Chlorogenic acid is poorly absorbed, independently of the food matrix: A Caco-2 cells and rat chronic absorption study

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CHLOROGENIC ACID IS POORLY ABSORBED, INDEPENDENTLY OF THE FOOD MATRIX : A CACO-2 CELLS AND RAT CHRONIC ABSORPTION STUDY.

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Abbreviations:
CQA: chlorogenic acid

ABSTRACT

According to epidemiologic studies, dietary phenolic antioxidants, such as chlorogenic acid (CQA), could prevent coronary heart diseases and some cancers. Coffee is the main source of CQA in the human diet. The aim of this study was to assess the effect of usual coffee consumption conditions, such as the addition of milk, on CQA bioavailability. Interactions between CQA and milk proteins of the beverage were shown, using an ultrafiltration technique. These interactions were proved to be slightly disrupted during an *in vitro* digestion process. CQA absorption and bioavailability were then studied *in vitro* using a Caco-2 cell model coupled with an *in vitro* digestion process, and *in vivo*, in a chronic supplementation study in which rats were fed daily coffee or coffee and milk for three weeks. Both experiments showed that CQA absorption under its native form is weak, but not altered by the addition of milk proteins, and slightly reduced by the addition of Maillard reaction products. These data show that there are some weak interactions between coffee phenolics and milk proteins, but these have no significant effect on CQA bioavailability from coffee in the rat. CQA is poorly but indeed absorbed under its native form in the body, when ingested in a realistic food matrix.

INTRODUCTION

Phenolic compounds are mainly found in fruits, vegetables and cereals, and in vegetal-matter based products such as wine or tea [1].The lower mortality rates from stroke and some cancers, among populations consuming phenolic compounds-rich diets, suggests that they could prevent coronary heart diseases [2] and some cancers [3, 4].

Their health benefits, closely related to their antioxidant power [5, 6] and gene regulation effects [7, 8], have been extensively demonstrated *in vitro*. However, before concluding on any potential health effect, their bioavailability should be investigated. During the past years, a lot of data concerning pure phenolic compounds absorption were generated, yet the influence of the food matrix on polyphenol absorption remains poorly considered. Indeed, the bioavailability of phenolic compounds may be modified as a consequence of interactions with food macronutrients, such as fibers in low-processed fruits and cereals [9] or proteins and polysaccharides in processed foods and beverages [10-14]. More, some interaction may appear in the mouth or gastrointestinal tract, since many different foods come in contact then. For example, addition of fat to a meal was clearly demonstrated to enhance quercetine bioavailability [15]. As a consequence, a better understanding of these interaction phenomenons appears to be essential.

With an average of 6 million tons produced per year, coffee is a widely consumed beverage, and the major source of caffeoyl-quinic acids in the human diet. Coffee drinkers would ingest up to 500 mg-1 g a day while coffee abstainers' daily intake is <100 mg/day [16]. These polyphenols and their hydrolyzed derivatives such as caffeic acid, are considered powerful antioxidant compounds *in vitro* [17]. They are able to inhibit LDL oxidation [18, 19] and DNA damage [20] *in vitro*, and to regulate gene

expression [21] ; more, they proved to be bioavailable in the rat when ingested out of a food matrix [22]. They are thus potent candidates for cancer and cardiovascular diseases prevention. As milk addition in coffee is a frequent consumption habit, coffee and milk beverage appears to be a relevant model system to study the influence of a food matrix containing both polyphenols and proteins, on polyphenol absorption.

In the present study, we first demonstrate the existence of interactions between coffee hydroxycinnamic acids and milk proteins. Then, their fate in the gastro-intestinal tract is investigated by means of an *in vitro* digestion of model solutions, coupled with Caco-2 cells absorption experiments. The influence of Maillard reaction products, that are also present in coffee and may as well interfere with CQA absorption, is also briefly investigated. Finally, the influence of milk addition on phenolic compounds bioavailability is investigated by means of a chronic rat feeding study.

