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Catechin Degradation with Concurrent Formation of Homo- and Heterocatechin Dimers during *in Vitro* Digestion

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Catechins were subjected to *in vitro* gastric and small intestinal digestion. EGCG, EGC, and ECG were significantly degraded at all concentrations tested, with losses of 71–91, 72–100, and 60–61%, respectively. EC and C were comparatively stable, with losses of 8–11 and 7–8%, respectively. HLPC-ESI-MS/MS indicated that EGCG degradation under simulated digestion resulted in production of theasinensins (THSNs) A and D (*m*/*z* 913) and P-2 (*m*/*z* 883), its autoxidation homodimers. EGC dimerization produced the homodimers THSN C and E (*m*/*z* 609) and homodimers analogous to P-2 (*m*/*z* 579). ECG homodimers were not observed. EGCG and EGC formed heterodimers analogous to the THSNs (*m*/*z* 761) and P-2 (*m*/*z* 731). EGCG and ECG formed homodimers analogous to the THSNs (*m*/*z* 897). This study provides an expanded profile of catechin dimers of digestive origin that may potentially form following consumption of catechins. These data provide a logical basis for initial screening to detect catechin digestive products *in vivo*.

KEYWORDS: Catechin; oxidation; digestion; dimer; theasinensin; P-2; gut; pH; HPLC; electrochemical detection; mass spectrometry

INTRODUCTION

Epidemiological and experimental evidences have highlighted the potential physiological activities of flavanols and flavanol-rich foods, including protection against oxidative stress and inflammation and reduced risk of cardiovascular disease and certain cancers (*1*–*9*). The major flavanols present in the human diet are (−)-epigallocatechin (EGC), (+)-epicatechin (EC), (±)-catechin (C), (−)-epigallocatechin gallate (EGCG), and (−)-epicatechin gallate (ECG), collectively referred to as catechins (**Figure 1**). Significant dietary sources of these catechins include grapes (EC, C, EGC, ECG), tea (EGCG, EGC, EC, C, ECG), cocoa (EC, C), and other fruits (*10*–*17*).

In vitro experiments have demonstrated that catechins are stable in acid but are extensively degraded in fluids of near-neutral or greater pH, such as intestinal juice, plasma, bile, cell culture media, or simulated digestive conditions (*18*–*31*). EGCG and EGC are the most sensitive to higher pH, ECG has intermediate stability, and EC and C are relatively stable. When exposed to elevated pH, EGCG degrades by epimerization and autoxidation reactions involving the B-ring, leading to the formation of homocatechin dimers such as theasinensin (THSN) A, THSN D, and P-2 (**Figure 2**), which are minor constituents of mildly fermented tea including oolong and black teas (*18*, *21*, *23*, *24*, *27*, *30*–*34*). These data suggest that catechins with similar structures may also undergo dimerization, resulting in the formation of products analogous to the THSNs and P-2. However, autoxidation of other homogenous catechin reactants (such as EGC) or complex heterogeneous mixtures of several catechin species have not been reported. As catechins are typically present in foods as heterogeneous mixtures, a more complete profile of expected homo- and heterocatechin autoxidation products is essential for the evaluation of catechin degradation in *in vivo* biological matrices relevant to human health.

Conditions promoting autoxidation are present in the upper small intestine during human digestion, including elevated pH (6.0–7.5), digestive secretions (bile, etc.), and dissolved O_2 and reactive oxygen species (ROS) (*35*–*37*). Therefore, the potential

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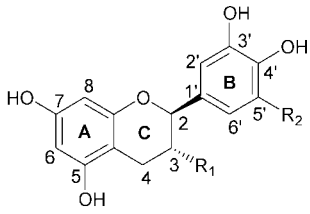
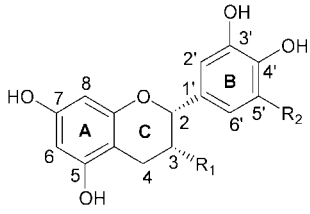
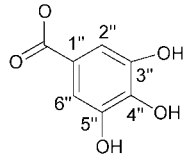


Catechins



Epicatechins



Gallate group

Compound	Abbreviation	R_1	R_2
Catechin	C	OH	H
Gallocatechin	GC	OH	OH
Catechin gallate	CG	Gallate	H
Gallocatechin gallate	GCG	Gallate	OH
Epicatechin	EC	OH	H
Epigallocatechin	EGC	OH	OH
Epicatechin gallate	ECG	Gallate	H
Epigallocatechin gallate	EGCG	Gallate	OH

Figure 1. Structures of the major monomeric catechins and their epimers present in the human diet.

exists for preabsorptive catechin autoxidation in the gut lumen prior to intestinal uptake, without *in vivo* protection of endogenous antioxidant systems present on the enterocyte surface and in body tissues. Previous studies of catechin dimerization under *in vitro* digestive conditions have focused on bile or authentic intestinal juice individually (*20*, *21*). Although catechin stability during exposure to the enzymes and pH conditions of gastric and small intestinal phases sequentially has been reported (*23*, *28*), characterization of catechin digestive profiles including identification of specific catechin degradation products under these conditions has not been accomplished.

Animal and human studies have shown that catechin bioavailability is relatively low and that catechins are subsequently present in tissues and biological fluids as native species and their phase-II conjugates (*33*, *38–47*). With the exception of colonic fermentation products, data regarding the potential preabsorptive formation, absorption, tissue levels, and *in vivo* physiological activity of catechin digestive degradation products remain limited (*21*), possibly due to incomplete information regarding potential compounds of interest to monitor. Furthermore, data regarding the potential for preabsorptive digestive formation and structural variation of expected homo- and heterocatechin dimers are required to effectively monitor catechin degradation and assess the physiological relevance of these dimers following oral administration to animals and humans.

The aim of this study was to monitor native catechin degradation and simultaneously characterize the structural variation of the resulting catechin degradation products using an *in vitro* digestion model to simulate preabsorptive digestion events. Characterization of these digestive species will provide valuable insight regarding the potential alteration of dietary catechin profiles during digestive transit and will provide a framework for future animal and human studies regarding preabsorptive formation, absorption, and subsequent physiological relevance of catechin dimers *in vivo*.

MATERIALS AND METHODS

Reagents and Standards. Pepsin from porcine stomach mucosa, pancreatin, and lipase (type II) from porcine pancreas, porcine bile extract, EGC (>98%), C, EC (>98%), ECG (>98%), and (−)-gallocatechin (GC) (>98%) were obtained from Sigma-Aldrich (St. Louis, MO). EGCG (>97%) was a gift from DSM Nutritional Products (Parsippany, NJ). All solvents were of HPLC grade. Acetic acid (AA) solutions were prepared by diluting glacial acetic acid in distilled–deionized water (ddH_2O). Saline (0.9% NaCl w/v) was prepared in ddH_2O. Catechin standard solutions for calibration curves were prepared in 2% AA to obtain serial dilutions covering 10–500 μM each.

Formulation of Raw Material. Raw material (RM) for individual catechin digestions (EGCG, EGC, EC, ECG, and C) was formulated in saline solution (pH 5.5) at 0.1, 0.05, and 0.01 mg/mL, simulating typical catechin concentrations in a cup of green or black tea (*10*, *16*), with sonication. RM for combined catechin digestions (EGCG and EGC; EGCG and ECG; EGC and ECG) was formulated similarly at 0.1 mg/mL of each species. RM for digestion background blanks was saline only. RM aliquots were acidified with 10% AA (1 mL/5 mL of RM) and stored under N_2 at −80 °C until analysis.

