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ORIGINAL CONTRIBUTION

Simultaneous ingestion of dietary proteins reduces the bioavailability of galloylated catechins from green tea in humans

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Abstract
Purpose To investigate the influence of dietary proteins (casein, soy protein) and skimmed milk on the plasma kinetics of green tea (GT) catechins.
Methods In a randomized cross-over design with one-week intervals, 24 healthy normal-weight women consumed a test drink containing 1.75 g GT extract with or without the addition of different proteins. Treatments were GT (control), GT with skimmed milk (GT + M), GT with caseinate (GT + CS), or GT with soy protein (GT + S). Venous blood samples were taken before and several times during a period of 4.5 h after consumption of the test drink. Plasma concentrations of catechins were analyzed by HPLC with electrochemical detection.
Results Compared to control, consumption of GT with milk, caseinate, or soy protein significantly reduced the bioavailability (mean area under the plasma concentration–time curve) of total catechins (means ± SEM; GT + M, 87 ± 5%; GT + CS, 79 ± 5%; GT + S, 88 ± 4%), epigallocatechin gallate (GT + M, 68 ± 4%; GT + CS, 63 ± 5%; GT + S, 76 ± 5%), and epicatechin gallate (GT + M, 68 ± 5%; GT + CS, 66 ± 6%; GT + S, 77 ± 6%), while the bioavailability of non-galloylated catechins such as epigallocatechin (GT + M, 134 ± 9%; GT + CS, 118 ± 9 %; GT + S, 123 ± 8%) and epicatechin (GT + M, 125 ± 10%; GT + CS, 114 ± 11%; GT + S, 110 ± 8%) significantly increased. No significant differences in bioavailability of GT catechins were observed between the treatments GT + M, GT + CS, or GT + S.
Conclusion Simultaneous ingestion of dietary proteins reduces the bioavailability of galloylated catechins from GT in humans.

Keywords Catechins · Flavan-3-ols · Bioavailability · Dietary protein · Human study

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Introduction

Evidence from epidemiological studies suggests that regular consumption of green tea (GT) might be associated with a reduced risk of coronary artery disease (CAD) [1], which has been ascribed to the marked antioxidant potential of polyphenolic compounds commonly known as catechins (flavan-3-ols) [2–4]. The main catechins found in GT are epigallocatechin gallate (EGCG), epigallocatechin

(EGC), epicatechin gallate (ECG), and epicatechin (EC). In addition, GT contains small amounts of catechin and gallocatechin [5].

Interestingly, the meta-analysis cited above [1] reported that different from green tea, black tea consumption is not significantly associated with a decreased risk of CAD. In case of the effects of black tea, however, the analysis showed a heterogeneity among the European countries involved, supposedly due to differences in black tea drinking habits, namely with or without the addition of milk [1]. Thus, Hertog et al. [6, 7] reported a reduced risk of coronary heart diseases in subjects consuming black tea, but did not observe such beneficial effects in subjects consuming tea with milk [8]. One explanation for the discrepancy could be a poor absorption of catechins from tea with milk. Bioavailability is a prerequisite for any systemic effects of dietary catechins. Numerous factors may influence the amount of intestinally absorbed catechins. One important aspect concerning the bioavailability of dietary catechins is the influence of various food-derived factors, including food matrix and composition. In this regard, evidence exists for a reduced bioavailability of tea catechins upon the addition of milk to tea infusions [9–12]. Other studies, however, did not report reduced plasma concentrations of total catechins when tea was ingested with milk [13, 14].

It has been speculated that milk casein, especially β-casein, might be responsible for the bioavailability-lowering effect of milk [12]. The observed inhibitory effect by β-casein was attributed to the relatively large number of proline groups in the protein [15]. It is well known that proline-rich proteins such as specific salivary proteins bind phenolic compounds including catechins, which are responsible for the astringency of tea and red wine [16–18]. Until now, the effect of casein or other dietary proteins on the bioavailability of individual catechins has not been systematically studied in humans. Thus, the aim of the present study was to examine the influence of skimmed milk, casein, and soy protein on the plasma kinetics of flavan-3-ols from GT in young healthy women.