MATERIAL AND METHODS

CHEMICALS

Sodium acetate trihydrate, caseins (technical grade, from bovine milk), β-lactoglobulin (90%, from bovine milk), pepsin (from porcine gastric mucosa, 3500U/mg solid), porcine bile extract, pancreatin (from porcine pancreas, x 8 U.S.P. specifications), HEPES, glucose (96 %), L-lysine (98 %) and Sulfatase type H-1 from *Helix pomatia* (EC 3.1.6.1, 15500 U/g, with β-glucuronidase activity 465000 U/g) were obtained from Sigma Aldrich (Saint-Quentin Fallavier, France). Chlorogenic acid (CQA, 99 %), sodium chloride (>99 %), lithium acetate dehydrate (98 %) and sodium dihydrogenophosphate dihydrate (>99 %) were from Acros Organics (Geel, Belgium).

Disodium hydrogenophosphate dehydrate (>99.5 %) and acetic acid (99.7 %) were from Labosi (Oulchy-le-château, France), and sodium hydroxide from Carlo Erba (Val de Reuil, France). Hydrochloric acid (37 %) was obtained from Merck (Darmstadt, Germany). Ultrapure water was obtained from a Millipore Elix filtration system (Saint-Quentin-en-Yvelines, France), and was further purified through a Millipore Simplicity 185 device (Saint-Quentin-en-Yvelines, France), to obtain 18.3 MΩ/cm resistivity water for HPLC applications.

Instant coffee (lyophilized decaffeinated Arabica coffee), skimmed or semi-skimmed (15 g/L lipids) milk, and sugar were purchased from local supermarket.

Maillard reaction products were prepared by incubating a solution containing 0.8 mol/L L-lysine and 0.5 mol/L glucose in ultrapure water, in sealed vials, at 103°C for 92 h. Their absorbance was then compared with absorbance of Maillard reaction products separated from C coffee solution using LH-20 chromatographic separation according to the protocol of Maillard [23]. Briefly, 500 µL of solution « C » were deposited onto a Sephadex LH-20 column (Pharmacia Biotech, length : 7 cm ; inner diameter : 5 mm). The column was then eluted by two volumes of pH3 acidified water (using 37 % hydrochloric acid), which yielded a Maillard reaction products rich fraction, followed by elution with two volumes of methanol, which yielded a polyphenol rich fraction. Both fractions were analysed using spectrophotometry (spectrophotometer UV-VIS Perkin Elmer, equiped with Lambda 9 software (Shelton, USA).

Appropriate dilutions were made to achieve the same final absorbance in both solutions.

IN VITRO STUDY

MODEL SOLUTIONS PREPARATION

For *in vitro* study, model solutions containing 7.73 mmol/L CQA were prepared in 0.1 mol/L pH 5.5 sodium acetate buffer, to fit CM solution pH and ionic strength conditions. CQA initial concentration was chosen in order to get 5 mmol/L solutions at the end of *in vitro* digestion process, taking into account dilutions due to sampling. This concentration was high enough to be detected in our system after Caco-2 cell uptake experiments, according to [24].

Several model solutions containing 7.73 mmol/L CQA were prepared: CQA only (control), CQA and milk, CQA and a mix of caseins (25.50 g/L) or purified β-lactoglobulin (1.02 g/L) and CQA and Maillard reaction products, carefully reproducing the CM solution coffee/milk (as described in the *in vivo* study section) components ratios.

ULTRAFILTRATION EXPERIMENTS

An ultrafiltration study on model solutions (except solution containing Maillard reaction products) was carried out according to Pedone *et al.* [25], with slight modifications. Control and sample solutions were first diluted in appropriate buffers, so that protein concentrations did not exceed 1 g/L, according to manufacturer instructions. Next, 10 mL diluted samples or controls were introduced in disposable Vivaspin 20 centrifuge ultrafiltration units (Vivascience, Germany) with a 5000 Da cut-off and centrifuged for 30 minutes at 6000 x g and 20°C in a Sorvall ST 21 centrifuge (Dupont, Les Ulis, France). Finally, CQA concentrations of permeates were determined

spectrophotometrically by measuring the absorbance at 324 nm. At this wavelength and concentrations, proteins or peptides from proteins that may have permeated did not interfere with CQA concentration determination, as checked with CQA free solutions.

Free and bound percentages were calculated as follows: the concentration of the permeate of the control was assumed as total ligand concentration; sample permeate concentration was assumed as free ligand concentration; and the percentage of bound ligand was calculated by difference.

