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Determination of Theaflavins Including Methylated Theaflavins in Black Tea Leaves by Solid-Phase Extraction and HPLC Analysis

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A quantitative method for four theaflavins and two methylated theaflavin derivatives in black tea leaves was developed by solid-phase extraction and a high-performance liquid chromatographic method with photodiode array detection. The theaflavins in black tea leaves were extracted three times with 40 vol 50% aqueous ethanol (mg dry tea powder/mL) containing 2% ascorbic acid. The ethanol extracts were diluted 4-fold with distilled water. All diluted extracts were directly applied to the solid-phase C_{18} cartridge column without concentration. The fraction of theaflavins was obtained by 40% ethanol extraction after rinsing with water followed with 15% ethanol extraction. An aliquot of theaflavins after concentration was injected onto an ODS C_{18} reversed-phase column, and four theaflavins and two methylated theaflavins were sufficiently separated by a linear gradient system using distilled water and acetonitrile with 0.5% acetic acid. This analytical method is sensitive for the determination of a small amount of methylated theaflavins, since various interfering substances produced during the fermentation process were eliminated in advance by solid-phase extraction. Using this analytical method, we also demonstrated that methylated theaflavins were easily produced during the manufacture of black tea.

KEYWORDS: Black tea; methylated theaflavin; solid-phase extraction; HPLC analysis

INTRODUCTION

(−)-Epigallocatechin gallate (EGCG), which is the major catechin in tea leaves (*Camellia sinensis* L.), has been reported to exert various biological effects such as antioxidative (*1*, *2*), antimutagenic/anticarcinogenic (*3*, *4*) and antibacterial (*5*). We have reported that 3-*O*- or 4-*O*-methylated derivatives of the galloyl moiety in EGCG exhibit strong anti-allergic properties (*6*−*8*) and antioxidant activities (*9*). One of the methylated catechins, eipgallocatehin-3-*O*-(3-*O*-methyl)gallate (EGCG3″Me), is concentrated in limited tea cultivars such as "Benihomare" and "Benifuuki", which are classified as "Assam" hybrids, but the concentrations are much lower than that of EGCG (*10*). Therefore, EGCG3″Me was also detected in fresh leaves of "Assam" cultivars used for the manufacture of black tea. Tea catechins are degraded markedly during the black tea manufacturing process (fermentation), and theaflavins (TFs) and high molecular weight products such as thearubigins are generated by fermentation. The biological activities of TFs, which are dimerization products of catechins, have been reported concerning antioxidant (*11*−*14*), antibacterial (*15*), antimutagenic (*16*), and hypocholesterolemic functions (*17*). The existence of methylated TFs in tea leaves and their biological activity remain unknown because an appropriate analytical method for methylated TFs has not been established. Although analytical methods for TFs using high-performance liquid chromatography (HPLC) techniques combined with UV detection (*18*) or electrospray mass spectrometry (*19*, *20*) have been reported, many of these methods are to determine TFs in an aqueous tea infusion. An amount of TFs and various oxidized breakdown products of catechins are still present in the used tea leaves after extraction with boiling water several times. The presence of oxidized products interferes with the determination of a small amount of methylated TFs in black tea leaves.

In this study, we report a specific determination method for TFs and methylated TFs in black tea leaves by solid-phase extraction, followed by HPLC analysis. We also investigated the conversion of EGCG or EGCG3″Me to the corresponding TFs or methylated TFs by using green tea, oolong tea, and black tea manufactured from the same lot of leaves.

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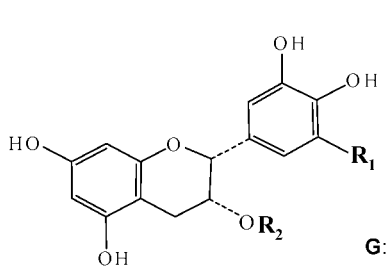
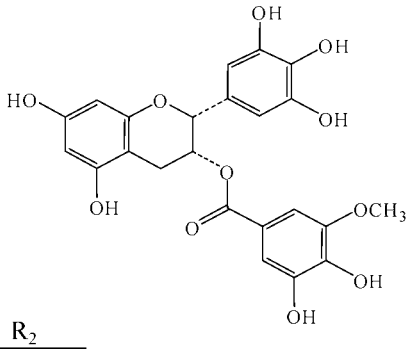
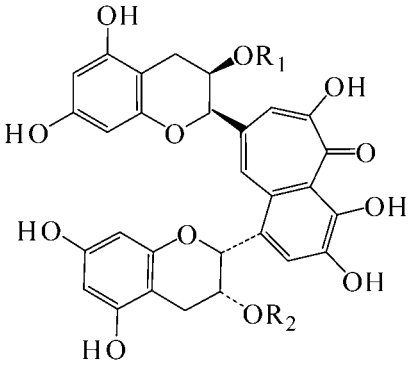
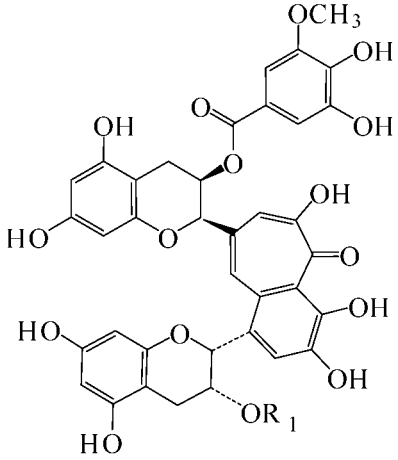