Participants and methods

Participants

A total of 24 non-smoking, normal-weight (body mass index [BMI] 18.5–24.9 kg/m^2) Caucasian women aged 23–32 years participated in this study. They were in good health as determined by a basic examination (body weight and height, blood pressure/pulse, dietary questionnaire, medical anamneses, and blood analyses; baseline characteristics presented in Table 1). Exclusion criteria were overweight (BMI 25–29.9 kg/m^2) or obesity (BMI $\geq$ 30.0 kg/m^2), metabolic or endocrine diseases, malabsorption syndromes, smoking, pregnancy/lactation, alcohol abuse, use of dietary supplements or any form of medication (with the exception of oral contraceptives), and high consumption of tea (>4 cups/day of green or black tea).

Table 1 Baseline characteristics of participants

Variable	$N = 24$ women
Age (years)	26.0 ± 0.5
Body height (cm)	168.6 ± 1.2
Body weight (kg)	63.7 ± 1.2
BMI (kg/m^2)	22.4 ± 0.3
Systolic blood pressure (mmHg)	117.0 ± 1.8
Diastolic blood pressure (mmHg)	83.0 ± 1.3
Blood hemoglobin (g/dl)	13.2 ± 0.2
Hematocrit (%)	37.9 ± 0.4
Fasting serum total cholesterol (mmol/l)	4.68 ± 0.15
Fasting serum LDL cholesterol (mmol/l)	2.29 ± 0.13
Fasting serum HDL cholesterol (mmol/l)	1.88 ± 0.06
Fasting serum triacylglycerols (mmol/l)	1.11 ± 0.12
Fasting plasma glucose (mmol/l)	4.52 ± 0.07

Values are means ± SEM

The study was conducted according to the guidelines laid down in the Declaration of Helsinki, and all procedures involving human participants were approved by the ethical committee of the Medical Faculty of the Christian-Albrechts-University of Kiel, Germany. Written informed consent was obtained from all participants.

Study design

In a randomized four-armed, diet-controlled cross-over design with one-week intervals between treatments, the participants consumed one of the four GT beverages (test drink): GT (control treatment), GT with skimmed milk (GT + M), GT with caseinate (GT + CS), and GT with soy protein (GT + S). The treatments were given in the morning after a 12-h overnight fast under standardized conditions.

Two days before the treatments, the participants refrained from flavonoid-rich food. For this purpose, a list of food items rich in flavonoids (e.g. tea [*Camellia sinensis*], wine, unpeeled apples, apricots, grapes, peaches, cherries, green leafy vegetables, onions, and chocolate) was given to each participant, and they were instructed to avoid such foods. Compliance with the dietary restrictions was controlled with a self-completed standardized 2-day dietary record. No deviation from the low flavonoid diet occurred. To avoid hypoglycemia during the experiment, the participants were given 6 g of glucose hourly and blood

glucose was continuously monitored. In no case, the blood glucose was lower than 3.5 mmol/l. One participant reported signs of gastrointestinal discomfort after consumption of the control treatment.

Tea preparation

The green tea beverage consisted of 1.75 g decaffeinated extract (Plantextrakt, Vestenbergsgreuth, Germany; composition [in mg]: 445 total catechins, 260.1 EGCG, 112.6 ECG, 42.5 EGC, 24.8 EC, and 5.3 catechin) dissolved in 300 ml water. This dosage is equivalent to 4–5 cups of green tea. In the GT + M group, 20% of water was replaced with commercial skimmed milk (fat content 0.1%), which corresponds to a protein content of 2.17 g. For the two other treatments, either caseinate (EMULAC, Molkerei Meggle Wasserburg GmbH & Co.KG, Wasserburg, Germany) or soy protein (Supro® 500E IP, Solae Company, St. Louis, USA) was added in equal amounts (2.17 g total protein) to the green tea beverage. The detailed compositions of the green tea beverages are given in Table 2.