IN VITRO DIGESTION

Model solutions were then subjected to an *in vitro* gastric and intestinal digestion [26]. Briefly, 20 mL of solution were allowed to equilibrate at 37°C, then pH was brought to 2 with 5 mol/L HCl, 2.5 mL of a pepsin solution in 0.1 mol/L HCl (enzyme/substrate ratio: 1/20) were added and the volume was adjusted to 25 mL. After 1 hour incubation at 37°C under orbital stirring, enzyme activity was stopped by cooling samples in ice for 10 minutes and CQA binding was analyzed by ultrafiltration. The remaining samples were then subjected to intestinal digestion: pH was brought to 6 using 0.1 mol/L NaOH, 0.5 mL of a mixture containing bile extract and pancreatin (enzyme/substrate ratio: 1/13 and 1/20 respectively) in 0.1 mol/L $NaHCO_3$ solution were added and the volume brought to 25 mL. After 2 hours incubation at 37°C under orbital stirring, enzyme activity was stopped by cooling samples in ice for 10 minutes and CQA binding was analyzed by ultrafiltration as described above. Percentages of bound CQA were corrected by the quantity bound to digestive enzymes (12 %).

Enzymatic activity was then definitely stopped by incubating digested solutions at 100°C for 4 minutes under pressure-sealed conditions. The solutions were then centrifuged at 3900 x g, 30 minutes at 4°C and supernatant collected. pH was adjusted to 6 using 1 mol/L HCl and 1 mol/L NaOH, and osmolarity was controlled to achieve a value between 290 and 330 mOsm/kg using an automatic device. CQA contents were then determined.

CELL CULTURE

Caco-2 cells were obtained from the American Type Culture collection (Rockville, U.S.A.) and used at passage 38-45. For the experiments, they were seeded at a density of 40000 cells/cm² on polyethylene terephtalate filters, which were inserted in polycarbonate twelve-wells plates. The cells were grown in Dulbecco's modified Eagle medium with 15 % v/v fetal calf serum, 25 mmol/L glucose, 1 % non-essential amino-acids, 6 mmol/L glutamine, 50 U/mL penicillin and 50 µg/mL streptomycin. The cells were kept at 37°C in an incubator with a 5 % CO_2, 95 % air atmosphere at constant humidity. The medium was changed every 2 days, and then daily after confluent cell cultures were established.

CACO-2 CELL UPTAKE EXPERIMENTS

Immediately before the uptake experiments, the growth medium was removed from each culture well and the cell layer was washed twice with MES buffer pH 6. 1.5 mL of HEPES buffer pH 7.4 was then added to the basal chamber while the apical chamber received 0.5 mL of the digested solution, according to the protocol of Konishi and Kobayashi [24]. Every 10 minutes for one hour, 150 µL of the basal solution were

collected and subsequently replaced by an equal volume of HEPES buffer. Three repetitions were performed for each assay.

Collected and initially loaded samples were investigated for phenolic acids (chlorogenic, caffeic and ferulic acid in particular) using an HPLC 600E Waters system coupled with an ESA Coulochem II detector equipped with an analytical cell model 5010 with 2 working electrodes. The electrochemical settings were as follows: electrode 1, 300 mV potential and 10 µA sensitivity; electrode 2, 600 mV potential and 10 µA sensitivity. Aliquots of 20 µL were injected onto a C_{18} Interchrom column (250 x 4.6 mm i.d.; 10 µm particle size, Interchim). The elution phase was a mixture of acetonitrile and 25 mmol/L pH 2.4 NaH_2PO_4 in water (15/85, v/v) at a flow rate of 1 mL/min.

IN VIVO STUDY

ANIMALS AND DIETS

18 male Wistar rats weighing 202 ± 3g were housed in individual cages in a temperature-controlled room (22 ± 2°C) with a 12 hours inverse light/dark cycle : « day » was from 9:00 p.m. to 9:00 a.m. and « night » from 9:00 a.m. to 9:00 p.m. Animals weight and total food consumption were recorded daily.