Individual and Combined Catechin *in Vitro* Digestions. *In vitro* digestions were performed following a static two-stage *in vitro* digestion model as originally described by Garrett et al. (*48*) and more recently adapted in our laboratory to investigate catechin degradation from tea (*28*). Gastric conditions were as follows: 3 mL of pepsin (40 mg/mL in 0.1 M HCl) was added to 20 mL of RM and the pH was adjusted to 2.0 ± 0.1 with 0.1 M HCl. Samples were blanketed with N_2 and incubated in a dark shaking water bath at 37 °C for 1 h. Gastric aliquots (3 mL) were then removed for analysis (from individual catechin digestions only). Small intestinal conditions were as follows: gastric digesta was neutralized to pH ≥5.3 with 0.1 M $NaHCO_3$, followed by the addition of 4.5 mL of pancreatin/lipase (4 mg/mL pancreatin and 2 mg/mL lipase in 0.1 M $NaHCO_3$) and 4.5 mL of bile (24 mg/mL bile extract in 0.1 M $NaHCO_3$), after which the pH was adjusted to 7.2 ± 0.1 with 0.1 M $NaHCO_3$ and/or 0.1 M NaOH. Samples were diluted to 50 mL total volume with saline, blanketed with N_2, and incubated as stated above for 2 h. Gastric aliquots and final digesta were acidified with 10% AA (0.2 mL/mL of sample) and then centrifuged at 18000*g* for 15 min. The supernatant was stored under N_2 at −80° until analysis.

Controls. Nondigested controls (NDC) were performed for each individual catechin RM to account for nondigestive catechin losses. Three milliliters of saline was added to 20 mL of RM, and the pH was adjusted to 5.5 with 0.1 M $NaHCO_3$. Samples were blanketed with N_2 and incubated as stated above for 1 h. Samples were then diluted to 50 mL with saline, flushed with N_2, and incubated as stated above for 2 h. NDC samples were then processed for storage and analysis as described above.

HPLC-ESI-MS/MS Sample Preparation. For HPLC-ESI-MS/MS analyses, representative digesta for each individual and combined RM (0.1 mg/mL only) and background blanks were generated as stated above. Replicate digesta were pooled, acidified to pH 3.5 with 1 N HCl, and divided into 6–15 mL aliquots, and then $(NH_4)_2SO_4$ was added to saturation. Aliquots were extracted twice with 15 mL of ethyl acetate/0.01% butylated hydroxytoluene (BHT) (w/v) by vortexing (15 s), centrifugation (1950*g* for 5 min), and collection of the organic layer. Extracts were pooled, dried in a rotary vacuum evaporator, solubilized in 3 mL of mobile phase A (see below), and centrifuged (1950*g* for 5 min). The supernatant was processed for storage and analysis as described above.

HPLC Analysis. HPLC–diode array detection (DAD) analysis was performed following the method of Neilson et al. (*49*) with modifications to quantify catechins. Separation was performed on a Waters Alliance 2695 separation module (Milford, MA) with a 3.9 × 100 mm

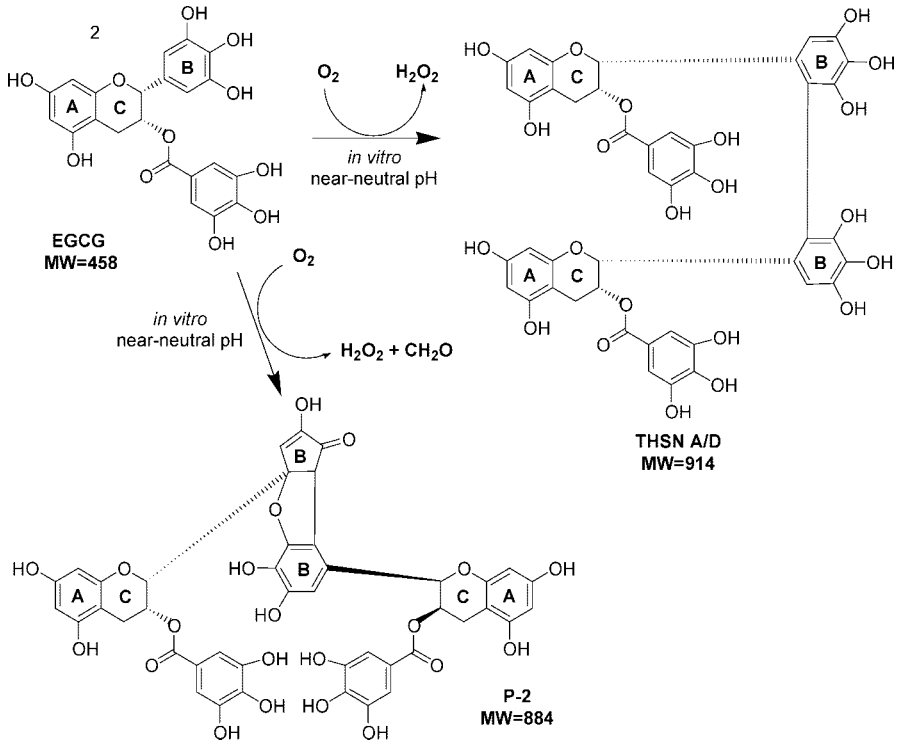


Figure 2. Autoxidation reactions of EGCG at near-neutral pH. Two EGCG monomers form a C–C bond in the B-ring, resulting in the net loss of two hydrogen atoms, to generate the homodimers theasinensin (THSN) A and THSN D. Two EGCG monomers also undergo B-ring opening and subsequent condensation, resulting in the net loss of two hydrogen atoms and formaldehyde (CH_2O), to generate the homodimer P-2.

(3.5 μm particle size) XTerra RP C_{18} column preceded by an identical 3.9 × 20 mm guard column. Columns were thermostated to 35 °C. Samples were filtered through a 0.45 μm filter and thermostated at 5 °C prior to injection. Injection volume was 50 μL. Linear gradient elution was performed using a binary mobile phase system: phase A, ddH_2O, acetonitrile (ACN), and formic acid (FA) (89.9:10:0.1 v/v); phase B, ddH_2O, ACN, and FA (69.9:30:0.1 v/v). The system flow rate was 0.9 mL/min. Following injection, the mobile phase gradient was 99:1 (A/B) at 0 min, 1:99 at 6 min, and 99:1 at 8 min and held to 10 min for a total analysis time of 10 min. DAD was performed with a Waters 2996 photodiode array detector (wavelength scan from 200 to 600 nm). Chromatographic data were collected using Waters Empower2 software.

To obtain more selective and sensitive detection of catechin-derived dimers, HPLC–electrochemical detection (ECD) analysis of all RM, extracted digesta, and background blanks was performed under similar conditions with the following modifications: columns were thermostated to 25 °C; injection volume, 1 μL; phase A, ddH_2O, ACN, and trifluoroacetic acid (TFA) (91.9:8:0.1 v/v); phase B, ddH_2O, ACN, methanol (MeOH), and TFA (69.9:27:3:0.1 v/v); the mobile phase gradient was 75:25 (A/B) at 0 min, 15:85 at 2 min, 0:100 at 4 min, held to 5 min, 75:25 at 6 min, and held to 12 min (all transitions linear) for a total analysis time of 12 min; 8-channel ECD was performed on an ESA CoulArray detector (ESA Biosciences, Chelmsford, MA) with cell potentials at −100, 60, 130, 200, 270, 340, 410, and 480 mV.

