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The Type and Concentration of Milk Increase the in Vitro Bioaccessibility of Coffee Chlorogenic Acids

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ABSTRACT: Coffee with different types and concentrations of milk was digested with pepsin (2 h) and pancreatin (2 h) to simulate gastropancreatic digestion. Chlorogenic acids (CGAs) were determined by high-performance liquid chromatography−electrospray ionization−tandem mass spectrometry in ultrafiltrate (cutoff 3 kDa) to evaluate their bioaccessibility. After digestion, bioaccessible CGAs decreased from 80.2 to 53.0 and 69.5 μmol/200 mL in coffee without milk and coffee-whole milk, respectively. When whole, semiskimmed, skimmed, or diluted milk were present, the increase in bioaccessibility was dependent on fat content (r = 0.99, p < 0.001). No relationship was observed between bioaccessibility and proteins, carbohydrates, and calcium content. The addition of milk to coffee caused an immediate decrease in the bioaccessibility due to CGAs binding to proteins. After digestion, 86−94% of bound CGAs remained in the high molecular weight fraction. Casein bound 5-caffeoylquinic acid with high affinity (K_D of 37.9 ± 2.3 μmol/L; n = 0.88 ± 0.06).

KEYWORDS: *coffee, chlorogenic acids, digestion, milk, fat, bioaccessibility, protein, casein*

INTRODUCTION

Coffee is among the most widely consumed pharmacologically active beverages in the world. Epidemiological studies have associated coffee consumption with a reduced risk of several diseases, including cardiovascular disorders and type 2 diabetes.[1,2] Coffee contains over a thousand chemicals, comprising the bioactive compounds caffeine, diterpenes, chlorogenic acids (CGAs), and melanoidins that are formed during the roasting process. It has been suggested that CGAs are the principal responsible for the protective effect of coffee in cardiovascular diseases and diabetes,[1,2] although a role for high molecular weight melanoidins has been recently reported.[3]

Several health benefits have been linked with the dietary introduction of CGAs in the human body, such as decrease in blood pressure,[4] and in the relative risk of cardiovascular disease,[5] chemoprotective and antigenotoxic activities,[6] and prevention of type 2 diabetes.[7] On the contrary, some other studies have shown that CGAs could induce genotoxic effects that may increase the risk of some types of cancer.[8] The genotoxic activity of CGAs is mediated by a pro-oxidant mechanism (involving H_2O_2 production) and resulted in the induction of DNA damage in cultured cells.[9] The genotoxic effect was found at millimolar concentrations of CGAs, but at the submicromolar concentrations found in the body, the antioxidant and chemopreventive activities may prevail.[9]

CGAs represent 4−12% of green coffee constituents in mass, but because of their thermal instability, they may be largely degraded during intense roasting.[10] CGAs are esters of hydroxycinnamic acid with quinic acid. The most representative are the isomers of caffeoylquinic acid (CQA), feruloylquinic acid (FQA), and *p*-coumaroylquinic acid (*p*-CoQA).[11,12] There are also low concentrations of dicaffeoylquinic acids (diCQA) and CQA lactones.[13] The bioavailability and metabolism of coffee CGAs in humans have been recently studied.[14−16] During the passage through the body, extensive metabolism of CGAs occurs. Stalmach et al.[14] found that the only unmetabolized compounds detected in human plasma were FQAs and traces of 5-CQA, after ingestion of a cup of coffee containing more than 400 μmol of total CGAs.

The Stalmach's studies[14,15] carried out on healthy subjects and ileostomists highlights the role of the small intestine and colon in the bioavailability of dietary CGAs. It was found that the small intestine is the primary site for the hydrolysis of CQAs and FQAs with the release of caffeic and ferulic acids, which are subsequently metabolized (methylate, sulfate, and glucuronide derivatives) mainly in the liver. The colon is the site for the conversion of ferulic acid and caffeic acid to dihydroferulic and dihydrocaffeic acids, which are further sulfated.[17] In addition, caffeic acid may be metabolized by the colonic microflora in 3-hydroxyphenylacetic, (3,4-dihydroxyphenyl)propionic, 2,4-dihydroxybenzoic, and *trans*-3-hydroxycinnamic acids.[18] Free and sulfated forms of dihydroferulic acid and dihydrocaffeic acid are found in plasma, suggesting that the CGAs not absorbed at stomach and small intestine level are modified by the intestinal microflora prior to the absorption by large bowel.[19] In human plasma, Monteiro et al.[20] detected micromole amounts of unmetabolized CQAs and diCQAs after ingestion of coffee containing more than 3 mmol of total CGAs.

Presently, many of the studies investigating the bioavailability of coffee CGAs have been focused on simple beverages, while only a few studies have been carried out to test the effect of formulations on their bioavailability.[21] Dupas et al.[22] showed that milk proteins (after 25% semiskimmed or skimmed milk addition to coffee) bound CGAs in vitro. Recently, a human

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study demonstrated that the addition of 10% whole milk to coffee had no significant impact on the bioavailability of CGAs, while the addition of lipid-derived nondairy creamer caused a delay in the plasmatic appearance of caffeic acid and isoferulic acid without affecting their bioavailability.[23] In a recent in vivo study, Duarte and Farah[18] demonstrated that the consumption of instant coffee directly dissolved in whole milk (100% milk beverage) may impair the bioavailability of coffee CGAs in humans. This decrease was mainly driven by the differences in the colonic metabolites hippuric and 3,4-dihydroxyphenylacetic acids. Duarte and Farah[18] suggest that the high fat content of milk affects protein digestion or microbial colonic activity.

The bioaccessibility can be defined as the amount of compound in the gut available for the absorption. The bioaccessibility definition comprises the release of compounds from food matrices and their stability under the gastrointestinal condition.[24] Regarding this, no data are present in the literature displaying the bioaccessibility of CGAs in coffee formulations. The bioavailability of a compound strongly depends on its bioaccessibility.

Several studies suggest that coffee is one of the main contributors to total polyphenol intake in the human diet.[13,25] Coffee is a major source of CGAs in the human diet, and the daily intake of CGAs in coffee consumers may reach 1 g/day versus an intake lower than 100 mg/day in coffee abstainers.[25] Coffee as well as other beverages (tea and wine) may be the most important source of polyphenols for subjects with a low intake of vegetables. Moreover, polyphenols of beverages are more accessible and bioavailable with respect to those in the matrix of vegetables. In many cases, coffee is consumed with milk. Therefore, the aim of the present work was to study the stability of coffee CGAs at the pH values of gastropancreatic digestion. The effect of the addition of different bovine milk types (whole, semiskimmed, and skimmed) and milk concentrations (from 10 to 50% of milk) to coffee on the bioaccessibility of CGAs was investigated. The bioaccessibility was evaluated by ultrafiltration of CGAs during simulated gastropancreatic digestion. The knowledge of the bioaccessibility of CGAs allows new formulations of food and beverages to increase their bioavailability.

MATERIALS AND METHODS

Materials. Pepsin from porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP specifications), bile salts (mixture of sodium cholate and sodium deoxycholate), 5-CQA (>95%), and casein from bovine milk were supplied by Sigma (Milan, Italy). Solvents for high-performance liquid chromatography−electrospray ionization−tandem mass spectrometry (HPLC-ESI-MS/MS) analysis of coffee CGAs were from Carlo Erba (Milan, Italy). Amicon Ultra-4 regenerated cellulose 3 kDa were supplied by Millipore (Italy). Instant coffee (Nescafe Classic) was purchased in a local supermarket (Reggio Emilia, Italy). From our determination of CGAs as well as from other studies,[19,26] it appears that the instant coffee that we have utilized is a blend of Arabica and Robusta in which Arabica prevails. The bovine milk (whole, semiskimmed, and skimmed milk), belonging to the same batch of raw milk, was obtained from a local producer. The three types of milk were homogenized. The different types of milk have the same total protein (3.1 g/100 mL), caseins (2.6 g/100 mL), carbohydrates (4.8 g/100 mL), and calcium (120 mg/100 mL) contents but differ in fat content (3.60, 1.55, and 0.05 g/100 mL in whole, semiskimmed, and skimmed milk, respectively).