Rats were divided into 3 groups, each one receiving 12.5 g of different experimental beverages, respectively water with sugar for control group, sweetened coffee drink prepared from commercial instant powder for "C" group, and sweetened coffee and milk drink for "CM" group (table 1) from 9:00 a.m. to 10:00 a.m. everyday for 3 weeks. The coffee solutions were prepared daily, using 22°C deionized water. The commercial

instant coffee powder was investigated for phenolic acids esters, according to the method of Nardini *et al.* [27] and exhibited a value of 14.21 ± 0.61 mg chlorogenic acid (CQA) per g of powder. Coffee solutions were prepared so that CQA concentrations consistent with freshly brewed coffee [16] were achieved. Solutions were sweetened using saccharose in order to enhance coffee acceptability to animals.

Rats were also fed a complete polyphenol free laboratory diet (P14) prepared at INRA (Jouy en Josas, France) (table 2) from 01:00 p.m. to 06:00 p.m., in order for this second meal not to interfere with the experimental beverage absorption. The P14 powder was mixed with water (1/1, w/w) so that food consumption could be followed, as rodents could not scatter food in cages.

After 3 weeks, the animals were sacrificed. They were deeply anesthetized at 01:00 p.m., 4 hours after receiving their last ration of experimental coffee beverage or sweetened water with an overdose of sodium pentobarbital and exsanguinated by section of the abdominal aorta and vena cava after the injection of heparin. Blood was collected and centrifuged at 3000 x g for 20 minutes at 4°C. The so-obtained plasma was acidified to pH 3 using 3 mol/L HCl, aliquoted, then frozen at -21°C until analysis.

PLASMA SAMPLES ANALYSIS :

Prior to HPLC analysis, plasma samples were extracted according to the protocol of Rondini *et al.* [28], slightly modified. 1.94 mg of sulfatase from *Helix pomatia* type H-1 (30 units) in 20 µL acetate buffer 1 mol/L pH 4.9, or 20 µL buffer only, were added to 100 µL plasma. The samples were then incubated for 2 hours at 37°C. 250 µL acetone were then added. The samples were vortexed for 30 seconds at 2400 vibrations/min and

centrifuged for 10 minutes at 7200 x g. Supernatants were collected and evaporated under nitrogen. The samples were then dissolved in 150 µL HPLC elution phase, and filtered onto 0.22 µm nylon syringe filters.

Extracted samples were then analysed using HPLC coupled with a coulometric detector Coulochem II (ESA, Chelmsford, USA) equipped with a 5010 analysis cell with 2 electrodes. A C18 ODS Interchrom (250 x 4.6 mm i.d. ; 10 µm) column (Interchim, France) was eluted with a elution phase consisting of 57 mmol/L lithium acetate/methanol/acetic acid 88/10/2 (v/v/v) with a 1 mL/min flow rate. Cell potentials were set as follows: E1: 300 mV (sensibility 1 µA), E2: 400 mV (sensibility 1 µA).

STATISTICAL ANALYSIS :

Results are mean ± S.E.M.. ANOVA, Student t-tests and Smallest Significant Difference Test were performed using Microsoft Excel 2003 ($p<0.05$).

RESULTS AND DISCUSSION

COFFEE AND MILK COMPONENTS INTERACTION STUDIES

The existence of interactions between proteic fraction from milk and coffee phenolics (mainly chlorogenic acid), was determined using ultrafiltration.

It was not possible to study coffee solutions themselves directly, for their high contents in Maillard reaction products interfered with the UV-VIS measurements, and with ultrafiltration process. For the same reason, CQA and Maillard reaction products interactions could not be assessed using this method. Thus, interactions were investigated from extemporaneously prepared solutions of CQA, coffee's main phenolic acid, and milk or milk protein. Ultrafiltration studies show that up to 39 ± 10 % of total CQA would be bound to milk protein (table 3). Milk fat does not seem to have any influence on the amount of bound CQA. Results found in model solutions proportions were identical to those found when leading the same experiment using the concentrations of CQA and milk found in the solutions used for *in vitro* study (CM solution, data not shown).

Our data show that most of the interaction is due to caseins (43 ± 7%), while β-lactoglobulin contributes little or nothing to bonding of CQA. The latter is poorly represented in milk, when compared with caseins, what may explain this result.

