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Research Article

Common tea formulations modulate *in vitro* digestive recovery of green tea catechins

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Epidemiological evidence suggests a role for tea catechins in reduction of chronic disease risk. However, stability of catechins under digestive conditions is poorly understood. The objective of this study was to characterize the effect of common food additives on digestive recovery of tea catechins. Green tea water extracts were formulated in beverages providing 4.5, 18, 23, and 3.5 mg *per* 100 mL epicatechin (EC), epigallocatechin (EGC), epigallocatechin-gallate (EGCG), and epicatechin-gallate (ECG), respectively. Common commercial beverage additives; citric acid (CA), BHT, EDTA, ascorbic acid (AA), milk (bovine, soy, and rice), and citrus juice (orange, grapefruit, lemon, and lime) were formulated into finished tea beverages at incremental dosages. Samples were then subjected to *in vitro* digestion simulating gastric and small intestinal conditions with pre- and post-digestion catechin profiles assessed by HPLC. Catechin stability in green tea was poor with <20% total catechins remaining post-digestion. EGC and EGCG were most sensitive with ≤10% recovery. Teas formulated with 50% bovine, soy, and rice milk increased total catechin recovery significantly to 52, 55, and 69% respectively. Including 30 mg AA in 250 mL of tea beverage significantly ($p < 0.05$) increased catechin recovery of EGC, EGCG, EC, and ECG to 74, 54, 82, and 45% respectively. Juice preparation resulted in the highest recovery of any formulation for EGC (81–98%), EGCG (56–76%), EC (86–95%), and ECG (30–55%). These data provide evidence that tea consumption practices and formulation factors likely impact catechin digestive recovery and may result in diverse physiological profiles.

Keywords: Citrus juice / Flavonoids / HPLC / Milk / Tea

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1 Introduction

Epidemiological studies have associated green tea (*Camellia sinensis*) consumption with a reduced risk of several chronic diseases including cancer and cardiovascular disorders [1–7]. Among the most commonly consumed beverages worldwide, teas and tea mixes could be of great value in reducing disease severity and risk if factors associated with its protective activity are identified. Catechins, including (–)-epicatechin (EC), (–)-epigallocatechin (EGC), (–)-epigallocatechin-gallate (EGCG), and (–)-epicatechin-gallate (ECG) (Fig. 1), are the most abundant polyphenols in tea accounting for approximately 80% of the flavonoid content of brewed tea. Catechins have attracted the interest of medical and nutritional researchers as their documented biological activities include scavenging of reactive oxygen and nitrogen species, chelation of redox active metals, inhibition of cancer-related transcriptional factors, and inhibition of oxidative enzymes [8–12].

A limiting factor is the poor bioavailability of tea catechins resulting from instability under digestive conditions, poor transcellular transport, and rapid metabolism followed by excretion [13–15]. Catechin losses of approximately 80%, including almost total degradation of EGCG, have been observed during simulated digestion of simple tea infusions [16]. Instability of catechins in authentic intestinal juice and buffered systems above pH 7.4 has also been previously demonstrated [17, 18], indicating that degrada-

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Abbreviations: AA, ascorbic acid; **BHT,** 2,6-di-*tert*-butyl-4-methylphenol; **CA,** citric acid; **CAF,** caffeine; **DG,** digested material; **EC,** epicatechin; **ECG,** epicatechin-gallate; **EGC,** epigallocatechin; **EGCG,** epigallocatechin-gallate; **RM,** raw material; **RTD,** ready-to-drink

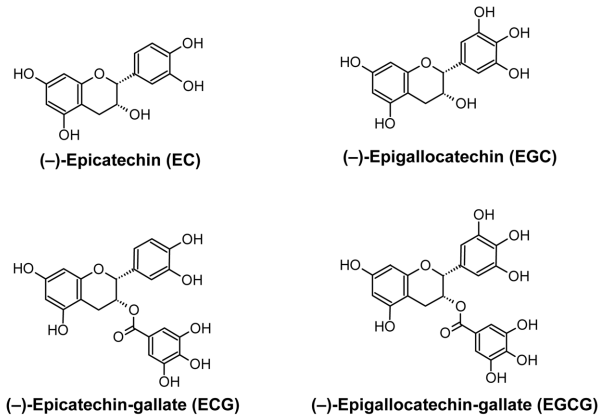

Figure 1. Structure of major catechins from green tea.

tion in the small intestine limits catechin uptake and bioavailability.

While the study of plain tea infusions has been useful in estimating the digestive fate of catechins, tea is commonly consumed in combination with additional ingredients such as sweeteners, juices, and creamers. For ready-to-drink (RTD) tea products, food additives are often included to modify sensory characteristics, provide process and storage stability, and aid in preserving color, flavor, and catechin content of the finished beverages. Common and effective food additives formulated within RTD tea beverages include citric acid (CA), EDTA, 2,6-di-*tert*-butyl-4-methylphenol (BHT), and ascorbic acid (AA) [19]. Furthermore, milk and citrus juices are increasingly being utilized as adjuncts to RTD and fresh brewed tea products to modify the sensory characteristics of the finished product. While the impact of these common formulation factors on the stability of tea catechins in beverage systems has been investigated, information on their ability to modulate catechin digestive behavior is still unknown.

The specific objective of this study was to determine the effect of common food additives and tea adjuncts on *in vitro* digestive recovery of catechins from tea in preparation for future assessments of food formulation factors that modulate catechin bioavailability *in vivo*.

2 Materials and methods

2.1 Chemicals and standards

ACN (Mallinckrodt-Baker, Phillipsburg, NJ), acetic acid (Mallinckrodt-Baker), and formic acid (Sigma – Aldrich) used in HPLC analysis were certified HPLC and ACS grade. Porcine pepsin, lipase, and pancreatin utilized for *in vtiro* digestion, CA, EDTA, gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox®), and BHT were purchased from Sigma (St. Louis, MO). NaCl, HCl, NaOH, $NaHCO_3$, and L-AA were purchased from Mallinckrodt (Phillipsburg, NJ). Bovine milk (The Kroger Company), rice milk (Rice Dream®) and soymilk (West Soy®) were purchased from a local market and stored at 4°C until use. Fresh, whole grapefruits, lemons, limes, and oranges were purchased from a local market, juiced and coarse filtered to provide clarified fresh juice. Aliquots of juice were stored frozen at –80°C under a blanket of nitrogen until use. Analytical standards of EC, EGC, EGCG, ECG, and caffeine (CAF) were purchased from Sigma (St. Louis, MO).

2.2 Tea beverage preparation

Powdered green tea extract (A gift from Nestlé R&D, Marysville, OH, USA) was dissolved in boiling water (100°C) to provide ~50 mg total catechins *per* 100 mL and combined with prescribed amounts of AA (6 – 80 mg/100 mL), EDTA (1 – 100 mg/100 mL), CA (12 – 80 mg/100 mL), and BHT (0.5 – 10 mg/100 mL). Citrus juices (grapefruit, lemon, lime, and orange) and creamers (bovine, rice, and soy milks) were prepared at 10, 20, and 50% (v/v) displacing water in the formulation and maintaining constant catechin levels between formulated and plain green tea preparations. Total AA content of each juice was determined by the 2,6-dichloroindophenol titration method and total solids of creamers and juices was determined by microwave moisture analysis (CEM Smart System 5, Matthews, NC) as previously reported [20]. Creamers were further analyzed for phenolic acid (methanol extract) and protein content using the Folin-Ciocalteau and Bicinchoninic acid (BCA) methods, respectively [21].