Preparation of Beverages. Coffee was prepared by dissolving 2.0 g of instant coffee in 100 mL of boiling water. To test the effect of milk type on the in vitro bioaccessibility of coffee CGAs, the following beverages were prepared. CM beverage was made by mixing 50% of coffee with 50% of whole milk, CSSM by mixing 50% of coffee with 50% of semiskimmed milk, CSM by mixing 50% of coffee with 50% of skimmed milk, and C beverage by mixing 50% of coffee with 50% of water to maintain constant CGA levels between formulations. To test the effect of milk concentration, beverages with 10 (CM/5) and 25 (CM/2) whole milk and with 10% semiskimmed milk (CSM/5) were prepared. To maintain constant CGA levels between beverages, water was added to CM/5, CM/2, and CSM/5 formulations until the 50% was reached. For the preparation of beverages, coffee was brought at room temperature (22 °C) before the addition of the milk or milk−water mixture kept at room temperature.

In Vitro Gastropancreatic Digestion of Beverages. Aliquots (200 mL) of freshly prepared beverages were subjected to consecutive simulated gastric and pancreatic digestion as reported in Tagliazucchi et al.[27] A fixed amount (1 mL) was withdrawn immediately after the mixing of beverage with pepsin. These samples are reported in the paper as after mixing beverage or before digestion beverage. At the end of 2 h of gastric digestion (postgastric beverage) and at the end of 2 h of pancreatic digestion (postpancreatic beverage), 1 mL of beverage was withdrawn. Samples removed after the pancreatic digestions were immediately acidified to pH 2 to ensure the stability of the phenolic compounds. Digestions without added enzymes were carried out to differentiate the effects due to the presence of enzymes from those caused by the chemical environment in the assay. In other experiments, the effect of pH was independently evaluated by incubating (2 h, 37 °C, under stirring) coffee beverage (C) at pH values at which the gastric and pancreatic digestion were carried out (pH 2 and pH 7.5). 5-CQA, at the same concentration found in C beverage (30 μmol/200 mL), was subjected to gastropancreatic digestion with or without added enzymes. The degree of protein hydrolysis in beverages has been determined according to Adler-Nissen[28] considering casein as the only protein in milk.

Ultrafiltration of Beverages for Determination of Bioaccessible CGAs. Beverages were subjected to ultrafiltration with Amicon Ultra-4 nominal cutoff 3 kDa, at 7500*g* for 120 min at 4 °C (as reported in the specifications sheet of the filter unit) to separate CGAs that are bound to high molecular weight material. The ultrafiltrated compounds are considered the bioaccessible CGAs and are reported as μmol/200 mL of beverage or as bioaccessibility. The bioaccessibility is expressed as the percentage of CGAs in each ultrafiltrate with respect to their amount in the ultrafiltrate of coffee beverage after mixing. The low molecular weight fraction, containing bioaccessible CGAs, was filled up to the original volume (1 mL) and utilized for the analysis.

Identification and Quantification of CGAs in the Instant Coffee and in Ultrafiltrates. The CGAs were separated and identified with HPLC-ESI-MS/MS analysis based on previously published work.[29] After HPLC separation and MS/MS identification, CGAs were quantified using selective ion monitoring (SIM) mode. The CGAs were all quantified as 5-CQA equivalents, monitoring the loss of caffeoyl moiety with the resultant ionization of quinic acid.

The SIM calibration curve was generated using 5-CQAs and was constructed by plotting the area of 5-CQA (y) versus analyte concentration (x). Linear detection responses were obtained for 5-CQA concentrations ranging from 1 to 60 μmol/200 mL ($y = 42149.3 + 39016.5x$; $R^2 = 0.998$).

The total CGAs were the sum of all of the identified peaks. The CGAs have been numbered according to IUPAC recommendations.[30]

Analysis of the Interactions between 5-CQA and Casein by Fluorescence Spectroscopy. Casein (a mixture of α-and β-caseins) was dissolved in 10 mM sodium phosphate buffer, pH 6.5, at 37 °C under continuous stirring for 2 h. The linear range of casein fluorescence was between 0 and 10 μmol/L. Therefore, 5 μmol/L was chosen as the concentration for fluorescence quenching experiments. Fluorescence spectra were recorded at 37 °C in the emission range of 290−500 nm at an excitation wavelength of 280 nm using Jasco, FP-6200 spectrofluorometer (Tokyo, Japan). The intensity at 340 nm (the maximum emission wavelength of tryptophan) was used to calculate the binding constant according to Dufour and Dangles.[31] Various solutions of 5-CQA at different concentrations were prepared in methanol. For each data point, 2 mL of casein solution was transferred

in a cuvette. After 5 min of equilibration at 37 °C, 0.01 mL of 5-caffeoylquinic solution was added to each cuvette to have a final 5-CQA concentration between 1 and 100 μmol/L.

The change in fluorescence emission intensity was measured after 10 min of the mixing with casein. The effect of the addition of methanol on the casein fluorescence emission spectrum was subtracted from the value of casein alone.

The type of binding was assessed using the Stern−Volmer equation.[32] For the kinetic analysis of ligand binding, nonlinear regression analysis was performed. K_D (dissociation constant) and n (number of binding site) were calculated according to Rawel et al.[33]

Statistics. All data are presented as the mean ± SD for three replicates for each prepared sample. The Student's t test was performed using Graph Pad Instat (GraphPad Software, San Diego, CA) when data were compared with controls. Univariate analysis of variance (ANOVA) with LSD posthoc test was applied using PASWStatistics 18.0 (SPSS Inc. Chicago, IL) when multiple comparisons were performed. The differences were considered significant with $P < 0.05$.

RESULTS

Total CGAs in Coffee Beverage and Their Bioaccessibility during Simulated Digestion. The CGAs content and composition in coffee beverage (50% coffee and 50% water) before ultrafiltration and in ultrafiltrate are reported in Table 1. When the total contents of CGAs in ultrafiltrate and in beverage before ultrafiltration were compared, a decrease in ultrafiltrate of about 8.1% was observed. This decrease may be due to the binding of CGAs to ultrafiltration apparatus or to high molecular weight material removed by ultrafiltration (retentate). To ensure that the ultrafiltration apparatus did not bind CGAs in another set of experiments, ultrafiltrates were ultrafiltrated again, and CGAs were determined by HPLC-MS/MS. All of the reported compounds as well as the standard 5-CQA were recovered by at least 95% after the second ultrafiltration step with respect to the first separation step. In a second set of experiments, the retentate of coffee was filled up with water and ultrafiltered again. This operation was repeated twice. The obtained ultrafiltrates were analyzed for the CGAs content. We recovered 4.9 ± 0.3 μmol/200 mL of total CGAs after the first washing and 1.5 ± 0.1 μmol/200 mL after the second washing. A total of 99.2% of the CGAs originally present in the coffee beverage were recovered in the ultrafiltrates, suggesting that the binding of CGAs to the ultrafiltration apparatus was negligible. Similar results were obtained when the coffee beverage was maintained in contact with the filter before ultrafiltration for 1 h.