HPLC-ESI-MS/MS Experiments. HPLC separation for MS experiments was performed on a Waters 2795 separations module employing parameters identical to those described for HPLC-DAD analysis. Following separation, the column effluent was split 1:1 (retained/waste) prior to introduction by negative mode electrospray ionization (ESI) into a Waters Q-TOF Micromass spectrometer. ESI capillary voltage was −3.5 kV, source temperature was 150 °C, sample cone voltage was 40 V, and extraction cone voltage was 1 V. The desolvation temperature was 300 °C. The desolvation and nebulizer cone gas was N_2 at flow rates of 500 and 50 L/h, respectively. Quadrupole pre- and postfilter voltages were 5.0 V, ion quadrupole energy was 5.0 V, and radio frequency/direct current offset voltages were 0.5 and 3.0 V, respectively. For collision-activated dissociation (CAD), the collision gas was Ar at 69 kPa, and ion kinetic energies (KE_{ion}) were 20–30 eV. TOF acceleration voltage was 200 V, reflectron voltage was 1780 V, and multichannel plate detector voltage was 2800 V. Mass data were collected from *m*/*z* 50 to 1500, with a scan time of 0.5 s and an interscan delay of 0.1 s. HPLC-MS/MS data were collected using Waters MassLynx (V4.0 SP2) software. The TOF was calibrated daily by direct infusion of 2 mM H_3PO_4 in MeOH/ddH_2O (50:50 v/v).

Statistical Analysis. Digestive and NDC native catechin losses were calculated from $n = 4$ independent replicate experiments and are reported as mean ± SE.

RESULTS

Individual Catechin Digestive and NDC Losses. All RM, background blanks, gastric aliquots, and catechin digesta from each individual species digestion experiment were analyzed by HPLC-DAD; digestive losses are reported in **Table 1**. EGCG, EGC, and ECG were significantly degraded by digestion at concentrations tested, with losses of 71–91, 72–100, and 60–61%, respectively. In contrast, EC and C were much more resistant to digestive degradation, with losses of only 8–11 and 7–8%, respectively. These results agree with previous studies which have shown that simple catechins (C, EC) are much more stable than the corresponding gallocatechins (EGC, EGCG) under digestive conditions (*23*, *28*), whereas catechin gallates (ECG) have intermediate stability. The majority of the loss for each species was due to intestinal phase degradation, in contrast to gastric phase losses of only 5–7, 3–12, and 5–6% for EGCG, EGC, and ECG, respectively. The more stable catechins (C and EC) experienced gastric phase losses of 1–2 and 0.1–0.2%, respectively. Thus, gastric phase losses for all species were generally 10–20-fold less than intestinal phase losses, suggesting that catechins are comparatively more stable under gastric phase conditions than under intestinal phase conditions of digestion.

NDC losses for all catechin species were much lower than total and intestinal digestive losses, but were also greater than gastric losses. Because NDC experiments were performed at a

Table 1. Percent Digestive and Nondigestive Control (NDC) Losses Observed for Individual Species Catechin Formulations at Different Raw Material Concentrations ([RM])

		loss from RM[a–c] (%)			
			digestion		
compound	[RM] (mg mL^{-1})	NDC[c]	gastric[c]	intestinal[d]	total
EGCG	0.10	15.0 ± 6.6	5.1 ± 1.7	85.9 ± 1.4	90.9 ± 1.0
	0.05	18.6 ± 6.5	5.7 ± 1.9	79.2 ± 4.0	84.8 ± 2.4
	0.01	18.1 ± 2.8	6.8 ± 4.1	64.4 ± 8.9	71.2 ± 6.1
EGC	0.10	15.2 ± 7.7	5.5 ± 5.3	66.8 ± 6.0	72.3 ± 1.0
	0.05	14.0 ± 6.0	2.6 ± 2.6	69.0 ± 3.3	71.6 ± 2.3
	0.01	27.1 ± 14.5	11.6 ± 3.3	88.4 ± 3.3	100[e]
EC	0.10	2.2 ± 1.1	1.6 ± 0.7	9.8 ± 4.0	11.4 ± 4.0
	0.05	10.1 ± 4.6	0.6 ± 0.4	7.6 ± 2.0	8.3 ± 1.9
	0.01	6.8 ± 3.6	1.0 ± 0.6	10.3 ± 2.2	11.3 ± 2.0
ECG	0.10	6.4 ± 1.3	5.3 ± 1.2	54.2 ± 1.2	59.5 ± 1.6
	0.05	7.7 ± 1.9	6.1 ± 0.6	54.0 ± 1.5	60.0 ± 1.7
	0.01	4.7 ± 1.1	5.5 ± 0.5	55.1 ± 0.8	60.6 ± 0.8
C	0.10	1.7 ± 1.1	0.1 ± 0.1	7.0 ± 0.3	7.1 ± 0.3
	0.05	3.4 ± 1.7	0.2 ± 0.2	7.8 ± 0.8	8.0 ± 0.7
	0.01	2.4 ± 1.2	0.0 ± 0.0	8.2 ± 5.3	8.2 ± 5.3

[a] Losses were calculated by HPLC-DAD analysis of RM and digesta. [b] Losses are reported as mean ± SE from $n = 4$ replicate experiments. [c] Some individual NDC or gastric losses were measured as <0; these values were taken to be 0 for calculation of means. [d] Intestinal losses were calculated by subtracting gastric losses from total losses. [e] Due to the extensive degradation of EGC, the low RM concentration, and coelution with digestive matrix peaks, EGC was not detectable in the final digesta for this dose.

higher pH than the gastric phase (5.5 vs 2.0) but did not include digestive enzymes, the degradation of catechins under digestive conditions appears to be most directly correlated to pH rather than to digestive enzyme activity. These results are in agreement with previous studies in model systems that have reported catechins to be stable in acidic conditions while being unstable in a variety of solutions at near-neutral or greater pH (*18*,*20*–*25*, *29*, *30*, *46*, *50*).

Identification of Catechin Digestive Degradation Products. Catechins exhibiting digestive instability (EGCG, EGC, ECG) were identified as likely dimer precursors and were digested individually and in pairs (EGCG–EGC; EGCG–ECG; EGC–ECG) at 0.1 mg/mL RM each. The RM and digesta extracts were analyzed by HPLC-ECD and HPLC-ESI-MS/MS to characterize the resulting products. HPLC-ESI-MS analyses were performed to identify deprotonated molecular ions ([M – H]$^-$) corresponding to native catechins and potential catechin degradation products. Ions of interest were selected and subsequently fragmented by CAD to obtain MS/MS spectra. Although HPLC-DAD was used to quantify native catechins, HPLC-ECD demonstrated superior sensitivity and selectivity for detection of dimers in complex matrices; ECD profiles are therefore provided. HPLC-ECD analysis of these digesta indicated significant digestive alteration of each RM profile; representative elution profiles of two selected RM (EGCG-EGC and EGCG-ECG) and the subsequent digesta are shown in **Figure 3**. Refer to available Supporting Information for all HPLC-ECD chromatograms and mass spectra not shown, and **Table 2** for HPLC retention times (t_R) and additional information. Compounds are referred to by the number assigned to each peak on chromatograms.

EGCG RM contained EGCG (**1**, *m*/*z* 457) and <2% ECG contaminant (**2**, *m*/*z* 441). EGC RM contained EGC (**3**, *m*/*z* 305) only. ECG RM contained ECG (**2**) only. Each combined RM contained only the two individual species RMs comprising it.

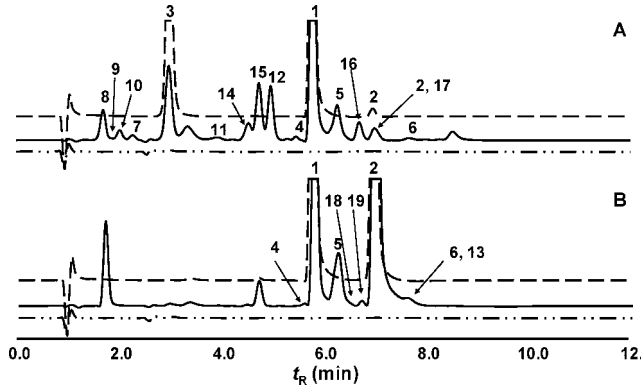


Figure 3. HPLC-ECD elution profiles (response at 200 mV) of undigested combined catechin raw material (- - -), the subsequent *in vitro* digesta extract (—), and a digestive blank extract (no RM added) (– - - –) from combined EGCG-EGC (**A**) and combined EGCG-ECG (**B**) raw material formulations. Peak scale is from −0.1 to 1.0 μA. Refer to **Table 2** for identities of numbered peaks. Note that *in vitro* digesta extracts were concentrated prior to HPLC-ECD analysis (see Materials and Methods) to facilitate peak detection due to low analyte levels in digesta following digestive losses.

