JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Simultaneous Analysis of Catechins, Gallic Acid, Strictinin, and Purine Alkaloids in Green Tea by Using Catechol as an Internal Standard

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We developed a high-performance liquid chromatography-based method for simultaneous analysis of nine catechins, gallic acid, strictinin, caffeine, and theobromine in green tea by using catechol as an internal standard. Although the high cost and instability of the catechin reference standards limit the application of this method, the addition of ascorbic acid to the standard stock solution preserved the stability of the reference standards in the solution for 1 year when stored at −30 °C. Furthermore, we found that the slopes of the calibration curves plotted were stable for a run time of 2000 h. Our method proved to be appropriate for quantification and yielded good correlation coefficients, detection levels, repeatability, reproducibility, and recovery rates. Quantitative data revealed that the contribution of only 200 mL of brewed tea to the total dietary catechins was approximately 220−420 mg, while that of 500 mL of bottled tea was approximately 170−900 mg.

KEYWORDS: Green tea; catechins; gallic acid; strictinin; alkaloid; HPLC

INTRODUCTION

The demand for green tea has increased due to human preferences and health concerns. Green tea contains many bioactive substances and is particularly rich in polyphenol compounds. The predominant polyphenols in green tea include epigallocatechin gallate (EGCg), epicatechin gallate (ECg), epigallocatechin (EGC), and epicatechin (EC) (*1*). These polyphenols are reported to have various functions that involve their antiviral, antioxidative, antimutagenic, anticarcinogenic, antiobesity, and hypocholesterolemic activities (*2*). During the manufacture of bottled and canned tea, some catechins undergo isomerization at the C-2 position of flavan-3-ol (*3*); for example, EGCg, ECg, EGC, and EC are converted into gallocatechin gallate (GCg), catechin gallate (Cg), gallocatechin (GC), and catechin (C), respectively. Similar to the original tea catechins, these epimers also exhibit antioxidative (*4*) and hypocholesterolemic activities (*5*). Proanthocyanidins have attracted considerable attention due to their possible contribution to the health benefits of green tea. Strictinin (ST), which belongs to the class of hydrolyzable tannins, was first detected in tea by Nonaka (*6*). It exhibits antioxidative (*7*) and antiallergic activities (*8*). Furthermore, epigallocatechin-3-*O*-(3-*O*-methyl)gallate (EGCg3″Me) and gallocatechin-3-*O*-(3-*O*-methyl)gallate (GCg3″Me) also exhibit antiallergic activities (*9*, *10*). In addition, tea contains caffeine (CAF) and a small amount of theobromine (TB) (*1*). These are the main methyl xanthines that constitute tea alkaloids, and they are important factors in determining the quality of green tea. The chemical structures of these compounds are shown in **Figure 1**. The determination of tea quality by routine quality control methods has recently gained substantial importance because of the pharmacological importance of tea and its application in the food industry. In addition, the high concentration of catechins in green tea, coupled with its widespread consumption, makes it a significant dietary product. Therefore, several studies in which tea catechins and alkaloids were separately analyzed have been reported in literature, and the related reviews are available (*11*−*13*). Recent studies have reported the absolute calibration curve method for the analysis of catechins, other phenolic compounds, and alkaloids in tea (*1*, *14*−*22*). Although these methods are practicable, their application is limited because some reference standards of catechin are very expensive and unstable. An internal standard method is a good alternative to the calibration curve method because the latter is extremely expensive and time-consuming when multiple compounds are required to be analyzed in a single sample. In one study, ethyl gallate was used as an internal standard for the analysis of tea catechins in human plasma (*23*). In another study, (+)-C was selected as the reference compound for determining the relative response factors of seven catechins and alkaloids in tea (*24*). Methods in which five or six tea catechins and CAF can be analyzed using phenylalanine (*25*), 4-amino-2-hydroxybenzoic acid (*26*), l-tryptophan (*27*), and naringenin (*28*) as internal standards are also available. In addition, 12 tea catechins without alkaloids were analyzed using propyl gallate (*29*). However, thus far, limited research has been conducted to determine methods by which tea catechins, their epimers, ST, and tea alkaloids can be simultaneously analyzed using an internal standard.