Table 1. Quantity of CGAs in Coffee Beverage (C Beverage; 50% Coffee and 50% Water) before Ultrafiltration and in Ultrafiltrate and Stability of Ultrafiltrate CGAs at pH 2.0 and 7.5[a]

	μmol in 200 mL of C beverage		ultrafiltrate recovery (%)	
compd[b]	before ultrafiltration	after ultrafiltration	pH 2	pH 7.5
3-CQA	18.5 ± 0.2	16.5 ± 0.7	101.2 ± 10.1	78.5 ± 8.2
4-CQA	17.5 ± 0.5	14.8 ± 0.8	85.0 ± 9.3	82.9 ± 9.3
5-CQA	29.8 ± 0.7	28.8 ± 1.0	95.8 ± 7.8	71.2 ± 6.2
total CQAs	65.8 ± 0.9	60.1 ± 1.5	94.7 ± 15.8	76.1 ± 13.9
3-FQA	1.3 ± 0.3	1.2 ± 0.5	100.0 ± 13.5	76.9 ± 4.5
4-FQA	6.0 ± 0.2	5.2 ± 0.3	100.0 ± 12.7	78.2 ± 7.1
5-FQA	7.3 ± 0.4	7.4 ± 0.4	81.5 ± 10.3	79.9 ± 2.6
total FQAs	14.6 ± 0.5	13.8 ± 0.7	89.9 ± 21.2	79.0 ± 8.8
3,4-diCQA	2.9 ± 0.1	2.5 ± 0.1	73.2 ± 8.6	98.8 ± 4.2
3,5-diCQA	1.2 ± 0.1	1.2 ± 0.1	76.7 ± 6.5	90.0 ± 3.3
4,5-diCQA	1.2 ± 0.2	1.2 ± 0.4	95.8 ± 5.3	90.7 ± 4.1
total diCQAs	5.3 ± 0.4	4.9 ± 0.4	79.6 ± 12.0	94.7 ± 6.7
5-CoQA	1.6 ± 0.1	1.4 ± 0.1	100 ± 8.1	100 ± 7.2
total CGAs	87.3 ± 1.1	80.2 ± 1.7	91.3 ± 20.1	78.1 ± 19.2

[a]To test the stability as a function of pH, aliquots of coffee beverage ultrafiltrate were incubated at 37 °C under stirring for 2 h at pH 2 or at pH 7.5. Data are means ± SDs (n = 3). [b]CGAs were identified and quantified with HPLC-ESI-MS/MS.

Coffee CGAs showed different stability under different pH conditions (Table 1). The amount of total CGAs in ultrafiltrate of coffee beverage after simulated gastropancreatic digestion is reported in Table 2.

The standard of 5-CQA was stable during in vitro gastric digestion (recovery of 100%), while it was degraded by 10% during pancreatic digestion. During the pancreatic digestion of standard 5-CQA, two additional peaks appeared in the chromatogram and were identified as the 3- and 4-isomers of CQA. Their concentration after pancreatic digestion was 0.4 and 0.2 μmol in 200 mL of 3-CQA and 4-CQA, respectively. Because we had obtained higher recovery when 5-CQA standard was digested (90% recovery after pancreatic digestion) with respect to the recovery with C beverage (62.5%), in another set of experiments, we digested the low molecular weight fraction of coffee separated from the high molecular weight material by ultrafiltration. The removal of high molecular weight material increased the recovery of all CGAs after gastropancreatic digestion (data not reported). The digestion experiments carried out without the addition of digestive enzymes showed similar results to those in the presence of enzymes.

Effect of Addition of Different Milk Types on Bioaccessibility. As can be seen in Figure 1A, in the presence of whole and semiskimmed milk (CM and CSSM beverages), the quantities of total CGAs in ultrafiltrates after pancreatic digestion were significantly higher as compared to the values obtained in the presence of coffee alone (C beverage). The addition of skimmed milk (CSM beverage) had no impact on the CGAs bioaccessibility with respect to C beverage. The addition of the three types of milk to coffee caused an immediate decrease in total CGAs in ultrafiltrate (Figure 1A, before digestion).

In the following experiment, we have evaluated the capacity of high molecular material to bind CGAs after gastropancreatic digestion. The degree of protein hydrolysis in CM and CSM beverages was 17.1 and 21.6, respectively, after gastropancreatic digestion. Whole and skimmed milk were digested at the same hydrolysis degree. At the end of digestion, 50% coffee solution was added, the resulting beverages were ultrafiltered, and the CGAs content in ultrafiltrates was determined. The concentration of CGAs in the ultrafiltrates was 67.2 ± 3.3 and 66.8 ± 3.4 μmol/200 mL, respectively. Partially digested whole and skimmed milk maintained CGAs binding capacity corresponding to 86 and 94% of the respective milk before digestion (after mixing ultrafiltrates). The concentrations of CQAs, FQAs, and diCQAs are reported in Figure 1B−D, respectively.

Table 2. Bioaccessibility Data after Gastropancreatic Digestion of Individual CGAs[a]

	CGAs in ultrafiltrate beverages (μmol/200 mL)							
	after mixing (before digestion)	after gastropancreatic digestion						
compd[b]	C	C	CSM	CSSM	CM	CM/2	CM/5	CSM/5
3-CQA	16.5 ± 0.7	12.0 ± 0.9	13.1 ± 0.6	15.2 ± 0.6[c]	15.9 ± 0.6[c]	14.4 ± 0.5[c]	14.4 ± 0.8[c]	11.1 ± 1.8
4-CQA	14.8 ± 0.8	9.3 ± 0.5	9.9 ± 0.5	10.6 ± 0.2[c]	11.2 ± 0.3[c]	11.6 ± 0.1[c]	10.2 ± 0.9	9.1 ± 1.0
5-CQA	28.8 ± 1.0	18.0 ± 1.0	18.3 ± 0.9	20.5 ± 1.0[c]	24.9 ± 1.0[c]	22.2 ± 0.3[c]	20.0 ± 2.0	18.1 ± 1.2
3-FQA	1.2 ± 0.5	0.5 ± 0.1	0.4 ± 0.1	0.8 ± 0.5	1.3 ± 0.1[c]	0.8 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
4-FQA	5.2 ± 0.3	3.9 ± 0.1	3.4 ± 0.3	3.8 ± 0.1	4.4 ± 0.1	3.8 ± 0.1	4.1 ± 0.3	4.4 ± 0.3
5-FQA	7.4 ± 0.4	4.7 ± 0.1	3.7 ± 0.5	4.5 ± 0.2	5.6 ± 0.1[c]	4.3 ± 0.2	4.7 ± 0.4	5.0 ± 0.4
3,4-diCQA	2.5 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.5 ± 0.1	2.1 ± 0.1[c]	1.7 ± 0.1	1.3 ± 0.1	1.3 ± 0.1
3,5-diCQA	1.2 ± 0.1	1.0 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	1.3 ± 0.1[c]	0.4 ± 0.1	0.4 ± 0.1	0.8 ± 0.1
4,5-diCQA	1.2 ± 0.4	1.0 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	1.6 ± 0.1[c]	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.1
5-CoQA	1.4 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.4 ± 0.1	1.4 ± 0.1
fat content (g/200 mL)	0	0	0.05	1.55	3.60	1.80	0.72	0.01

[a]All beverages contained 50% coffee. CSM, CSSM, and CM contained 50% skimmed, 50% semiskimmed, and 50% whole milk, respectively. C, CM/2, CM/5, and CSM/5 contained 50% water, 25% water−25% whole milk, 40% water−10% whole milk, and 40% water−10% skimmed milk, respectively. Results are expressed as μmol in 200 mL of the specific beverage after ultrafiltration. Data are means ± SDs (n = 3). [b]CGAs were identified and quantified after ultrafiltration with HPLC-ESI-MS/MS. [c]Denotes P value <0.05 with respect to coffee beverage (C).