2.3 *In vitro* digestion model

The two-stage *in vitro* digestive model was adapted from Ferruzzi *et al.* [22] with minor adjustments. Aliquots (20 mL) of formulated tea beverages, as described above, were diluted with 10 mL of 0.9% saline and the initial pH was recorded. The gastric phase was initiated by addition of 3 mL porcine pepsin solution (40 mg/mL in 0.1 N HCl) and adjustment of the pH to 2.0 ± 0.1 with 1.0 N HCl. Samples were blanketed with N_2 and incubated at 37°C in a covered shaking water bath for 1 h. The small intestinal phase was initiated by adjusting the pH of the gastric digesta (GD) to ~5.3 with combinations of 100 mM $NaHCO_3$ and 1.0 N NaOH followed by the addition of small intestinal enzyme solution (9 mL, porcine lipase (1 mg/mL), pancreatin (2 mg/mL), and bile (12 mg/mL) in 100 mM $NaHCO_3$). The final sample pH was adjusted to 7.2 ± 0.1 with 1.0 N NaOH, volume standardized to 50 mL with saline and samples blanketed with N_2 and placed in a 37°C shaking water bath for 2 h. After completion of the small intestinal phase, samples were centrifuged at 10, 000 × *g* for 1 h at 4°C. Aliquots of raw material (RM), GD and small intestinal digesta (DG) were collected, acidified with 2% aqueous acetic acid (1:1), and stored frozen at –80°C under a blanket of nitrogen until analysis by HPLC.

2.4 Catechin analysis by HPLC

Aliquots from RM and aqueous digesta were thawed and centrifuged at 14 000 × *g* for 10 min. Supernatants for both RM and DG were collected and filtered through a 0.45 μm PTFE filter prior to analysis. RM tea-creamer beverages (bovine, rice, and soy milks) were extracted and prepared for analysis following the protocol previously reported [23]. Catechin analysis was accomplished on a Waters Alliance 2695 HPLC system equipped with a model 2996 photodiode array detector (PDA) (Milford, MA, USA). Separation was as described by Neilson *et al.* [24] with minor modification. A Waters Xterra RP-C18 column (3.9 mm id × 100 mm) preceded by a guard column packed with the same stationary phase were housed and thermostated at 35°C. Catechins were eluted under gradient conditions at a flow rate of 0.9 mL/min using a binary mobile phase of ddH_2O, ACN, formic acid (899:100:1 v:v) in reservoir A and ddH_2O, ACN, formic acid (699:300:1 v:v) in reservoir B. Initial conditions of 99:1 A/B followed a linear gradient to 1:99 A/B (0–6 min) and an immediate linear gradient back to initial conditions (6–8 min) followed by a 2 min re-equilibration. Detection and tentative identification of major tea catechins was accomplished using inline PDA data between 220 and 600 nm. Calibration plots for quantification were constructed from 280 nm absorbances resulting from injection of authentic standards of CAF, EC, EGC, EGCG, and ECG.

2.5 Data analysis

Catechin content, measured from quantitative HPLC analysis as mg/100 mL, was determined for both RM and DG of each formulation factor. The RM provided a reference for the starting concentration of available catechins before the *in vitro* digestion procedure for which to compare the final DG catechin contents. Four individual digestions ($n = 4$) were performed *per* variable with duplicate digestion lots analyzed on separate occasions. Total catechin values were the summation of individual EGC, EC, EGCG, and ECG contents in the beverage *per* 100 mL. Analysis by one-way ANOVA was performed using SAS 9.1.3 (SAS Institute, Cary, NC) for each beverage CAF, EGC, EC, EGCG, ECG, and total catechin contents and significant differences were evaluated by a Tukey's *post hoc* test ($\alpha < 0.05$). Differences between the individual catechin contents of RM and DG were attributed to digestive instability.

3 Results and discussion

3.1 *In vitro* digestive recovery of catechins from plain green tea preparations

Efficient separation of four major catechins and CAF from RM and final green tea digesta was achieved within 10 min (Fig. 2A). Average levels (±SEM, $n = 50$) of CAF, EGC, EC, EGCG, and ECG were 9.2 ± 0.11, 18.2 ± 0.24, 4.4 ± 0.18, 22.7 ± 0.43, and 3.6 ± 0.07 mg/100 mL, respectively, in green tea preparations prior to digestion (RM) (Fig. 2B). These results are consistent with catechin levels reported for typical green tea infusions [25]. Following simulated gastric and small intestinal digestion overall catechin digestive recovery from green tea preparations was found to be poor with approximately 80.4 ± 1.46% loss of total catechins (Fig. 2B). This degree of green tea catechin *in vitro* digestive sensitivity is similar to that previously reported in simplified gastric and small intestinal model systems without presence of digestive enzymes [16]. Furthermore, preliminary experiments confirmed that addition of digestive enzymes did not significantly alter catechin digestive recovery from green tea (data not shown). Individual catechin levels in DG (±SEM, $n = 50$), were determined to be 8.97 ± 0.11, 2.39 ± 0.15, 2.97 ± 0.13, 2.77 ± 0.45 and 1.40 ± 0.06 mg/100 mL for CAF, EGC, EC, EGCG, and ECG, respectively. CAF recovery was greater than 97% and consistent with previous reports of its stability to simulated digestive conditions [16]. Catechin degradation through the gastric portion of the *in vitro* digestive model was not significant (data not shown) and degradation occurred primarily during the small intestinal phase. Both EGC and EGCG were the most susceptible of the catechins to degradation with 86.9 ± 0.85% and 87.9 ± 2.29% digestive losses respectively, while EC and ECG remained relatively stable with 31.8 ± 2.78% and 61.3 ± 2.63% losses, respectively. The poor recovery of EGC and EGCG is particularly interesting considering these catechins account for > 80% of the total catechin content of the green tea utilized in this study.

The sensitivity of catechins to small intestinal conditions has been previously reported [16]. Upper small intestine conditions are particularly favorable for catechin degradative reactions. The elevated pH (6.0–8.0), residual dissolved oxygen, and likely presence of reactive oxygen species from normal digestive function may facilitate several reactions including epimerization and auto-oxidation in the intestinal lumen. Instability of catechins to near neutral pH (> 6) has been previously established [17, 18, 26]. Auto-oxidation reactions are believed to proceed due to vulnerability of adjacent 3′, 4′, and 5′ hydroxyl groups (pyrogallol moiety) in the B ring of EGC and EGCG [18] leading to deprotonation [27] and subsequent semiquinone free radical formation in the B ring [28, 29]. Elevated pH conditions further propagate auto-oxidation reactions by stabilizing catechin semiquinone species [30] allowing for these intermediates to react further forming various end-products that potentially include irreversible homo- and hetero-dimerization products such as theasinensins and P-2 (EGCG dimer species) and EGC dimer analogs [17, 31, 32]. While these reactions have been established following incubation of EGCG in authentic intestinal fluid and cell culture media [17, 31], the extent to which these dimerized end-products

