since then, a large number of studies have shown favourable changes in oxidative measurements in plasma following cocoa or chocolate consumption [10–17].

Trolox-equivalent antioxidant capacity value (TEAC) assay based on the ability of the antioxidants to scavenge the long-life radical cation $ABTS^+$ is one of the most common methods used to determine the antioxidant activity of a given compound [18,19].

The TEAC assay was used due to its operational simplicity and because it was a method proposed by Prior et al. [20] to be standardized for use in a routine quality control for TEAC determination in many compounds and food samples in research laboratories. TEAC is not affected by ionic strength, so it can be used in different media to determine antioxidant capacities of extracts and body fluids. Furthermore, ABTS radical use is not apparent in mammalian biology and thus represents a "non-physiological" radical source, thus it can be used in urine with good results as was described in the previous studies [21]. Thermodynamically, a compound can reduce $ABTS^+$ if it has a redox potential lower than that of ABTS (0.68 V). Many phenolic compounds have low redox potentials and thus can react with $ABTS^+$ [20].

Flavonoids are extensively metabolized *in vivo*, resulting in a significant alteration in their redox potentials. It has become clear that the bioactive forms of flavonoids *in vivo* are not those found in plants; thus, their beneficial actions could be affected during their metabolism. The objectives of this study were to determine the (−)-epicatechin metabolites excreted in human urine after the intake of a standard portion of cocoa beverage and also to study the effect of these metabolites on the total antioxidant activity (TAC) of these urine samples, thereby providing the opportunity to analyse whether all the conjugations that cocoa flavonoids undergo during their metabolism affect their bioactivity and hence their beneficial effects, especially their antioxidant activity.

Materials and methods

Materials

Reagents were obtained from the following sources: methanol and acetonitrile (HPLC grade) from Scharlau (Barcelona, Spain), *o*-phosphoric acid from Panreac (Barcelona, Spain) and formic acid from Sigma (Steinheim, Germany). Standards were obtained as follows: (−)-epicatechin from Sigma (St Louis, MO, USA) and taxifolin from Extrasynthese (Genay, France), creatinine from Fluka (Seelze, Germany), ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt and potassium persulphate (dipotassium peroxydisulphate) from Sigma (St Louis, MO, USA). Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) and phosphate buffered saline, pH 7.4, (PBS) from Aldrich Chemical (Steinheim, Germany).

All the chemicals used were of analytical or chromatographic grade. The water was purified in a Milli-Q water purification system (Millipore, Molsheim, France). Working standard solutions were filtered with waters 4 mm PTFE 0.45 μm (Mildford, MA, USA) before being injected into the column. The extraction cartridges were Waters Oasis™ HLB 3 cm^3 (60 mg) (Mildford, MA, USA).

Subjects

Twenty-one non-smoking healthy volunteers (nine women and 12 men) aged between 18 and 50 years

with a body mass index (BMI) average of 21.59 ± 2.1 (range 19.1–27.7) were selected. None of them reported any history of heart disease, homeostatic disorders or other medical disease. None of the subjects were receiving any medication or taking any vitamin supplements. The Institutional Review Board of the Hospital Clinic, Barcelona, approved the study protocol and all the volunteers gave written informed consent before their inclusion in the trial.

Test meals

Two different test meals were used to study the cocoa (−)-Ec metabolites excreted in urine. All the volunteers were submitted to both the interventions in a random order: (a) polyphenol rich meal (PRM); cocoa beverage containing 40 g of cocoa powder (Nutrexpa, Spain) and 250 ml water, and (b) polyphenol free food (PFF); 250 ml of whole milk as a control. Milk is a PFF and, unlike water, it provides calories from its sugar, fat and proteins. To avoid variations in the results of the present study, we used whole milk as a control due to the following reasons: (a) whole milk provides the same amounts of calories as test meal CC-M; (b) it avoids variations in the rate of stomach emptying, (c) to give the subjects the same volume of liquid as test meals and (d) to avoid keeping the subjects from fasting for long hours.

The cocoa powder administered in this study had the following composition: 47 g/100 g of carbohydrates (sucrose, 46 g/100 g; starch, 1 g/100 g), 16 g/100 g of fibre, 5.4 g/100 g of fat, 70.5 mg/100 g of (−)-Ec, 63.75 mg/100 g of procyanidin B_2, 21 mg/100 g of catechin and 5 mg/100 g of flavonols, including isoquercitrin, quercetin, quercetin-3-glucoside and quercetin-3-arabinoside. Phenolic compounds were not detected in the milk that was used as a control meal.