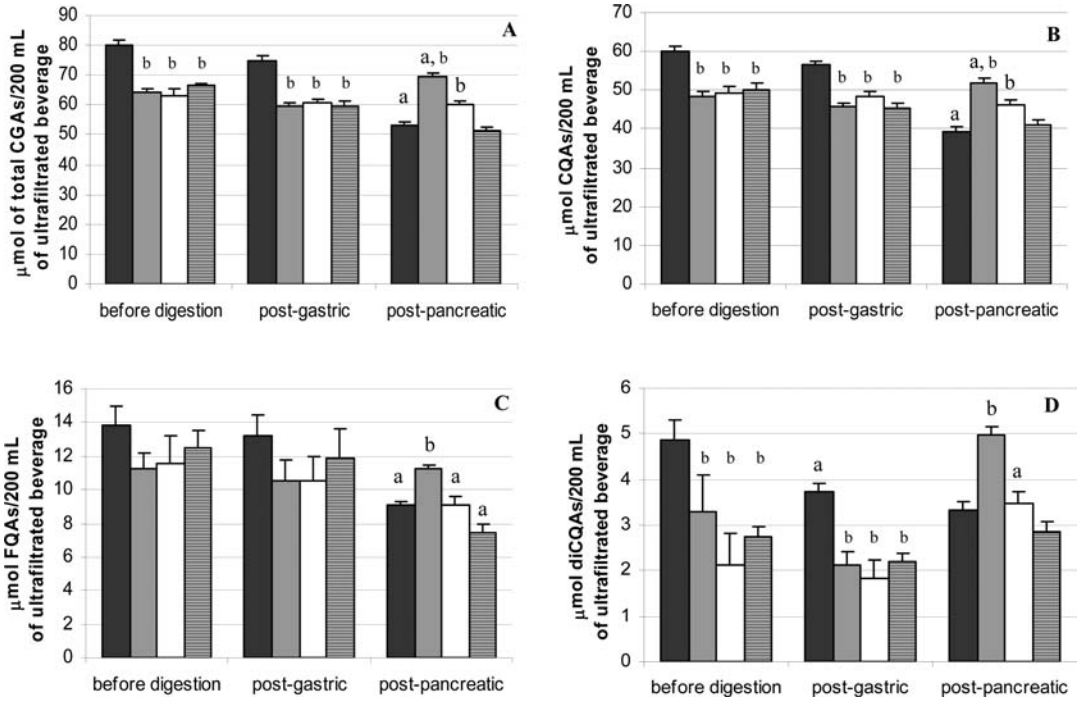

Figure 1. Effect of simulated gastropancreatic digestion on the recovery of CGAs in ultrafiltrates of beverages with different types of milk. (A) Total CGAs, (B) total CQAs, (C) total FQAs, and (D) total diCQAs. Black bar, 50% coffee and 50% water (C beverage); gray bar, 50% coffee and 50% whole milk (CM beverage); white bar, 50% coffee and 50% semiskimmed milk (CSSM beverage); and striped bar, 50% coffee and 50% skimmed milk (CSM beverage). Data are means ± SDs (n = 3). Samples of beverages for ultrafiltration were taken immediately after mixing with pepsin (before digestion), after 2 h of gastric (postgastric), and after 2 h of pancreatic (postpancreatic) digestion. [a]Denotes P value <0.05 with respect to the previous time. [b]Denotes P value <0.05 with respect to the C sample at the same time. CGAs were identified and quantified after ultrafiltration with HPLC-ESI-MS/MS.

The bioaccessibility of the individual CGAs in coffee milk beverages at the end of the digestion is shown in Table 2. There were no significant differences in the postpancreatic bioaccessibility of CGAs between coffee beverage (C) and coffee-casein beverage in which casein was added at the same concentration as in milk (data not shown). The decrease of total CGAs in the ultrafiltrate of coffee-casein beverage after mixing was 9.3 ± 1.1 μmol/200 mL.

Effect of Addition of Different Quantities of Milk. In Figure 2A, the effect of the addition of different quantities of

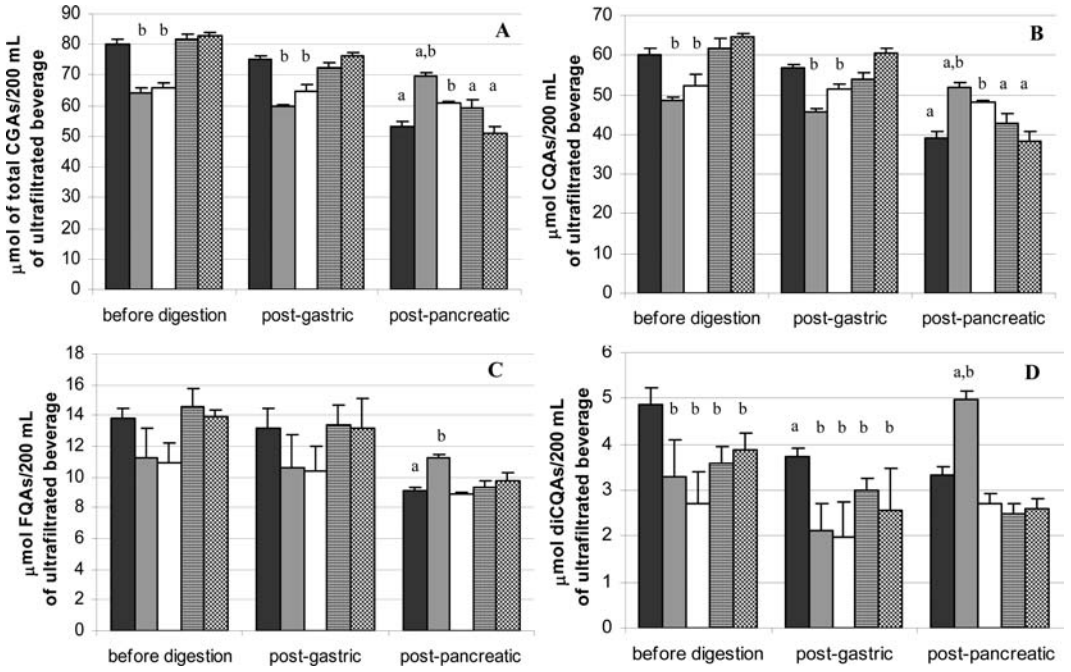

Figure 2. Effect of simulated gastropancreatic digestion on the recovery of CGAs in ultrafiltrates of beverages with different concentration of milk. (A) Total CGAs, (B) total CQAs, (C) total FQAs, and (D) total diCQAs. Black bar, 50% coffee and 50% water (C beverage); gray bar, 50% coffee and 50% whole milk (CM beverage); white bar, 50% coffee, 25% whole milk, and 25% water (CM/2 beverage); striped bar, 50% coffee, 10% whole milk, and 40% water (CM/5 beverage); and checkered bar, 50% coffee, 10% skimmed milk, and 40% water (CSM/5 beverage). Data are means ± SDs (n = 3). Samples of beverages for ultrafiltration were taken immediately after mixing with pepsin (before digestion), after 2 h of gastric (postgastric), and after 2 h of pancreatic (postpancreatic) digestion. [a]Denotes P value <0.05 with respect to the previous time. [b]Denotes P value <0.05 with respect to the C sample at the same time. CGAs were identified and quantified after ultrafiltration with HPLC-ESI-MS/MS.

whole milk on the bioaccessibility of total CGAs is shown. In the 10% whole milk beverage (CM/5) and 10% skimmed milk beverage (CSM/5), the amount of total CGAs before digestion (after mixing) was not different as compared to C beverage. This was due to the low concentration of proteins in CM/5 and CSM/5 beverages (0.62 mg/200 mL). In the CM/5 and CSM/5 beverages, we did not find any changes in the bioaccessibility of CGAs with respect to C beverage at the end of the pancreatic digestion. The postpancreatic bioaccessibility of CGAs in the CM/2 beverage was similar to that in semiskimmed milk coffee beverage but lower than that in coffee whole milk beverage. The concentrations of CQAs, FQAs, and diCQAs are reported in Figure 2B–D, respectively.