EGCG3″Me

	R_1	R_2
Epigallocatechin-3-*O*-gallate (EGCG)	OH	G
Epigcatechin-3-*O*- gallate (ECG)	H	G
Epigallocatechin (EGC)	OH	H
Epicatechin (EC)	H	H





	R_1	R_2
Theaflavin (TF)	H	H
Theaflavin 3-gallate (TF3G)	G	H
Theaflavin 3'-gallate (TF3'G)	H	G
Theafiavin3,3'-digallate (TF3,3'diG)	G	G

	R_1
Theaflavin 3-*O*-(3-*O*-methyl)gallate (TF3MeG)	H
Theaflavin 3-*O*-(3-*O*-methly)gallate,3'-gallate (TF3MediG)	G

Figure 1. Chemical structures of theaflavins and methylated theaflavins.

MATERIALS AND METHODS

Reagents and Samples. Commercial black tea leaves (Assam, Ceylon, Darjeeling, and Earl Grey), manufactured by Lipton or Twinings, were purchased from markets in Japan. Green tea, oolong tea, and black tea manufactured from the same lot leaves of "Benihomare", which contains a relatively high level of EGCG3″Me, were obtained from the National Research Institute of Vegetable and Tea Science (Kanaya, Shizuoka, Japan). Authentic samples of theaflavin (TF), theaflavin 3-*O*-gallate (TF3G), theaflavin 3′-*O*-gallate (TF3′G), and theaflavin 3,3′-di-*O*-gallate (TF3,3′diG) at 94∼99% purity were provided by Lipton Unilever Japan (Tokyo, Japan).

EGCG3″Me, EGCG, and the other tea catechins were prepared according to the method previously reported (*6*). Two methylated TFs, theaflavin-3-*O*-(3-*O*-methyl)gallate (TF3MeG) and theaflavin-3-*O*-(3-*O*-methyl)gallate-3′gallate (TF3MediG), were synthesized by the reaction of EGCG3″Me and catechol-type catechins such as EC and ECG in the presence of cocklebur polyphenol oxidase according to the method described by Tanaka et al. (*21*). Their purities (>91%) were confirmed by ^{1}H NMR. The two methylated TFs were used as reference standard for calibration and identification purposes. The chemical structures of catechins and TFs used in this study are shown in **Figure 1**. Ethanol, acetonitrile, and acetic acid for extraction or HPLC mobile phase were purchased from Wako Pure Chemical Co., Ltd. (Osaka, Japan). Water was purified in a Milli-Q system (Millipore, Bedford, MA). Other chemicals were of reagent grade.

Apparatus. A C_{18} Bond Elut solid-phase cartridge column (Varian Inc. Co., Harbor City, CA) set in vacuum manifold apparatus (GL Science, Tokyo, Japan) and high-speed shaker (CM-1000, Eyela, Tokyo, Japan) were used for TFs extraction and fractionation. The solid-phase cartridge column was previously rinsed with 4 mL of ethanol, then 5 mL of water for activation just before use. TFs were analyzed using a Shimazu LC−MS system (Shimazu, Kyoto, Japan) consisting of two pumps (LC-20AD), an automated sample injector (SIL-20AC), a column oven (Shimadzu, CTO-20AC), a system controller (CBM-20A), a photodiode array detection (SPD-M20A), and mass detection (MS2010EV) with a computer (LC−MS solution software). The HPLC separation column was a Shimazu ODS 80Ts column (2.0 × 250 mm i.d., Shimazu, Kyoto, Japan).