Table 2. Catechins and Their Degradation Products Observed in *in Vitro* Digestion: HPLC-ECD and HPLC-DAD Retention Times (t_R), Deprotonated Molecule ([M – H]$^-$) Mass-to-Charge Ratios (*m*/*z*) from Negative Mode ESI-MS, and Fragment Ions Observed in CAD-MS/MS[a] Spectra of Each Compound

	HPLC t_R (min)		[M – H]$^-$	fragment ions
compd[a]	ECD	DAD	(*m*/*z*)	(*m*/*z*)
1	5.8	5.7	457	125,169,193,305
2	7.0	6.7	441	125, 169, 203, 245, 289
3	3.0	3.6	305	111, 125, 165, 169, 219, 261
4, **5**	5.5, 6.3	5.2, 6.0	913	169, 435, 573, 591, 743, 761
6	7.7	7.3	883	125, 169, 405, 525, 543, 561, 713
7	2.3	2.9	305	111, 125, 167, 219, 261
8, **9**	1.7,1.9	2.3, 2.5	609	167, 333, 427, 453, 471, 591
10–**12**	2.0, 3.9, 5.0	2.7, 4.6, 4.8	579	125, 167, 405, 423, 435, 543, 561
13	7.7	7.2	441	109, 125, 169, 245, 289
14, **15**	4.5, 4.8	4.7, 5.2	761	125, 167, 327, 435,453, 591, 609
16, **17**	6.7, 7.0	6.2, 6.6	731	125, 167, 395, 405, 525, 543, 561
18, **19**	6.4, 6.7	6.3, 6.5	897	289, 437, 455, 607, 625, 727, 745

[a] Ion kinetic energies (KE_{ion}) used for CAD-MS/MS analysis of each ionized parent compound were 30 eV for *m*/*z* 441, 457, 731, 761, 883, and 897; 25 eV for *m*/*z* 609; and 20 eV for *m*/*z* 305, 579, and 913.

Observed MS/MS spectra of native catechins were similar to published spectra (data not shown) (*30*, *51*). Catechin degradation products were not found in RM formulations.

Individual EGCG digesta contained residual EGCG (**1**), residual ECG (**2**), two catechin-derived degradation products with *m*/*z* 913 (**4**, **5**), and one with *m*/*z* 883 (**6**). Compounds **4** and **5** have similar MS/MS spectra and are the previously reported EGCG C_2'–C_2' homodimers THSN A and THSN D (**Figure 2**). It is likely that **4** is THSN A and that **5** is THSN D, as THSN A has been shown to elute more rapidly under RP HPLC conditions (*21*). Compound **6** was identified as the previously reported EGCG B-ring condensation–elimination homodimer P-2 (**Figure 2**). MS/MS spectra for **4**–**6** were

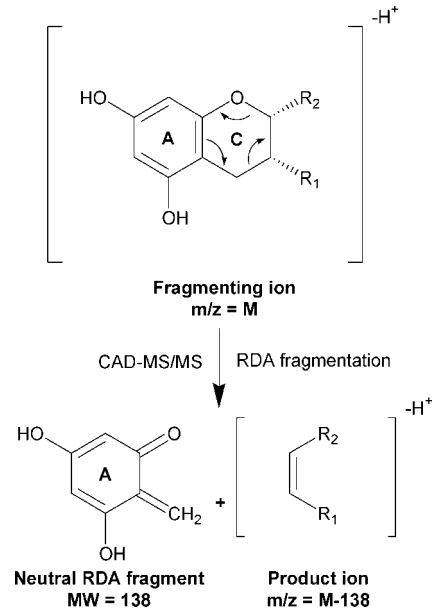


Figure 4. Mechanism of the retro-Diels–Alder cleavage reaction typically observed during fragmentation of flavonoids during MS/MS analysis.

identical to previously published spectra (data not shown) (*24, 30*). Major MS/MS fragmentations for both THSNs correspond to sequential loss of two gallic acid residues from $[M - H]^-$. Major fragmentations for P-2 correspond to sequential loss of two gallic acid residues from the $[M - H]^-$, followed by a retro-Diels–Alder (RDA) cleavage (loss of a neutral A-ring derivative, with MW 138, by breaking the A/C-ring system) typically observed during fragmentations of flavonoids (**Figure 4**) (*15, 52–55*).

Individual EGC digesta contained residual EGC (**3**), another compound with *m/z* 305 (**7**) not present in the RM, and several degradation products with *m/z* 609 (**8**, **9**) and *m/z* 579 (**10**, **11**, **12**). Compound **7** was tentatively identified as GC (see **Figure 1**) as HPLC-MS and MS/MS analysis of authentic GC standard material resulted in a $[M - H]^-$ with identical t_R, *m/z*, and MS/MS spectrum as that of **7**(data not shown). MS/MS analysis of **8**–**12** suggests that they are EGC homodimers analogous to those observed for EGCG. Compounds **8** and **9** have similar MS/MS spectra (**Figure 5**), with major fragmentations corresponding to consecutive RDA fragmentations of the molecular ion. Compounds **8** and **9** were tentatively identified collectively as THSNs C and E, previously reported as minor enzymatic oxidation products in mildly fermented tea leaves (*31, 56*); **8** and **9** have never been identified as potential digestive degradation products of tea catechins until now. Compounds **10**–**12** appear to be P-2 analogues (**Figure 5**) and have similar MS/MS spectra with major fragmentations corresponding to loss of H_2O from $[M - H]^-$ followed by RDA fragmentation. Compounds **10**–**12** have not previously been reported in the literature.

Individual ECG digesta contained residual ECG (**2**) and another product with *m/z* 441 (**13**) not present in the RM. Compound **13** was tentatively identified as (−)-catechin gallate (CG) (see **Figure 1**), as the MS/MS spectra for both ECG and **7** were identical (data not shown). ECG homodimer products analogous to THSN or P-2 (predicted *m/z* 881 and 851, respectively) were not observed.

Analysis of combined EGCG–EGC digesta indicated the presence of residual EGCG (**1**), residual EGC (**3**), residual ECG (**2**), GC (**7**), and each of the EGCG and EGC homodimers previously identified in the individual species digesta (**4**–**6** and **8**–**12**, respectively). Additionally, several products with *m/z* 761

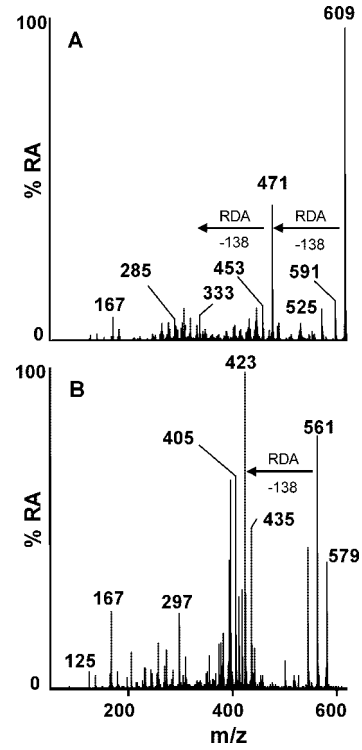
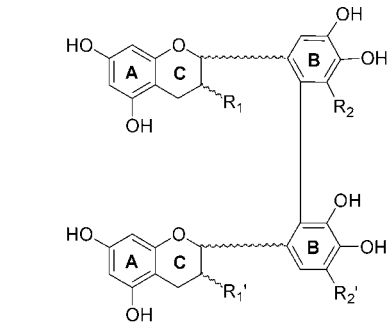


Figure 5. CAD-MS/MS spectra (fragment ion relative abundance, %RA, versus mass-to-charge ratio, *m/z*) obtained by HPLC-ESI-MS/MS analysis of selected EGC homodimers formed during *in vitro* digestion of EGC: compounds **8** (**A**) and **10** (**B**). Refer to **Table 2** for compound identities and CAD parent ion collision energies.