INTERACTIONS DURING *IN VITRO* DIGESTION

The CQA and milk (or milk protein) model solutions were then subjected to an *in vitro* acidic and enzymatic digestion. CQA degradation proved to be negligible (<2%) in all

tested beverages during the digestion process. The amount of CQA bound to gastric and intestinal enzymes was <12 %. It was yet deducted to total bound CQA amount determined by ultrafiltration, in order to assess the real quantity of CQA bound to milk proteins. This method implies that no competition exists between milk proteins and digestive enzymes for fixation to CQA. The results clearly suggest that during the digestion process, the interactions between milk components and coffee polyphenols significantly decrease, but do not disappear (table 3).

According to literature, β-lactoglobulin remains unchanged during gastric digestion, thus suggesting that it can interact with CQA during this step [29]. However, our results do not clearly show that new interaction formation extensively occurred during this step (5 ± 5 % bound CQA, table 3). During intestinal digestion, trypsin and chymotrypsin hydrolyse β-lactoglobulin into 17 and 10 peptides respectively [29]. No increase in bound CQA percentage could be detected either during the second step, suggesting that peptides obtained from the whole protein either do not interact with CQA or that complexes that may have formed could not be retained on ultrafiltration membrane, being smaller than membrane cut-off.

Caseins precipitation at acidic pH caused high standard deviation values. Surprisingly, this pitfall was not encountered with milk or skimmed milk based models, suggesting that some components from milk, that are not present in the casein-based model, have a stabilizing effect on the solution. Still, in spite of these high standard deviations, the results only show a drop in interactions, during both gastric and intestinal phases. Even considering the strong standard deviations, it seems that a part of CQA remains bound to caseins at the end of the digestion process.

The interaction between milk proteins and CQA may be partially dissociated during digestion, but the formation of new interactions between CQA and peptides obtained from digestion, cannot be excluded, thus partially balancing the dissociation and being a potential source of hindrance to CQA and/or peptides absorption through the intestinal brush border. More, 17 ± 2 % of the initially introduced CQA seem to remain bound to milk proteins or peptides (table 3) at the end of *in vitro* digestion process, thus suggesting that milk addition could modify coffee antioxidants absorption in the body.

CACO-2 CELLS UPTAKE EXPERIMENTS

In order to simulate chlorogenic acid absorption in presence of milk proteins, Caco-2 cells were used. This model has proved to be efficient for polyphenols absorption studies [24, 30, 31]. It is also interesting since it can be combined with *in vitro* digestion [26, 32]. The *in vitro* digested samples were applied on the cells in order to investigate absorption modification due to milk proteins or Maillard reaction products from coffee.

Previous data showed that chlorogenic acid was poorly absorbed in this kind of model [24] : 0.06 ± 0.01 % and 0.12 ± 0.02 % of initial CQA were found in the basolateral phase, respectively with or without apical to basolateral proton gradient. But in this study, *in vitro* digestion steps were not performed. In our experiments, the cumulated permeate value for control CQA solution is 0.14 ± 0.04 % of initial quantity (figure 1a), which is in the same range. Samples were also investigated for other molecules possibly found in plasma following the ingestion of chlorogenic acid rich foods, such as caffeic acid, obtained by chlorogenic acid hydrolysis by intestinal esterases, or ferulic acid, the

290 methylated form of caffeic acid [33]. None of these molecules could be detected in our system.

This suggests that CQA, consumed out of a food matrix background, is not affected by digestive conditions, and shows that CQA under its native form, is poorly, but indeed absorbed in the body. More, metabolization to glucuronidated or sulfated forms that
295 takes places in the body as described in literature [34], was neglected in this part of our study, thus suggesting that CQA absorption was slightly underestimated in this experiment (0.14 ± 0.04 % of initial quantity).

The same experiments, carried out in presence of milk proteins (β-lactoglobulin or caseins), show slight modifications in absorption kinetics (figures 1c and 1d), but no
300 significant difference can be observed, compared with control CQA : the final percentages of initially loaded CQA are 0.09 ± 0.03 % in presence of β-lactoglobulin, and 0.25 ± 0.09 % in presence of caseins. Milk proteins do not seem to modify CQA absorption in a significant way.