Blood sample processing and analyses

Fasting venous blood samples were collected before (baseline) and 30, 60, 90, 120, 150, 180, 210, 240, and 270 min after ingestion of the tea preparations. Blood was obtained by an indwelling cannula placed into the median cubital vein. Blood was drawn into tubes containing lithium heparin (Sarstedt, Nümbrecht, Germany). Plasma was immediately separated by centrifugation at 2,000×*g*; 10 min at 4 °C and aliquoted. Thereafter, 1 ml of each plasma sample was mixed with 20 µl of an ascorbate-EDTA solution (0.4 mol/l NaH_2PO_4 buffer containing 20% ascorbic acid, 0.1% EDTA, pH 3.6) and stored at −80 °C until analysis.

EGCG, EGC, ECG, EC, catechin, gallocatechin, and all other reagents and HPLC-grade solvents were purchased from Carl Roth (Karlsruhe, Germany). The plasma concentrations of individual catechins (unconjugated and glucuronidated/sulfated metabolites) were determined by HPLC as described by Lee et al. [19] with some modifications. Briefly, total catechins (non-conjugated and conjugated catechins) were determined after hydrolysis by incubating 0.5 ml plasma for 45 min with an enzyme mixture of β-D-glucuronidase (2,000 units) and sulfatase (70 units) (Sigma-Aldrich, Steinheim, Germany). Then, 1 ml of methylene chloride was added, and the sample was shaken and centrifuged at 3,220×*g* for 15 min at 4 °C. The supernatant was transferred to a new test tube and mixed with 1 ml ethyl acetate. After that, the sample was mixed and centrifuged (3,220×*g*; 20 min at 4 °C) again. An 800-µl volume of the organic phase was transferred to a fresh test tube, and the ethyl acetate extraction was repeated twice. The combined supernatants were mixed with 10 µl of 1% aqueous ascorbic acid and dried by vacuum centrifugation. The dried sample was dissolved in 150 µl of mobile phase A by vortex mixing and sonication. The sample was centrifuged, and 30 µl of the supernatant injected into the HPLC.

Analysis was performed on a LC-2000Plus series HPLC system (JASCO, Gross-Umstadt, Germany; pump, model PU-2080Plus; column oven, model Jetstream II Plus) linked with a 4-channel coulometric electrochemical detector (CoulArray; ESA Inc., Massachusetts, USA). Separation was carried out on a reversed-phase C_{18} Kromasil 100 column (25 × 4.6 mm, 5 µm; JASCO) maintained at 30 °C and protected by a C_{18} Inertsil ODS-2 guard column (10 × 4 mm, 5 µm; JASCO). Mobile phase A and B (pH 2.5) contained water, acetonitrile, and trifluoroacetic acid (TFA) at the ratio of 92:8:0.1 and 65:35:0.1 (v/v/v), respectively. The flow rate was 0.9 ml/min, and the eluent was monitored by electrochemical detection with potential settings at 0, 120, 240, and 360 mV. The dominant signals, which were used for quantification, were 0 mV for EGC, 120 mV for GC, EC, and EGCG, and 240 mV for C and ECG. The detection limit was ∼10 nmol/l. The coefficient of variation (repeated analysis of standard solutions) was 2.4 ± 2.6–6.5 ± 4.7% for all catechins analyzed. The individual plasma catechins were quantified using external standards. Standard curves were generated by adding C, GC, EGC, EGCG, ECG, and EC to blank plasma at final

Table 2 Ingredients (all in g) used to prepare the green tea test drinks GT (control, green tea beverage without any additions), GT + M (green tea beverage with skimmed milk), GT + CS (green tea beverage with caseinate), and GT + S (green tea beverage with soy protein)

	GT (control)	GT + M	GT + CS	GT + S
Water (g)	300	240	300	300
Green tea extract[a,b] (g)	1.75	1.75	1.75	1.75
Protein matrix added (g)				
Skimmed milk	–	60	–	–
Caseinate	–	–	2.38	–
Soy protein	–	–	–	2.40
Total protein (g)		2.17	2.17	2.17

[a] The nutrient composition of the green tea extract was as follows: protein, 10; carbohydrates, 65; fat, 0.1; and dietary fiber, 15 g/100 g dry mass

[b] Catechin composition of the standardized green tea extract [in mg per 1.75 g extract] and therefore of the test beverages (in mg/beverage): total catechins, 445; epigallocatechin gallate, 260.1; epicatechin gallate, 112.6; epigallocatechin, 42.5; (−)-epicatechin, 24.8; and (+)-catechin, 5.3

concentrations of 0.125, 0.25, 0.5, 1, 2.5, 5, 7.5, and 10 μmol/l. All calibration samples were treated the same way as the experimental samples. The standard curves displayed linearity for all catechins with $r \geq 0.99$. The recovery of the individual catechins from plasma was between 50 and 60%.