Fluorescence Spectroscopy Analysis of 5-CQA–Casein Interaction. Figure 3 shows the emission spectra of casein before and after the addition of different concentrations of 5-CQA. The binding of 5-CQA to casein caused a concentration-dependent decrease in the tryptophan emission. The results from fluorescence spectroscopy analysis demonstrated that the type of quenching between 5-CQA and casein was static (Ksv = 27.9 ± 0.6 × 10^3 M^{-1}; K_q = 465.0 ± 10.0 × 10^{10} M^{-1} s^{-1}, about 3 orders of magnitude more than the diffusion-limited quenching in water ~10^{10} M^{-1} s^{-1}), suggesting the formation of a complex between the 5-CQA and the protein. The calculated K_D value was 37.9 ± 2.3 μmol/L, and the number of binding sites was 0.88 ± 0.06. A red shift of emission spectrum is observed as the 5-CQA concentration increases.

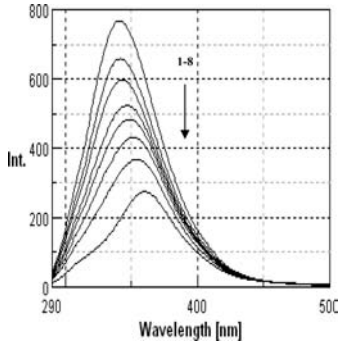

Figure 3. Fluorescence emission spectra of casein in the presence of different concentrations of 5-CQA in 10 mM phosphate buffer, pH 6.5, at 37 °C. Free casein 5 μM spectra (1) and casein spectra recorded after 5-CQA additions at final concentrations of 1, 2, 5, 10, 20, 50, and 100 μM (2–8).

DISCUSSION

Considering the mean of all of the seven different beverages investigated, CQAs were the class of CGAs with the highest bioaccessibility at the end of the digestion (mean 73.2%) followed by FQA class (mean 67.1%) and by diCQA class (mean 65.3%). Regarding the individual CGAs, the 5-CoQA was the most bioaccessible compound with a mean of 89.8%. We found a higher bioaccessibility for 3-CQA with respect to 4-CQA and 5-CQA. This may be due to a greater stability of the 3-CQA during digestion. It has been shown that 3-CQA was more stable under in vitro intestinal digestion than 5-CQA.[34,35] A known pathway of transformation of CGAs is acyl migration.[36] According to Farrell et al.,[19] approximately 2% of 5-CQA was converted to 3- and 4-isomers after 2 h of incubation at pH 7.4. The isomerization of 5-CQA to 3- and 4-CQA has been demonstrated in the digestion of 5-caffeoylquinic standard.[36] It is possible that isomerization

takes place during the digestion of beverages forming small amounts of isomers, which increase the recovery of 3- and 4-CQA. The 5-CQA was more bioaccessible when incubated alone than when present in coffee, suggesting that some compounds of coffee beverage decrease its bioaccessibility during simulated digestion. Among FQAs, 3-FQA was less bioaccessible than the other two isomers. We found that, on the contrary to the other CGAs, peptic digestion had a significant effect on the bioaccessibility of diCQAs. The diCQAs decreased in ultrafiltrates more in the gastric phase than in the pancreatic one.

The decrease in CGAs bioaccessibility after gastropancreatic digestion may be due to degradation. Siracusa et al.[37] found that a general degradation of CGAs takes place during a two-step in vitro digestion of caper and sea fennel.

In the present work, caffeic acid (hydrolysis product of CQAs and diCQAs) and ferulic acid (hydrolysis product of FQAs) were not found in the chromatograms after pancreatic digestion, suggesting that relevant hydrolysis of ester bond did not occur during simulated digestion. CGAs are easily oxidized in alkaline solutions to its corresponding quinone.[38] The quinones react with other quinones to produce dark polymerized compounds. The oxidation and polymerization of CGAs are a possible pathway of loss of these compounds in the alkaline pancreatic media. The stability of CGAs depends on the presence of antioxidant or prooxidant compounds. For example, the presence of flavonols stabilizes CGAs during simulated gastrointestinal digestion.[37]

The food matrix plays a relevant role on the bioaccessibility in our experimental conditions. The removal of high molecular weight material including melanoidins, before the digestion, increased the CGAs bioaccessibility. This increase was probably due to the presence in the high molecular weight fraction (>3 kDa) of prooxidant or CGAs binding compounds. High molecular weight material of coffee brew has been isolated by Bekedam et al.[39,40] by diafiltration with a MW cutoff of 3 kDa to purify melanoidins. The further fractionation by ethanol precipitation allows us to prepare different fractions of melanoidin-rich material free of LMW compounds. The formation of high, intermediate, and low molecular weight materials rich in melanoidins from coffee beans depends on the roasting degree.[41,42] Melanoidins have antioxidative capacity, Fe^{2+} chelating capacity, heme-binding ability, and radical-scavenging activity and may have a role in oxidative damage prevention.[3,39−43]

The role of the food matrix is also shown by the results with coffee-milk beverages. In our experimental conditions, we found an immediate decrease of bioaccessible CGAs (about 19−20%) when the three different types of milk were mixed with coffee (data after mixing) that was probably due to the binding of CGAs to milk proteins. There were no statistical differences in the amount of bound CGAs among the different types of milk since all three types of milk used had the same amount of proteins (3.1 g/200 mL beverage), while the amount of fat changes from 3.6 to 0.05 g/200 mL beverage. The amount of total CGAs bound to milk proteins, evaluated by ultrafiltration data of coffee-whole milk beverage (CM), was 16.4 μmol/200 mL beverage. With casein-coffee beverage, in which casein is at the same concentration as in whole milk-coffee beverage, the decrease after mixing of ultrafiltrate total CGAs was 9.3 μmol/200 mL. The amount of CQAs bound to milk proteins, evaluated by ultrafiltration data of CM, was 10.8 μmol/200 mL beverage. The fluorescence spectroscopy study showed that 5-CQA, which constituted in our beverages about 50% of the total CQA isomers, bound casein with high affinity determining a variation of fluorescence emission spectrum of casein due to tryptophan residues. The n value found was 0.88, which is indicative of a single binding with a K_D of 37.9 μmol/L. Hasni et al.[44] showed the presence of a preferred binding site in α- and β-caseins for tea catechins and suggested that the binding involves hydrogen bonding and hydrophobic interactions. From K_D values, we have calculated that at the concentrations of CQAs and casein as in CM beverage, only 3.6 μmol/200 mL of CQAs are bound to high affinity sites of casein. The observed difference in the binding may be due to several reasons, which are the difference in composition and affinity for 5-CQA between the commercial casein used and the whole milk casein and the difference in affinity between 5-CQA and the other CQA isomers for the high affinity binding site of casein. The presence of binding sites that are not revealed by a fluorescence study in casein and the presence of the binding sites in the other milk proteins are, however, the most important factors. Polyphenols in fact have a great capacity to bind proteins. Polyphenols are responsible for the astringent taste observed in food and beverages. The binding is rather nonspecific, although sequences containing proline are preferred.[45−48] In any case, our study shows that in CM beverage about 30% of CQAs bound to high molecular weight material is bound to the high affinity binding site of casein. The binding of 5-CQA to high affinity binding site of casein determines a decrease in fluorescence emission and a shift to higher wavelength with the increase of the 5-CQA concentration. The red shift indicates that tryptophan residues are more exposed to the solvent when 5-CQA is bound, suggesting that the binding of 5-CQA determines a change of casein conformation.[49]