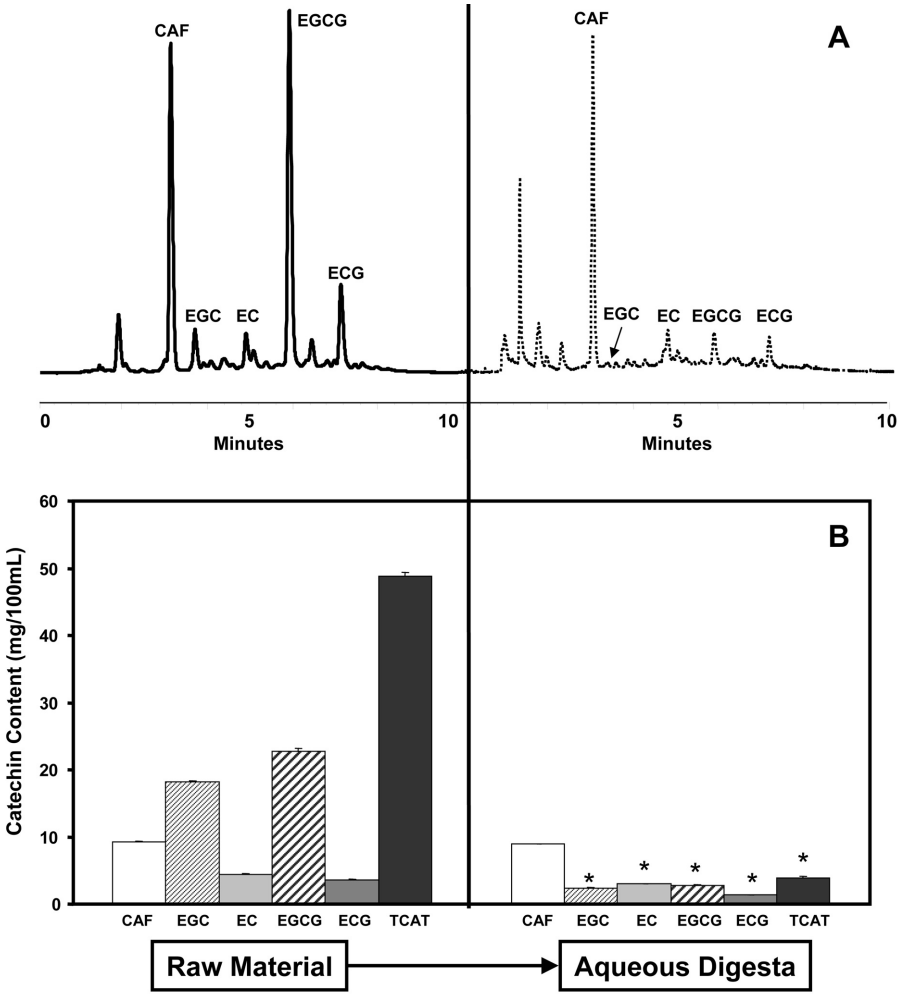

Figure 2. (A) HPLC separation of major green tea catechins pre- and post-*in vitro* digestion with corresponding tea components. Online UV – Vis (ultraviolet and visible) spectra were collected from 220 to 600 nm. Response at 280 nm shown. (B) Catechin profile of raw material and post-digestion (aqueous digesta) tea beverages for CAF (□), EGC (▨), EC (▩), EGCG (▨), ECG (■), and total catechin (TCAT) (■) ($n = 50$, ±SEM, (*) indicates significant decrease in recovery ($p < 0.01$) relative to RM).

are formed during simulated digestive conditions is currently being investigated.

EC and ECG were found to be less sensitive to simulated digestion. In fact, these derivatives have been shown to be less susceptible than EGC and EGCG to similar auto-oxidation reactions due to the dihydroxyl (catechol moiety) B ring in these catechins. At near neutral pH the electron donating capacity of the pyrogallol groups for EGCG and EGC is significantly higher than for catechol groups on EC and ECG resulting in an increased rate of oxidation [30]. These differences in B ring structure may therefore be useful in predicting intestinal sensitivity of catechins.

3.2 Impact of common food ingredients on catechin digestive recovery

Considering the poor digestive stability of catechins from plain green tea infusions, the ability of common food ingredients to modulate small intestinal recovery of green tea catechins was assessed. CA, BHT, EDTA, and AA were selected and formulated into tea preparations, as these are commonly integrated into RTD beverages and foods for preserving labile components and maintaining sensorial qualities. The recovery of catechins in formulated green teas relative to plain green tea preparations was compared following simulated digestions. BHT, EDTA, and CA, common antioxidant and chelating agents had minimal impact on tea catechin digestive recovery *in vitro* (Fig. 3). Among these, statistically significant changes only occurred with EGC and EC upon addition of BHT at 10 mg *per* 100 mL of tea beverage. Higher levels of BHT could have the potential for increased catechin recoveries within the tea beverages through simulated digestion, however its insolubility in water systems prevents these investigations. Only addition of AA significantly increased tea catechin digestive recoveries (Fig. 4). A dose dependent increase in total catechin recovery was noted with AA at 0, 6, and 12 mg/100 mL. Addition of AA to tea beverages beyond 12 mg/100 mL did not provide any notable increase in protection for susceptible catechins. The most notable increases in recovery were for EGC and EGCG with observed recoveries increasing from $12.2 \pm 0.73\%$ to $74.3 \pm 3.48\%$ and $4.0 \pm 0.88\%$ to $53.9 \pm 2.04\%$, respectively. Significant differences were also observed with EC and ECG between treatments, how-

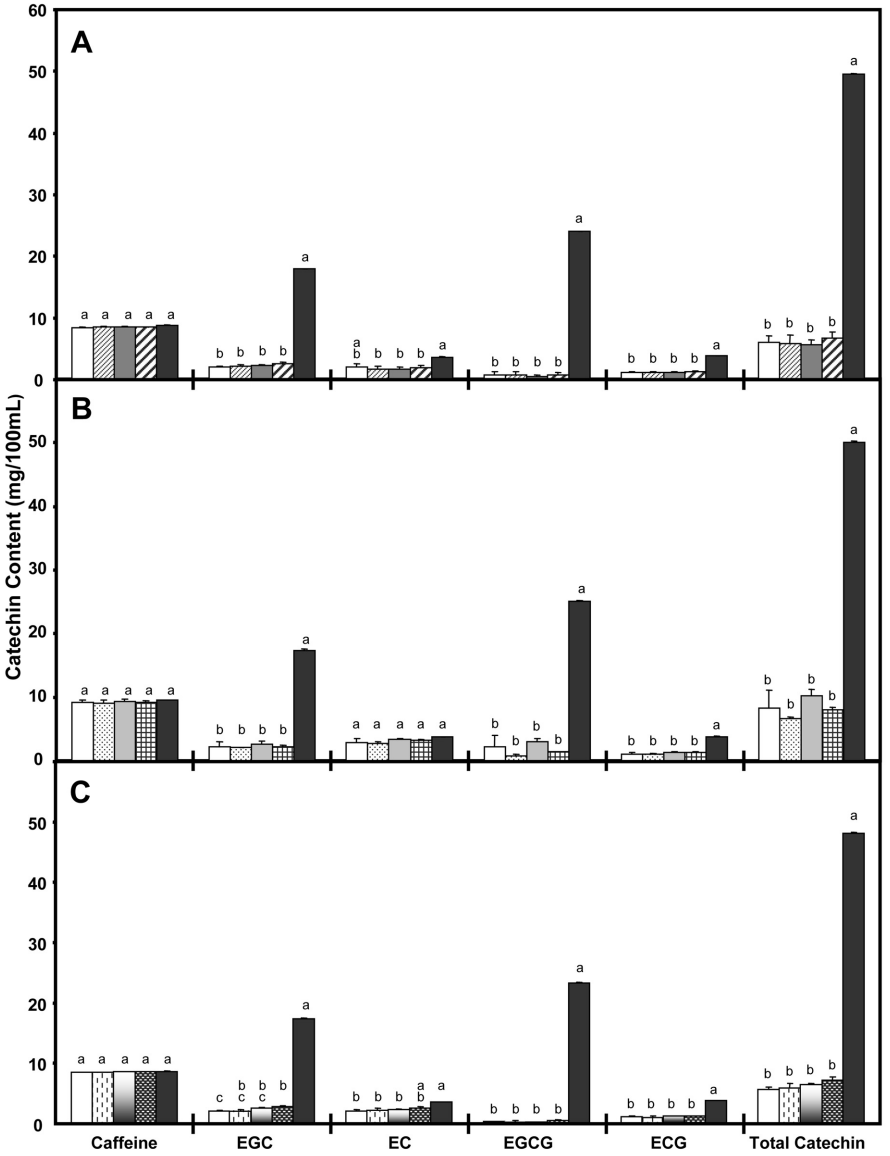

Figure 3. Comparison of *in vitro* digestive recovery of green tea catechins evaluated with the addition of (A) CA at 0 (□), 12 (▨), 36 (■), and 80 (▨) mg *per* 100 mL, (B) EDTA at 0 (□), 1 (⬚), 10 (■) and 100 (▦) mg *per* 100 mL, and (C) BHT at 0 (□), 0.5 (⬚), 1 (■) and 10 (■) mg *per* 100 mL matched with respective predigested RM (■) from each trial set. Data represent catechin content recovered following *in vitro* digestion of tea solutions with specified formulations. Each bar represents the mean ± SEM of four independent experiments. Bars not sharing a common superscript letter are significantly different ($p < 0.05$).