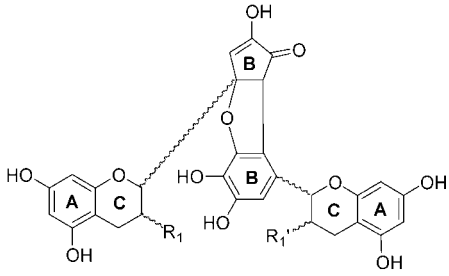
(**14**, **15**) and *m/z* 731 (**16**, **17**) were detected. MS/MS analysis of **14**–**17** suggested that they are EGCG–EGC heterodimers, analogous to the homodimers produced by degradation of each compound. Compounds **14** and **15** have similar MS/MS spectra and are C_2'–C_2' heterodimers structurally analogous to the THSNs. Major MS/MS fragmentations of **14** and **15** correspond to sequential loss of gallic acid and a RDA fragmentation. Compounds **16** and **17** have similar MS/MS spectra and are B-ring condensation–elimination heterodimers structurally analogous to P-2. Major fragmentations of **16** and **17** correspond to consecutive loss of gallic acid, H_2O, and a RDA fragmentation. A compound with this structure has previously been reported in mildly fermented tea leaves treated with phenazine and subsequently heated (*56*); compounds **14**–**17** have not been previously reported as potential digestive degradation products of tea catechins until now, although their existence has been suggested by several studies (*31, 32, 56*).

Analysis of combined EGCG–ECG digesta indicated that residual EGCG (**1**), residual ECG (**2**), CG (**13**), and each of the EGCG homodimers previously identified in individual species digesta (**4**–**6**) were present. No ECG homodimers were observed, consistent with the fact that none were observed in individual ECG digesta. However, products with *m/z* 897 were detected (**18**, **19**). Compounds **18** and **19** have similar MS/MS spectra, and MS/MS analysis suggests that they are EGCG–ECG heterodimers analogous to the THSNs. Major MS/MS fragmentations of **18** and **19** correspond to consecutive losses of gallic acid, a RDA fragmentation, and loss of another gallic acid residue. EGCG–ECG P-2 analogues were not observed (predicted *m/z* 867).

Analysis of combined EGC–ECG digesta indicated that residual EGC (**3**), residual ECG (**2**), GC (**7**), and each of the EGC homodimers previously identified in individual species



C_2'-C_2' dimers
(THSN and THSN analogs)



B-ring opening dimers
(P-2 and P-2 analogs)

Type	Compound	R_1	R_1'	R_2	R_2'
C_2'-C_2' dimers	4,5	Gallate	Gallate	OH	OH
	8,9	OH	OH	OH	OH
	14,15	Gallate	OH	OH	OH
	18,19	Gallate	Gallate	OH	H
B-ring opening dimers	6	Gallate	Gallate	-	-
	10-12	OH	OH	-	-
	16,17	Gallate/OH	OH/Gallate	-	-

Figure 6. Proposed structures of homo- and heterocatechin autoxidation dimers observed during *in vitro* digestion of EGCG, EGC, and ECG individually and in pairs. Refer to **Table 2** and available Supporting Information for compound identities and CAD-MS/MS spectra. Wavy bonds indicate ambiguous stereochemistry, as these are preliminary structures based solely on mass spectral analysis.

digesta (**8**–**12**) were present. Neither ECG homodimers nor EGC–ECG heterodimer products corresponding to THSN analogues (predicted *m*/*z* 745) nor P-2 analogues (predicted *m*/*z* 715) were observed.

To verify that these dimers were formed under digestive conditions and not during subsequent extraction and concentration, EGCG standard material was formulated at 0.1 mg/mL in saline, acidified, and extracted according to the procedure detailed previously. No dimers were detected by HPLC-ESI-MS/MS analysis, indicating that EGCG was stable during extraction and that the dimers we have reported are indeed *in vitro* digestive degradation products and not artifacts from subsequent sample preparation.

DISCUSSION

Proposed dimer structures are shown in **Figure 6**. These structures are based upon catechin autoxidation reactions analogous to those of EGCG, as formation of these previously characterized products was observed under the conditions of the present study. Further analysis (NMR, MS^n, etc.) is required to confirm the proposed structures and assign stereochemistry; however, the stereochemistry of THSNs A, D, C, and E has been previously described (*21*, *31*, *32*, *56*). The presence of two or more compounds with identical MS/MS spectra for several dimers strongly suggests that stereoisomers or other isomers are formed, consistent with EGCG dimerization to form THSNs A and D and EGC dimerization to form THSNs C and E (*21*, *31*, *56*). Additionally, epimerization of catechin monomers (*26*, *27*, *29*, *30*) prior to dimerization may contribute to the stereochemical variation in subsequently formed dimers.

Degradation of EGCG under *in vitro* digestive conditions appears to proceed similarly to that found in other *in vitro* systems. THSNs A and D (**4**, **5**) are produced when two EGCG molecules dimerize at C_2' on the B-ring, resulting in the net loss of 2 mass units, and P-2 (**6**) is produced when two EGCG molecules undergo a condensation and elimination reaction in the B-ring, resulting in the net loss of 32 mass units. Thus, the masses of the homo- and heterocatechin analogues reported here are $[M_1 + M_2 - 2]$ for the THSN analogues and $[M_1 + M_2 - 32]$ for the P-2 analogues (where M_1 and M_2 are monomer masses).

Degradation of EGC appears to proceed similarly to that of EGCG, resulting in two homodimers analogous to the THSNs (**8**, **9**) and three homodimers analogous to P-2 (**10**–**12**). A possible explanation for the presence of multiple P-2 analogues may be that significant quantities of GC (**7**) were produced by isomerization of EGC. Therefore, the three resulting P-2 analogues could be dimers of EGC–EGC, EGC–GC, and GC–GC, all with identical masses and MS/MS fragmentation patterns. Hatano et al. (*34*) have suggested that formation of P-2 analogues may be slower than that of THSN analogues, thus resulting in greater potential for inclusion of epimerized catechins formed by reactions competing with dimerization.

The behavior of ECG is consistent with previous data that demonstrated its stability to be intermediate between the labile (EGCG and EGC) and the stable (C and EC) catechins (*18*, *19*, *21*, *25*). Epimerization to CG (**13**) was observed rather than formation of ECG homodimers, which is consistent with previous studies showing that epimerization predominates at neutral pH when autoxidation is slow or prohibited (either by structural limitations of the monomer or by the reaction environment) (*29*, *30*).

When combined, the digestive behavior of EGCG and EGC remain consistent with their individual behavior. Formation of all eight EGCG and EGC homodimers (**4**–**6**, **8**–**12**) is observed, as well as formation of EGCG–EGC heterodimers: two THSN analogues (**14**, **15**) and two P-2 analogues (**16**, **17**). Similar reactions of ECGC and EGC to form THSN analogues have been reported via enzymatic oxidation of tea leaves (*31*, *32*, *56*).

Surprisingly, the degradation of ECG when combined with EGCG did not proceed as predicted by the individual behavior of ECG. In addition to the EGCG homodimers, two EGCG–ECG heterodimers analogous to THSN-type products were detected (**18**, **19**). This finding was unexpected because homodimers of this type did not appear in ECG digesta, which suggested that ECG does not undergo dimerization to the same extent as EGC and EGCG. Despite the formation of EGCG–ECG THSN analogues, EGCG–ECG heterodimer products corresponding to a P-2 analogue were not detected.