Green et al.[50] found that the addition of 50% bovine milk to green tea resulted in a significant improvement in total catechins recovery after gastropancreatic digestion, suggesting that the protective effect is a consequence of the binding between green tea catechins and milk proteins. The mechanism for the stabilization of polyphenols by milk is believed to involve protein−polyphenol interactions, which provide a physical trapping of the reactive polyphenolic species.[44] Our results show that the protein content in milk beverages investigated does not correlate with the amount of CGAs in ultrafiltrates after gastropancreatic digestion. The CM, CSSM, and CSM beverages contained the same amount of proteins but had very different bioaccessibility of CGAs. The CGAs bioaccessibility is about the same for CM/2 and CSSM beverages even if they have 1.55 and 3.1 g of protein in 200 mL, respectively. The CGAs bioaccessibility is about the same for coffee alone, coffee-skimmed milk, and coffee-casein beverages, which contain very different quantities of proteins. All of these experimental results show that there is not a relationship between the protein content of added milk and the CGAs in ultrafiltrates at the end of the gastropancreatic digestion, suggesting that milk proteins are not responsible for the increased bioaccessibility.

Van der Burg-Koorevaar and co-workers[51] showed that during simulated digestion, tea catechins are released from the protein−polyphenol complexes. Because also peptides may bind polyphenols,[45] we have evaluated the binding capacity of partially digested milk, showing that partially digested whole and skimmed milk maintained a CGAs binding capacity

corresponding to 86 and 94% of the respective nondigested milk.

Besides the protein hydrolysis, another condition that may modify the binding between CGAs and proteins (or peptides) is the concentration of surfactants, which increased at the end of the digestion. In pancreatic digestion, bile salts are added to beverages, and the presence of lipase activity in pancreatin determines the formation of monoglycerides, diglycerides, and free fatty acids from triglycerides, which is higher in beverages with a higher lipid content. Surfactants may also modify the size and composition of lipid and protein (or peptide) aggregates and their interaction with CGAs.

From the above-reported results, it appears that after gastropancreatic digestion, in our experimental conditions, most (86−94%) of CGAs bound to proteins remain bound to high molecular weight materials and are not bioaccessible. CGAs bound to proteins are probably protected as suggested by Green et al.,[50] and they may become bioaccessible as the hydrolysis of peptide bonds proceeds as in vivo conditions. In this case, a further increase of bioaccessibility due to the addition of milk to coffee will be observed when the digestion is complete.

A good relationship was observed between the fat concentration of beverages and the increase of bioaccessibility at the end of gastropancreatic digestion. The presence of 50% whole or semiskimmed milk or 25% whole milk, as in CM, CSSM, or CM/2 beverages, increased the amount of CGAs in ultrafiltrate (i.e., bioaccessibility) at the end of the simulated digestion process. Figure 4 shows the clear relationship

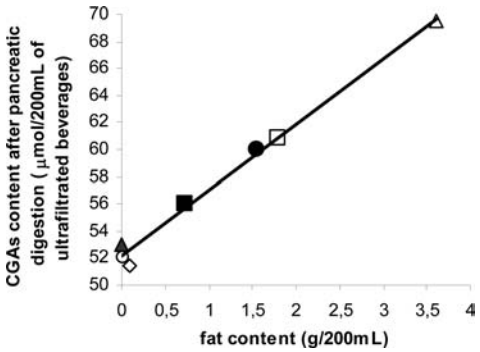

Figure 4. Relationship between the fat content in beverages and the recovery of coffee CGAs (CGAs) in ultrafiltrates after gastropancreatic digestion. C (▲), CM (Δ), CSSM (●), CSM (◊), CM/2 (■), CM/5 (□), and CSSM/5 (○).

between the fat content in beverages and the increase of CGAs concentration in beverage ultrafiltrates after gastropancreatic digestion (r = 0.9914; P < 0.001).

While the increase of bioaccessibility depends on the concentration of fat, no relationship in the increase of bioaccessibility was observed not only with the variation of the protein content but also of carbohydrates and calcium in beverages (data not shown). Because a great part of CGAs remains bound to proteins after gastropancreatic digestion in our experimental conditions, we conclude that the observed increase of bioaccessibility with beverage containing fats is mainly due to the protective effect of fats against CGAs degradation.

Milk fat may increase CGAs bioaccessibility with different mechanisms. First of all, we must consider the higher concentration of antioxidant in milk-containing fats,[52] which may increase the protection of CGAs by oxidative degradation. Hydrophobic interactions between milk fat and CGAs that favor their stability are possible. We separated and determined the CGAs by chromatography on C-18 column eluted by gradients of acetonitrile as hydrophobic component of mobile phase. The elution of CGAs from C-18 column gives us a good evaluation of their hydrophobicity. From elution data, it results that the rank order of hydrophobicity of the four classes is diCQA > FQA = CoQA > CQA. There was no good relationship between the bioaccessibility of the four classes of CQAs at the end of the digestion of whole milk-coffee beverage (CM) with respect to C beverage and their elution profile. These data suggest that other factors, besides hydrophobicity, are relevant to explain the observed increase in the bioaccessibility, for example, the increase of surfactant in fat-containing beverages.

Besides CGAs degradation and their temporary binding to proteins and peptides, the formation of aggregates between CGAs and caffeine, which remain with high molecular materials on filter, may decrease bioaccessibility. For their chemical properties, CGAs bind not only macromolecules as proteins and polysaccharides but also small molecules as caffeine.[44−48,53−56] Instant coffee used in our research contains CGAs and caffeine.[19,26] The polydentate complex formation of polyphenols with caffeine allows, at the end, the formation of precipitable aggregates.[46] The structure of the caffeine−potassium chlorogenate complex and the bonds involved have been investigated.[46,57] If aggregates of caffeine and CGAs are formed in beverages, it is possible that the presence of fat and surfactants formed by fat hydrolysis interferes with aggregate formation and precipitation, increasing bioaccessibility.

To conclude, our results clearly show that there is a good relationship between the amount of fat in added milk and the increase of coffee CGAs bioaccessibility. We have also shown that after gastropancreatic digestion in our experimental conditions, significant quantities of CGAs still remain bound to proteins and peptides in the high molecular fraction. Bound CGAs that are not bioaccessible as determined by our ultrafiltration method may become bioaccessible as proteins digestion proceeds. A high affinity binding site for 5-CQA (K_D = 37.9 μmol/L; n = 0.88) was demonstrated in casein. The food matrix, presence of prooxidant and antioxidant, and pH value were shown to be very important to determine CGA bioaccessibility during in vitro gastropancreatic digestion.

We considered this study a starting point for further investigation directed to increase the bioaccessibility and bioavailability of the CGAs of coffee beverages. The method of evaluation of in vitro bioaccessibility by ultrafiltration allows us to evaluate several factors that modulate bioaccessibility of low molecular weight bioactive compounds including the role of the food matrix, the effect of oxidants and antioxidants, the degree of protein digestion, and the formation of surfactant from lipid digestion. This method may be utilized to investigate the factors that influence the bioaccessibility of other bioactive molecules in other beverages and food. Although in vivo studies are the best and most complete methods to evaluate bioavailability, in vitro methods are important since they allow us to investigate some variables that determine bioaccessibility as well as bioavailability.