Analytical HPLC Method. The optimal mobile phase for the separation of four TFs (TF, TF3G, TF3′G, and TF3,3′diG) and two methylated TFs (TF3MeG and TF3MediG) was a gradient system consisting of solvent A (15% acetonitrile/water containing 0.5% acetic acid) and solvent B (80% acetonitrile/water containing 0.5% acetic acid). These TFs were eluted from the column at 25 °C with a linear

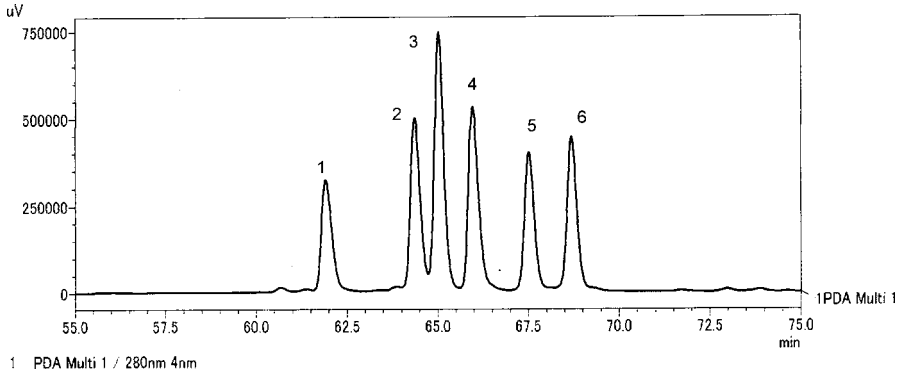


Figure 2. HPLC chromatogram of authentic theaflavins and methylated theaflavins. A mixture of authentic theaflavins (0.5 nmol μL^{-1}) was used for HPLC-UV analysis. Peaks are 1, TF (theaflavin); 2, TF3G; 3, TF3,3′diG; 4, TF3′G; 5, TF3MeG; 6, TF3MediG.

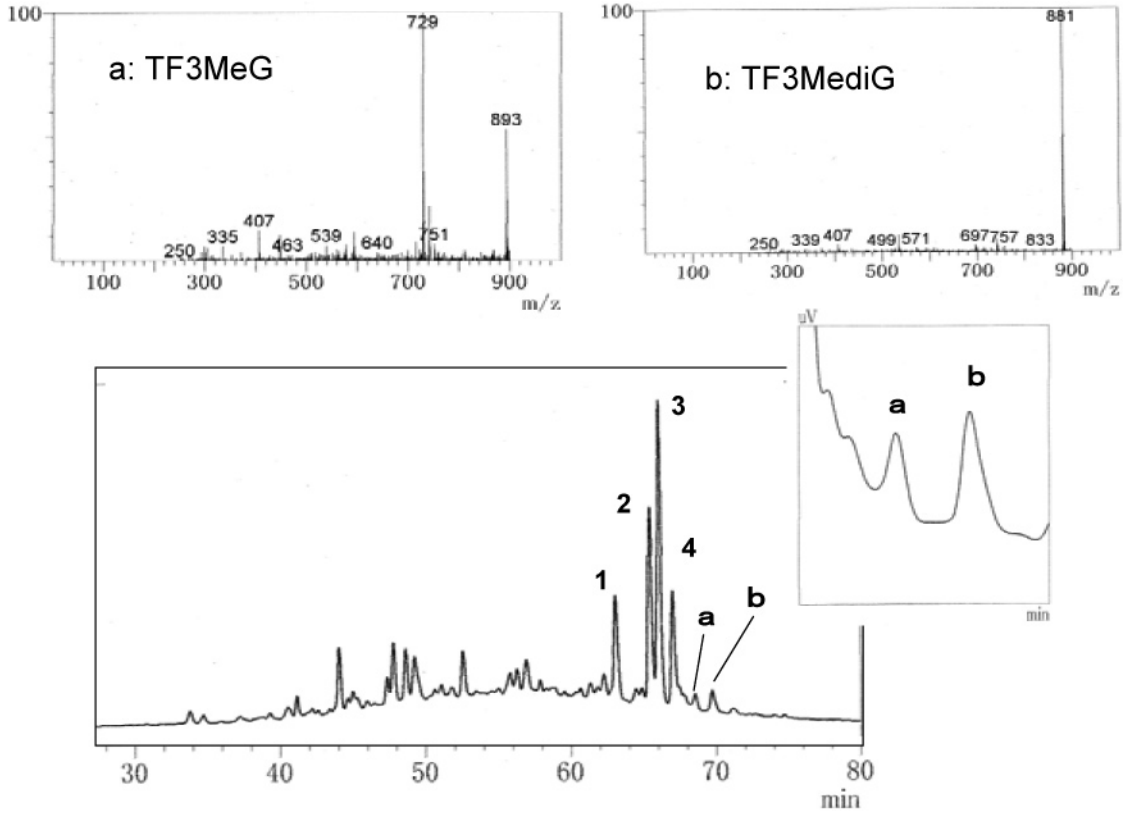


Figure 3. Chromatogram of Ceylon black tea extracts and mass spectra of peak a and peak b in ESI-negative mode. Peaks are 1, TF; 2, TF3G; 3, TF3,3′diG; 4, TF3′G; a, TF3MeG; b, TF3MediG.