Kinetic calculations and statistical analyses

Plasma kinetic parameters as well as statistical analyses were performed using GraphPad Prism Software (version 4.01, San Diego, CA, USA). Plasma concentrations of individual catechins obtained at the different time points (t30–t270) were corrected for individual baseline catechin concentrations (t0). Total plasma catechins (nmol/l) were calculated as the sum of the respective concentrations of EC, EGC, EGCG, ECG, and gallocatechin. Maximum plasma concentrations (c_{max}) and times to achieve maximum plasma concentrations (t_{max}) were directly obtained from the plasma concentration–time profiles. As a measure for the bioavailability of catechins, the area under the plasma concentration–time curve (AUC) was estimated by using the linear trapezoidal rule.

Data were checked for deviation from normal distribution by D'Agostino and Pearson test. Because most data were skewed, a nonparametric analysis of variance for dependent samples according to Friedman with subsequent comparisons of group means using Dunn's post hoc test was performed. All tests were 2-tailed, and the level of significance was set at $P < 0.05$. Results are expressed as means ± SEM.

Results

The major tea catechins detected in human plasma samples after consumption of the various test drinks were EGCG, ECG, EGC, and EC. In addition, small amounts of catechin and gallocatechin were detected. Plasma concentrations of total catechins, EGCG, and ECG were consistently higher after bolus consumption of green tea (control) compared to that of green tea with milk, caseinate, or soy protein (Fig. 1a–c).

Compared to control, c_{max} and AUC values for total catechins, EGCG, and ECG were significantly lower after bolus consumption of green tea with milk and green tea with caseinate (Table 3) with no significant differences in these parameters regarding the protein source (milk, caseinate, and soy protein, respectively).

Bolus consumption of green tea with soy protein resulted in a significant decrease in c_{max} of total catechins and of EGCG and ECG, as well as in a significant decrease in the AUC of EGCG as compared to controls (Table 3).

Fig. 1 Mean (±SEM) plasma concentration–time profile of total (**a**) and individual (**b–e**) catechins (sum of unconjugated and glucuronidated/sulfated metabolites) after oral bolus ingestion of a green tea beverage without (GT) or with the addition of skimmed milk (GT + M), caseinate (GT + CS), or soy protein (GT + S) ($n = 22$–23). Total catechins are the sum of all quantified catechins after enzymatic treatment (catechin; gallocatechin; *EC* epicatechin; *ECG* epicatechin gallate; *EGC* epigallocatechin; *EGCG* epigallocatechin gallate). Plasma concentrations of individual catechins obtained at the different time points were corrected for individual baseline catechin concentrations. Note that the ordinates show different scales due to substantial different concentrations of individual catechins

In addition, consumption of green tea with soy protein significantly increased t_{max} values for EGCG and ECG when compared to control (Table 3). Green tea with caseinate significantly increased t_{max} values for ECG and EC when compared to control (Table 3).