Study design

The study was an open, prospective, randomized and crossover trial. The participants were instructed to abstain from vitamin supplements, drugs, alcoholic beverages and any polyphenol-rich foods (PRF) for at least 48 h before and during the day of the study. A list delineating the allowed and the forbidden foods and two menus were given to all the participants to help them to follow correctly the polyphenol-free diet the day before the study. Subjects were made to fast for at least 8 h before test meal consumption.

On two different days (with a week in between), following a crossover experimental design, the 21 subjects were submitted to two different treatments (PRF and PFF). Urine samples were obtained before and during 6, 12 and 24 h after the consumption of the test meals. All subjects were given a light meal consisting of bread and cheese 4 h after the consumption of the beverage. The volunteers remained in the experimental clinical ward during the first study period (>6 h) to avoid the possibility of transgressing the prescribed diet. For the remaining 18 h, the all volunteers followed a standardized free polyphenol diet (as the day before the study).

Urine metabolites determination by LC-MS/MS analysis

Urine samples were analysed after collection and storage at −80°C as described in a previous study [22] with a few modifications. In brief, 1 ml of the homogenized urine was submitted to a solid phase extraction procedure (SPE) with an Oasis® HLB 96-well SPE plate (30 mg) (Waters, Mildford, MA, USA), preconditioned with 1 ml of methanol and equilibrated with 1 ml 1.5 M formic acid. The plate was washed with 1 ml 1.5 M formic acid and 1 ml of methanol in water (5%). The analytes were collected in a 96-well collection plate by elution with 1.5 ml of methanol containing 1 ml/l formic acid. The eluate was evaporated to dryness using a Techne sample concentrator (Duxford, Cambridge, UK) at 30°C under a nitrogen stream. The residue was reconstituted with an aliquot of 100 μl of taxifolin dissolved in a mobile phase as an additional Internal Standard to assess the performance of the mass spectrometer. It was then vortexed briefly and left in a refrigerated autosampler for LC-MS/MS analysis.

A triple-quadrupole mass spectrometer was used for the analysis and quantification of (−)-Ec metabolites. LC analyses were performed using a Perkin–Elmer series 200 (Norwalk, CT, USA) with a quaternary pump. An API 3000 triple-quadrupole mass spectrometer (Perkin–Elmer Sciex, Concord, ON, Canada) equipped with a TurboionSpray source in negative ion mode was used to obtain the MS and MS/MS data. The column that was selected for the analyses was a Luna C18 column (50 × 2 mm i.d., 5 μm) (Phenomenex, Torrance, CA, USA).

The MS/MS analyses are rigorous and described in detail in a previous study [22]. Briefly, a urine sample from 6 h after the cocoa beverage intake was investigated using LC-MS/MS in MRM mode to check the traces of all cocoa (−)-Ec metabolites described in the literature, to identify the metabolites and to establish their retention time (RT). Once identified, the urine metabolites they were confirmed in a second experiment, a product-ion scan (PIS) which only confirmed the sulphates (*m*/*z* 369/289) and glucuronides (*m*/*z* 465/289) metabolites.

The elution gradient was carried out with binary solvent system consistent in water–0.1% formic acid (solvent A) and acetonitrile–0.1% formic acid (solvent B) at a constant flow rate of 800 μl/min. A linear gradient profile with the following proportions (v/v) of solvent B was applied (*t* (min), %B): (0, 9), (4, 20), (9, 100). The injection volume was

15 μl and the turbo ionspray source settings were: capillary voltage, −3500 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); focusing potential, −200 V; entrance potential, −10 V; drying gas (N_2) heated to 300°C and introduced at a flow rate of 6000 cm^3/min. The declustering potential was −50 and the collision energy −20. In all experiments, the Q1 and Q3 quadrupoles both operated at unit resolution.

Urine antioxidant capacity determination by TEAC assay

The method used to study the total antioxidant capacity of the urine samples (TAC) was the one described by Re et al. [23], which gave a measure of the antioxidant capacity on the range of carotenoids, phenolics and some plasma antioxidants, determined by the decolourization of the $ABTS^+$ through measuring the reduction of the radical cations as the percentage inhibition of absorbance at 734 nm.