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10.1021/jf070323f CCC: $37.00
Published on Web 05/26/2007

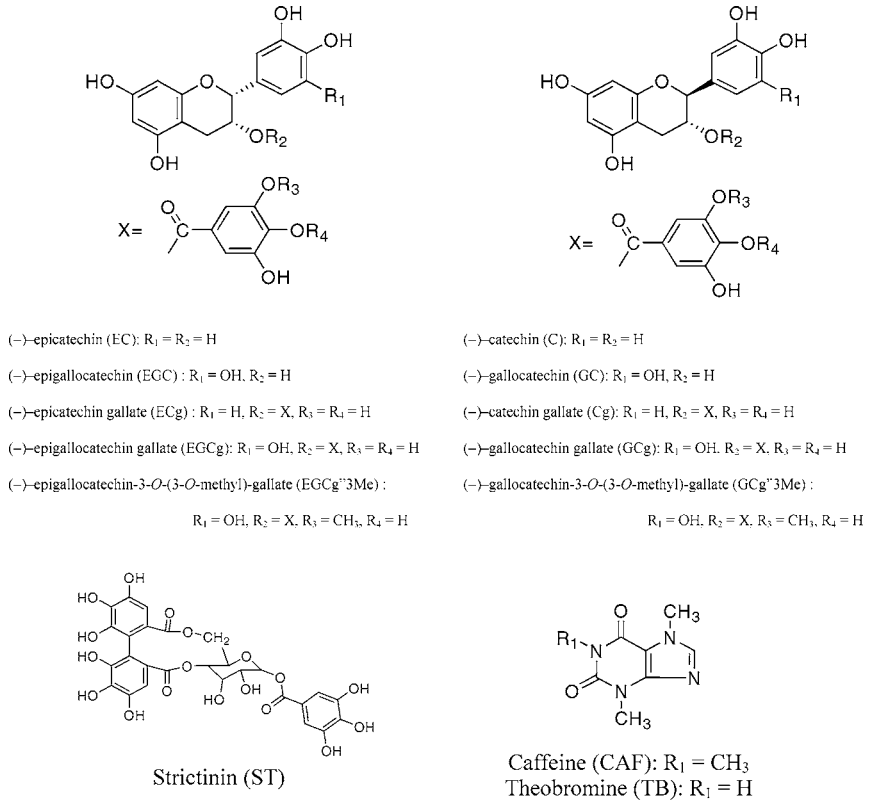

Figure 1. Chemical structure of tea catechins, ST, CAF, and TB.

We have developed an efficient method by which catechins (EGC, EC, EGCg, ECg, EGCg3″Me, GC, C, GCg, and Cg), gallic acid (GA), and ST as well as CAF and TB in green tea can be simultaneously analyzed by high-performance liquid chromatography (HPLC) using catechol as an internal standard. Although the analytical method discussed in this study requires approximately 1 h, it could greatly contribute to the analysis of these compounds and facilitate quality control. This is because our method proved to be appropriate for quantification and yielded good correlation coefficients, detection levels, repeatability, reproducibility, and recovery rates.

MATERIALS AND METHODS

Materials. Green tea leaves ($n = 20$) and bottled green tea ($n = 20$) were purchased from local markets in Shizuoka Prefecture, Japan, from January to December 2006. The bottled beverage contained 500 mL of tea infusion. Brewed tea was prepared according to standardized methods (*30*) established by the Japan Science and Technology Agency. Briefly, green tea leaves (10 g) were weighed in a beaker (1 L), and 430 mL of hot water (90 °C) was added. The mixture was left to stand for 1 min in a hot water bath (KZ-1000T; National Co. Ltd., Tokyo, Japan) at 90 °C. It was filtered through a tea strainer (mesh size, <1 mm^2), and the filtrate was cooled to room temperature in an ice bath. The filtrate was then centrifuged at 20000 rpm for 10 min at 20 °C, and the supernatant obtained was used as the analyte. For quantification, this treatment was conducted in triplicate.