ever the practical magnitude of change in these catechins were miniscule compared with more labile catechins, EGC and EGCG. At higher concentrations of AA additions, variability was observed over all the catechins, however overall recovery of EGC and EGCG at higher AA contents provided the large percentage of total catechin recovery shown. These findings are in agreement with the stabilizing effects of AA on green tea catechins in other alkaline systems [18, 19]. AA is a common additive to food and beverages both as a vitamin and as an antioxidant to stabilize beverage systems. Therefore, its use in stabilization of tea beverage color during processing and storage may have an added benefit of increasing digestive recovery of labile catechins.

The lack of response for the levels tested of common chelating and antioxidant additives provides further insight into the mechanism of catechin digestive sensitivity pointing to pH dependent oxidative reactions (Fig. 3). EDTA and CA are efficient food grade metal ion sequestrants. The inability of these compounds to modulate digestive recovery indicates that metal ion catalyzed oxidation may not be a predominant degradation pathway in this *in vitro* model. However, poor aqueous solubility of BHT likely limited its

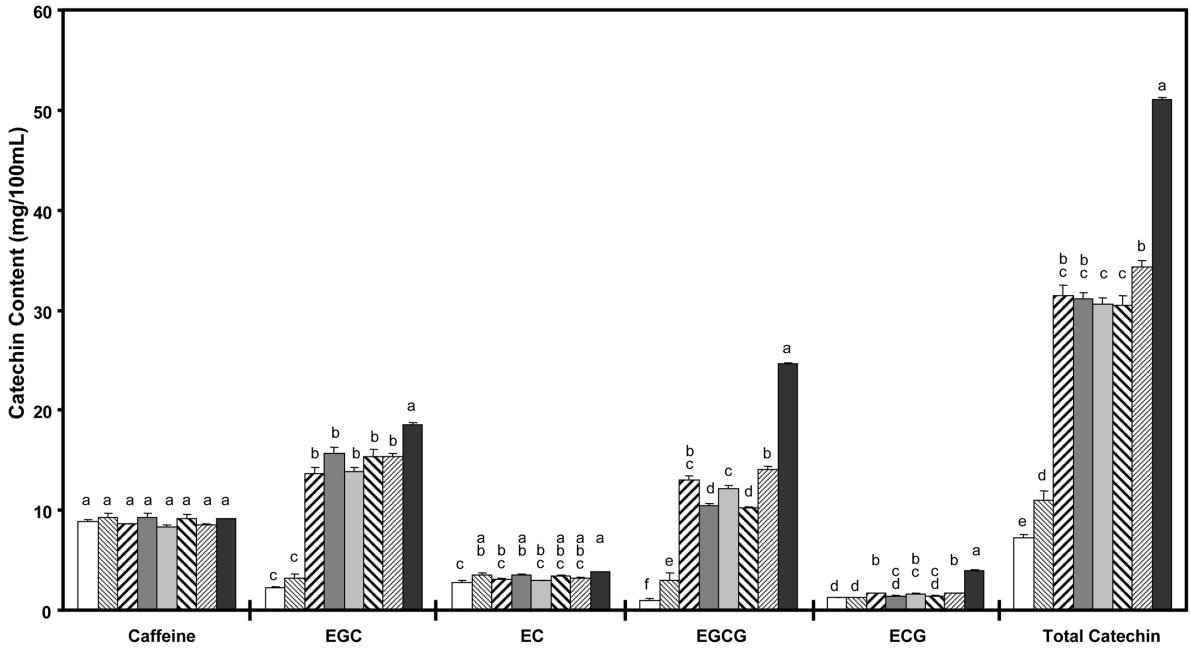

Figure 4. Effects of AA addition to green tea formulation at 0 (□), 6 (▨), 12 (▨), 24 (■), 36 (▩), 48 (▨), and 80 (▨) mg *per* 100 mL on *in vitro* digestive recovery of catechins with corresponding predigested RM (■) catechin content. Each bar represents the mean ± SEM of four independent experiments. Bars not sharing a common superscript letter are significantly different ($p < 0.05$).

ability to provide stabilizing activity to tea catechins during simulated digestions. In our confirmatory experiments, the addition of other water-soluble antioxidants such as gallic acid and Trolox, a vitamin E derivative, did not protect tea catechin to digestive degradation (data not shown). On the other hand, AA has been shown to actively quench phenoxyl radicals formed by polyphenols preferentially to other reducing compounds [33]. At the pH of the small intestine (pH 7 – 8) catechins are deprotonated to form a semiquinone. AA is able to reduce reactive catechin semiquinone free radicals back to native forms prior to progression to further degradation.

Only moderate levels of AA are required to significantly improve digestive stability of catechins *in vitro*. The current recommended daily intake for vitamin C is 60 mg (U.S. Food and Drug Administration. 1994. A Food Labeling Guide, www.cfsan.fda.gov.). In perspective, this would equate to the 24 mg per 100 mL AA formulation based on a 250 mL serving size. This dose is shown to provide significant protection of catechins through simulated digestion. At this dosage, beverages prepared with AA contents as low as 50% of the RDI would likely provide effective protection. This quantity of vitamin C is typically found in RTD tea beverages where vitamin C is claimed and/or utilized as an antioxidant ingredient. While additional work is needed, formulation of RTD tea beverages with AA or supplementing brewed teas with vitamin C rich ingredients may potentially increase intestinal stability *in vivo* thereby increasing native catechin content in the intestinal lumen available for absorption.

3.3 Impact of citrus juice on catechin digestive recovery

The impact of common tea adjuncts on digestive recovery of catechins from green tea was investigated by formulating green tea preparations with citrus juices (grapefruit, lemon, lime, and orange) prior to *in vitro* digestion. Juices were prepared and formulated from 10 – 50% (v/v) displacing water in the formulation in order to maintain constant catechin levels between formulated and plain green tea preparations. For citrus juices, significant increases in individual and total catechin recoveries were noted with increased level of juice in the formulations (Fig. 5). Significant increases in individual catechins were generally noted after addition of 20 and 50% juices and were most notable among the relatively instable EGC and EGCG. Maximum total catechin recoveries were observed for lemon (77.9 ± 2.9%) followed by orange (71.2 ± 1.5%), lime (67.1 ± 6.8%) and grapefruit juice (62.3 ± 4.1%) at the 50% juice relative to the RM values. With the exception of orange juice, the AA content (Table 1) appears to account for only a small percentage of the observed enhancement in digestive recovery of catechins in tea-juice formulations (Fig. 6) indicating that other factors from juice may be responsible, in part, for observed digestive stabilization. Other components such as