gradient of solvent B starting from 0% to 60% in 80 min at a flow rate of 0.2 mL/min. Each peak of TFs and methylated TFs was monitored by a photodiode array UV detector at 280 nm. The TF peak was confirmed by the retention times of authentic samples and mass spectrometric analysis with an electrospray interface (ESI). The molecular weight of TFs was analyzed in negative and positive ionization modes.

LC−MS conditions are as follows: ODS80Ts column, 2.0 × 250 mm Shimadzu; flow rate, 0.2 mL/min; injection volume, 10 μL; column temperature, 25 °C; probe voltage, +4.5 V (ESI-negative mode); nebulizing gas flow, 1.5 L/min; drying gas pressure, 0.1 MPa; CDL temperature, 250 °C; CDL voltage, −1.5 V; Q-array voltage, DC + 5.0 V, RF + 150 V; SIM, 563 (TF), 715 (TF3G, TF3′G), 729 (TF3MeG), 867 (TF3,3′diG), 881 (TF3MediG) (negative).

The analysis of EGCG3′′Me and major catechins (EGCG, EGC, EC, and ECG) was carried out by the method of HPLC with electrochemical detection as previously reported (*11*).

Statistical analysis was performed by the Student's *t* test, with significance at $P < 0.05$.

RESULTS AND DISCUSSION

Preparation of Tea Extracts for HPLC Analysis. Black tea leaves were pulverized into powder for good extraction. TFs were extracted from 50 mg of tea leaf powder three times with 2 mL of 50% ethanol solution containing 2% ascorbic acid (w/v) at room temperature by shaking at 1500 rpm for 20 min. Almost all theaflavins in tea powder were extracted under the extraction conditions shown in **Table 1**. The three extractions with 50% aqueous ethanol showed good efficiency, ranging from 92% to 105%. The three extracts were combined and centrifuged at 5000 rpm at 4 °C for 10 min. The supernatant was diluted 4-fold with distilled water, and then, the diluted extracts were applied to the solid-phase C_{18} cartridge column set in a vacuum manifold apparatus. The column was rinsed with 6 mL of water, followed with 6 mL of 15% ethanol solution. These two rinses eliminated polar compounds and ascorbic acid added as an antioxidant to the ethanol extraction. Almost all theaflavins were

Table 1. Extraction of Theaflavins and Methylated Theaflavins from Black Tea Leaves[a]

	theaflavins (mg/g dry tea leaves)					
extraction	TF	TF3G	TF3′G	TF3,3′diG	TF3MeG	TF3MediG
1st	2.17	3.04	3.80	1.74	0.19	0.54
2nd	0.40	0.58	0.69	0.36	0.03	0.09
3rd	0.05	0.07	0.08	0.05	0.01	0.01
4th	0.002	0.003	0.00	0.00	0.003	0.00

[a] Theaflavins in black tea powder (50 mg) were extracted with 2 mL of 50% aqueous ethanol containing 2% ascorbic acid at room temperature for 20 min using a shaker at 1500 rpm.

Table 2. Separation of Theaflavins from Solid-Phase C_{18} Cartridge Column by Ethanol Eluate[a]

	separation (mg/g dry tea leaves)			
theaflavins	H_2O	15% EtOH	40% EtOH	99.5% EtOH
TF	nd	0.03	2.06	nd
TF3G	nd	0.02	3.01	nd
TF3,3′diG	nd	0.02	3.89	0.01
TF3′G	nd	0.01	1.79	nd
TF3MeG	nd	0.01	0.20	nd
TF3MediG	nd	0.01	0.60	nd

[a] nd, not detected.

eluted with 6 mL of 40% ethanol solution (**Table 2**). The eluate was evaporated to dryness under vacuum. An aliquot (10 μL) of the residue, redissolved with 500 μL of 50% ethanol, was used for HPLC analysis.