Chemicals. Water was purified using a Milli-Q system (Millipore, Bedford, MA) with a resistivity of >17.5 MΩm. HPLC-grade methanol and acetonitrile were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Analytical grade catechol (internal standard), ascorbic acid, CAF, TB, GA, and phosphoric acid were also obtained from Wako Pure Chemical Industries. ST, (−)-EGC, (−)-EC, (−)-EGCg, (−)-ECg, (−)-EGCg3″Me, (−)-GC, (−)-C, (−)-GCg, and (−)-Cg were purchased from Nagara Science (Gifu, Japan).

The optical rotation properties of authentic artificial tea catechins may differ from those of catechins in natural tea flush. For example, GC occurring in fresh tea flush is in the positive form (*31*), while that obtained from Nagara Science was in the negative form. However, this discrepancy did not influence the identification of tea catechins in our experiment because the HPLC method described below could not distinguish the catechins based on the differences in their optical rotation properties. Therefore, the (+) or (−) optical rotation property for each catechin has not been addressed in this study.

HPLC Analysis. HPLC analysis was performed using a Hitachi HPLC system (Tokyo, Japan) equipped with a vacuum degasser (L-5090), quaternary pump to generate gradients (L-6300), temperature-controlled column chamber (L-5025), UV−vis detector (L-4250), and an autosampler (AS-2000). Resolution was achieved at 40 °C by using a series of 2 Wakopack Navi C18-5 columns (5 μm, 4.6 mm × 150 mm; Wako Pure Chemical Industries) equipped with a guard column (5 μm, 4.6 mm × 10 mm). Mobile phase A comprised 0.25% v/v phosphoric acid and acetonitrile (20:1 v/v, pH 2.4), and mobile phase B comprised mobile phase A and methanol (5:1 v/v, pH 2.5). A gradient was established as follows: 0−10 min, 10% B; 10−20 min, linear gradient of B from 10 to 50%; 20−30 min, linear gradient of B from 50 to 95%; and 30−65 min, 95% B. The flow rate was 1.0 mL/min, and the injection volume was 10 μL. It has been reported that the use of a detection wavelength of 210 nm considerably improves the signal-to-noise ratio of the chromatograms obtained in the analysis (*27*); therefore, the wavelength of the UV−vis detector was set at 210 nm. The peak area was calculated using the Empower 2 (revision A) software (Waters Co. Ltd., Milford, MA). The identification and peak purity of each compound were confirmed using a photodiode array detector (SPD-M10Avp; Shimadzu, Kyoto, Japan) with the Class-M10A (version 1.61) software (Shimadzu). The peaks were identified based on a combination of retention time and spectral matching over 99% from 200 to 300 nm.

Standard Solution. The internal standard catechol was weighed (69.4 mg), transferred into a 100 mL volumetric flask containing ascorbic acid (176 mg), and dissolved in 90 mL of water by sonication for 1 h at 20−25 °C; the volume was made up to 100% with water. All of the authentic references were accurately weighed (4.25−14.48 mg), transferred into a 100 mL volumetric flask containing ascorbic acid (176 mg), and dissolved in 90 mL of water by sonication for 1 h at 20−25 °C; the final volume was made to 100% with water. The

stock standard and internal standard solutions were stored at −30 °C until use. The addition of ascorbic acid into the stock solution preserves the stability of its components for approximately 1 year at −30 °C. The stability of the compounds in the solution is described in the Results and Discussion. Working standard solutions for calibration and validation tests were prepared by diluting the stock solution 1−200-fold with water and adding the internal standard solution to these diluted solutions.