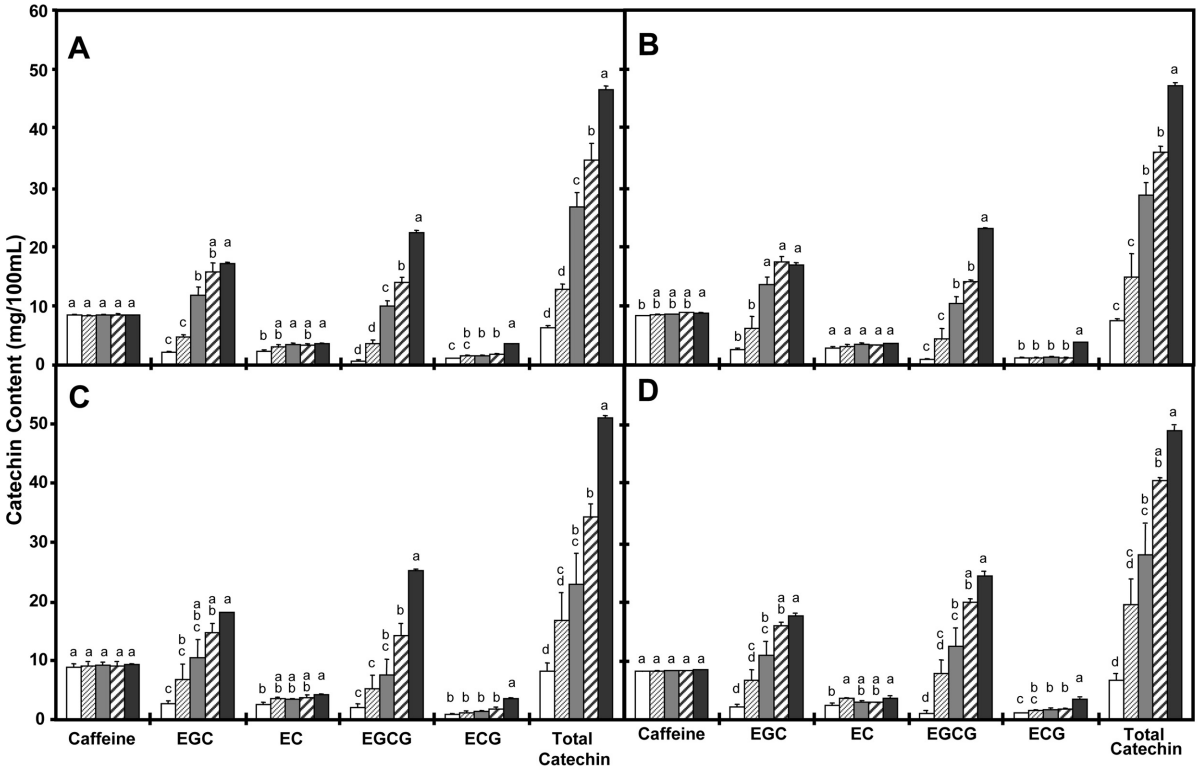

Figure 5. Effects on green tea catechin digestive stability with the addition of (A) orange, (B) grapefruit, (C) lime, (D) lemon juices at 0% (□), 10% (▨), 20% (▩), and 50% (▨) (v/v) with corresponding pre-digested RM (■) catechin content from each trial. Each bar represents the mean ± SEM of four independent experiments. Bars not sharing a common superscript letter are significantly different ($p < 0.05$).

Table 1. Percent solids and AA contents of individual juices utilized in this study (±SEM, $n = 3$).

	% Solids	AA[a)] (mg/mL)
Orange	10.6 ± 0.03	0.51 ± 0.006
Grapefruit	9.4 ± 0.09	0.34 ± 0.002
Lemon	7.7 ± 0.06	0.34 ± 0.002
Lime	8.2 ± 0.03	0.24 ± 0.004

a) Ascorbic acid content.

flavonols (kaempherol, myricetin, quercetin), flavanones (hesperetin, naringenin), or terpenes (*d*-limonene) are known to be abundant in citrus juices [34–36]. These phytochemicals may be capable of stabilizing catechins by quenching catechin free radicals formed under high pH conditions of the small intestine or indirectly by synergistically sparing AA from oxidative damage. While, possible the extent to which specific citrus polyphenols may stabilize digestive catechin reactions is still unknown and remains to be investigated.

Furthermore, juices also contain a small amount of natural proteins, of which have potential to interact strongly with polyphenols [37] physically trapping individual catechins and limiting their availability for reaction. Nondigestible fiber and polysaccharides present in the fresh squeezed juices may also prove to aid in recovery of catechins with similar mechanistic approaches. Further analysis of the specific component and combinations on tea catechin recoveries remains to be completed.

3.4 Impact of creaming agents on catechin digestive recovery

As with juices, creaming systems were incorporated into tea preparations prior to *in vitro* digestion. Creamers (bovine, rice, and soy milks) were formulated from 10–50% (v/v) displacing water in the formulation in order to maintain constant catechin levels between formulated and plain green tea preparations. Bovine, rice, and soy milks all resulted in moderate, but significant ($p < 0.05$) improvement in total catechin recovery as compared to control plain green tea (Fig. 7). Specific improvement in recovery of EGC (58.2 ± 17.8% and 47.0 ± 8.2%) and EGCG (30.8 ± 3.9% and 20.9 ± 0.5%) were noted at highest levels of bovine and soymilk, respectively. Formulating tea with 50% rice milk also significantly ($p < 0.05$) altered recovery of EGC and EGCG to 74.0 ± 3.6% and 69.7 ± 3.3%, respectively. Significant decreases in catechins at higher levels of

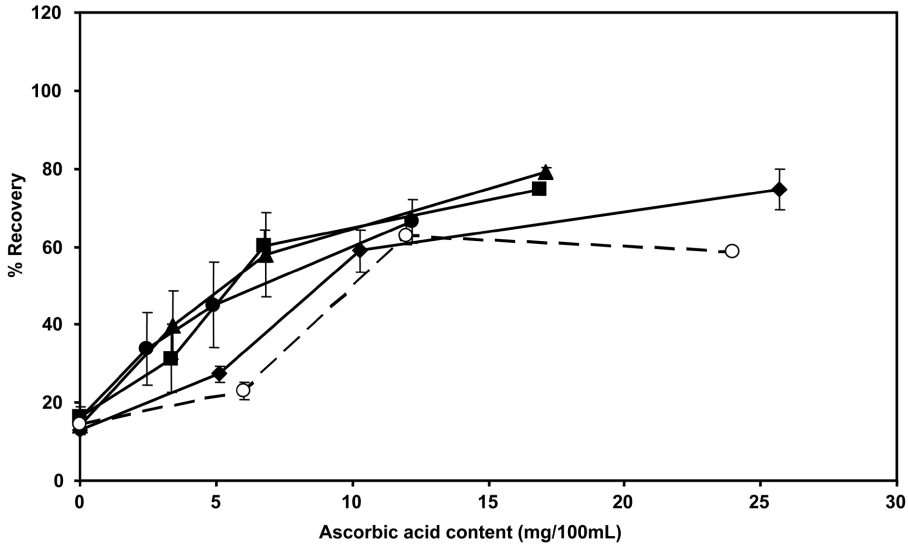

Figure 6. Relationship between the AA content of tea formulations and the percent of total catechin recovery following *in vitro* digestion. Orange (–◆–), grapefruit (–■–), lemon (–▲–), lime (–●–), and AA (–○–) values represent mean of four independent trials ±SEM.

milk formulations were likely due to inherent extraction losses of catechins from the more complex matrices compared to the food ingredients [23]. In particular, bovine and soy milks saw significant decreases in recovery of ECG and EGCG through simulated digestion in 50% milk–tea formulation. As stated in the methods, RM samples were extracted as previously report [23]; however DG samples were not extracted further. Understanding that larger undigested peptides may still be present in the DG sample, these could bind and decrease available catechins for quantification.