After the addition of 20 μl of sample (urine in water 1:100 v/v) or Trolox standard to 2 ml of diluted $ABTS^+$ solution (10 ml of a solution of potassium persulphate 2.45 mM mixed with ABTS 7.00 mM in distilled water and left for 12–16 h in darkness at room temperature, followed by dilution of the solution with PBS 1:100 v/v, pH 7.4 up to a final absorbance of 0.7 ± 0.02 and equilibration at 30°C), absorbance readings were taken every minute in a 1-cm cell using a Hewlett Packard 8452A Diode Array Spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). Duplicate determinations were made in triplicate for the samples. The percentage inhibition was calculated from the absorbance values at 5 min. The absorbance was read at 30°C, exactly 1 min after initial mixing and up to 5 min. PBS solution was used as a blank in every run during all the assays. The percentage of absorbance inhibition at 734 nm was calculated for Trolox (0–15 μM) and plotted as a function of the concentration and finally the absorbance inhibition of antioxidants in urine was determined with Trolox concentration being equivalent. The relative change of sample absorbance, ΔA_{sample}, was calculated for solvent correction, according to the following equation:

$$\Delta A_{\text{sample}} = \frac{A_{t=0(\text{sample})} - A_{t=5(\text{sample})}}{A_{t=0(\text{sample})}} - \frac{A_{t=0(\text{PBS})} - A_{t=5(\text{PBS})}}{A_{t=0(\text{PBS})}}$$

The values of percentage inhibition were obtained by multiplying ΔA_{sample} values by 100. The TEAC was determined by previous calibration with Trolox.

Uric acid quantification

The uric acid excreted in urine was determined with a colourimetric enzymatic method based on the uric acid oxidation to allantoin and hydrogen peroxide by the action of an uricase. In the presence of peroxidase, ADPS and 4-aminoantipirina (4-AA) mixture condensed due to the action of hydrogen peroxide, forming a coloured quinonamine being proportional to the uric acid concentration of the sample. The reaction was conducted using a standard enzymatic kit from Cromatest, Linear Chemicals, S.L. (Monlab, Barcelona) and the absorbance reading was measured at 550 nm [24].

Statistical analysis

All statistical analyses were performed using the SPSS software for Windows Ver. 11.5 (SPSS Japan Inc., Tokyo, Japan). Two-tailed paired *t*-test was also used to compare (−)-Ec metabolites concentration excreted following 6, 12 and 24 h after intake of the test meals and to compare the urine antioxidant capacity. Differences between the two interventions were also tested by an analysis of covariance using general linear models; baseline values and sex were used as covariates. Significance was recognized at $p < 0.05$ and the variables are presented as arithmetic means ± SD. The effects of each intervention as well as the differences between interventions are expressed as mean changes (95% confidence intervals, CI). Pearson's correlations were used to examine the associations between epicatechin metabolites excreted in urine and TAC.

Results

The results were expressed as mass or equivalents of convenient [(−)-Ec or Trolox]/g de creatinine excreted in urine. Creatinine was determined by the classical Jaffé alkaline picrate method [25].

Urine (−)-Ec metabolites

One (−)-epicatechin-glucuronide and three (−)-epicatechin-sulphates were identified and confirmed in all the urine samples after the intake of cocoa beverage. (−)-Ec metabolites were not detected either in urine samples after the intake of the control meal (PFF) or before the consumption of the test meal. The (−)-Ec metabolites excreted in urine samples at different time periods from the 21 subjects after intake of the cocoa beverage are shown in Table I.

The maximum metabolite concentration was found in the 6–12 h period after PRF ingestion, although there were no significant differences in the values excreted during the first 6 h. In the urine samples

Table I. (−)-Ec metabolites (μg(−)-Ec/g creatinine) excreted in urine at different time periods after intake of a cocoa beverage (PRF).

		Metabolites concentration (μg(−)-Ec/g creat)					
Beverage	Excretion period (h)	Ec-S^1	Ec-S^2	Ec-S^3	$\Sigma^{\text{Ec-S1.Ec-S2.Ec-S3}}$	Ec-G	Total (SD)
PRF	0–6	48.83	29.61	5.07	83.52	194.95	278.47 (252.47)
	6–12	18.57	195.29	1.30	215.16	76.48	291.64 (215.33)
	12–24	2.74	32.30	nd	35.04	10.23	45.83 (45.83)

nd, not detected.
Ec-S^1; (−)-epicatechin-sulphate 1, Ec-S^2; (−)-epicatechin-sulphate 2, Ec-S^3; epicatechin-sulphate 3, $\Sigma^{\text{Ec-S1,Ec-S2,Ec-S3}}$; sum of Ec-S^1,Ec-S^2,Ec-S^3, Ec-G; (−)-epicatechin-glucuronide and total; is the sum of the four excreted metabolites (Ec-S^1, Ec-S^{2}Ec-S^3 and Ec-G).

collected during the period of 12–24 h, metabolite concentration had a tendency to reach basal levels, indicating that at ~24 h after the PRF intake, there were no metabolite concentrations in the urine.

