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ARTICLE

Influence of Chocolate Matrix Composition on Cocoa Flavan-3-ol Bioaccessibility In Vitro and Bioavailability in Humans

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Conflicting data exist regarding the influence of chocolate matrices on the bioavailability of epicatechin (EC) from cocoa. The objective of this study was to assess the bioavailability of EC from matrices varying in macronutrient composition and physical form. EC bioavailability was assessed from chocolate confections [reference dark chocolate (CDK), high sucrose (CHS), high milk protein (CMP)] and cocoa beverages [sucrose milk protein (BSMP), non-nutritive sweetener milk protein (BNMP)], in humans and in vitro. Six subjects consumed each product in a randomized crossover design, with serum EC concentrations monitored over 6 h post consumption. Areas under the serum concentration−time curve (AUC) were similar among chocolate matrices. However, AUCs were significantly increased for BSMP and BNMP (132 and 143 nM h) versus CMP (101 nM h). Peak serum concentrations (C_{MAX}) were also increased for BSMP and BNMP (43 and 42 nM) compared to CDK and CMP (32 and 25 nM). Mean T_{MAX} values were lower, although not statistically different, for beverages (0.9−1.1h) versus confections (1.8−2.3h), reflecting distinct shapes of the pharmacokinetic curves for beverages and confections. In vitro bioaccessibility and Caco-2 accumulation did not differ between treatments. These data suggest that bioavailability of cocoa flavan-3-ols is likely similar from typical commercial cocoa based foods and beverages, but that the physical form and sucrose content may influence T_{MAX} and C_{MAX}.

KEYWORDS: *Theobroma cacao*; food matrix; chocolate; catechin; epicatechin; bioavailability; in vitro digestion; Caco-2 cells

INTRODUCTION

Cocoa and chocolate products have generated significant interest due to their association with various health-protective and therapeutic activities. Cocoa and chocolate are rich sources of flavan-3-ols, which are present in monomeric and polymeric forms (*1*−*5*). Although the concentration of specific flavan-3-ol species in chocolate varies greatly depending upon the raw material, the type of processing, and the nature of the finished product (*4*, *6*−*8*), monomers are one of the predominant forms present (*4*, *6*). Major monomeric flavan-3-ols present in chocolate include (+)-catechin and (−)-catechin (referred to collectively as C), and (−)-epicatechin (EC, **Figure 1**) (*1*). Many of the proposed health-protective activities associated with the consumption of cocoa and chocolate have been attributed to flavan-3-ols, including monomers (*9*−*12*). Such activities include decreased blood pressure (*13*−*16*), increased circulating nitric oxide (NO) species (*10*, *17*), decreased platelet adhesion (*18*), improved serum cholesterol profiles (*15*, *16*, *19*−*21*), improved endothelial function (*10*, *13*, *16*−*18*, *22*), increased serum and/or plasma antioxidant activity (*21*, *23*), decreased susceptibility to low density lipoprotein (LDL) oxidation (*12*, *19*, *21*, *23*, *24*), and increased insulin sensitivity and decreased insulin resistance (*14*−*16*). Interest in these activities has resulted in the need to better understand the bioavailability of flavan-3-ol monomers, especially when consumed at levels reasonably found in commercial chocolate matrices such as confections and beverages. Due to the fact that the net absorption and resulting circulating levels of flavan-3-ols are regarded to be generally low (*25*−*27*), designing matrices to optimize the flavan-3-ol bioavailability resulting from cocoa consumption could prove to be an important strategy for maximizing the in vivo health benefits from cocoa products.

Although data exist regarding the bioavailability of C and EC from chocolate, previous investigations of the influence of food matrices have generated conflicting data regarding the influence of factors such as milk protein, carbohydrate, and physical form of the product as consumed (solid chocolate confection vs cocoa beverage). Serafini et al. (*28*) reported that the area under the EC plasma pharmacokinetic curve (AUC) was significantly lower for

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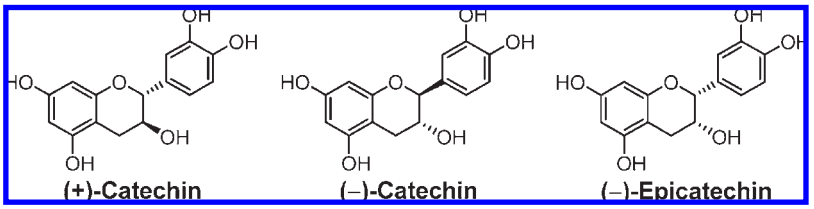

Figure 1. Structures of (+)-catechin and (−)-catechin (collectively referred to as C) and (−)-epicatechin (EC), the predominant flavan-3-ol monomers present in cocoa powder and chocolate products.

milk chocolate compared to dark chocolate of the same flavan-3-ol content, while consumption of liquid milk with dark chocolate produced an intermediate AUC, which was also statistically lower than the dark chocolate. However, Schramm et al. (*29*), Schroeter et al. (*30*), and Roura et al. (*31*) reported that liquid milk consumed with cocoa resulted in slightly but not significantly higher AUCs for EC than consumption of cocoa with water. Additionally, Keogh et al. (*32*) found essentially no difference in the bioavailability and plasma levels of C and EC between cocoa consumed with liquid milk versus water. Interestingly, the study by Serafini et al. (*28*) involved confection chocolate, while the other studies employed cocoa beverages. These data suggest that the physical form of the chocolate, in addition to the ingredient profile, could influence the plasma kinetics of cocoa flavan-3-ols.

Schramm et al. (*29*) investigated the effect of carbohydrate on the absorption of cocoa flavan-3-ols and reported that the presence of carbohydrate resulted in increased plasma AUC and maximal plasma concentrations of EC in a dose-dependent manner following consumption of cocoa with water, while T_{MAX} appeared to be similar regardless of carbohydrate content. However, limited data exist regarding whether carbohydrate levels similarly affect EC bioavailability when confection chocolate is consumed.