The mechanism for the stabilization by creamer systems is believed to involve the protein–catechin interactions which provide a physical trapping of the reactive catechin species. Strong protein–flavonoid interactions are known to exist and are mediated by hydrophobic association between flavonoids with proline rich regions of intact proteins and peptides fragments [38]. Interaction between catechins and specific bovine milk proteins including α-casein, β-casein, κ-casein, and albumin have been characterized [39–41]. Furthermore, catechin interactions with protein is believed to diminish specific reactivity of the native catechin molecules as evidenced by a reported loss of antimutagenic and antioxidant activity in milk systems [39, 42, 43]. Gallated catechins such as EGCG and ECG have been previously shown to have the strongest association with milk protein [23]. In each of the creamer systems, the gallated catechin EGC recoveries were significantly increased with milk addition (Fig. 7).

To better elucidate the true protective effects of greater protein contents in creamer formulations, pepsin and pancreatic enzyme concentrations were increased to meet the demands of additional solids in the preparation. This adjustment would ensure a more complete protein digestion, further simulating *in vivo* digestion under high protein load. Concentrations of pepsin (20, 40, 80, and 160 mg/mL), pancreatin (1, 2, 4, and 8 mg/mL), lipase (0.5, 1, 2, 4 mg/mL), and bile (6, 12, 24, 48 mg/mL) were applied to the *in vitro* digestion of a 50% bovine milk-tea beverage. As pancreatic enzyme/bile concentrations were increased from 0.5 to 4 × standard doses, individual catechin *in vitro* digestive stability decreased significantly (Fig. 8). Most interestingly significant decreases were observed for EGC and EGCG while EC and ECG maintain their digestive stability. These data indicate efficient protein digestion, as would be expected in normal healthy subjects, would disrupt catechin–protein interactions sufficiently enough to expose native catechins to digestive conditions thereby facilitating there degradation. Therefore, while catechin digestive degradation appears to be primarily driven by pH dependent auto-oxidative reactions, inclusion of digestive enzymes is critical to accurate estimation of catechin digestive stability and bioaccessibility from complex food matrices where catechin–protein interactions may mask digestive sensitivities.

In vivo observations have demonstrated no significant difference in catechin bioavailability and plasma antioxidant activity between tea and tea-milk beverage in humans [44–47]. However, previous reports on disease preventative activity have shown both a potentiated activity of black tea on colon cancer formation in mutagen-fed rats [48] and a negative effect on vascular protection from black tea when consumed with milk [47]. Although the potential for protective effects of protein–catechin interaction exists, the stability of the native tea catechins in the mild alkaline matrix still requires consideration during trials based on consumption. Conversely, these differential activities in vascular and cancer preventative activities upon consumption of tea-milk beverages could point to disease-specific byproducts produced from catechins with, or in the presence of milk polypeptides [49].

Increased recovery of tea catechins from rice milk-tea beverages could not fully be explained by protein content,

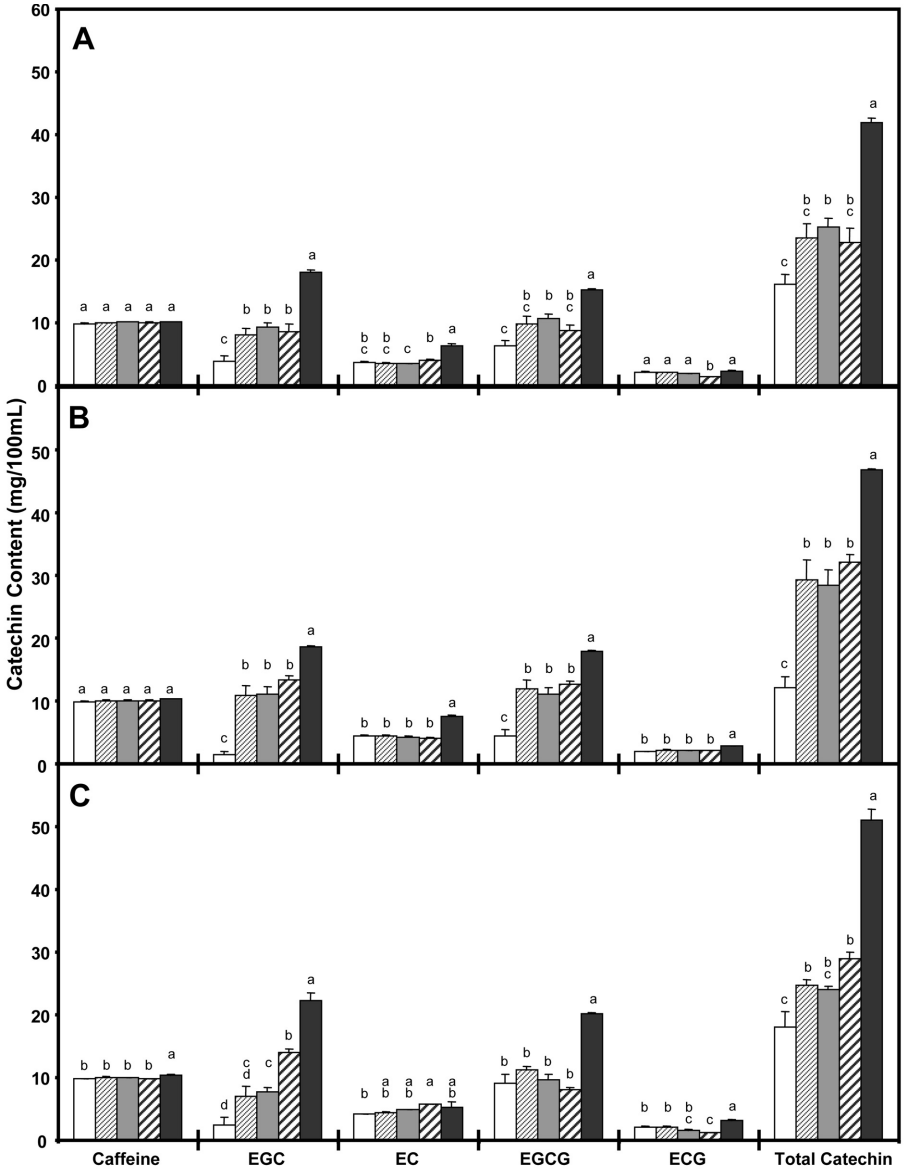

Figure 7. Green tea catechin digestive stability with the addition of (A) soy milk, (B) rice milk, and (C) bovine milk at 0% (□), 10% (▨), 20% (▩), and 50% (▨) v/v with corresponding predigested RM (■) catechin content from each trial. Each bar represents the mean ± SEM of four independent experiments. Bars not sharing a common superscript letter are significantly different ($p < 0.05$).

as it was relatively low compared to the bovine and soy milks (Table 2). Phenolic content, measured as total polyphenols, was higher in the rice milk compared to soy and bovine milks, indicating a potential for rice phenolic interactions during digestion as a protective mechanism. The rice milk utilized in this study according to its dietary label contained a small amount of lipid (2 g *per* 240 mL) and a significant amount of carbohydrate (27 g *per* 240 mL), the majority of which from rice origin would be complex starches like amylopectin and amylase [50]. The porcine pancreatin used during this investigation contains several enzymes including amylase, trypsin, and protease enzymes expected to hydrolyze the starch components present in the rice milk product. However, the potential for a lipid-starch complexation in rice has been shown to decrease breakdown by amylases [50] and may be analogous in protective

Table 2. The percent solids and phenolic acid contents (reported as gallic acid equivalents (GAE)) of individual creamers utilized in this study (±SEM, $n = 3$).