This urinary excretion of (−)-Ec metabolites corresponded to ~1.6% of ingestion. This low value may be due to the partial absorption of (−)-Ec in the small intestine and reaching the colon where the microbiota metabolized the aglycones into aromatic acids or due to a pronounced biliary excretion and/or to suffer an extensive metabolism [26].

Urine antioxidant capacity (TEAC)

TAC of all the urine samples is shown in Figure 1A and B. The TAC of urine samples that corresponded to the volunteers following control (PFF) intake did not vary significantly throughout the day of study and there were no significant changes between the TAC values that corresponded to urine obtained before control intake and TAC corresponding to urine samples obtained after milk consumption. However, the TAC of urine samples following cocoa shake ingestion (PRF) experienced a significant increase ($p < 0.01$) regarding 0 h urine TAC values, the one prior to cocoa milkshake intake, indicating that the higher urine TAC values were due to cocoa consumption. The antioxidant capacity of urine samples taken during the 6 h following cocoa milkshake ingestion experienced an increase of 66.6% [increase from 148.97 (38.44) to 248.18 (158.73) mol Trolox/g of creatinine] and during the subsequent 6 h, that is the 6–12 h period after ingestion, where there was an increase of 72.67% [increase from 148.97 (38.44) to 257.23 (85.02) mol Trolox/g of creatinine].

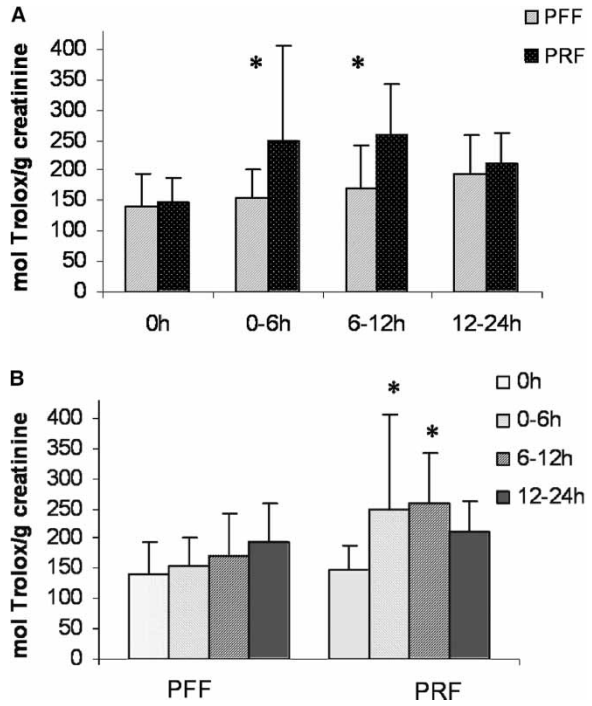

Figure 1. Total antioxidant activity determined by TEAC assay, mean (mol Trolox/g creatinine) and SD as the vertical lines of all the urine samples excreted at different time periods after an intake of two test meals, milk as a PFF and cocoa beverage as a PRF.

Baseline urinary TAC values (0 h) were the same for the PFF and PRF groups. However, the 0–6 h urine following the cocoa milkshake intake experienced a significant increase with respect to the control, PFF 0–6 h urine, $p = 0.0073$ and an increase of 62.35% [increase from 152.85 (47.41) to 248.18 (158.73) mol Trolox/g of creatinine]. This increase lasted until 12 h after its intake, with minor differences (Figure 1A and B). These results would indicate that urine TAC values, even after 12 h following antioxidant rich food ingestion, were significantly higher than the polyphenol-free diet. They also demonstrated that cocoa polyphenols provide a better urine antioxidant capacity. Therefore, we could affirm that the intake of a polyphenol rich diet provided the organism with a better antioxidant capacity to scavenge free radicals because the cumulative amount of drug excreted in the urine is directly related to the total amount of drug absorbed [27].

However, the values of urine samples collected at 12–24 h following the ingestion of both treatments (PFF and PRF) were not significantly different, a fact that indicates that the TAC values of 24 h urine following food intake were not due to cocoa antioxidant compounds but to the rest of the components of the diet.