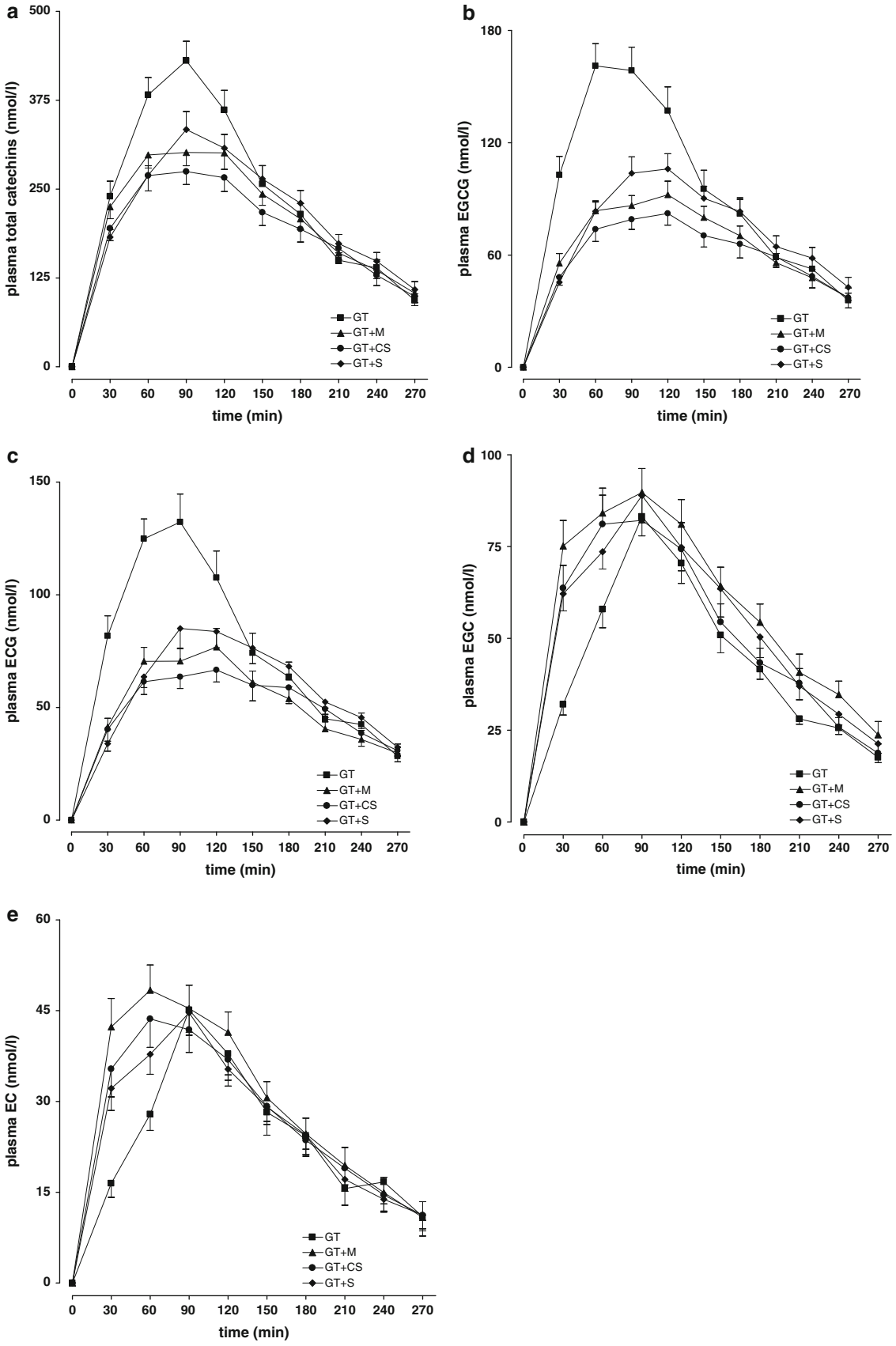
Relative bioavailability

Compared to controls, bolus consumption of green tea with milk, caseinate, or soy protein significantly reduced the AUC of total catechins (means ± SEM, in %; GT + M, 87 ± 5; GT + CS, 79 ± 5; GT + S, 88 ± 4), of EGCG (GT + M, 68 ± 4; GT + CS, 63 ± 5; GT + S, 76 ± 5), and of ECG (GT + M, 68 ± 5, GT + CS, 66 ± 6; GT + S, 77 ± 6), while the bioavailability of non-galloylated catechins such as EGC (GT + M, 134 ± 9; GT + CS, 118 ± 9; GT + S, 123 ± 8) and EC (GT + M, 125 ± 10; GT + CS 114 ± 11; GT + S, 110 ± 8) significantly increased.