The bioaccessibility of chlorogenic lactones and caffeine is not reported. These compounds will be the subject of another investigation. We underline that the chlorogenic lactones, which are about the 6−10% of the total CGAs in the instant

coffee,[19] have a good bioavailability. Recently, it has been shown by Redeuil et al.[58] that unmodified FQA lactones together with their sulfate and glucuronide derivatives and CQA lactones sulfate are present in human plasma after instant coffee ingestion. It also has been shown that CQA lactones are absorbed by the gastric cell monolayer.[19] We can speculate that lactones have a different bioaccessibility with respect to their acids because they are more lipophilic. However, further specific investigations are needed to evaluate their behavior in simulated gastropancreatic digestion.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Bonita, J. S.; Mandarano, M.; Shuta, D.; Vinson, J. Coffee and cardiovascular disease: in vitro, cellular, animal and human studies. *Pharmacol. Res.* **2007**, *55*, 187−198.

(2) Van Dam, R. M. Coffee and type 2 diabetes: From beans to beta-cells. *Nutr. Metab. Cardiovasc. Dis.* **2006**, *16*, 69−77.

(3) Tagliazucchi, D.; Verzelloni, E.; Conte, A. Effect of dietary melanoidins on lipid peroxidation during simulated gastric digestion of meat: Their possible role in the prevention of oxidative damage. *J. Agric. Food Chem.* **2010**, *58*, 2513−2519.

(4) Watanabe, T.; Arai, Y.; Mitsui, Y.; Kusaura, T.; Okawa, W.; Kajihara, Y.; Saito, I. The blood pressure-lowering effect and safety of chlorogenic acids from green coffee bean extract in essential hypertension. *Clin. Exp. Hypertens.* **2006**, *28*, 439−449.

(5) Olthof, M. R.; Hollman, P. C.; Zock, P. L.; Katan, M. B. Consumptions of high doses of chlorogenic acid, present in coffee, or of black tea increases plasma total homocysteine concentrations in humans. *Am. J. Clin. Nutr.* **2001**, *73*, 532−538.

(6) Glei, M.; Kirmse, A.; Habermann, N.; Persin, C.; Pool-Zobel, B. L. Bread enriched with green coffee extract has chemoprotective and antigenotoxic activities in human cells. *Nutr. Cancer* **2006**, *56*, 182−192.

(7) Natella, F; Scaccini, C. Role of coffee in modulation of diabetes risk. *Nutr. Rev.* **2012**, *70*, 207−217.

(8) Zheng, L. F.; Dai, F.; Zhou, B.; Yang, L.; Liu, Z. L. Prooxidant activity of hydroxycinnamic acids on DNA damage in the presence of Cu(II) ions: mechanism and structure-activity relationship. *Food Chem. Toxicol.* **2008**, *46*, 149−156.

(9) Burgos-Moron, E.; Calderon-Montaño, J. M.; Orta, M. L.; Pastor, N.; Perez-Guerrero, C.; Austin, C.; Mateos, S.; Lopez-Lazaro, M. The coffee constituent chlorogenic acid induces cellular DNA damage and formation of topoisomerase I- and II-DNA complex in cells. *J. Agric. Food Chem.* **2012**, *60*, 7384−7391.

(10) Perrone, D.; Donangelo, R.; Donangelo, C. M.; Farah, A. Modeling weight loss and chlorogenic acids content in coffee during roasting. *J. Agric. Food Chem.* **2010**, *58*, 12238−12243.

(11) Clifford, M. N.; Johnston, K. L.; Knight, S.; Kuhnert, N. Hierarchical scheme for LC-MS^n identification of chlorogenic acids. *J. Agric. Food Chem.* **2003**, *51*, 2900−2911.

(12) Jaiswal, R.; Kuhnert, N. Identification and characterization of two new classes of chlorogenic acids in arnica (*Arnica Montana* L.) flowers by high-performance liquid chromatography/tandem mass spectrometry. *J. Agric. Food Chem.* **2011**, *59*, 4033−4039.

(13) Clifford, M. N. Chlorogenic acids and other cinnamates-nature, occurrence and dietary burden. *J. Sci. Food Agric.* **1999**, *79*, 362−372.

(14) Stalmach, A.; Mullen, W.; Barron, D.; Uchida, K.; Yokota, T.; Cavin, C.; Steiling, H.; Williamson, G.; Crozier, A. Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: Identification of biomarkers of coffee consumption. *Drug Metab. Dispos.* **2009**, *37*, 1749−1758.

(15) Stalmach, A.; Steiling, H.; Williamson, G.; Crozier, A. Bioavailability of chlorogenic acids following acute ingestion of coffee by humans with an ileostomy. *Arch. Biochem. Biophys.* **2010**, *501*, 98−105.

(16) Del Rio, D.; Stalmach, A.; Calani, L.; Crozier, A. Bioavailability of coffee chlorogenic acids and green tea flavan-3-ols. *Nutrients* **2010**, *2*, 820−833.

(17) Verzelloni, E.; Pellacani, C.; Tagliazucchi, D.; Tagliaferri, L.; Calani, L.; Costa, L. G.; Brighenti, F.; Borges, G.; Crozier, A.; Conte, A.; Del Rio, D. Antiglycative and neuroprotective activity of colon-derived polyphenol catabolites. *Mol. Nutr. Food Res.* **2011**, *55*, S35−S43.

(18) Duarte, S. G.; Farah, A. Effect of simultaneous consumption of milk and coffee on chlorogenic acids' bioavailability in humans. *J. Agric. Food Chem.* **2011**, *59*, 7925−7931.

(19) Farrell, T. J.; Dew, T. P.; Poquet, L.; Hanson, P.; Williamson, G. Absorption and metabolism of chlorogenic acids in cultured gastric epithelial monolayers. *Drug Metab. Dispos.* **2011**, *39*, 2338−2346.

(20) Monteiro, M.; Farah, A.; Perrone, D.; Trugo, L. C.; Donangelo, C. Chlorogenic acid compounds from coffee are differentially absorbed and metabolized in humans. *J. Nutr.* **2007**, *137*, 2196−2201.

(21) Ferruzzi, M. G. The influence of beverage composition on delivery of phenolic compounds from coffee and tea. *Phys. Behav.* **2010**, *100*, 33−41.

(22) Dupas, C.; Marsset Baglieri, A.; Ordonaud, C.; Tomé, D.; Maillard, M. N. Chlorogenic acid is poorly absorbed, independently of the food matrix: A Caco-2 cells and rat chronic absorption study. *Mol. Nutr. Food Res.* **2006**, *50*, 1053−1060.

(23) Renouf, M.; Marmet, C.; Guy, P.; Fraering, A. L.; Longet, K.; aaMoulin, J.; Enslen, M.; Barron, D.; Cavin, C.; Dionisi, F.; Rezzi, S.; Kochhar, S.; Steiling, H.; Williamson, G. Nondairy creamer, but not milk, delays the appearance of coffee phenolic acid equivalents in human plasma. *J. Nutr.* **2010**, *140*, 259−263.

(24) Palafox-Carlos, H.; Ayala-Zavala, J. F.; González-Aguilar, G. A. The role of dietary fiber in the bioaccessibility and bioavailability of fruit and vegetable antioxidants. *J. Food Sci.* **2011**, *76*, R6−R11.

(25) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130*, 2073S−2085S.

(26) Trugo, L. C.; Macrae, R. Chlorogenic acid composition of instant coffee. *Analyst* **1984**, *109*, 263−266.

(27) Tagliazucchi, D.; Verzelloni, E.; Bertolini, D.; Conte, A. In vitro bioaccessibility and antioxidant activity of grape polyphenols. *Food Chem.* **2010**, *120*, 599−606.