However, when Maillard reaction products are added to CQA and subjected to the same
305 digestion and absorption steps, CQA absorption on Caco-2 cells is significantly lowered (figure 1b), and the final percentage of initially loaded CQA found in basolateral phase is significantly lower ($p<0.05$) than control (0.05 ± 0.03 %). This result clearly suggests that CQA absorption in a coffee beverage matrix is lower than when the compound is consumed pure in a buffered solution.

310 The Caco-2 cells method returns quick and reliable results, but requires the use of purified molecules in model solutions, and can barely be used with a fully complex food

matrix, such as coffee and milk, as is. Therefore, the other hydroxycinnamate esters that are present in coffee beverage were neglected in this experiment. Moreover, *in vivo*, small intestine mucosal esterases or colonic flora may hydrolyze CQA to caffeic acid [35], which is better absorbed [24]. In order to get a more realistic overview of the phenomenon, carrying out an *in vivo* study remained an essential point.

IN VIVO BIOAVAILABILITY STUDY

3 groups of 6 rats were fed water (« control »), coffee (« C »), or coffee with 25 % semi-skimmed milk (« CM ») for three weeks. This experimental diet, coupled with a semi-synthetic daily diet, did not induce significant differences in weight gain, growth or diet efficiency between the different groups, in spite of discrepancies in coffee consumption between the different groups (rats consumed 50 to 100% of the meals containing coffee, daily), due to the poor palatability of coffee to rats. For this reason, phenolics found in plasma were divided by the average consumption for each animal, and are shown individually.

In our experiment, only very small amounts of chlorogenic acid (table 4) could be detected in plasmas of the rats that consumed “C” or “CM” drink, as well as another compound that evidence from HPLC/DAD analysis suggested was benzoic acid, in the range of 0.1 to 0.3 µmol/L CQA equivalents (table 5), whereas neither phenolics nor benzoic acid were present in plasma from control groups (table 5).

As phenolics may be found in sulfated or glucuronided forms in plasma, a preliminary enzymatic hydrolysis was carried out. After this step, detected CQA did not increase significantly, and no other cinnamates derived from coffee phenolic esters, such as

caffeic or ferulic acid, or their glucuronidated or sulfated forms, could be detected, although HPLC conditions had been optimized to detect them. This result is quite unexpected, but may be explained by the fact that CQA was detected only at extremely low levels, and the other phenolic compounds may be below the detection limits of our system (0.2 µmol/L for CQA). Indeed, 41 % of plasmatic metabolites found after caffeic acid ingestion in the rat are glucuronided forms [36], whereas ~20 % are sulfoglucuronidated, the aglycone being negligible. Such data are not available for chlorogenic acid.

In a more global way, only little data is available concerning CQA bioavailability, most of it concerning its urinary excretion, mostly because this molecule is very difficult to extract from plasma, and is probably poorly absorbed [22]. We tested several published phenolic acids extraction methods from plasma and found a range of weak recoveries CQA, although recoveries were excellent for other phenolic acids such as ferulic acid. The main difficulty is that CQA is easily hydrolysed by any residual esterase activity in the enzyme mixture used, or precipitates with proteins during their removal, leading to poor recovery rates in extracted plasma samples. We developed a method of CQA extraction from rat plasma that was rapid, reliable and with reasonable recoveries: 71 ± 4 % without enzyme and 64 ± 10 % with enzyme.

CQA absorption under native form remained controversial for a long time. Some authors found 0.86 % of ingested CQA in rat urine under its native form [33], 0.2 % to 0.46 % in ileostomic man [34, 37] or up to 1.7 % in healthy volunteers [34], whereas others did not find any [38-42] or only under hydrolyzed or metabolized forms [43]. According to Olthof *et al*. [34] and Gonthier *et al.* [33], respectively 36 % and 50 % of

the ingested dose could be found in the form of metabolites from colonic flora, mainly hippuric and 3-hydroxyphenylpropionic acids. Up to 60 different metabolites were detected in urine in this study.

The majority of publications concerned with CQA absorption and metabolism suggest that most of the ingested CQA is extensively hydrolyzed and metabolized. However, a recent publication clearly established that a small amount is absorbed under its native form [22], while only 15–32% of ingested CQA would be hydrolyzed into caffeic acid in the cecum [22]. The metabolization pathways remains uncertain. To our knowledge, the effect of food matrix on CQA absorption and metabolization was never investigated either.