While promising, significant questions remain regarding the effects of chocolate matrices on cocoa flavan-3-ol bioavailability. A challenge in interpreting these studies stems from the fact that studies have focused on the differences between specific ingredients, while differences between confection and beverage products has not been directly examined. Also, some studies have been performed with high levels of chocolate or cocoa extracts, resulting in polyphenol doses that are in some cases significantly higher than those obtained when typical amounts of commercial chocolate are consumed (*28*, *31*−*35*).

The objectives of this study were to assess the impact of the physical form and composition of chocolate matrices on monomeric flavan-3-ol bioavailability following consumption of commercially relevant chocolate confections or cocoa beverages, and to investigate the digestive mechanisms by which matrix factors may influence the pharmacokinetic behavior of cocoa flavan-3-ols in vivo. Identification of digestive and absorptive mechanisms modulated by beverage and confection matrix factors could potentially lead to the development of formulation strategies designed specifically to optimize the bioavailability and, by extension, the in vivo activities of monomeric cocoa flavan-3-ols.

MATERIALS AND METHODS

Solvents and Standards. All solvents and acids were obtained from Mallinckrodt Baker (Phillipsburg, NJ) and were HPLC grade (solvents) or AR-ACS (analytical reagent-American Chemical Society) grade (acids) or higher, unless otherwise specified. All H_2O was distilled/deionized using a Barnstead MegaPure MP-1 system (Dubuque, IA). Authentic (±)-C and (−)-EC standards were obtained from Sigma-Aldrich (St. Louis, MO).

Chocolate Matrices. Chocolate matrices were prepared by Kraft Foods Research and Development (Munich, Germany). Three solid confection [reference dark chocolate (CDK), high sucrose (CHS), high milk protein (CMP)] and two beverage [sucrose milk protein (BSMP), non-nutritive sweetener milk protein (BNMP)] matrices providing commercially relevant levels of C and EC (approximately 36 mg C+EC/serving based on analysis of raw materials) from cocoa were formulated (refer to **Table 1** for matrix formulation information). Serving size for bars was 40 g, and serving size for beverages was 250 mL (all solid ingredients were dissolved in 250 mL boiling water and consumed as a beverage).

Human In Vivo Pharmacokinetics. Clinical protocols were developed in accordance with Purdue University Human Research Protection Program guidelines and were approved by the University's Biomedical Institutional Review Board. Six healthy subjects (three male, three female, all Caucasian) with a mean age of 23.8 ± 1.7 y and a mean body mass index (BMI) of 25.5 ± 0.7 kg/m^2 were recruited from the greater Lafayette, IN area. Each subject consumed all five products once in a crossover design, with a seven d washout period between samples. The order of consumption was randomized for each individual. Following an overnight fast, a catheter was placed in a vein in the antecubetal space of the dominant arm of each subject and kept patent by continuous drip of sterile saline solution (due to the low circulating levels of the compounds of interest observed for the majority of subjects when consuming a normal diet, the overnight fast sufficiently reduced baseline serum concentrations relative to the concentrations observed during the acute pharmacokinetic experiment). Baseline blood samples were collected (0 h), and subjects then consumed one serving of chocolate (40 g confection or 250 mL beverage, see **Table 1** for composition) over ≤10 min. Blood samples were drawn at 0.5, 1, 2, 4, and 6 h after dosing. Blood samples were centrifuged (92 × *g*, 10 min, 4 °C), and the resulting serum was stabilized by addition of 0.06 M L-ascorbic acid (L-AA, Mallinckrodt Baker) in 0.15 M aqueous NaCl (25 μL/100 μL serum) and stored at −80 °C under N_2 prior to analysis.

Serum extractions were performed following the method of Wang et al. (*23*) with minor modifications. Aliquots (400 μL) of serum were combined with 50 μL of a solution containing β-glucuronidase (type H-1 from *Helix pomatia*, Sigma-Aldrich) (*36*−*40*) and L-AA (80 kU mL^{-1} and 2.74 M, respectively) in aqueous $Na_2EDTA \cdot 2H_2O$ (2.15 mM, Sigma-Aldrich) was added to facilitate quantification of total EC after deconjugation of glucuronide and sulfate metabolites present in plasma. The sample was blanketed with N_2 and incubated in a dark shaking water bath at 37 °C for 45 min. Following incubation, 0.5 mL of acetonitrile (ACN) was added, and the sample was vortexed and centrifuged (17970 × *g*, 5 min, 25 °C). The supernatant was collected and mixed with 1 mL of an aqueous suspension containing 0.1 g mL^{-1} Brockman I weakly acidic activated alumina (Al_2O_3, surface area: 155 m^2 g^{-1}, Sigma-Aldrich) in Tris-HCl buffer (50 mM, pH 7.0, Fisher Scientific, Pittsburgh, PA). The mixture was incubated at room temperature for 30 min with intermittent vortexing. Following incubation, the sample was centrifuged and the supernatant was discarded. The pellet was washed with 1 mL of the Tris-HCL buffer, vortexed, centrifuged, and the supernatant was discarded. The pellet was washed again with 1 mL methanol (MeOH), vortexed, centrifuged, and the supernatant was discarded. The pellet was then dried under N_2, 100 μL of 0.25 M $HClO_4$ (ACS analytical reagent grade, Mallinckrodt Baker) was added, and the mixture was vortexed, sonicated, and centrifuged. The supernatant was then filtered through a 17 mm diameter (0.45 μm) PTFE filter (National Scientific, Rockwood, TN) and analyzed by reverse-phase (RP)-HPLC. The efficiency of extraction was assessed by spiking baseline serum samples (400 μL) with known concentrations of EC in water (40 μL) to obtain concentration on the order of