	Protein (mg/mL)	Phenolic content (GAE)[a)]
Milk	34.4 ± 3.54	0.19 ± 0.003
Ricemilk	27.1 ± 4.23	0.36 ± 0.058
Soymilk	28.8 ± 4.77	0.08 ± 0.005

a) Phenolic content presented as GAE.

activities by proteins in bovine milk from the *in vitro* model. Cocoa flavanol absorption has been shown to increase when consumed with bread and proposed to be a ramification of carbohydrate content during the meal [45]. While carbohydrate has been postulated to impact catechin bioavailability

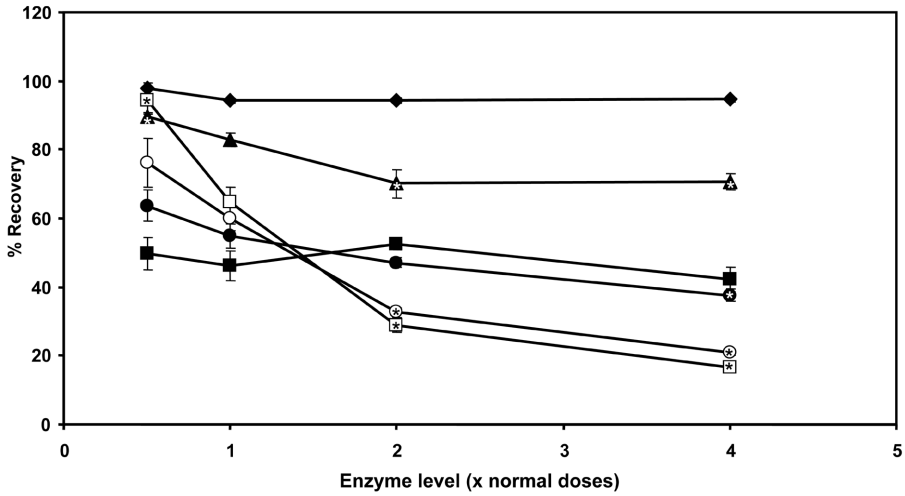

Figure 8. Green tea beverage profile formulated with 50% bovine milk following *in vitro* digestion with increasing enzyme concentration. Components are indicated as follows, CAF (–♦–), EGC (–■–), EC (–▲–), EGCG (–○–), ECG (–□–), and total catechin (–●–). Values represent the mean of four independent trials ± SEM. (* indicates significant difference ($p < 0.05$) in recovery relative to the 1× dose of enzymes among individual catechins; porcine pepsin (40 mg/mL), lipase (1 mg/mL), pancreatin (2 mg/mL) and bile (12 mg/mL)).

from cocoa, the extent to which these complex carbohydrates and proteins may impact either digestive release or bioaccessibility of catechin from tea is still unknown.

4 Concluding remarks

Catechins in model tea preparations exhibited poor stability to simulated gastric and small intestinal digestive conditions. Among the catechins, EGCG and EGC were most sensitive to *in vitro* digestive conditions. Common industrial food additives and preparation methods (juice and creamer addition) can significantly improve *in vitro* digestive recovery of native catechins. However, the static nature of the *in vitro* model employed in these studies may not fully account for the dynamic nature and heterogeneity of the gastrointestinal (GI) tract *in vivo*. Considering the pH dependence of observed catechin degradative reactions, *in vivo* differences in GI transit time and local pH extremes may further alter catechin intestinal profiles. Therefore, while these *in vitro* results cannot be fully extended directly to *in vivo* conditions, these data provide evidence that formulation factors may potentially impact the tea polyphenol profile in the small intestine. The extent to which these effects on digestive catechin recovery may impact ultimate catechin bioavailability and physiological profiles *in vivo* warrant further investigation.

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5 References

[1] Hollman, P. C. H., Feskens, E. J. M., Katan, M. B., Tea flavonols in cardiovascular disease and cancer epidemiology, *Proc. Soc. Exp. Biol. Med.* 1999, *220*, 198 – 202.

[2] Borelli, F., Capasso, R., Russo, A., Ernst, E., Systematic review: Green tea and gastrointestinal cancer risk, *Aliment. Pharmacol. Ther.* 2004, *19*, 497 – 510.

[3] Scalbert, A., Manach, C., Morand, C., Remesy, C., Jimenez, L., Dietary polyphenols and the prevention of diseases, *Crit. Rev. Food Sci. Nutr.* 2005, *45*, 287 – 306.

[4] Sesso, H. D., Gaziano, J. M., Buring, J. E., Hennekens, C. H., Coffee and tea intake and the risk of myocardial infarction, *Am. J. Epidemiol.* 1999, *149*, 162 – 167.

[5] Geleijnse, J. M., Launer, L. J., van der Kuip, D. A. M., Hofman, A., Witteman, J. C. M., Inverse association of tea flavonoid intakes with incident myocardial infarction: The Rotterdam study, *Am. J. Clin. Nutr.* 2002, *75*, 880 – 886.

[6] Mukamal, K. J., Maclure, M., Muller, J. E., Sherwood, J. B., Mittleman, M. A., Tea consumption and mortality after acute myocardial infarction, *Circulation* 2002, *105*, 2476 – 2481.

[7] Yang, C., Maliakal, P., Meng, X., Inhibition of carcinogenesis by tea, *Annu. Rev. Pharmacol. Toxicol.* 2002, *42*, 25 – 54.

[8] Higdon, J. V., Frei, B., Tea catechins and polyphenols: Health effects, metabolism, and antioxidant functions, *Crit. Rev. Food Sci. Nutr.* 2003, *43*, 89 – 143.

[9] Chung, J. H., Kim, M., Kim, H. K., Green tea polyphenols suppress nitric oxide-induced apoptosis and acetylcholinesterase activity in human neuroblastoma cells, *Nutr. Res.* 2005, *25*, 477 – 483.

[10] Lin, S. C., Li, W. C., Shih, J. W., Hong, K. F., *et al.*, The tea polyphenols EGCG and EGC repress mRNA expression of human telomerase reverse transcriptase (h*TERT*) in carcinoma cells, *Cancer Lett.* 2006, *236*, 80 – 88.

[11] Mandel, S., Amit, T., Reznichenko, L., Weinreb, O., Youdim, M. B. H., Green tea catechins as brain-permeable, natural iron chelators-antioxidants for the treatment of neurodegenerative disorders, *Mol. Nutr. Food Res.* 2006, *50*, 229 – 234.

[12] Nichenametla, S. N., Taruscio, T. G., Barney, D. L., Exon, J. H., A review of the effects and mechanisms of polyphenolics in cancer, *Crit. Rev. Food Sci. Nutr.* 2006, *46*, 161 – 183.

[13] Feng, W. Y., Metabolism of green tea catechins: An overview, *Curr. Drug Metab.* 2006, *7*, 755 – 809.

[14] Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C., Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies, *Am. J. Clin. Nutr.* 2005, *81*, 230S – 242S.

[15] Arts, I. C. W., Hollman, P. C. H., Polyphenols and disease risk in epidemiologic studies, *Am. J. Clin. Nutr.* 2005, *81*, 317S – 325S.

[16] Record, I. R., Lane, J. M., Simulated intestinal digestion of green and black teas, *Food Chem.* 2001, *73*, 481 – 486.

[17] Yoshino, K., Suzuki, M., Sasaki, K., Miyase, T., Sano, M., Formation of antioxidants from (–)-epigallocatechin gallate in mild alkaline fluids, such as authentic intestinal juice and mouse plasma, *J. Nutr. Biochem.* 1999, *10*, 223 – 229.

[18] Zhu, Q. Y., Zhang, A. Q., Tsang, D., Huang, Y., Chen, Z. Y., Stability of green tea catechins, *J. Agric. Food Chem.* 1997, *45*, 4624 – 4628.

[19] Chen, Z. Y., Zhu, Q. Y., Wong, Y. F., Zhang, Z. S., Chung, H. Y., Stabilizing effect of ascorbic acid on green tea catechins, *J. Agric. Food Chem.* 1998, *46*, 2512 – 2516.