HPLC Separation and Detection of TFs and Methylated TFs. The authentic theaflavin mixtures consisting of four TFs (TF, TF3G, TF3′G, and TF3,3′diG) and two methylated TFs (TF3MeG and TF3MediG) were sufficiently separated under the conditions described in the analytical HPLC method section (**Figure 2**). The detection limits (S/N = 3) of these theaflavins were 20−25 pmol μL^{-1}. **Figure 3** shows typical HPLC chromatograms of extracts from Ceylon black tea. The peak assignments of the two methylated TFs were based on retention times relative to authentic samples and were confirmed by using the sensitive technique of selective ion monitoring of mass spectrometry. The results suggested that TF3MeG and TF3MediG were also produced by the enzymatic oxidative reaction between EGCG3″Me and catechol-type catechins (EC or ECG) during the fermentation process. Linear calibration curves for standard TFs and methylated TFs were obtained in the 2.5−10 nmol range for the determination of theaflavins in tea leaves (**Figure 4**). This method could determine a small amount of TFs and methylated TFs present in tea leaves by ethanol extraction and solid-phase extraction treatment.

Theaflavin Concentrations of Black Tea Leaves. Theaflavins in various black tea leaves obtained from markets were examined by the proposed HPLC method (**Table 3**).

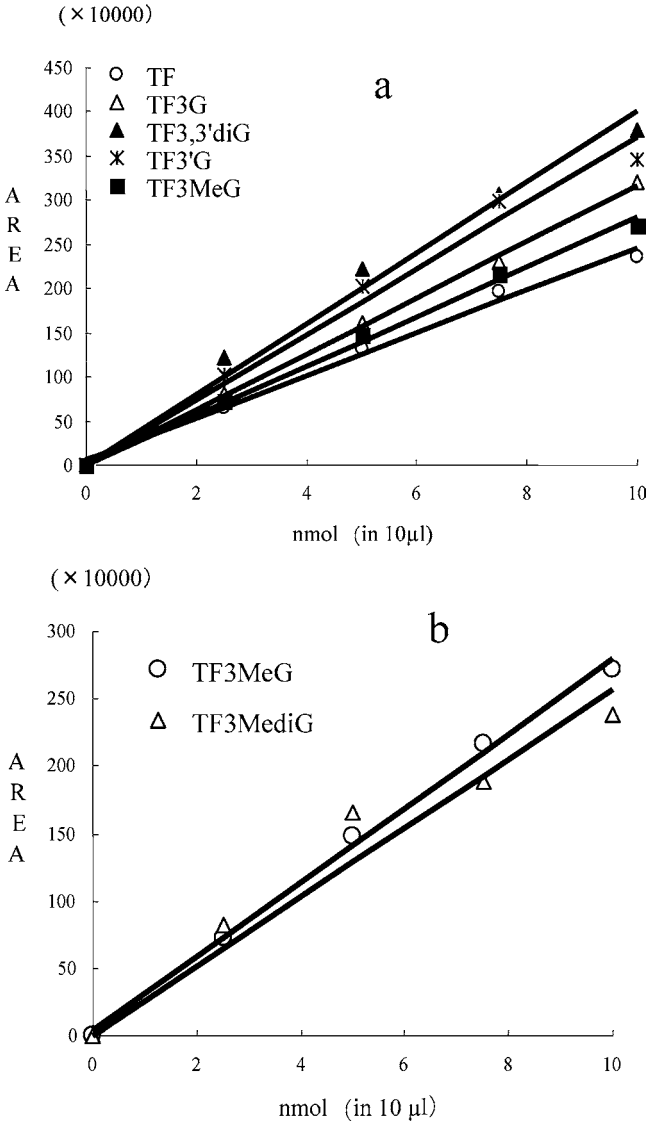


Figure 4. Calibration curves of standard TFs and methylated TFs Ten microliters of standard TFs (**a**) and methylated TFs (**b**) was injected into HPLC with photdiode array detection.

The levels of all TFs in Darjeeling teas were lower, whereas TF3,3′diG in Assam teas was much higher than in the other tea cultivars used. These results suggest that in Darjeeling tea the degree of oxidation in the fermentation process is lower or the contents of catechins is lower, and in Assam green tea leaves, the contents of EGCG and ECG, which are materials of TF3,3′diG, are higher than those of other tea cultivars (*22*).

Methylated TFs were recognized in all black tea leaves used. The levels in Ceylon and Assam teas were higher than those of Darjeeling and Earl Grey tea. The contents of TF3MeG and TF3MediG were 130∼ 200 μg/g dry tea leaf in Ceylon and Assam black tea and 440∼540 μg/g in Ceylon tea, respectively.