Discussion

The aim of the present human study was to systematically investigate the influence of dietary proteins (casein, soy protein) and skimmed milk on the plasma kinetics of various individual flavan-3-ols from green tea. The study was conducted with a decaffeinated green tea extract with a specified content of catechins (445 mg total catechins). The main flavanols found in green tea, and to a lesser extent in black tea, are monomeric compounds like EGCG, ECG, EGC, EC, and catechin (sorted in descending order by content in green tea extract) [5, 20]. These catechins represent more than half of total catechins in green tea [5, 21].

Consumption of green tea with skimmed milk significantly reduced the bioavailability (AUC) as well as the maximum plasma concentration of total catechins, most likely due to a decreased absorption of these compounds. The reduction was comparable to the data reported by Reddy et al. [11] in volunteers who consumed tea with milk. Interestingly, only the bioavailability of galloylated


a
plasma total catechins (nmol/l)
500
375
250
125
0
0 30 60 90 120 150 180 210 240 270
time (min)
GT
GT+M
GT+CS
GT+S
b
plasma EGCG (nmol/l)
180
120
60
0
0 30 60 90 120 150 180 210 240 270
time (min)
GT
GT+M
GT+CS
GT+S
c
plasma ECG (nmol/l)
150
100
50
0
0 30 60 90 120 150 180 210 240 270
time (min)
GT
GT+M
GT+CS
GT+S
d
plasma EGC (nmol/l)
100
75
50
25
0
0 30 60 90 120 150 180 210 240 270
time (min)
GT
GT+M
GT+CS
GT+S
e
plasma EC (nmol/l)
60
45
30
15
0
0 30 60 90 120 150 180 210 240 270
time (min)
GT
GT+M
GT+CS
GT+S

catechins like EGCG and ECG was reduced by milk, whereas the bioavailability of non-galloylated catechins (EGC and EC) was rather increased. The inhibitory effect of milk on the absorption of esterified catechins was also shown by a later occurrence of t_{max}.

It has been speculated that casein, especially β-casein, might be responsible for the decreased bioavailability of tea catechins when consumed with milk [12, 15]. Thus, we investigated the influence of the addition of caseinate (containing α-, β-, and κ-casein fractions) on the bioavailability of green tea catechins. Plasma concentrations of catechins, in particular of EGCG and ECG, were significantly lower after the consumption of green tea with caseinate than after green tea alone. The effects of caseinate were similar to those observed after the addition of milk. Previous studies demonstrated the non-covalent cross-linking of EGCG and other catechins with casein, emphasizing the interaction of tea catechins with milk caseins [7, 15, 18, 22]. The strong inhibitory effect observed with caseinate, which contained 12% of the amino acid proline, in our study might be explained by the relatively large number of prolyl residues within casein [18]. The cyclic structure of proline results in a reduced number of hydrogen bonds within the peptide backbone leading to a more open and flexible conformation of prolyl-rich proteins compared to proteins with low proline content [23].

Interestingly, the observed effects were not specific for caseinate, because soy protein had a similar effect. No statistically significant differences in the bioavailability of catechins were observed after consumption of green tea with a protein with a high (caseinate) or low proline (soy protein) content. Similar to milk and caseinate, the consumption of green tea with soy protein also resulted in a significant reduction of the bioavailability of total catechins, in particular of the galloylated catechins EGCG and ECG. The effect is the most likely attributable to a reduced absorption (deduced from reduced c_{max} values), as elimination kinetics (plasma concentrations $\geq$ 150 min) appeared to be unaltered (Fig. 1). Thus, the inhibitory effect by proteins on the bioavailability of galloylated can

Table 3 Plasma kinetic parameters of total and individual catechins (sum of free and conjugated forms) after bolus consumption of a green tea beverage without (GT) or with the addition of skimmed milk (GT + M), caseinate (GT + CS), or soy protein (GT + S)