Table 1. Formulation Descriptions of the Five Chocolate Matrices

	matrix				
	CDK	CHS	CMP	BSMP	BNMP
consumed as formulation	solid reference dark chocolate	solid high sucrose	solid milk protein	liquid sucrose milk protein	liquid non-nutritive sweetener milk protein
Ingredient	Solid Components (g/serving)				
cocoa butter	20.00	12.00	14.00	0.00	0.00
cocoa powder	13.40	13.40	13.40	13.53	13.55
sucrose	6.55	14.55	6.55	6.55	0.00
vanilla flavor	0.03	0.03	0.03	0.03	0.03
soya lecithin	0.02	0.02	0.02	0.02	0.02
milk protein[a]	0.00	0.00	6.00	6.06	6.07
sucralose	0.00	0.00	0.00	0.00	0.03
total	40.00	40.00	40.00	28.20	19.67
Flavan-3-ol	Composition (mg/serving)[b]				
C	9.35	9.35	9.35	9.44	9.46
EC	27.13	27.13	27.13	27.39	27.44
total monomer	36.49	36.49	36.49	36.84	36.90
dimer-octomer	78.76	78.76	78.76	78.76	78.76
total	115.25	115.25	115.25	115.25	115.25

[a] Milk protein concentrate (80%). [b] Flavan-3-ol monomer composition was determined by reverse-phase HPLC, as described in Materials and Methods. Data represent mean of four independent analyses. Total flavan-3-ol and dimer-octomer flavan-3-ol composition was determined using normal-phase HPLC by Kraft Foods Research and Development (Munich, Germany).

typical serum values, extracting as stated above, and analyzing by RP-HPLC. The extraction efficiency of EC was calculated to be 106 ± 13%. To validate the effectiveness of the enzymatic deconjugation, HPLC-(−)-ESI-MS analysis, which was not possible for individual samples due to the low concentrations of individual metabolites, was performed on pooled samples of serum treated with or without β-glucuronidase. Analysis indicated that samples not treated with β-glucuronidase contained various O-methyl glucuronides (m/z 479) and O-glucuronides (m/z 465), whereas these conjugates were not detected in samples treated with β-glucuronidase (data not shown). The contribution of O-methyl metabolites to total EC response was not measured. However, these appear to be present in blood at low levels for EC doses similar to those used in the present study (< 30 mg), present at < 25% of the total metabolites at intermediate EC doses (30−55 mg), and only appear in large amounts relative to the other metabolites when higher EC doses are consumed (≥ 90 mg) (*10*, *17*, *31*, *34*).

Extraction of Catechins from Chocolate Products. Extractions of food matrices were performed (n = 4 per matrix) to assess flavan-3-ol monomer content. For confections, 1 g chocolate was melted at 50 °C for 15 min. For beverage products, 5 mL formulated beverage was employed; additionally, $(NH_4)_2SO_2$ (ACS reagent grade, Sigma-Aldrich) was added to saturation (for beverage extractions only) to break any emulsions. Products were defatted 3× by vortexing with 5 mL of hexane, sonicating for 5 min, centrifugation (3901 × g, 5 min, 4 °C), and discarding the organic layer. Products were then extracted 3× by vortexing with 5 mL of an acetone/H_2O/glacial acetic acid mixture (70:28:2 v/v), sonicating for 5 min, centrifugation (5 min, 4 °C), and pooling of the supernatants. Pooled supernatants were diluted to 100 mL with the acetone/water/glacial acetic acid mixture, filtered with a 0.45 μm PTFE filter, and analyzed by RP-HPLC.

In Vitro Digestion. A two-phase in vitro digestion protocol employing porcine digestive enzymes originally developed by Garrett et al. (*41*) was performed with modifications to mimic the gastric and small intestinal phases of digestion as a means to assess the bioaccessibility of C and EC from the food matrices (n = 4 replicate digestions per matrix). Food matrices were diluted to provide the equivalent of one serving (40 g confection or 250 mL beverage) in 2 L (approximate digestive volume) and scaled to 50 mL total in vitro digestive volume. For confections, 1 g chocolate was melted at 50 °C for 15 min and mixed with 29 mL of 0.15 M aqueous NaCl at room temperature to mimic the melting and chewing that occurs during the oral phase of digestion when confections are consumed. For beverages, 6.25 mL of beverage was mixed with 23.75 mL 0.15 M aqueous NaCl. The gastric phase was simulated as follows: 3 mL porcine pepsin solution (40 mg mL^{-1} in 0.1 M HCl) was added, the pH was adjusted to 2.5 with 0.1 M HCL, and the sample was blanketed with N_2 and incubated in a dark shaking water bath (37 °C) for 1 h. The small intestinal phase was then simulated as follows: gastric digesta was neutralized to pH > 5.3 with 1 M NaOH, 4.5 mL of porcine bile solution (48 mg mL^{-1} in 0.1 M HCO_3) and 4.5 mL of porcine pancreatin/lipase solution (400 mg mL^{-1} each in 0.1 M HCO_3) were added, the pH was adjusted to 6.5 with 0.1 M NaOH, the volume was adjusted to 50 mL total with 0.15 M aqueous NaCl, and the sample was blanketed with N_2 and incubated as described above for 2 h. Following digestion, the crude digesta was centrifuged (29200 × g, 1 h, 4 °C). Aliquots of the resulting aqueous (bioaccessible) fraction were pooled for each matrix, blanketed with N_2, and stored at −80 °C for cell culture experiments. Additionally, 0.4 mL aliquots of each aqueous fraction were acidified with 1.6 mL of 2% acetic acid (v/v), blanketed with N_2, and stored at −80 °C prior to RP-HPLC analysis. Pepsin from porcine stomach mucosa, porcine bile extract, pancreatin, and lipase (type II) from porcine pancreas were all obtained from Sigma-Aldrich.