The degradation of ECG when combined with EGC proceeded as predicted: the EGC homodimers were observed, but no EGC–ECG heterodimers were detected (predicted *m*/*z* 745 and 715 for THSN and P-2 analogues, respectively). The absence of a P-2 analogue was expected because no analogous product was formed between EGCG–ECG. However, the fact that THSN analogues were not observed during digestion of ECG with EGC was surprising because this reaction was observed for EGCG–ECG digesta, and the reactivity seems to be nearly identical for EGC and EGCG.

The dimers we have observed under *in vitro* digestive conditions are catechin autoxidation products. Studies have shown that these products are produced at near-neutral or greater pH when catechins scavenge O_2 to produce superoxide ($O_2^{\bullet-}$), resulting in a semiquinone intermediate. A second catechin monomer then scavenges O_2 or $O_2^{\bullet-}$, and the two semiquinone intermediates dimerize. Overall, O_2 is reduced to H_2O_2 and catechins are autoxidized to THSN or P-2 analogues (P-2 analogue formation requires additional elimination of formaldehyde, CH_2O). This mechanism is supported by data suggesting that the use of O_2-free solutions, or addition of superoxide dismutase (SOD) to quench $O_2^{\bullet-}$, stabilizes catechins, reduces evolution of H_2O_2, and reduces the formation of dimers (*24, 29, 50, 57–63*). Additionally, a quinone trapping study further verified this mechanism by isolating quinone intermediates produced by polyphenol oxidase-catalyzed degradation of catechins (*56*). The reactivity of catechins is also explained by this model. First, H_2O_2 generation in solution is much greater for EGCG and EGC than for ECG, whereas EC produces very little (*62*). Second, the rate of H_2O_2 generation increases with increasing pH (*50, 62*). Third, EGCG and EGC scavenge $O_2^{\bullet-}$ much more effectively than EC and C, whereas ECG shows intermediate scavenging capacity (*58, 64, 65*).

On the basis of mechanism alone, catechins containing either pyrogallol (e.g., EGCG, EGC) or catechol (e.g., ECG) B-ring structures should be adequate to allow formation of the quinone intermediates necessary for formation of THSN and P-2 analogues by the mechanism described above (*29, 34, 50, 56*). It is likely that the kinetics of $O_2/O_2^{\bullet-}$ scavenging to form semiquinones and subsequent semiquinone dimerization play a role in which products are observed. Catechol and related derivatives (ECG, EC, C) are weaker scavengers of $O_2/O_2^{\bullet-}$ than pyrogallol and its derivatives (EGCG, EGC) and, therefore, would be expected to react more slowly to produce semiquinones and dimerization products (*50, 57, 58, 64*). Additionally, slow ECG semiquinone formation would exert a doubled inhibition for the formation of ECG homodimers, which require two ECG semiquinone residues. This, coupled with the slower rate of P-2 analogue formation relative to THSN formation (*34*), agrees with our observed result that neither ECG homodimers (THSN or P-2 analogues) nor ECG-containing heterodimers analogous to P-2 were observed, but that certain THSN-type heterodimers containing one ECG monomer and one EGCG monomer (likely to scavenge $O_2/O_2^{\bullet-}$ quickly and hence speed dimerization) were observed. A possible explanation for the absence of the EGC–ECG THSN-type dimers may be that EGC semiquinone formation may be too fast to allow competition between homo- and heterocatechin dimerization when ECG is present. Studies have shown that EGC may be a better scavenger of $O_2^{\bullet-}$, with a slightly higher initial scavenging rate, than EGCG (*50, 58, 65*). The present data support the conclusion that EGCG and EGC semiquinone formation kinetics allow competition for dimerization between each other but that EGC reacts too quickly to allow appreciable incorporation of ECG in heterodimer formation over the timeframe of our *in vitro* digestion experiment. It is therefore likely that all catechin species may produce THSN and P-2 analogues (both homo- and heterodimers); observation of a specific dimer may then depend upon the factors governing reaction rates (monomer concentration, reaction activation energies, temperature, catalysis) and reaction time.

The reaction mechanism also suggests that low levels of dissolved O_2 or ROS could limit the extent to which dimers are observed *in vivo*. Our model accounted for the controlled O_2 content of the gastrointestinal tract by blanketing samples with N_2. Additional research is required to determine whether the concentration of dissolved O_2 and ROS in small intestinal digesta *in vivo* is sufficient to facilitate formation of appreciable quantities of dimers during the timeframe of small intestinal transit.

Quantification of dimers was not performed in this study due to lack of appropriate authentic standards. Detection and quantification may be even more challenging in complex biological matrices (gastrointestinal contents, plasma, etc.) in which analyte concentration and interferences become problematic. HPLC-MS/MS and selected ion monitoring HPLC-MS appear to be the best methods for definitive identification of these compounds. However, this study demonstrates that ECD also provides sufficient sensitivity and selectivity to detect these products at low concentrations without significant matrix interferences, facilitating the use of HPLC-ECD for high-throughput quantitative analysis once peak identities have been confirmed by HPLC-MS/MS and/or authentic standards have been generated.

The discovery that both homo- and heterocatechin autoxidation dimers are formed under simulated digestive conditions suggests that luminal alteration of ingested catechin profiles *in vivo* warrants further consideration. This work facilitates future *in vivo* studies of catechin digestive behavior and bioavailability by providing analytical methods specifically designed to identify and characterize catechin autoxidation dimers. Additionally, by demonstrating that catechin autoxidation is not limited to EGCG, this work provides a more complete profile of the homo- and heterocatechin dimers that may be formed when various native catechins are consumed. If present *in vivo*, some biological activities currently attributed to native catechins may be due, in part, to specific dimers formed under normal digestive conditions.

ABBREVIATIONS USED

AA, glacial acetic acid; ACN, acetonitrile; BHT, butylated hydroxytoluene; C, (±)-catechin; CAD, collision-activated dissociation; CG, (−)-catechin gallate; DAD, diode array detection; ddH_2O, distilled deionized water; EC, (+)-epicatechin; ECD, electrochemical detection; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; EGCG, (−)-epigallocatechin gallate; ESI, electrospray ionization; FA, formic acid; GC, (−)-gallocatechin; KE_{ion}, ion collision kinetic energy; MeOH, methanol; NDC, nondigested control; Q-TOF, quadrupole time-of-flight; RDA, retro-Diels–Alder; RM, raw material; RP, reverse phase; ROS, reactive oxygen species; SOD, superoxide dismutase; TFA, trifluoroacetic acid; THSN, theasinensin.

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Supporting Information Available: HPLC-ECD chromatograms of all RM formulations; HPLC-ECD chromatograms of ethyl acetate extracts from all digesta; CAD-MS/MS spectra of EGCG (**1**), ECG (**2**), EGC (**3**), GC (**7**), CG (**13**), and dimer compounds (**4**–**6**, **8**–**12**, **14**–**19**). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) 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.

(2) Geleijnse, J. M.; Launer, L. J.; van der Kuip, D. A. M.; Hofman, A.; Witteman, J. C. M. Inverse association of tea and flavonoid intakes with incident myocardial infarction: the Rotterdam study. *Am. J. Clin. Nutr.* **2002**, *75*, 880–886.

(3) Crespy, V.; Williamson, G. A review of the health effects of green tea catechins in *in vivo* animal models. *J. Nutr.* **2004**, *134*, 3431S–3440S.

(4) Gross, M. Flavonoids and cardiovascular disease. *Pharm. Biol.* **2004**, *42S*, 21–35.

(5) Jeong, W. S.; Kong, A. N. T. Biological properties of monomeric and polymeric catechins: green tea catechins and procyanidins. *Pharm. Biol.* **2004**, *42S*, 84–93.