Calibration and Validation. For all of the calibration and validation calculations, the analyte concentration was defined in terms of μg/mL of the injection volume (10 μL). Calibration curves were plotted for all of the authentic references by performing HPLC-UV analysis in triplicate on eight incremental dilutions (ranging from 0.45 to 86.88 μg/mL) of the stock solution containing the internal standard. The detection limit (DL) and quantification limit (QL) were also determined by performing HPLC-UV analysis in triplicate on six incremental dilutions (ranging from 0 and 0.11 μg/mL, respectively, to 9.01 μg/mL) of the stock solution containing the internal standard. The intraday variation (repeatability) in the analytical results was assessed by performing HPLC-UV analysis of the brewed tea and bottled tea. The bottled tea was analyzed six times on the same day, and the relative standard deviation (RSD %) was calculated. The brewed tea was prepared in triplicate, and each aliquot was measured in triplicate on the same day; the RSDs were calculated based on the nine measurements obtained for the brewed tea. Furthermore, the interday variation (reproducibility) in the analytical results was assessed by performing HPLC-UV analysis of the brewed tea and bottled tea on three consecutive days. The triplicate analyses of the bottled tea that were conducted on each day were treated as single data points to calculate the RSD between days for each compound. Similarly, the brewed tea was prepared in triplicate on the same day, and each aliquot was analyzed in triplicate; the nine measurements obtained for the brewed tea on each day were treated as single data points. To verify the accuracy of our analyses, we carried out a recovery test by adding three incremental dilutions (ranging from 23 to 120 μg/mL) of the stock solution to the brewed tea and bottled tea in triplicate and performed three independent measurements.

Sample Preparation for HPLC Analysis. For preparing the brewed tea and bottled tea samples for HPLC, the internal standard solution (1 mL) containing ascorbic acid was added to each sample (2 mL) and mixed by shaking for 1 min. When the peak area ratio (analyte/catechol) was over the linear range described in the Results and Discussion, the additional amount of internal standard solution was increased. The mixture was diluted with water to achieve a volume of 10 mL and passed through a 0.45 μm filter disk (Advantec, Tokyo, Japan) prior to injection.

RESULTS AND DISCUSSION

Identification. The chromatograms obtained for the resolution of the standard solution, brewed tea, and bottled tea are shown in **Figure 2**. No impurities were detected by the peak purity software that was used to analyze the diode array data; the signal ratios (relative absorbance) were constant across each peak profile examined in the range of 200−300 nm. In a previous study, a considerable amount of GCg3″Me was formed from EGCg3″Me when the tea was brewed at 90 °C for 9−15 min (*10*); however, the GCg3″Me peak was not detected in the brewed tea (90 °C for 1 min) and commercial bottled tea samples. In contrast, the GCg3″Me peak was detected at a retention time of 61 min by using our method.

Stability of the Standard Solution. The stability of the standard solution containing the catechins, GA, ST, TB, and CAF was tested. The solution was found to be stable for approximately 1 month when stored at −30 °C. We expect that the solution would remain stable for at least a year since expensive reference standards of catechins were used. It was reported that the addition of ascorbic acid (11 mg/mL) protects green tea catechins in the solution (pH 2.55) for a month at room temperature (*32*). Hence, we added ascorbic acid (10 mM, 176 mg) to the 100 mL stock solution. The response factor (peak area/μg mL^{-1}) of each solution component was stable for approximately 1 year when the solution was stored at −30 °C. Therefore, we added 176 mg of ascorbic acid to the 100 mL stock solution. The stability was also investigated during the HPLC analysis. This investigation revealed that after the standard solution was maintained at room temperature (20 ± 3 °C) in the autosampler for a day, the response factor of its components decreased by approximately 0−10%. However, the addition of ascorbic acid at a concentration greater than 1 mM rendered the solution stable for approximately more than 2 days at room temperature. Therefore, we added ascorbic acid (176 mg) to the internal standard solution (100 mL) to achieve a concentration of 1 mM ascorbic acid in the mixture (internal standard, 1 mL; sample, 2 mL; and water, 7 mL). This information proved to be useful in the experimental design.

The antioxidant effect of ascorbic acid significantly contributes to the stabilization of compounds in the solution. Although a decrease in the pH of the solution was also an important effect of the protection (*33*), catechins in a solution mixture of green tea and Coca Cola or Pepsi soft drinks at pH 2.6 exhibited poor stability (*32*). This suggests that other ingredients in tea and/or Coca Cola or Pepsi soft drinks may interact with catechins and affect their stability. Although ascorbic acid is an ingredient of green tea, the addition of ascorbic acid to the standard stock solution is valuable for the analysis of expensive catechin reference standards.