Caco-2 Cell Culture Experiments. Caco-2 human intestinal epithelial cells (TC7 clone) were employed as a model of flavan-3-ol intestinal absorption. Cells (passage 80−90) were seeded at a density of 6.4 × 10^4 cells/well in six-well polystyrene plates (9.5 cm^2/well, Corning Inc., Corning, NY) and cultured to 10 d postconfluence to obtain highly differentiated polarized monolayers. Cells were cultured in 2 mL/well complete Dulbecco's Modified Eagle's Medium (DMEM, Lonza Group, Basel, Switzerland) containing 10% (v/v) fetal bovine serum (FBS, JR Scientific Inc., Woodland, CA), with media replaced every 2 d. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Experimental media for accumulation studies were prepared by diluting pooled aqueous (bioaccessible) fractions of in vitro digesta with pH 5 phosphate-buffered saline (PBS, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.14 M NaCl, 8.1 mM Na_2HPO_4, Invitrogen, Carlsbad, CA, 10 mL digesta + 40 mL PBS). Dilute digesta were then filter-sterilized through 0.22 μm Millipore Express PLUS membranes (Millipore Corp., Billerica, MA). For accumulation experiments, monolayers were washed with 2 mL of PBS (pH 7.4), and then 1.8 mL of dilute digesta/well was applied and incubated at 37 °C for 1 h (n = 5 wells per matrix). Monolayers were then washed 2× with 2 mL of cold PBS (pH 5), covered with 1 mL of cold

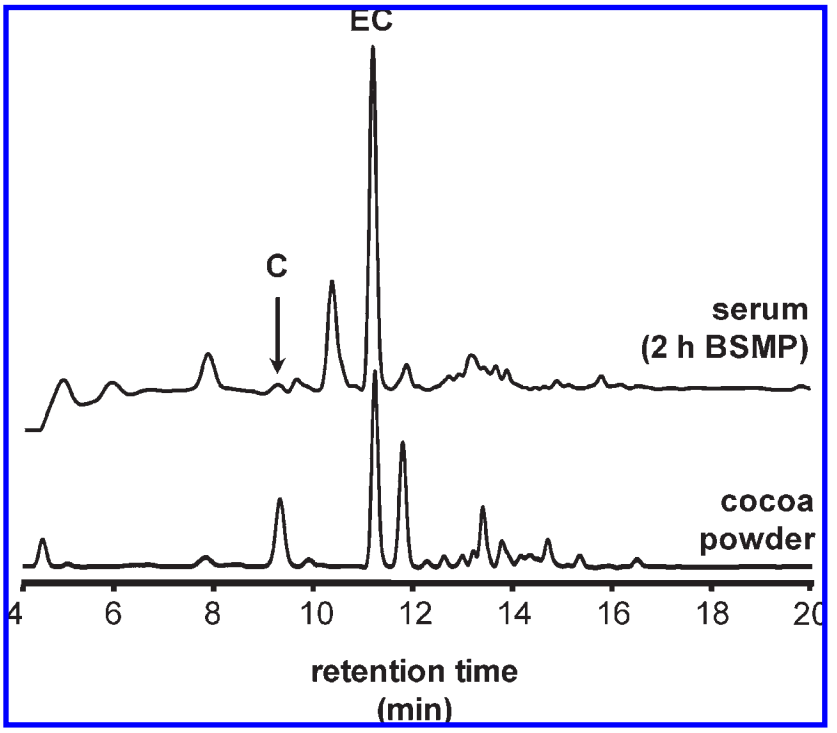

Figure 2. Reverse-phase (RP)-HPLC chromatograms of an extract of serum collected from a subject 2 h after consumption of the beverage product formulated with sucrose and milk protein (BSMP) (top) and an extract of the cocoa powder used to formulate chocolate confections and cocoa beverages (bottom; response at 200 mV shown, scales are distinct for each chromatogram as flavan-3-ols are present at very low levels in serum). Note that any sulfate and glucuronide phase-II metabolites present in serum have been converted to the native forms by deconjugation during extraction.

PBS (pH 5), scraped from the plate, and stored at −80 °C under N_2 prior to analysis. Protein values were determined by the bicinchoninic acid (BCA) method using a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA), and were found to be 1.96 ± 0.09 mg/well. For extraction of flavan-3-ols, cells were thawed, ultrasonicated in 3 mL of 4.5 mM butylated hydroxytoluene (BHT, Sigma-Aldrich) in ethyl acetate for 30 s on ice, centrifuged (3901 × *g*, 10 min, 4 °C), and the organic layer was collected. The samples were extracted 2× more with 3 mL of 4.5 mM BHT in ethyl acetate by vortexing, centrifugation, and pooling of the organic layers. Extracts were dried under vacuum, dissolved in 200 μL of RP-HPLC mobile phase A (see below) with sonication, filtered with a PTFE filter, and analyzed by RP-HPLC.

RP-HPLC Analysis. Catechins were quantified by RP-HPLC with electrochemical array detection (ECD). RP-HPLC separation was performed on an HP1050 liquid chromatograph (Agilent Technologies, Santa Clara, CA) equipped with a Waters XTerra RP-C_{18} column (3.9 × 100 mm, 3.5 μm particles, Milford, MA). Binary elution was employed using the following mobile phases: ACN/H_20/trifluoroacetic acid (TFA, reagent grade, Sigma-Aldrich), 8:91.9:0.1 (v/v, phase A) and MeOH/ACN/H_20/TFA, 3:27:69.9:0.1 (v/v, phase B). Linear gradient transitions were achieved by the following program: 99% A from 0 to 3 min, 50% A at 8 min, 10% A from 10 to 13 min, 99% A from 15 to 20 min. System flow rate was 0.8 mL min^{-1} and column temperature was 25 °C. Injection volumes were 5 μL for chocolate extracts, aqueous digesta fractions, and cell culture media, 40 μL for Caco-2 cell extracts, and 75 μL for serum extracts. Detection was performed on an 8-channel CoulArray ECD (ESA Biosciences, Chelmsford, MA) with cell potentials at −100, 60, 130, 200, 270, 340, 410, and 480 mV, and summation of peak areas from the three-channel cluster of greatest response for each peak was performed. Quantification was performed from standard response curves prepared using three-channel cluster peak areas from analysis of known concentrations of authentic standard material.