Our results suggest that the presence of milk proteins is not a hindrance to the absorption of CQA, for no difference could be observed between the “C” and “CM” groups, in agreement with the data from Caco-2 cell absorption experiments. This is in agreement with previous work lead on tea polyphenol absorption in presence of milk, that showed no significant effect of milk on tea polyphenol absorption [44, 45]. Moreover, the coffee beverage doses used in this *in vivo* experiment were relevant to human nutrition (calculated to be consistent with the daily consumption of an heavy coffee drinker [46]).

CONCLUDING REMARKS

Chlorogenic acid is poorly absorbed in the body from the gut its native form, when consumed within a realistic food matrix such as coffee or coffee and milk. Some interactions between CQA and milk proteins do exist, but do not seem to hinder

chlorogenic acid absorption. On the other hand, Maillard reaction products also contained in coffee influence CQA absorption and further research is required to assess this possibility.

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FIGURES AND TABLES

Table 1 : Experimental beverages for *in vivo* study.

Group	Water (g)	Sugar (g)	Instant Coffee powder (g)	Semi-skimmed milk (g)
Control	80	20	-	-
C	80	20	4	-
CM	60	20	4	20

Table 2. Composition of the standard complete P14 diet.

Ingredient	**wt (g / kg)**	**Ingredient**	**wt (g / kg)**
Milk proteins	140	Vitamins	10
Starch	623	Cellulose	50
Saccharose	100	Choline	2
Soy oil	40		
Mineral salts	35	Total	1000

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Table 3: Percentage of chlorogenic acid bound to milk proteins in coffee and milk model solutions, determined by ultrafiltration

Tested solution	**Initial solution**	**After gastric digestion**	**After intestinal digestion**
Semi-skimmed milk	42 ± 13%	N.D.	N.D.
skimmed milk	39 ± 10%	21 ± 2%*	17 ± 2%*
β-lactoglobulin	0 ± 0%	5 ± 5%	0 ± 0%
caseins	43 ± 7%	36 ± 21%	27 ± 19%

N.D.: Not Determined. *: $p<0.05$ when compared with corresponding initial solution. Data are mean ± S.E.M..

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Table 4 : CQA found in plasmas of the rats that consumed C or CM meals for 3 weeks, with or without enzymatic hydrolysis by sulfatase and β-glucuronidase.

CQA in µmol/L plasma/mg average daily consumed CQA			
Rat group	**Rat number**	**plasma**	**hydrolyzed plasma**
Control	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	6	0	0
C	1	Traces	Traces
	2	0.031 ± 0.001	0.000 ± 0.000
	3	0.017 ± 0.001	0.031 ± 0.003
	4	0.000 ± 0.000	0.011 ± 0.001
	5	0.051 ± 0.002	0.028 ± 0.003
	6	Traces	Traces
CM	1	0.029 ± 0.001	0.032 ± 0.003
	2	0.010 ± 0.000	0.012 ± 0.001
	3	0.014 ± 0.001	0.019 ± 0.002
	4	Traces	Traces
	5	0.018 ± 0.001	Traces
	6	Traces	0.017 ± 0.002

"traces" indicates a signal/noise ratio < 3. « C » : Coffee, « CM » : coffee and milk.. Data are mean ± S.E.M..

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Table 5 : Benzoic acid, expressed as CQA equivalents, found in plasmas of the rats that consumed C or
490 CM meals for 3 weeks, with or without enzymatic hydrolysis by sulfatase and β-glucuronidase.