	GT (control)	GT + M	GT + CS	GT + S
Total catechins[a]				
c_{max} (nmol/l)	461.8 ± 27.4	351.4 ± 19.7***	326 ± 19.4***	374.0 ± 21.0*
t_{max} (min)	90.0 ± 4.2	95.5 ± 8.8	94.1 ± 8.9	100.9 ± 6.7
AUC (μmol min/l)	66.7 ± 3.8	57.7 ± 3.2*	52.8 ± 3.5**	58.9 ± 2.9
EGCG				
c_{max} (nmol/l)	178.9 ± 12.7	104.5 ± 6.3***	96.1 ± 6.0***	119.3 ± 8.2*
t_{max} (min)	73.0 ± 4.9	105.0 ± 10.4	106.4 ± 12.1	110.5 ± 7.5*
AUC (μmol min/l)	26.0 ± 1.8	17.7 ± 1.1***	16.3 ± 1.2***	19.7 ± 1.3**
ECG				
c_{max} (nmol/l)	147.3 ± 11.4	91.6 ± 7.3***	83.2 ± 6.2***	101.8 ± 7.3**
t_{max} (min)	77.0 ± 4.9	98.2 ± 8.7	111.8 ± 9.5*	114.5 ± 7.6*
AUC (μmol min/l)	20.6 ± 1.7	13.9 ± 1.0***	13.6 ± 1.2***	15.8 ± 1.2
EGC				
c_{max} (nmol/l)	86.5 ± 5.1	102.6 ± 6.9	98.3 ± 7.9	104.0 ± 7.0
t_{max} (min)	93.9 ± 4.3	77.7 ± 8.1	75.0 ± 6.5	90.0 ± 8.1
AUC (μmol min/l)	12.0 ± 0.7	16.0 ± 1.1**	14.1 ± 1.0	14.7 ± 1.0
EC				
c_{max} (nmol/l)	48.2 ± 3.8	55.9 ± 4.6	53.9 ± 4.4	49.9 ± 3.2
t_{max} (min)	99.1 ± 4.0	75.0 ± 7.3	75.0 ± 7.6*	83.2 ± 6.5
AUC (μmol min/l)	6.5 ± 0.7	8.2 ± 0.6	7.5 ± 0.7	7.2 ± 0.5

Values are means ± SEM; n = 22–23 participants in a randomized cross-over design

Plasma concentrations were corrected for baseline catechin concentrations

AUC area under the plasma concentration–time curve, c_{max} the maximum plasma concentration, and t_{max} the time to reach the maximum

* Different from control, $P < 0.05$; ** different from control, $P < 0.01$; *** different from control, $P < 0.001$ (Friedman test followed by Dunn's post hoc test)

[a] Total plasma catechins were calculated according to: total catechins (nmol/l) = catechin (nmol/l) + EC (nmol/l) + EGC (nmol/l) + EGCG (nmol/l) + ECG (nmol/l) + gallocatechin (nmol/l)

probably not be (solely) explained by the content of the amino acid proline.

The observed inhibitory effect of proteins on the bioavailability of green tea catechins reported in the present study only affected the galloylated catechins. In vitro experiments support the interaction of milk proteins with EGCG and ECG [12, 15]. We further observed that the addition of milk or proteins to green tea increased the absorption of non-galloylated catechins (Table 2). After consumption of green tea with milk, the bioavailability of EGC was significantly increased by 34% compared to control. One might speculate that the difference in bioavailability between galloylated and non-galloylated catechins might be due to some kind of competition between individual catechins during intestinal absorption. Complexation of galloylated catechins with proteins would result in a delayed liberation/absorption of these catechins, promoting the absorption of non-galloylated catechins concomitantly present in the intestinal contents. However, until so far, no evidence does exist that carrier-mediated absorption mechanisms competitively used by different catechins are involved in the intestinal absorption of catechins.

In the production of black tea, most monomeric catechins are oxidized, giving polymeric compounds such as theaflavins and thearubigins [5]. Therefore, green tea contains more reactive catechins especially EGCG and ECG than black tea. This is probably the reason why previous studies [13, 14, 24, 25] have been unable to demonstrate an inhibitory effect of milk on the bioavailability of catechins from black tea.

In conclusion, consumption of green tea with milk or dietary proteins reduced the bioavailability of catechins, most likely due to an interaction of proteins and catechins during the absorptive phase. The observed inhibitory effects are not specific for casein, because soy protein had a similar effect. As the effects were only observed during the first 2.5 h after ingestion, it remains to be established whether or not this transiently reduced bioavailability might result in a reduced biological activity.

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Conflict of interest None.

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