Data Analysis. Statistical analyses were performed using SAS 9.1.3 software (SAS Institute Inc., Cary, NC). In vivo serum EC levels, in vitro bioaccessibility, and Caco-2 intracellular levels are reported as mean ± standard error of the mean (SEM). Area under the serum concentration vs time curve (AUC) for EC was calculated using the linear trapezoidal rule. The maximum serum concentration (C_{MAX}), and the time at which the maximum serum concentration was observed (T_{MAX}) were determined from individual serum concentration versus time curves and expressed as mean ± SEM. Human pharmacokinetic parameters were analyzed using repeated-measures analysis of variance (ANOVA), and significance was determined using a Fisher's protected least significant difference (Fisher's PLSD) test (α = 0.05). Differences in in vitro digestion recoveries and Caco-2 cell accumulation data were determined by ANOVA using Tukey's pairwise comparisons of least-squares means (α = 0.05).

RESULTS AND DISCUSSION

In Vivo Pharmacokinetics. A representative chromatogram demonstrating the RP-HPLC separation of cocoa polyphenols detected in human serum compared to the polyphenol profile from the cocoa powder used to formulate the chocolate matrices is presented in **Figure 2**. The profile of detected compounds represents the sum of free C and EC as well as glucuronide and sulfate metabolites, due to the deconjugation step in the extraction. The contribution of O-methyl metabolites was not determined due to the limitations of ECD detection, which was necessary due to the low level of C, EC, and their metabolites in serum. However, O-methyl forms appear to be present in blood at low levels for EC doses similar to those used in the present study (< 30 mg), and the contribution of O-methyl forms increases with higher doses of EC (*10*, *17*, *31*, *34*). Although both C and EC were detected in serum, C was present at extremely low concentrations in serum for the majority of samples [it should be noted that the RP-HPLC method used in this study did not resolve (+)- and (−)-C and, therefore, both elute as one peak and are designated collectively as C]. This is likely due to both the lower C content of most cocoa powders (~9 mg C vs ~27 mg EC per serving in the cocoa used in the present study) and the reported lower bioavailability of (−)-C (which accounts for roughly 89% of the C in chocolate) compared to (+)-C and (−)-EC (*1*, *42*, *43*). As a result of the low serum response of C, the

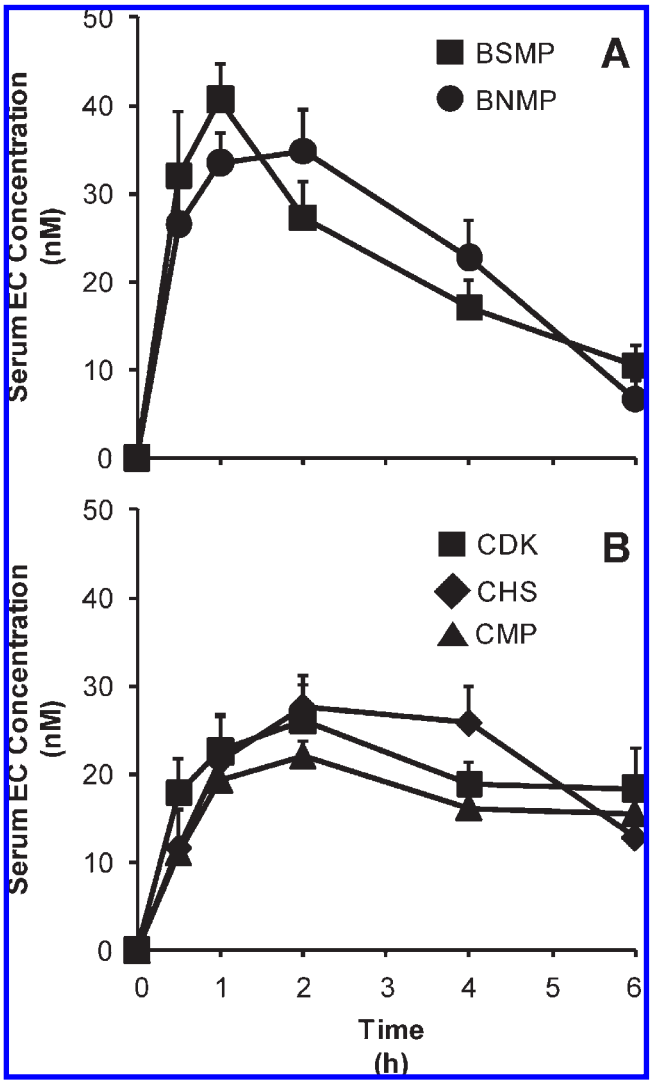

Figure 3. EC serum pharmacokinetic curves (0−6 h) in subjects fed each of the (A) cocoa beverages and (B) chocolate confections. Curve values represent the mean serum concentration from $n = 6$ subjects per time point for each matrix and error bars represent the SEM.

majority of published data regarding the bioavailability of monomeric flavan-3-ols from chocolate have focused exclusively on EC. Similarly, only EC data are reported here for the in vivo experiments.

Serum pharmacokinetic curves of EC for 0−6 h postconsumption are presented in **Figure 3**, and calculated pharmacokinetic parameters (AUC, T_{MAX}, and C_{MAX}) are listed in **Table 2**. While low, EC pharmacokinetic parameters including C_{MAX} were consistent with previously reports of the bioavailability of EC from cocoa and chocolate, once normalized by EC dose (*10*, *17*, *21*−*23*, *28*, *29*, *31*−*34*, *44*−*50*). Shapes of the pharmacokinetic curves indicate distinct serum EC responses for beverage (BSMP, BNMP) versus confection (CDK, CHS, CMP) matrices.