Table 3. Theaflavin Concentrations in Various Black Tea Leaves Obtained from Markets

	(mg/g dry tea leaves)					
	TF	TF3G	TF3,3′diG	TF3′G	TF3MeG	TF3MediG
Assam	1.90 ± 0.30^{a}	3.59 ± 0.40^{a}	11.31 ± 0.39^{a}	2.27 ± 0.16^{a}	0.15 ± 0.02^{a}	0.37 ± 0.03^{a}
Earl Grey	$1.86 \pm 0.70^{a,b}$	2.78 ± 0.84^{a}	3.79 ± 0.43^{b}	1.25 ± 0.48^{b}	0.17 ± 0.09^{a}	$0.38 \pm 0.26^{a,b}$
Ceylon	2.26 ± 0.20^{a}	2.54 ± 1.13^{a}	$3.02 \pm 1.70^{b,c}$	1.43 ± 0.26^{b}	0.16 ± 0.06^{a}	$0.35 \pm 0.20^{a,b}$
Darjeelin	1.24 ± 0.05^{b}	0.97 ± 0.07^{b}	1.54 ± 0.17^{c}	0.63 ± 0.05^{c}	0.05 ± 0.01^{b}	0.13 ± 0.01^{b}

[a−c] Values are mean ± SD (n = 3–5 samples). Values with the same superscript notation are not significantly different ($P > 0.05$).

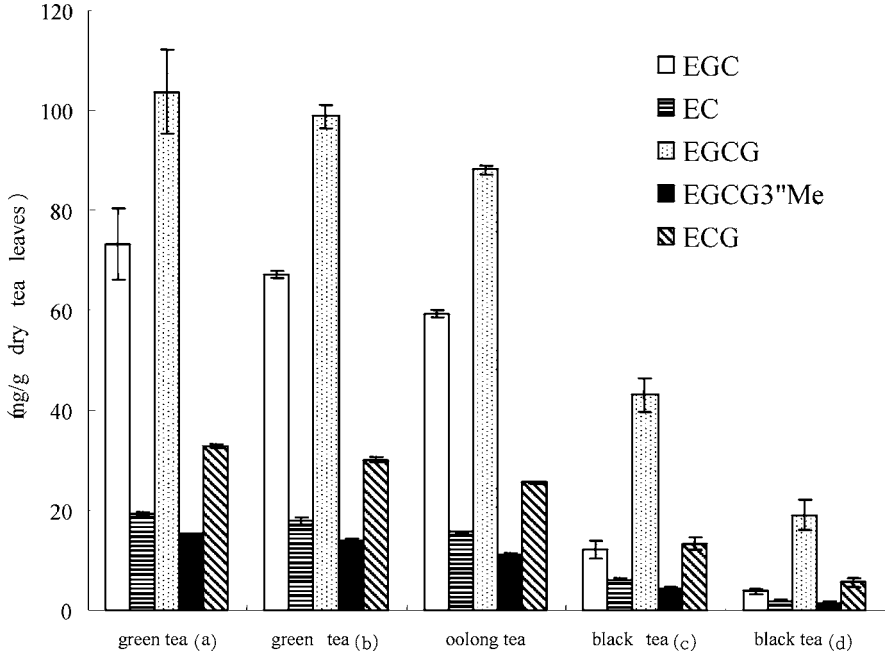


Figure 5. Quantitative changes of catechins during the fermentation process. Tea leaves used were prepared from the same lot of fresh leaves of "Benihomare" cultivars. (**a**) The tea ("Sen-cha") was prepared by steaming treatment which inactivates polyphenol oxidase in first step of green tea production. (**b**) The tea ("Kamairi-cha", panning green tea) was prepared by parching treatment instead of steaming. (**c** and **d**) These teas were prepared by the fermentation for 20 min (**c**) and 60 min (**d**) during the process of black tea production, respectively.