(6) Neuhouser, M. L. Flavonoids and cancer prevention: what is the evidence in humans. *Pharm. Biol.* **2004**, *42S*, 36–45.

(7) Yang, Y. C.; Lu, F. H.; Wu, J. S.; Wu, C. H.; Chang, C. J. The protective effect of habitual tea consumption on hypertension. *Arch. Intern. Med.* **2004**, *164*, 1534–1540.

(8) Scalbert, A.; Manach, C.; Morand, C.; Remesy, C. Dietary polyphenols and the prevention of diseases. *Crit. Rev. Food Sci. Nutr.* **2005**, *45*, 287–306.

(9) Williamson, G.; Manach, C. Bioavailability and bioefficacy of polyphenols in humans. II. Review of 93 intervention studies. *Am. J. Clin. Nutr.* **2005**, *81*, 243S–255S.

(10) Lakenbrink, C.; Lapczynski, S.; Maiwald, B.; Engelhardt, U. H. Flavonoids and other polyphenols in consumer brews of tea and other caffeinated beverages. *J. Agric. Food Chem.* **2000**, *48*, 2848–2852.

(11) Khokhar, S.; Magnusdottir, S. G. M. Total phenol, catechin, and caffeine contents of teas commonly consumed in the United Kingdom. *J. Agric. Food Chem.* **2002**, *50*, 565–570.

(12) Monagas, M.; Gomez-Cordoves, C.; Bartolome, B.; Laureano, O.; Da Silva, J. M. R. Monomeric, oligomeric, and polymeric flavan-3-ol composition of wines and grapes from *Vitis vinifera* L. Cv. Graciano, Tempranillo, and Cabernet Sauvignon. *J. Agric. Food Chem.* **2003**, *51*, 6475–6481.

(13) Fuleki, T.; Da Silva, J. M. R. Effects of cultivar and processing method on the contents catechins and procyanidins in grape juice. *J. Agric. Food Chem.* **2003**, *51*, 640–646.

(14) Nelson, B. C.; Sharpless, K. E. Quantification of the predominant monomeric catechins in baking chocolate standard reference material by LC/APCI-MS. *J. Agric. Food Chem.* **2003**, *51*, 531–537.

(15) Sanchez-Rabaneda, F.; Jauregui, O.; Casals, I.; Andres-Lacueva, C.; Izquierdo-Pulido, M.; Lamuela-Ravento, R. M. Liquid chromatographic/electrospray ionization tandem mass spectrometric study of the phenolic composition of cocoa (*Theobroma cacao*). *J. Mass Spectrom.* **2003**, *38*, 35–42.

(16) Friedman, M.; Kim, S. Y.; Lee, S. J.; Han, G. P.; Han, J. S.; Lee, K. R.; Kozukue, N. Distribution of catechins, theaflavins, caffeine, and theobromine in 77 teas consumed in the United States. *J. Food Sci.* **2005**, *70*, C550–C559.

(17) Guendez, R.; Kallithraka, S.; Makris, D. P.; Kefalas, P. Determination of low molecular weight polyphenolic constituents in grape (*Vitis vinifera* sp.) seed extracts: correlation with antiradical activity. *Food Chem.* **2005**, *89*, 1–9.

(18) Zhu, Q. Y.; Zhang, A.; 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.; Chung, H. Y. Stabilizing effect of ascorbic acid on green tea catechins. *J. Agric. Food Chem.* **1998**, *46*, 2512–2516.

(20) Tomita, I.; Sano, M.; Sasaki, K.; Miyase, T. Tea catechin (EGCG) and its metabolites as bioantioxidants. In *Functional Foods for Disease Prevention I: Fruits, Vegetables, and Teas*; Shibamoto, T., Terao, J., Osawa, T., Eds.; ACS Symposium Series 701; American Chemical Society: Washington, DC, 1998; pp 209–216.

(21) 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.

(22) Chen, Z. Y.; Zhu, Q. Y.; Tsang, D.; Huang, Y. Degradation of green tea catechins in tea drinks. *J. Agric. Food Chem.* **2001**, *49*, 477–482.

(23) Record, I. R.; Lane, J. M. Simulated intestinal digestion of green and black teas. *Food Chem.* **2001**, *73*, 481–486.

(24) Hong, J.; Lu, H.; Meng, X.; Ryu, J. H.; Hara, Y.; Yang, C. S. 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.

(25) Su, Y. L.; Leung, Y. K.; Huang, Y.; Chen, Z. Y. Stability of tea theaflavins and catechins. *Food Chem.* **2003**, *83*, 189–195.

(26) Suzuki, M.; Sano, M.; Yoshida, R.; Miyase, M. T.; Maeda-Yamamoto, M. Epimerization of tea catechins and o-methylated derivatives of (−)-epigallocatechin-3-*O*-gallate: relationship between epimerization and chemical structure. *J. Agric. Food Chem.* **2003**, *51*, 510–514.

(27) Hatano, T.; Hori, M.; Kusuda, M.; Ohyabu, T.; Ito, H.; Yoshida, T. Characterization of the oxidation products of (−)-epigallocatechin gallate, a bioactive tea polyphenol, on incubation in neutral solution. *Heterocycles* **2004**, *63*, 1547–1554.

(28) Green, R. J.; Murphy, A. S.; Watkins, B. A.; Ferruzzi, M. G. Common tea formulations modulate *in vitro* digestive recovery of green tea catechins. *Mol. Nutr. Food Res.* **2007**, *51*, 1152–1162.

(29) Hou, Z.; Sang, S.; You, H.; Lee, M. J.; Hong, J.; Chin, K. V.; Yang, C. S. Mechanism of action of (−)-epigallocatechin-3-gallate: auto-oxidation–dependent inactivation of epidermal growth factor receptor and direct effects on growth inhibition in human esophageal cancer KYSE150 cells. *Cancer Res.* **2005**, *65*, 8049–8056.

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

(31) Hashimoto, F.; Nonaka, G.; Nishioka, I. Tannins and related compounds. LXIX. Isolation and structure elucidation of B,B′-linked bisflavonoids, theasinensins D-G and oolongtheanin from oolong tea. *Chem. Pharm. Bull.* **1988**, *36*, 1676–1684.

(32) Hashimoto, F.; Nonaka, G.; Nishioka, I. Tannins and related compounds. CXIV. Structures of novel fermentation products, theogallinin, theaflavonin, and desgalloyl theaflavonin from black tea, and changes of leaf tea polyphenols during fermentation. *Chem. Pharm. Bull.* **1992**, *40*, 1383–1389.

(33) Xu, J. Z.; Yeung, S. Y. V.; Chang, Q.; Huang, Y.; Chen, Z. Y. Comparison of antioxidant activity and bioavailability of tea epicatechins with their epimers. *Br. J. Nutr.* **2004**, *91*, 873–881.

(34) Hatano, T.; Ohyabu, T.; Yoshida, T. The structural variation in the incubation products of (−)-epigallocatechin gallate in neutral solution suggest its breakdown pathways. *Heterocyles* **2005**, *65*, 303–310.

(35) Parks, D. A. Oxygen radicals: mediators of gastrointestinal pathophysiology. *Gut* **1989**, *30*, 293–298.

(36) Aw, T. Y. Luminal peroxides in intestinal thiol–disulfide balance and cell turnover. *Comp. Biochem. Physiol.* **1997**, *118B*, 479–485.

(37) Prabhu, R.; Balasubramanian, K. A. Effect of oxidants on small intestinal brush border membranes and colonic apical membranes: a comparative study. *Comp. Biochem. Physiol. Part C* **2004**, *134*, 329–339.

(38) Donovan, J. L.; Bell, J. R.; Kasim-Karakas, S.; German, J. B.; Walzem, R. L.; Hansen, R. J.; Waterhouse, A. L. Catechin is present as metabolites in human plasma after consumption of red wine. *J. Nutr.* **1999**, *129*, 1662–1668.