Serum AUC is the measure of EC bioavailability from the food matrices assessed in this study. AUC values were not significantly different between confections, in agreement with the studies suggesting that the presence of milk does not negatively affect the bioavailability of cocoa flavan-3-ols. However, the AUC from milk protein-containing CMP (100.7 ± 11.1 nM h) was the lowest of the three confections (121.1 ± 12.6 and 128.1 ± 13.1 nM h for CDK and CHS, respectively). The AUCs were not significantly different between the beverages. The AUC from CMP, but not CDK or CHS, was significantly lower than both milk-containing beverages (132.1 ± 14.8 and 142.7 ± 9.3 nM h for BSMP and BNMP, respectively). These results suggest that the presence of milk solids does not significantly impact the bioavailability (as measured by AUC) of EC among chocolate confection products. However, the physical state of the cocoa-containing product does appear to have an impact on overall EC bioavailability.

Table 2. EC Pharmacokinetic Parameters (0-6 h) in Serum of Subjects Fed Each of the Chocolate Formulations[a]

	pharmacokinetic parameters		
formulation	AUC (nM h)	T_{MAX} (h)	C_{MAX} (nM)
CDK	121.1 ± 12.6^{ab}	2.3 ± 0.8^{a}	31.6 ± 2.8^{bc}
CHS	128.1 ± 13.1^{ab}	1.8 ± 0.5^{a}	34.0 ± 3.3^{ab}
CMP	100.7 ± 11.1^{b}	2.3 ± 0.8^{a}	24.7 ± 1.9^{c}
BSMP	132.1 ± 14.8^{a}	0.9 ± 0.1^{a}	42.5 ± 4.3^{a}
BNMP	142.7 ± 9.3^{a}	1.1 ± 0.3^{a}	41.6 ± 2.1^{a}

[a] The area under the pharmacokinetic curve (AUC), the time at which the maximum serum concentration was observed (T_{MAX}), and the maximum serum concentrations observed (C_{MAX}). Values represent the mean ± SEM from $n = 6$ subjects for each formulation. Common superscripts in the same column indicate no significant difference ($P > 0.05$) between formulations.

This observed effect should be considered in the context of 6 h pharmacokinetic curves only. The 6 h experimental time frame was chosen based on both practical clinical considerations and previous literature, indicating a return of EC blood concentrations to baseline values between 6 and 8 h postconsumption of catechin-rich foods, including chocolate (*28*, *29*, *31*, *32*, *35*, *51*). While beverages had lower mean serum concentrations (10.5 ± 2.2 and 6.7 ± 2.1 nM for BSMP and BNMP, respectively) at 6 h, confections (18.7 ± 4.6, 12.8 ± 2.7, and 15.5 ± 3.0 nM for CDK, CHS, and CMP, respectively) remained significantly higher than original baseline levels. The incomplete clearance of EC suggests that absorption may not have been complete from confections, especially CDK and CMP. Thus, the AUC from CMP would likely have increased relative to the beverage products if serum samples were collected at 8 and 10 h and factored into the pharmacokinetic calculations. Therefore, observed differences between the AUC of CMP and BSMP/BNMP may in fact be minimized once the serum EC returns to baseline for all treatments. This leaves open the possibility that absorption at later time points would have normalized the relative bioavailability of EC for all matrices.

Although not significantly different between matrices, T_{MAX} values tended to be lower from beverages versus confections and lower from treatments containing sucrose (BSMP vs BNMP, and CHS vs CDK and CMP). Specifically, comparison of T_{MAX} between CMP and the beverages BSMP and BNMP, which all contained milk and therefore facilitate the best comparison, approached significance ($p = 0.08$ and 0.12, respectively). The distinct shapes of the pharmacokinetic curves for confections versus beverages, the distinct grouping of T_{MAX} values for confections (1.8−2.3 h) versus beverages (0.9−1.1 h), as well as the fact that T_{MAX} is only observed as a discrete variable, suggest that the T_{MAX} differences between treatments may still be of practical significance in vivo, albeit not found to be statistically different in the current study. The extent to which T_{MAX} may be influenced by cocoa product form thus merits further study. Combined, these data suggest that the physical state of the product may be the principal factor governing serum T_{MAX}, while sucrose appears to lower T_{MAX} to a lesser extent. It is likely that the physical state may influence gastric emptying and, potentially, the rate of solubilization of flavan-3-ols in the gut lumen from chocolate matrices.

C_{MAX} values for beverages were generally higher than those of confections. Both beverages had statistically higher C_{MAX} values (42.5 ± 4.3 and 41.6 ± 2.1 nM for BSMP and BNMP, respectively) than those of CDK and CMP (31.6 ± 2.8 and 24.7 ± 1.9 nM, respectively) but were not different from CHS (34.0 ± 3.3). Though not significantly different, the high sucrose confection (CHS) had the highest C_{MAX} value among the confections, while the sucrose-containing beverage (BSMP) had the highest C_{MAX} value among the beverages, which roughly agrees with the results of Schraam et al. that carbohydrate increases C_{MAX} relative to low or carbohydrate-free matrices

Among the confections, CDK and CHS C_{MAX} values were not significantly different, but CHS was significantly higher than CMP. The presence of milk protein (CMP) appears to coincide with a decreased C_{MAX} compared to the high sucrose among confection matrices, but this effect is not observed between the beverage matrices (which both contained the same amount of milk solids as CMP) and the confections. Therefore, as with the other pharmacokinetic parameters, the physical state of the product as consumed appears to be the principal factor governing serum EC C_{MAX}.

When considered together, these pharmacokinetic data suggest that the physical state of the product does influence the kinetics of EC absorption. However, overall bioavailability of EC as measured by AUC is likely to be similar.