Uric acid concentration

The uric acid was determined to exclude the possibility that the large increase in urine antioxidant capacity observed after the consumption of PRF was not directly related to the flavonoids metabolites excreted, but was instead due to an increase in the uric acid concentration. Lotito and Frei [6] concluded that a significant increase of plasma antioxidant capacity observed after the consumption of flavonoid-rich foods is not caused by the flavonoids themselves, but is probably the consequence of increased uric acid levels.

No statistical differences were found between the urine excretes after the intake of the PRF test meal and the urine excretes before the two test meals and after PFF meal ingestion in any period excretion time. The means (SD) of the uric acid concentration (mg/g creatinine) in the urine samples were 396.13 (147.8) and 342.83 (173.2) mg uric acid/g creatinine, respectively, ($p > 0.05$). This result indicated that the intake of PRF did not increase the uric acid levels in urine; therefore, the observed increase of TAC values in the urine samples cannot be due to an increase in uric acid.

Discussion

Flavonoids are present mainly in plant foods and have attracted much attention in relation to oxidative stress-related diseases. Circulating metabolites of flavonoids, such as glucuronides, sulphates and methylated forms have a reduced ability to donate hydrogen and are less effective scavengers of reactive oxygen and nitrogen species relative to their parent aglycone forms [28]; however, in the body, the majority of flavonoids are found in the form of their conjugates; thus, it is essential to know the bioavailability of flavonoids involving intestinal absorption, metabolic conversion and urinary excretion, in order to evaluate their *in vivo* antioxidant activity after their intake.

The *in vitro* radical scavenging activity of the flavonoids metabolites has been demonstrated in some studies. Moon et al. [29], in 2001, showed that the quercetin-3-*O*-ß-D-glucuronide found in rat plasma after oral administration of quercetin possesses 1,1-diphenil-2picrylhydrazyl (DPPH) radical-scavenging activity and Harada et al. [30], in 1999, also demonstrated that (+)-catechin-5-*O*-ß-glucuronide and (−)-epicatechin-5-*O*-ß-glucuronide purified from rat urine samples after oral administration of catechin and epicatechin exhibited high antioxidative activities as superoxide anion radical scavengers like their parent compounds. Rimbach et al. [31], in 2003, showed that isoflavone metabolites also exhibited higher antioxidant activity than the parent compounds in standard antioxidant (FRAP and TEAC) assays and Natsume et al. [32], in 2004, described that the (−)-Ec glucuronides metabolites have less antioxidant activities than their parent compound epicatechin, but concentrations of 42.9 μM of epicatechin—7- glucuronide and epicatechin-3-glucuronide caused 61 and 20% inhibition of TBARS production.

In the present study, we analysed *in vivo* the relationship between the consumption of cocoa beverages with the excretion of (−)-Ec metabolites in urine and their corresponding radical scavenging activity. It has been clearly observed that the antioxidant activity of urine samples increases significantly during the12 h after the intake of a cocoa beverage and this increase in antioxidant capacity is also positively related to the excreted metabolites. These results would indicate that the intake of cocoa products increases the antioxidant capacity of the human organism even though we already know that the antioxidant capacity of metabolites is inferior to that of the aglycones. Terao [7] has found that (−)-Ec and quercetin are mostly accumulated as glucuronide and sulphate conjugates in blood plasma after oral administration in rats, while quercetin in its intact form is not detected in the circulation. However, after the oral administration of these flavonoids, the antioxidative ability of rat plasma was shown to be enhanced, indicating that conjugated metabolites participate in the antioxidant defense in blood plasma. These results are in agreement with a previous study [25] where a significant increase in total polyphenol excretion was observed by a Folin-Ciocalteu assay in urine excretes during the 6 h immediately following consumption of a cocoa beverage.

In a recent review, Lotito and Frei [6] concluded that the large increase of plasma antioxidant capacity observed after the consumption of flavonoid-rich foods is not caused by the flavonoids themselves, but is probably the consequence of increased uric acid levels. In this study, we determined the uric acid to corroborate whether the large increase in urine antioxidant capacity observed after the consumption of PRF was directly related to the flavonoids metabolite excreted.

According to Huang et al. [4], a valid *in vitro* assay is an invaluable tool for clinical studies if it is combined with bioavailability data, thus here we demonstrate that TEAC assay in urine is correlated with the phenols excreted in urine.

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