[20] Ruiz, R. P., in: Wrolstad, R. E. (Ed.), *Current Protocols in Food Analytical Chemistry – Gravimetric Determination of Water by Drying and Weighing*, John Wiley and Sons Inc., New York 2002, ppA1.1.1 – A1.1.6.

[21] Waterhouse, A. L., in: Wrolstad, R. E. (Ed.), *Current Protocols in Food Analytical Chemistry – Determination of Total Phenolics*, John Wiley and Sons Inc., New York 2002, pp I1.1.1 – I1.1.8.

[22] Ferruzzi, M. G., Failla, M. L., Schwartz, S. J., Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an in vitro digestion and caco-2 human cell model, *J. Agric. Food Chem.* 2001, *49*, 2082 – 2089.

[23] Ferruzzi, M. G., Green, R. J., Analysis of catechins from milk-tea beverages by enzyme assisted extraction followed by high performance liquid chromatography, *Food Chem.* 2006, *99*, 484 – 491.

[24] Neilson, A. P., Green, R. J., Wood, K. V., Ferruzzi, M. G., High-throughput analysis of catechins and theaflavins by high performance liquid chromatography with diode array detection, *J. Chromatogr. A.* 2006, *1132*, 132 – 140.

[25] Henning, S. M., Fajardo-Lira, C., Lee, H. W., Youssefian, A. A. *et al.*, Catechin content of 18 teas and a green tea extract supplement correlates with the antioxidant capacity, *Nutr. Cancer.* 2003, *45*, 226 – 235.

[26] Su, Y. L., Leung, L. K., Huang, Y., Chen, Z. Y., Stability of tea theaflavins and catechins, *Food Chem.* 2003, *83*, 189 – 195.

[27] Mizooku, Y., Yoshikawa, M., Tsuneyoshi, T., Arakawa, R., Analysis of oxidized epigallocatechin gallate by liquid chromatography/mass spectrometry, *Rapid Comm. Mass Spectrom.* 2003, *17*, 1915 – 1918.

[28] Guo, Q. N., Zhao, B. L., Li, M. F., Shen, S. R., Xin, W. J., Studies on protective mechanisms of four components of green tea polyphenols against lipid peroxidation in synaptosomes, *Biochim. Biophys. Acta, Lipid Metabol.* 1996, *1304*, 210 – 222.

[29] Lambert, J. D., Sang, S., Yang, C. S., Possible controversy over dietary polyphenols: Benefits vs. risks, *Chem. Res. Toxicol.* 2007, *20*, 583 – 585.

[30] Mochizuki, M., Yamazaki, S., Kano, K., Ikeda, T., Kinetic analysis and mechanistic aspects of autoxidation of catechins, *Biochim. Biophys. Acta.* 2002, *1569*, 35 – 44.

[31] Sang, S. M., Lee, M. J., Hou, Z., Ho, C. T., Yang, C. S., Stability of tea polyphenol (–)-epigallotcatechin-3-gallate and formation of dimers and epimers under common experimental conditions, *J. Agric. Food Chem.* 2005, *53*, 9478 – 9484.

[32] Hong, J., Lu, H., Meng, X. F., Ryu, J. H. *et al.*, Stability, cellular uptake, biotransformation, and efflux of tea polyphenol (–)-epigallocatechin-3-gallate in HT-29 human colon adenocarcinoma cells, *Cancer Res.* 2002, *62*, 7241 – 7246.

[33] Kagan, V. E., Yalowich, J. C., Day, B. W., Goldman, R. *et al.*, Ascorbate is the primary reductant of the phenoxyl radical of etoposide in the presence of thiols both in cell homogenates and in model systems, *Biochemistry* 1994, *33*, 9651 – 9660.

[34] Peterson, J. J., Beecher, G. R., Bhagwat, S. A., Dwyer, J. T. *et al.*, Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature, *J. Food Compost. Anal.* 2006, *19*, S74 – S80.

[35] Peterson, J. J., Dwyer, J. T., Beecher, G. R., Bhagwat, S. A., *et al.*, Flavanones in oranges, tangerines (mandarins), tangors, and tangelos: A compilation and review of the data from the analytical literature, *J. Food Compost. Anal.* 2006, *19*, S66 – S73.

[36] Aggarwal, B. B., Shishodia, S., Molecular targets of dietary agents for prevention and therapy of cancer, *Biochem. Pharmacol.* 2006, *71*, 1397 – 1421.

[37] Siebert, K. J., Troukhanova, N. V., Lynn, P. Y., Nature of polyphenol-protein interactions, *J. Agric. Food Chem.* 1996, *44*, 80 – 85.

[38] Papadopoulou, A., Frazier, R. A., Characterization of protein-polyphenol interactions, *Trends Food Sci. Technol.* 2004, *15*, 186 – 190.

[39] Arts, M. J. T. J., Haenen, G. R. M. M., Wilms, L. C., Beetstra, S. A. J. N. *et al.*, Interactions between flavonoids and proteins: Effect on the total antioxidant capacity, *J. Agric. Food Chem.* 2002, *50*, 1184 – 1187.

[40] Jobstl, E., O'Connell, J., Fairclough, J. P. A., Williamson, M. P., Molecular model for astringency produced by polyphenol/protein interactions, *Biomacromolecules.* 2004, *5*, 942 – 949.

[41] Papadopoulou, A., Green, R. J., Frazier, R. A., Interactions of flavonoids with bovine serum albumin: A fluorescence quenching study, *J. Agric. Food Chem.* 2005, *53*, 158 – 163.

[42] Catterall, F., Kassimi, A. I., Clifford, M. N., Ioannides, C., Influence of milk on antimutagenic potential of green and black teas, *Anticancer Res.* 2003, *23*, 3863 – 3868.

[43] Krul, C., Luiten-Schuite, A., Tenfelde, A., van Ommen, B., *et al.*, Antimutagenic activity of green tea and black tea extracts studied in a dynamic *in vitro* gastrointestinal model, *Mutat. Res., Fundam. Mol. Mech. Mutagenesis.* 2001, *474*, 71 – 85.

[44] Reddy, V. C., Sagar, G. V. V., Sreeramulu, D., Venu, L., Raghunath, M., Addition of milk does not alter the antioxidant activity of black tea, *Ann. Nutr. Metab.* 2005, *49*, 189 – 195.

[45] Schramm, D. D., Karim, M., Schrader, H. R., Holt, R. R., *et al.*, Food effects on the absorption and pharmacokinetics of cocoa flavanols, *Life Sci.* 2003, *73*, 857 – 869.

[46] Leenen, R., Roodenburg, A. J. C., Tijburg, L. B. M., Wiseman, S. A., A single dose of tea with or without milk increases plasma antioxidant activity in humans, *Eur. J. Clin. Nutr.* 2000, *54*, 87 – 92.

[47] van het Hof, K. H., Kivits, G. A. A., Weststrate, J. A., Tijburg, L. B. M., Bioavailability of catechins from tea: The effect of milk, *Eur. J. Clin. Nutr.* 1998, *52*, 356 – 359.

[48] Weisburger, J. H., Rivenson, A., Garr, K., Aliaga, C., Tea, or tea and milk, inhibit mammary gland and colon cancer carcinogenesis in rats, *Cancer Lett.* 1997, *114*, 323 – 327.

[49] Korhonen, H., Pihlanto, A., Food-derived bioactive peptides – Opportunities for designing future foods, *Curr. Pharm. Des.* 2003, *9*, 1297 – 1308.

[50] Guraya, H. S., Kadan, R. S., Champagne, E. T., Effect of rice starch-lipid complexes on *in vitro* digestibility, complexing index, and viscosity, *Cereal Chem.* 1997, *74*, 561 – 565.