In Vitro Digestion. While the influence of product matrix formulation (physical state and ingredient composition) on flavan-3-ol bioavailability (AUC) was modest, significant differences in the relative rate of absorption (T_{MAX}) and peak serum levels of EC (C_{MAX}) were observed between confection and beverage products, although differences in T_{MAX} were not statistically significant. It is possible that differences in flavan-3-ol bioaccessibility between confections and beverages may have influenced the kinetics of absorption. In this context, bioaccessibility is defined as the proportion of C and EC released from the food matrix, solubilized in the gut lumen, and made available for absorption by the intestinal epithelia (*41*, *52*). To assess the influence of food matrix factors on digestive events occurring in the lumen of the upper gastrointestinal (GI) tract (i.e., preabsorption), a two-phase in vitro digestion protocol designed to mimic the pH and enzyme conditions of the stomach and small intestinal regions sequentially was employed.

For the food matrices, in vitro bioaccessibility was defined as the % of C or EC present in the food prior to in vitro digestion that was subsequently recovered in the aqueous phase following digestion and centrifugation (**Figure 4**). The in vitro bioaccessibilities of EC were not significantly different among confection and beverage products, with recoveries of 77.1 ± 2.3, 78.6 ± 1.1, 77.9 ± 1.3, 79.4 ± 1.1, and 77.6 ± 0.8% of EC from CDK, CHS, CMP, BSMP, and BNMP, respectively, following digestion. EC bioaccessibility was similar for all matrices, regardless of physical state or presence/absence of sucrose or milk protein (note that in vitro digestion predicts the extent of solubilization, not the rate). Therefore, it is likely that differences observed in key pharmacokinetic parameters, including T_{MAX} and C_{MAX}, are not a result of limitations to the extent of EC solubility or digestive release from the different food matricies.

In vitro bioaccessibility of C was 77.4 ± 1.5, 76.2 ± 2.4, 72.9 ± 1.4, 91.4 ± 4.1, and 88.4 ± 3.8% of C from CDK, CHS, CMP, BSMP, and BNMP, respectively. For C, the recoveries from the beverages were significantly higher than the recoveries from the confections. This suggests that the bioaccessibility of C may be enhanced in beverages. While serum C concentrations were below the limits of quantification, this observation that matrix formulation may have differential effects on C and EC bioaccessibility within the same food matrix merits further study.

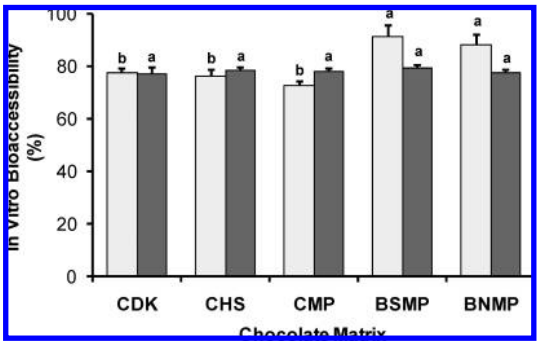

Figure 4. In vitro bioaccessibility of (light gray bar) C and (dark gray bar) EC (defined as the % of C or EC from the undigested food matrix that is subsequently recovered in the aqueous fraction following in vitro digestion and centrifugation) from the chocolate confections and cocoa beverages, reported as the mean from $n = 4$ replicates per matrix. Error bars represent the SEM. Common superscripts for the same compound indicate no significant difference ($P > 0.05$) between matricess.

Caco-2 Cell Experiments. Matrix formulation factors appear to have had minimal impact on preabsorptive events that may have altered EC pharmacokinetic behavior observed in vivo. The extent to which matrix factors may influence intestinal uptake was investigated using the Caco-2 human intestinal cell line. A coupled model linking the in vitro digestion to the cell culture experiments was employed to represent the exposure of enterocytes to concentrations of C and EC based on the assessment of bioaccessibility from the various matrices (**Figure 4**). Highly differentiated Caco-2 cell monolayers (10 days postconfluency) were exposed to dilute in vitro digesta from each matrix, and accumulation was defined as the intracellular concentration resulting from exposure to digesta for 1 h. The 1 h time point was selected based upon preliminary kinetic experiments illustrating linear uptake of C and EC up to 1 h followed by decreased uptake up to 3 h by Caco-2 monolayers (data not shown).

Caco-2 accumulation of both C and EC are shown in **Figure 5**. Intracellular levels of C were much lower than levels of EC, consistent with the profile observed in the cocoa powder as well as serum. Accumulation of C was 3.3 ± 0.3, 3.4 ± 0.5, 2.3 ± 0.2, 2.3 ± 0.5, and 3.1 ± 0.2 pmol mg^{-1} cell protein for CDK, CHS, CMP, BSMP, and BNMP, respectively (0.12−0.18% of C in the media). Accumulation of EC was 16.8 ± 1.7, 16.8 ± 2.1, 11.7 ± 0.8, 13.2 ± 3.3, and 16.0 ± 1.2 pmol mg^{-1} cell protein for CDK, CHS, CMP, BSMP, and BNMP, respectively (**Figure 5**). These values represent accumulation of roughly 0.16−0.26% of the EC from the media to which the monolayers were exposed, indicating generally poor intestinal uptake efficiency for C and EC, consistent with previously published reports (*53*−*57*). While not statistically significant, accumulation of EC from the CMP matrix appears to be somewhat lower than accumulation from the other matrices, as reflected in the % accumulation from media, which was 16% for CMP vs 20−26% for the other treatments (see **Figure 5**). This in vitro trend roughly corresponds to the observed in vivo bioavailability of EC, where the lowest serum AUC was observed for CMP. Therefore, although matrix differences do not appear to significantly alter the net accumulation of EC by intestinal epithelial cells in our in vitro model, the data suggest that modestly lower intestinal absorption of EC from the CMP matrix in vivo may contribute, in part, to the lower overall bioavailability observed for this matrix in vivo. The extent to which these factors might impact transcellular transport warrants a more detailed investigation. Modulation of intestinal uptake and subsequent transcellular transport rather