Calibration and Validation. We next performed experiments to calibrate and validate our method. Methods in which tea catechins and CAF can be analyzed using phenylalanine (*25*), 4-amino-2-hydroxybenzoic acid (*26*), L-tryptophan (*27*), and naringenin (*28*) as internal standards are available. However, phenylalanine and tryptophan are ingredients of green tea (*34*). Ethyl gallate (*23*) was used as an internal standard for the analysis of tea catechins in human plasma. Propyl gallate (*29*) has also been used for the analysis of 12 catechins. Catechol is more soluble in water than naringenin, 4-amino-2-hydroxybenzoic acid, ethyl gallate, and propyl gallate. The UV spectra of catechol, catechins, ST, and CAF at 200−300 nm are shown in **Figure 3**. Because the UV spectrum of each catechin is the same as that of its isomer, the UV spectra of five catechins are shown in the figure. Each spectrum had two peaks, and strong absorbance of each spectrum was detected at 200−220 nm. Furthermore, HPLC profiles of the reference mixture, brewed tea, and bottled tea showed no impurities (**Figure 2**). These findings prompted us to select catechol as the internal standard for our experiments.

Calibration curves of the peak area ratio (analyte/internal standard) vs the ratio of concentration in μg/mL (analyte/internal standard) were obtained for all of the authentic references at the detection wavelength of 210 nm. The least-squares method was used to calculate the regression equations. The characteristics of the calibration curves, including the slope of the peak area ratio vs the ratio of concentration in μg/mL, range of linearity, and correlation coefficient (*r*), are listed in **Table 1**. All of the calibration curves were linear over the tested concentration ranges, with $r \geq 0.998$. Both the DL and the QL were evaluated according to the International Conference on Harmonization (ICH) guidelines (*35*). The DL and QL are expressed as $3.3 \times \sigma/S$ and $10 \times \sigma/S$, respectively, where S is the slope of the peak area ratio vs the ratio of concentration in μg/mL, and σ is the standard deviation of the results based on the values obtained from the triplicate analyses using six standard solutions. The DL and QL values obtained are also