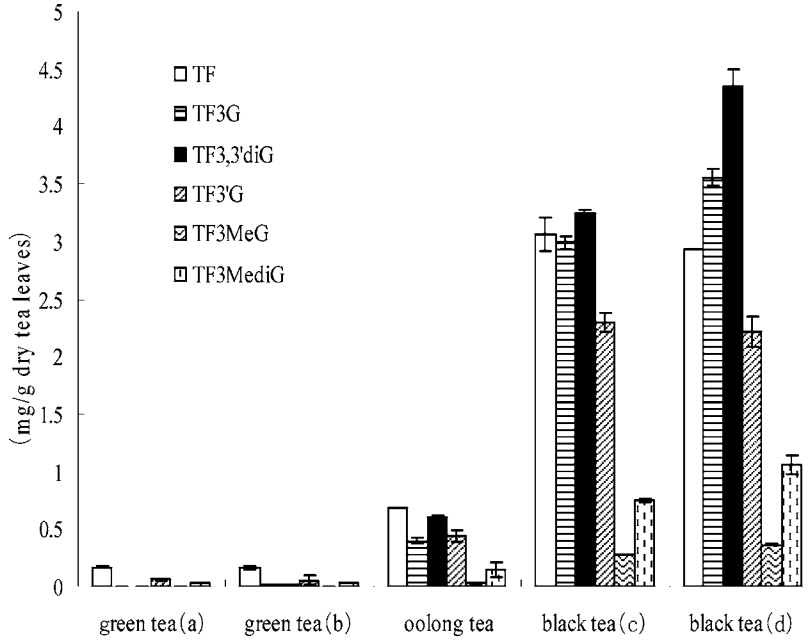


Figure 6. Quantitative changes of theaflavins during the fermentation process. Tea leaves used were prepared from the same lot of fresh leaves of "Benihomare" cultivars. (**a**) The tea ("Sen-cha") was prepared by steaming treatment which inactivates polyphenol oxidase in first step of green tea production. (**b**) The tea ("Kamairi-cha", panning green tea) was prepared by parching treatment instead of steaming. (**c** and **d**) These tea were prepared by the fermentation for 20 min (**c**) and 60 min (**d**) during the process of black tea production, respectively.

Quantitative Changes of Catechins and Theaflavins during the Fermentation Process. TF3G, TF3,3′diG, TF, and TF3′G are produced by oxidative reaction between EC and EGCG, between ECG and EGCG, between EC and EGC, and between ECG and EGC, respectively (*23*, *24*). In addition, this study suggests that TF3MeG and TF3MediG are also produced from the reaction between EGCG3″Me and catechins of the catechol type on the B ring. The content of catechins and TFs in tea leaves varies considerably among tea species and cultivation areas; therefore, tea leaves (green tea, oolong tea, and black tea) from the same lot of leaves of "Benihomare" cultivars were used in this examination to assess quantitative changes of tea catechins during the fermentation process.

All TFs, including methylated TFs in tea leaves, increased with the decrease of catechins following the development of fermentation (**Figures 5** and **6**). A marked change in catechin and theaflavin levels was observed in the manufacturing process of oolong tea to black tea. The quantity of TF3MediG produced from EGCG3″Me and ECG was higher than that of TF3MeG from EGCG3″Me and EC. The production ratio of TF3MediG to TF3,3′diG in black tea leaves was about 25%, but the ratio of EGCG3″Me to EGCG in green tea was about 14%. This

result suggests that EGCG3″Me is liable to cause dimerization with ECG compared to EGCG.

The proposed extraction and HPLC analysis method is useful for the small amount of theaflavins and methylated theaflavins present in black tea leaves. This extraction method with ethanol and a solid-phase column could also be applicable to isolate theaflavins from black tea leaves.

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LITERATURE CITED

(1) Nanjo, F.; Mori, M.; Goto, K.; Hara, Y. Radical scavenging activity of tea catechins and their related compounds. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1621−1623.

(2) Rice-Evans, C. A.; Millar, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(3) Tachibana, H.; Koga, K.; Fujimura, Y.; Yamada, K. A receptor for green tea polyphenol EGCG. *Nat. Struct. Mol. Biol.* **2004**, *11*, 380−381.

(4) Shankel, D. M.; Pillai, S. P.; Telikepalli, H.; Menon, S. R.; Pillai, C. A.; Mitscher, L. A. Role of antimutagens/anticarcinogens in cancer prevention. *Biofactors* **2000**, *12*, 113−121.

(5) Yam, T. S.; Shanh, S.; Hamilton-Miller, J. M. Microbiological activity of whole and fractionated crude extracts of tea (*Camellia sinensis*), and of tea components. *FEMS Microbiol. Lett.* **1997**, *152*, 169−174.

(6) Sano, M.; Suzuki, M.; Miyase, T.; Yoshino, K.; Yamamoto, M. M. Novel antiallergic catechin derivatives isolated from oolong tea. *J. Agric. Food Chem.* **1999**, *47*, 1906−1910.

(7) Suzuki, M.; Yoshino, K.: Yamamoto, M. M.; Miyase, T.; Sano, M. Inhibitory effects of tea catechins and *O*-methylated derivatives of (−)-epigallocatechin-3-*O*-gallate on mouse type IV allergy. *J. Agric. Food Chem.* **2000**, *48*, 5649−5653.