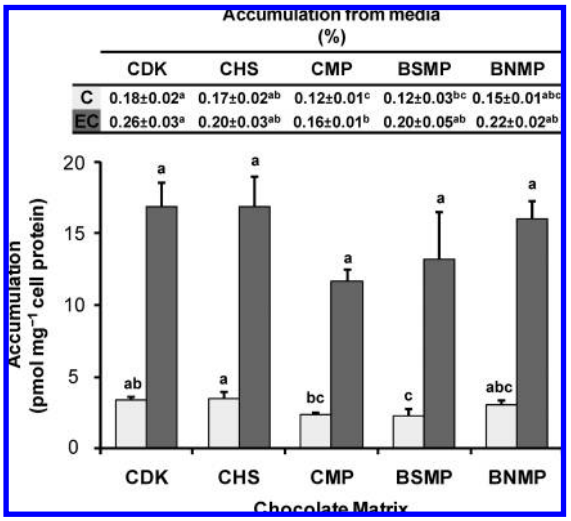

Figure 5. Accumulation of C (light gray bar) and EC (dark gray bar) in highly differentiated Caco-2 human intestinal epithelial cell monolayers exposed to diluted aqueous (bioaccessible) fractions resulting from in vitro digestion of the chocolate confections and cocoa beverages. Accumulation was defined as the intracellular concentration resulting from exposure to the diluted digesta for 1 h. Values represent the mean from $n = 5$ wells per matrix. Error bars represent the SEM. Values in the table above each bar indicate the % (mean ± SEM) of C or EC present in the media to which the cells were exposed that was subsequently accumulated in the cell monolayer for that matrix. Common superscripts for the same compound in the graph and the table indicate no significant difference ($P > 0.05$) between matrices.

than digestive release and bioaccessibility by milk may explain, in part, the lower systemic bioavailability of EC observed in vivo.

Our data suggest that the overall bioavailability of EC (as measured by AUC) following consumption of physiologically relevant doses of EC from chocolate is not likely to be significantly influenced by the ingredient composition of confection or beverage food matrices. However, differences in physical state of the matrix may play a large role in determining the relative pharmacokinetic properties of cocoa-containing products, specifically the rate of absorption and the subsequent C_{MAX}. This was illustrated in our finding that peak serum concentrations of EC were generally higher in cocoa beverages compared to that of a chocolate confections. Specifically, beverages and matrices containing higher levels of sucrose appear to lower T_{MAX} and raise C_{MAX} values relative to lower sucrose and milk formulations.

In vitro digestion data suggest that the extent of digestive release from the food matrix and solubilization of EC in the GI lumen does not differ significantly between matrices and, therefore, is unlikely to influence the overall bioavailability of EC. However, the in vitro digestion model utilized in these studies predicts only the magnitude of solubilization, and not the rate. It is therefore possible that the physical state of the food matrix may modulate GI mobility and the rate of EC solubilization, resulting in the observed distinct pharmacokinetic curve shapes, T_{MAX} (though not statistically different), and C_{MAX} behavior between beverage and confection matrices. The extent to which GI transit and resulting bioaccessibility may differ between physical state of chocolate product merits further investigation.

Beverage products (BSMP, BNMP) appear to more readily transfer EC to the soluble (bioaccessible) fraction in the GI lumen, as reflected by the lower T_{MAX} and greater C_{MAX} values (**Table 2**) and distinct pharmacokinetic curve shapes (**Figure 3**) observed for these matrices compared to the confections (CDK, CHS, CMP). Thus, it appears that beverage formulation may alter the pharmacokinetic behavior of EC through more rapid gastric emptying and more rapid transfer to the aqueous phase in the upper GI tract, resulting in lower T_{MAX} and higher C_{MAX}, but similar AUC values over 6 h (with the exception of statistically lower AUC in confections containing milk protein relative to beverages, and lower but not statistically significant AUC between confections containing milk protein relative to confections lacking milk). Caco-2 absorption experiments suggest that the net intestinal absorption of EC generally does not differ greatly between matrix formulations. However, the overall trends in these data roughly correspond to the observed bioavailability data: CMP had roughly lower, though generally not statistically different, mean accumulation of EC (total pmol/mg as well as % of media), which corresponds with the lower AUC and C_{MAX} observed in vivo for this product. Additionally, other factors not investigated in this study (phase II metabolism, elimination rate, etc.) may be modulated by the physical form or the presence of carbohydrate and milk. The influence of these factors also merits further investigation.

Overall, these data indicate that the bioavailability of EC is similar from cocoa-containing food matrices with reasonable and commercially practical levels of milk, sugar, or fat. Therefore, the flavan-3-ol content of the chocolate, rather than matrix factors, may be the most critical parameter for modulation of systemic EC delivery and, by extension, biological activity from cocoa based products. However, T_{MAX} and C_{MAX} values appear to be readily influenced by the physical state of the product. Therefore, it seems plausible that beverage and/or high sucrose matrices could be designed to facilitate rapid appearance of flavan-3-ols in blood at higher maximal concentrations. Confection chocolate formulations could be designed to maintain lower flavan-3-ol concentrations for extended periods of time.

ABBREVIATIONS USED

ACN, acetonitrile; AR-ACS, analytical reagent-American Chemical Society; AUC, area under the serum concentration−time curve; BCA, bicinchoninic acid; BHT, butylated hydroxytoluene; BMI, body mass index; BNMP, beverage non-nutritive sweetener milk protein; BSMP, beverage sucrose milk protein; C, (+)-catechin and (−)-catechin; CDK, confection reference dark chocolate; CHS, confection high sucrose; C_{MAX}, maximum serum concentration; CMP, confection high milk protein; DMEM, Dulbeco's Modified Eagle's Medium; EC, (−)-epicatechin; ECD, electrochemical array detection; FBS, fetal bovine serum; Fisher's PLSD, Fisher's protected least significant difference; GI, gastrointestinal; L-AA, L-ascorbic acid; LDL, low density lipoprotein; MeOH, methanol; NO, nitric oxide; PBS, phosphate-buffered saline; RP, reverse-phase; TFA, trifluoroacetic acid; T_{MAX}, time at which the maximum serum concentration was observed.

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