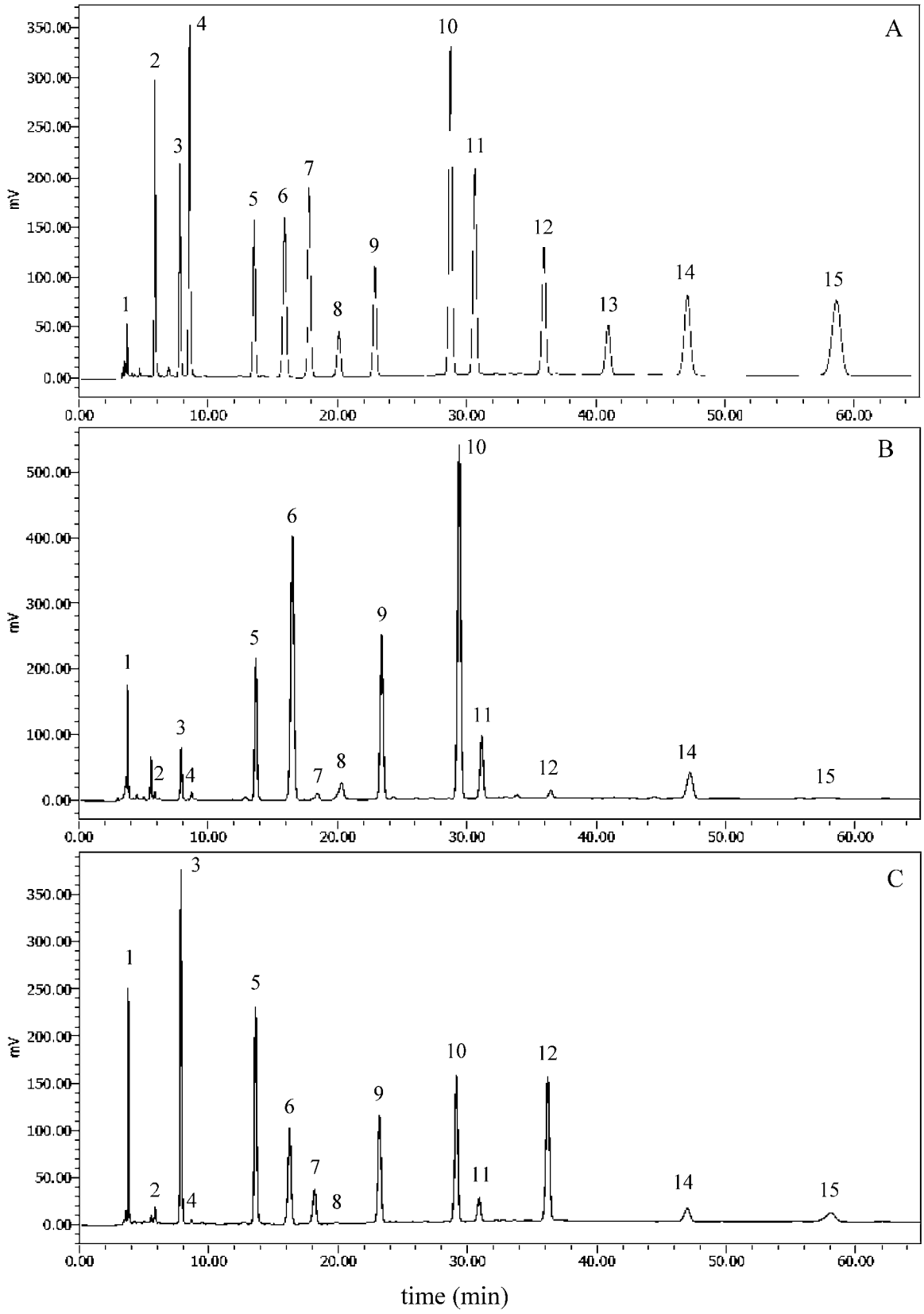

Figure 2. HPLC profiles of the reference mixture (**A**), brewed tea (**B**), and bottled tea (**C**). The chemicals are numbered, and the exact amounts of compounds without ascorbic acid injected into the column are as follows: 1, ascorbic acid; 2, GA (**A**, 334 ng; **B**, 9 ng; **C**, trace); 3, GC (**A**, 271 ng; **B**, 60 ng; **C**, 251 ng); 4, TB (**A**, 679 ng; **B**, 13 ng; **C**, trace); 5, catechol (**A**, 694 ng; **B** and **C**, 1388 ng); 6, EGC (**A**, 497 ng; **B**, 711 ng; **C**, 130 ng); 7, C (**A**, 540 ng; **B**, 19 ng; **C**, 49 ng); 8, ST (**A**, 425 ng; **B**, 125 ng; **C**, not detected); 9, CAF (**A**, 465 ng; **B**, 488 ng; **C**, 223 ng); 10, EGCg (**A**, 869 ng; **B**, 816 ng; **C**, 188 ng); 11, EC (**A**, 624 ng; **B**, 169 ng; **C**, 32 ng); 12, GCg (**A**, 348 ng; **B**, 19 ng; **C**, 207 ng); 13, EGCg″3Me (**A**, 257 ng; **B** and **C**, not detected); 14, ECg (**A**, 420 ng; **B**, 121 ng; **C**, 37 ng); and 15, Cg (**A**, 552 ng; **B**, not detected; **C**, 37 ng).