(39) Kuhnle, G.; Spencer, J. P. E.; Schroeter, H.; Shenoy, B.; Debnam, E. S.; Srai, S. K. S.; Rice-Evans, C.; Hahn, U. Epicatechin and catechin are o-methylated and glucuronidated in the small intestine. *Biochem. Biophys. Res. Commun.* **2000**, *277*, 507–512.
(40) Kohri, T.; Matsumoto, N.; Yamakawa, M.; Suzuki, M.; Nanjo, F.; Hara, Y.; Oku, N. Metabolic fate of (−)-epigallocatechin gallate in rats after oral administration. *J. Agric. Food Chem.* **2001**, *49*, 4102–4112.
(41) Donovan, J. L.; Crespy, V.; Manach, C.; Morand, C.; Besson, C.; Scalbert, A.; Remesy, C. Catechin is metabolized by both the small intestine and liver of rats. *J. Nutr.* **2001**, *131*, 1753–1757.
(42) Lee, M. J.; Maliakal, P.; Chen, L.; Meng, X.; Bondoc, F. Y.; Prabhu, S.; Lambert, G.; Mohr, S.; Yang, C. S. Pharmacokinetics of tea catechins after ingestion of green tea and (−)-epigallocatechin-3-gallate by humans: formation of different metabolites and individual variability. *Cancer Epidemiol. Biomarkers Prev.* **2002**, *11*, 1025–1032.
(43) Meng, X.; Sang, S.; Zhu, N.; Lu, H.; Sheng, S.; Lee, M. J.; Ho, C. T.; Yang, C. S. Identification and characterization of methylated and ring-fission metabolites of tea catechins formed in humans, mice, and rats. *Chem. Res. Toxicol.* **2002**, *15*, 1042–1050.
(44) Kohri, T.; Suzuki, M.; Nanjo, F. Identification of metabolites of (−)-epicatechin gallate and their metabolic fate in the rat. *J. Agric. Food Chem.* **2003**, *51*, 5561–5566.
(45) Lambert, J. D.; Lee, M. J.; Lu, H.; Meng, X.; Hong, J. J. J.; Seril, D. N.; Sturgill, M. G.; Yang, C.S. Epigallocatechin-3-gallate is absorbed but extensively glucuronidated following oral administration to mice. *J. Nutr.* **2003**, *133*, 4172–4177.
(46) Nakamura, Y.; Tonogai, Y. Metabolism of grape seed polyphenol in the rat. *J. Agric. Food Chem.* **2003**, *51*, 7215–7225.
(47) Tsang, C.; Auger, C.; Mullen, W.; Bornet, A.; Rouanet, J. M.; Croziert, A.; Teissedre, P. L. The absorption, metabolism and excretion of flavan-3-ols and procyanidins following the ingestion of a grape seed extract by rats. *Br. J. Nutr.* **2005**, *94*, 170–181.
(48) Garrett, D. A.; Failla, M. L.; Sarama, R. J. Development of an *in vitro* digestion method to assess carotenoid bioavailability from meals. *J. Agric. Food Chem.* **1999**, *47*, 4301–4309.
(49) 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.
(50) Mochizuki, M.; Yamazaki, S. I.; Kano, K.; Ikeda, T. Kinetic analysis and mechanistic aspects of autoxidation of catechins. *Biochim. Biophys. Acta, Gen. Subj.* **2002**, *1569*, 35–44.
(51) Miketova, P.; Schram, K. H.; Whitney, J.; Li, M.; Huang, R.; Kerns, E.; Valcic, S.; Timmermann, B. N.; Rourick, R.; Klohr, S. Tandem mass spectrometry studies of green tea catechins. Identification of three minor components in the polyphenolic extract of green tea. *J. Mass Spectrom.* **2000**, *35*, 860–869.
(52) Zeeb, D. J.; Nelson, B. C.; Albert, K.; Dalluge, J. J. Separation and identification of twelve catechins in tea using liquid chromatography/atmospheric pressure chemical ionization–mass spectrometry. *Anal. Chem.* **2000**, *72*, 5020–5026.
(53) Mateus, N.; Silva, A. M. S.; Santos-Buelga, C.; Rivas-Gonzalo, J. C.; de Freitas, V. Identification of anthocyanin–flavanol pigments in red wines by NMR and mass spectrometry. *J. Agric. Food Chem.* **2002**, *50*, 2110–2116.
(54) Remy-Tanneau, S.; Le Guerneve, C.; Meudec, E.; Cheynier, V. Characterization of a colorless anthocyanin–flavan-3-ol dimer containing both carbon–carbon and ether interflavanoid linkages by NMR and mass spectrometry. *J. Agric. Food Chem.* **2003**, *51*, 3592–3597.
(55) Sun, W.; Miller, J. M. Tandem mass spectrometry of the B-type procyanidins in wine and B-type dehydrodicatechins in an autoxidation mixture of (+)-catechin and (−)-epicatechin. *J. Mass Spectrom.* 2003*38*, 438–446.
(56) Tanaka, T.; Mine, C.; Watarumi, S.; Fujioka, T.; Mihashi, K.; Zhang, Y. J.; Kouno, I. Accumulation of epigallocatechin quinone dimers during tea fermentation and formation of theasinensins. *J. Nat. Prod.* **2002**, *65*, 1582–1587.
(57) Miura, Y. H.; Tomita, I.; Watanabe, T.; Hirayama, T.; Fukui, S. Active oxygens generation by flavonoids. *Biol. Pharm. Bull.* **1998**, *21*, 93–96.
(58) Unno, T.; Sugimoto, A.; Kakuda, T. Scavenging effect of tea catechins and their epimers on superoxide anion radicals generated by a hypoxanthine and xanthine oxidase system. *J. Sci. Food Agric.* **2000**, *80*, 601–606.
(59) Arakawa, H.; Kanemitsu, M.; Tajima, N.; Maeda, M. Chemiluminescence assay for catechin based on generation of hydrogen peroxide in basic solution. *Anal. Chim. Acta* **2002**, *472*, 75–82.
(60) Dashwood, W. M.; Orner, G. A.; Dashwood, R. H. Inhibition of β-catenin/Tcf activity by white tea, green tea, and epigallocatechin-3-gallate (EGCG): minor contribution of H_2O_2 at physiologically relevant EGCG concentrations. *Biochem. Biophys. Res. Commun.* **2002**, *296*, 584–588.
(61) Nakagawa, T.; Yokozawa, T. Direct scavenging of nitric oxide and superoxide by green tea. *Food Chem. Toxicol.* **2002**, *40*, 1745–1750.
(62) Nakagawa, H.; Hasumi, K.; Woo, J. T.; Nagai, K.; Wachi, M. Generation of hydrogen peroxide primarily contributes to the induction of Fe(II)-dependent apoptosis in Jurkat cells by (−)-epigallocatechin gallate. *Carcinogenesis* **2004**, *25*, 1567–1574.
(63) Chan, M. M.; Soprano, K. J.; Weinstein, K.; Fong, D. Epigallocatechin-3-gallate delivers hydrogen peroxide to induce death of ovarian cancer cells and enhances their cisplatin susceptibility. *J. Cell. Physiol.* **2006**, *207*, 389–396.
(64) Unno, T.; Yayabe, F.; Hayakawa, T.; Tsuge, H. Electron spin resonance spectroscopic evaluation of scavenging activity of tea catechins on superoxide radicals generated by a phenazine methosulfate and NADH system. *Food Chem.* **2002**, *76*, 259–265.
(65) Toyo'oka, T.; Kashiwazaki, T.; Kato, M. On-line screening methods for antioxidants scavenging superoxide anion radical and hydrogen peroxide by liquid chromatography with indirect chemiluminescence detection. *Talanta* **2003**, *60*, 467–475.

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