(28) Adler-Niessen, J. Determination of the degree of hydrolysis of food protein hydrolysates by trinitrobenzensulfonic acid. *J. Agric. Food Chem.* **1979**, *27*, 1256−1262.

(29) Verzelloni, E.; Tagliazucchi, D.; Del Rio, D.; Calani, L.; Conte, A. Antiglycative and antioxidative properties of coffee fractions. *Food Chem.* **2011**, *124*, 1430−1435.

(30) IUPAC.. Nomenclature of cyclotols. *Biochem. J.* **1976**, *153*, 23−31.

(31) Dufour, C.; Dangles, O. Flavonoid-serum albumin complexation: determination of binding constants and binding sites by fluorescence spectroscopy. *Biochim. Biophys. Acta* **2005**, *1721*, 164−173.

(32) Lacowitz, J. R. Quenching of fluorescence. *Principles of Fluorescence Spectroscopy*, 4th ed.; Springer-Verlag: New York, NY, 2006; Vol. *1*, pp 278−284.

(33) Rawel, H. M.; Frey, S. K.; Meidtner, K.; Kroll, J.; Schweigert, F. J. Determining the binding affinities of phenolic compounds to proteins by quenching of the intrinsic tryptophan fluorescence. *Mol. Nutr. Food Res.* **2006**, *50*, 705−713.

(34) Fazzarri, M.; Fukumoto, L.; Mazza, G.; Livrea, M. A.; Tesoriere, L.; Di Marco, L. In vitro bioavailability of phenolic compounds from

five cultivars of frozen sweet cherries (*Prunus avium* L.). *J. Agric. Food Chem.* **2008**, *56*, 3561−3568.
(35) Cilla, A.; González-Sarría, A.; Tomás-Barberán, F. A.; Espín, J. C.; Barberá, R. Availability of polyphenols in fruit beverages subjected to *in vitro* gastrointestinal digestion and their effect on proliferation, cell cycle and apoptosis in human colon cancer Caco-2 cells. *Food Chem.* **2009**, *114*, 813−820.
(36) Xie, C.; Yu, K.; Zhong, D.; Yuan, T.; Ye, F.; Jarrel, J.; Millar, A.; Chen, X. Investigation of isomeric transformation of chlorogenic acid in buffers and biological matrices by ultra performance liquid chromatography coupled with hybrid quadrupole/ion mobility/orthogonal acceleration time-of-flight mass spectrometry. *J. Agric. Food Chem.* **2011**, *59*, 11078−11087.
(37) Siracusa, L.; Kulisic-Bilusic, T.; Politeo, O.; Krause, I.; Dejanovic, B.; Ruperto, G. Phenolic composition and antioxidant activity of aqueous infusions from *Capparis spinosa* L. and *Crithmum maritimum* L. before and after submission to a two-step in vitro digestion model. *J. Agric. Food Chem.* **2011**, *59*, 12453−12459.
(38) Rawel, H. M.; Kroll, J.; Riese, B. Reactions of chlorogenic acid with lysozyme: physicochemical characterization and proteolytic digestion of the derivatives. *J. Food Sci.* **2000**, *65*, 1091−1098.
(39) Bekedam, E. K.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. High molecular weight melanoidins from coffee brew. *J. Agric. Food Chem.* **2006**, *54*, 7658−7666.
(40) Bekedam, E. K.; Roos, E.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. Low molecular weight melanoidins from coffee brew. *J. Agric. Food Chem.* **2008**, *56*, 4060−4067.
(41) Bekedam, E. K.; Loots, M. J.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. Roasting effects on formation mechanisms of coffee brew melanoidins. *J. Agric. Food Chem.* **2008**, *56*, 7138−7145.
(42) Bekedam, E. K.; Roos, E.; Schols, H. A.; van Boekel, M. A. J. S.; Smit, G. Incorporation of chlorogenic acids in coffee brew melanoidins. *J. Agric. Food Chem.* **2008**, *56*, 2055−2063.
(43) Verzelloni, E.; Tagliazucchi, D.; Conte, A. From balsamic to healthy: traditional balsamic vinegar melanoidins inhibit lipid peroxidation during simulated gastric digestion. *Food Chem. Toxicol.* **2011**, *48*, 2097−2102.
(44) Hasni, I.; Bourassa, P.; Hamdani, S.; Samson, G.; Carpentier, R.; Tajmir-Rihai, H. A. Interaction of milk α- and β-caseins with tea polyphenols. *Food Chem.* **2011**, *126*, 630−639.
(45) Charlton, A. J.; Baxter, N. J.; Khan, M. L.; Moir, A. J. G.; Haslam, E.; Davies, A. P.; Williamson, M. P. Polyphenols/peptide binding and precipitation. *J. Agric. Food Chem.* **2002**, *50*, 1593−1601.
(46) Haslam, E. Polyphenol-protein interactions. *Biochem. J.* **1976**, *139*, 285−288.
(47) Martin, R.; Lilley, T. H.; Falshaw, C. P.; Haslam, E.; Begley, M. J.; Magnolato, D. The caffeine-potassium chlorogenate molecular complex. *Phytochemistry* **1987**, *26*, 273−279.
(48) aaClifford, M. N.; Ohiokpehai, O. Coffee astringency. *Anal. Proc.* **1983**, *29*, 83−86.
(49) Callis, P. R. ^1L_a and ^1L_b transitions of tryptophan: Applications of theory and experimental observation to fluorescence of proteins. *Methods Enzymol.* **1997**, *237*, 113−150.
(50) Green, R. J.; Murphy, A. S.; Schulz, B.; Watkins, B. A.; Ferruzzi, M. G. Common tea formulations modulate *in vitro* digestive recovery of green tea catechins. *Mol. Nutr. Food Res.* **2007**, *51*, 1152−1162.
(51) Van der Burg-Koorevaar, M. C. D.; Miret, S.; Duchateau, G. S. M. J. E. Effect of milk and brewing method on black tea catechin bioaccessibility. *J. Agric. Food Chem.* **2011**, *59*, 7752−7758.
(52) Zulueta, A.; Maurizi, A.; Frigola, A.; Esteve, M. J.; Coli, R.; Burini, G. Antioxidant capacity of cow milk, whey and deproteinized milk. *Int. Dairy J.* **2009**, *19*, 380−385.
(53) Clifford, M. N. Chlorogenic acids. In *Coffee 1. Chemistry*; Clarke, R. J., Macrae, R., Eds.; Elsevier Applied Science: London, 1985; pp 153−202.
(54) Clifford, M. N. Chlorogenic acids and other cinnamates - nature, occurrence and dietary burden, absorption and metabolism. *J. Sci. Food Agric.* **2000**, *80*, 1033−1042.
(55) Haslam, E. *Practical Polyphenolics. From Structure to Molecular Recognition and Physiological Action*; Cambridge University Press: Cambridge, 1999; pp 1−438.
(56) Clifford, M. N. Phenol-protein interactions and their possible significance for astringency. In *Interactions of Food Components*; Birch, G. G., Lindley, M. G., Eds.; Elsevier Applied Science: London, 1986; pp 143−164.
(57) Horman, I.; Viani, R. The nature and conformation of the caffeine-chlorogenate complex of coffee. *J. Food Sci.* **1972**, *37*, 925−927.
(58) Redeuil, K.; Smarrito-Menozzi, C.; Guy, P.; Rezzi, S.; Dionisi, F.; Williamson, G.; Nagy, K.; Renouf, M. identification of novel circulating coffee metabolites in human plasma by liquid chromatography-mass spectrometry. *J. Chromatogr., A* **2011**, *1218*, 4678−4688.