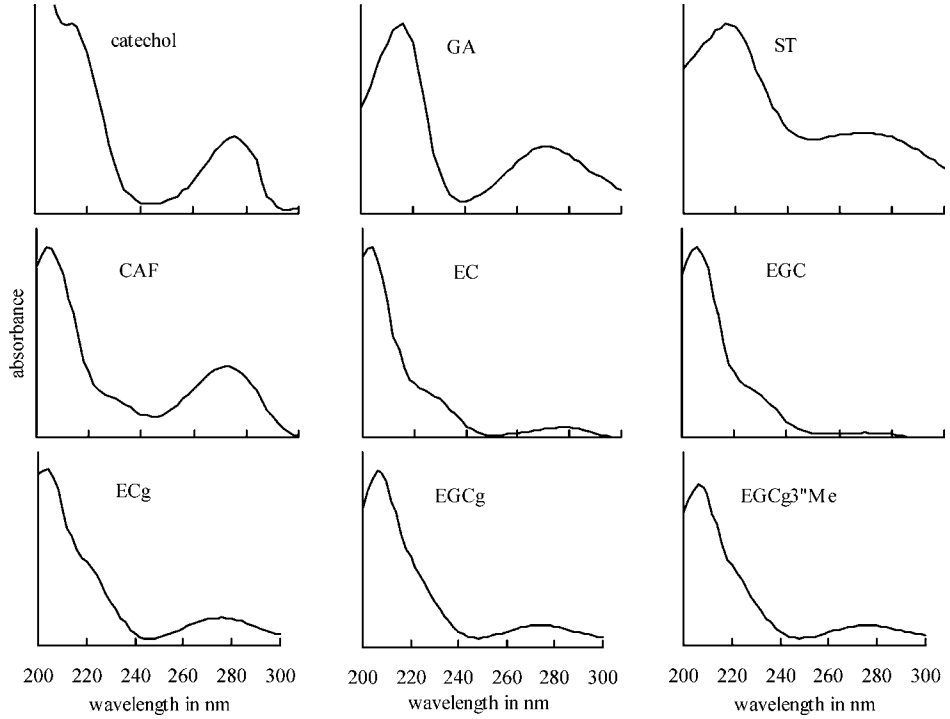

Figure 3. Absorption spectra of catechol, catechins, ST, and CAF ranging from 200 to 300 nm.

Table 1. Slope of the Peak Area Ratio vs the Ratio of Concentration in μg/mL, Linear Range, *r*, QL, and DL Obtained in the HPLC-Based Method Developed for the Analysis of Nine Catechins, GA, ST, CAF, and TB Using Catechol as an Internal Standard

					μg/mL	
compound	slope[a]	linear range (μg/mL)	peak area ratio	*r*	DL	QL
EGC	0.692	0.82–49.68	0.11–1.49	0.998	0.49	1.49
EGCg	0.596	1.45–86.88	0.01–2.24	0.998	0.81	2.44
EC	0.696	1.04–62.40	0.04–1.28	0.998	0.49	1.51
EGCg3′′Me	1.194	0.43–25.68	0.06–0.96	0.998	0.21	0.63
ECg	1.204	0.70–42.00	0.07–0.87	0.998	0.32	0.98
GC	0.292	0.45–27.12	0.02–1.36	0.999	0.45	1.38
GCg	0.627	0.58–34.80	0.08–1.34	0.999	0.27	0.83
C	0.656	0.90–54.00	0.04–1.11	0.998	0.46	1.34
Cg	1.691	0.92–55.20	0.06–0.88	0.998	0.41	1.24
GA	0.257	0.57–33.43	0.08–1.86	0.998	0.46	1.39
ST	1.883	0.71–42.48	0.02–1.05	0.998	0.41	1.24
TB	0.442	1.13–67.92	0.03–2.21	0.998	0.44	1.35
CAF	0.766	0.61–46.48	0.08–1.39	0.998	0.24	0.72

[a] Peak area ratio (analyte/internal standard) vs the ratio of concentration in μg/mL (analyte/internal standard).

Table 2. RSD of the Intraday (Repeatability) and Interday (reproducibility) Measurements Obtained by HPLC Analysis Using Catechol as an Internal Standard

	brewed tea[a]		bottled tea	
compound	intraday RSD (%)[b]	interday RSD (%)[c]	intraday RSD (%)	interday RSD (%)
EGC	3.4	6.1	0.3	0.2
EGCg	4.1	3.3	0.2	0.4
EC	2.8	3.4	0.4	0.8
EGCg3′′Me	–[d]	–	–	–
ECg	3.5	5.7	0.3	0.2
GC	4.8	6.1	0.1	0.2
GCg	6.1	5.6	0.5	0.4
C	9.2	9.7	0.8	1.1
Cg	–	–	0.7	0.6
GA	–	–	2.5	2.7
ST	6.3	5.7	–	–
TB	8.4	8.3	3.8	3.5
CAF	2.9	3.4	0.4	0.6