(8) Maeda-Yamamoto, M.; Inagaki, N.; Kitaura, J.; Chikumoto, T.; Kawahara, H.; Kawakami, Y.; Sano, M.; Miyase, T.; Tachibana, H.; Nagai, H.; Kawakami, T. O-methylated catechins from tea leaves inhibit multiple protein kinases in mast cells. *J. Immunol.* **2004**, *172*, 4486−4492.

(9) Sano, M.; Yoshida, R.; Degawa, M.; Miyase, T.; Yoshino, K.; Determination of peroxyl radical scavenging activity of flavonoids and plant extracts using an automatic potentiometric titrator. *J. Agric. Food Chem.* **2003**, *51*, 2912−2916.

(10) Sano, M.; Tabata, M.; Suzuki, M.; Degawa, M.; Miyase, T.; Yamamoto, M. M. Simultaneous determination of twelve tea catechins by high-performance liquid chromatography with electrochemical detection. *Analyst* **2001**, *126*, 816−820.

(11) Frei, B.; Higdon, J. V. Antioxidant activity of tea polyphenols in vivo: evidence from animal studies. *J. Nutr.* **2003**, *133*, 3275S−3284S.

(12) Yoshida, H.; Ishikawa, T.; Tomiyasu, K.; Nishiwaki, M.; Ohsuzu, F.; Nakamura, H. Inhibitory effect of tea flavonoids on the ability of cells to oxidize low density lipoprotein. *Biochem. Pharmacol.* **1999**, *58*, 1695−1703.

(13) Rechner, A. R.; Wagner, E.; Van Buren, L.; Van De Put, F.; Wiseman, S.; Rice-Evans, C. A. Black tea represents a major source of dietary phenolics among regular tea drinkers. *Free Radical Res.* **2002**, *36*, 1127−1135.

(14) Leung, L. K.; Su, Y.; Chen, R.; Zhang, Z.; Huang, Y.; Chen, Z. Y. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J. Nutr.* **2001**, *131*, 2248−2251.

(15) Friedman, M.; Henika, P. R.; Levin, C. E.; Mandrell, R. E.; Kozukue, N. Antimicrobial activities of tea catechins and theaflavins and tea extracts against Bacillus cereus. *J. Food Prot.* **2006**, *69*, 354−361.

(16) Shiraki, M.; Hara, Y.; Osawa, T.; Kumon, H.; Nakamura, T.; Kawakishi, S. Antioxidative and antimutagenic effects of theaflavins from black tea. *Mutat. Res.* **1994**, *323*, 29−34.

(17) Maron, D. J.; Lu, G. P.; Cai, N. S.; Wu, Z. G.; Li, Y. H.; Chen, H.; Zhu, J. Q.; Jin, X. J.; Wouters, B. C.; Zhao, J. Cholesterol-lowering effect of a theaflavin-enriched green tea extract: a randomized controlled trial. *Arch. Intern. Med.* **2003**, *163*, 1448−1453.

(18) Lee, M. J.; Lambert, J. D.; Prabhu, S.; Meng, X.; Lu, H.; Maliakal, P.; Ho, C. T.; Yang, C. S. Delivery of tea polyphenols to the oral cavity by green tea leaves and black tea extract. *Cancer Epidemiol. Biomakers Prev.* **2004**, *13*, 132−137.

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

(20) Mulder, T. P.; Van Platerink, C. J.; Wijnand, Schuyl, P. J.; Van Amelsvoort, J. M. Analysis of theaflavins in biological fluids using liquid chromatograpy-electrospray mass spectrometry. *J. Chromatogr., B* **2001**, *760*, 271−279.

(21) Tanaka, T.; Matsuo, Y.; Kouno, I. A novel black tea pigment and two new oxidation products of epigallocatechin-3-*O*-gallate. *J. Agric. Food Chem.* **2005**, *53*, 7571−7578.

(22) Takeda, Y. Genetic variation in caffeine and tannin contents of tea in Japan. *Proceedings of 2004 International Conference O-CHA(tea) Culture and Science*, 2004, pp 812−815.

(23) Takino, Y.; Ozawa, T.; Sanada, H.; Masaki, S.; Fukunaga, T. Studies on the mechanism of oxidation of catechins part V. Isolation of crystalline theaflavin gallates from black tea and their formation from catechins. *Nippon Nougeikagaku Kaishi* **1971**, *4*, 176−183.

(24) Tanaka, T.; Mine, C.; Inoue, K.; Matsuda, M.; Kouno, I. Synthesis of theaflavin from epicatechin and epigallocatechin by plant homogenates and role of epicatechin quinone in the synthesis and degradation of theaflavin. *J. Agric. Food Chem.* **2002**, *50*, 2142−2148.

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