Equivalent CQA in µmol/L plasma/mg average daily consumed CQA			
Rat group	**Rat number**	**plasma**	**hydrolyzed plasma**
Control	1	0	0
	2	0	0
	3	0	0
	4	0	0
	5	0	0
	6	0	0
C	1	0.031 ± 0.001	0.042 ± 0.004
	2	0.047 ± 0.002	0.069 ± 0.007
	3	0.061 ± 0.002	0.092 ± 0.009
	4	0.028 ± 0.001	0.039 ± 0.004
	5	0.000 ± 0.000	0.000 ± 0.000
	6	Traces	Traces
CM	1	0.038 ± 0.002	0.047 ± 0.005
	2	0.040 ± 0.002	0.028 ± 0.003
	3	0.028 ± 0.001	0.039 ± 0.004
	4	0.018 ± 0.001	0.025 ± 0.002
	5	Traces	Traces
	6	Traces	0.026 ± 0.003

"traces" indicates a signal/noise ratio < 3. « C » : Coffee, « CM » : coffee and milk. Data are mean ± S.E.M..

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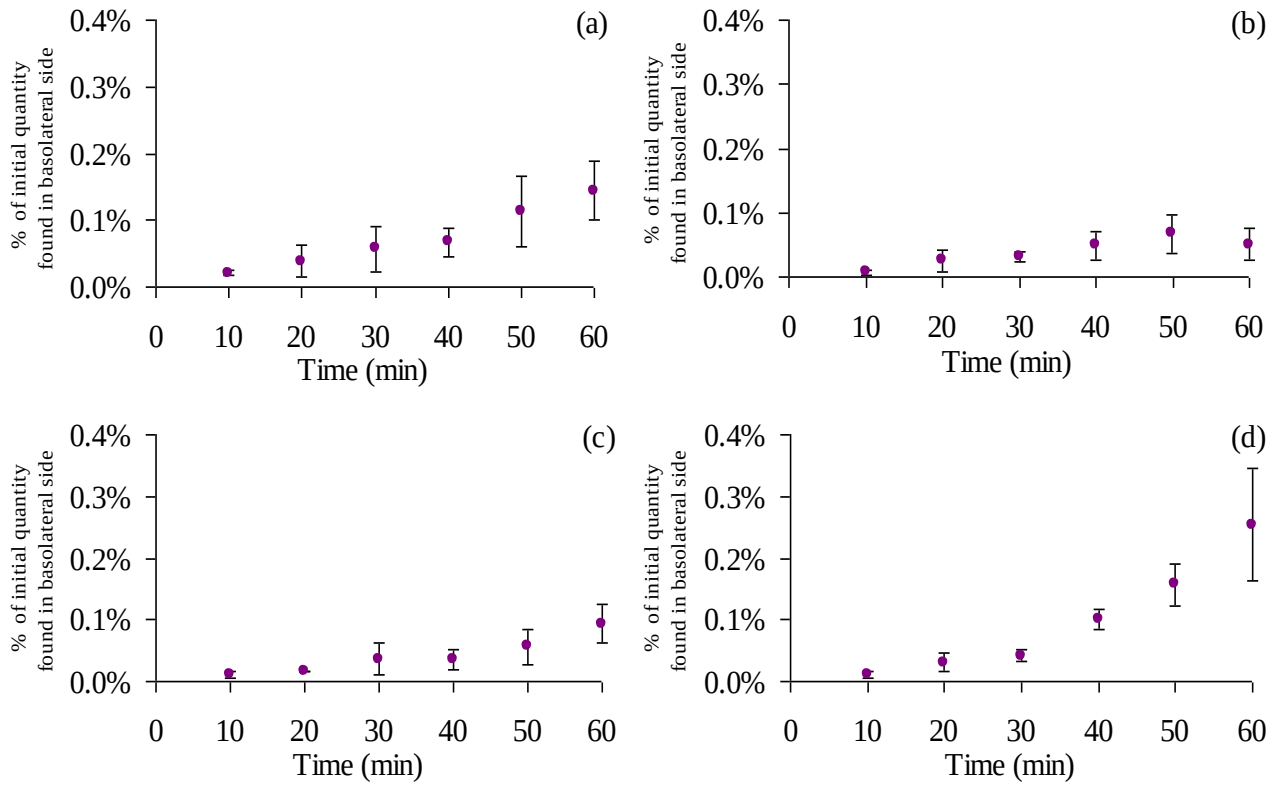

Figure 1: Caco-2 uptake of chlorogenic acid, after *in vitro* digestion, (a) alone (b) in presence of Maillard reaction products, (c) in presence of β-lactoglobulin, (d) in presence of caseins. . Data are mean ± S.E.M..