[a] Hot water (430 mL) at 90 °C was added to green tea leaves (10 g), and the mixture was allowed to stand at 90 °C for 1 min. [b] Intraday RSDs represent data obtained for nine measurements for brewed tea (brewing in triplicate with three independent measurements) and six measurements for bottled tea performed on the same day. [c] Interday RSDs represent data obtained for nine measurements of bottled tea and 27 measurements of brewed tea. The triplicate analyses conducted on each day for bottled tea were treated as single data points. Brewed tea was prepared three times on the same day, and each aliquot was analyzed in triplicate. The nine measurements conducted on each day for brewed tea were considered as single data points. [d] The peak area was small because the concentration of each compound was lower than the corresponding QL.

listed in **Table 1**. The DL and QL of the compounds were in the range of 0.21−0.81 and 0.63−2.44 μg/mL, respectively.

The chromatograms obtained for the brewed tea and bottled tea in the intra- and interday tests are shown in **Figure 2**. The exact amounts of compounds injected into the column are also presented in the figure caption. No significant difference was observed in the RSD between the intra- and the interday experiments (**Table 2**). The intra- and interday RSDs of GA and TB that yielded low signals in the bottled tea ranged from 2.5 to 3.8%. However, the other compounds that yielded higher signals exhibited low RSD values ranging from 0.1 to 1.1%. The intra- and interday RSDs for the brewed tea ranged from 2.8 to 9.7%; these values were approximately 10 times higher than those obtained for the bottled tea. Furthermore, we assessed the intraday experiment in which brewed tea that was not subjected to repeated brewing treatments was used. The RSD values obtained in this experiment ranged from 0.1 to 2.3%. Thus, the high RSD values obtained in the intra- and interday experiments using brewed tea included the errors that occurred due to the brewing treatment.

Furthermore, to verify the accuracy of our results, we carried out a recovery test by adding three incremental dilutions of the standard solution to the brewed and bottled tea samples. The overall recovery rate that was calculated by averaging the three

Table 3. Stability of the Slope of the Peak Area Ratio vs the Ratio of Concentration Obtained by HPLC Analysis Using Catechol as an Internal Standard

		slope of the peak area ratio vs the ratio of concentration												
relative reference lamp energy (%)[a]	run time (h)	EGC	EGCg	EC	EGCg3″Me	ECg	GC	GCg	C	Cg	GA	ST	TB	CAF
100	50	0.692	0.596	0.696	1.194	1.204	0.292	0.627	0.656	1.691	0.257	1.883	0.442	0.766
87	1000	0.694	0.601	0.697	1.202	1.203	0.297	0.631	0.667	1.695	0.261	1.891	0.448	0.769
64	2000	0.691	0.594	0.693	1.191	1.195	0.288	0.629	0.657	1.691	0.254	1.886	0.448	0.764

[a] Relative reference = (reference lamp energy × 100)/reference lamp energy at 50 h of run time.

measurements obtained for each test ranged from 96 to 105% (data not shown).

Stability of the Slope of the Peak Area Ratio vs the Ratio of Concentration. The response factor of the solution components is not always stable because of the influence of instrumental conditions and other factors. Therefore, when using the absolute standard curve method, the standard solution should be analyzed at regular intervals to compensate for the instability of the response factor. The internal standard method is well-known to compensate for these errors. In theory, the slope of the peak area ratio vs the ratio of concentration is stable; however, the stability of the slope for HPLC analysis has not been verified experimentally. We tested the stability of the slope of the peak area ratio vs the ratio of concentration by using the standard stock solution. Using the method described in the Materials and Methods, the slope was obtained after approximately 50, 1000, and 2000 h of run time. After the measurements, we also assessed the reference energy of the lamp (deuterium lamp, 635−0946; Hitachi, Tokyo, Japan), and the relative reference energy (%) of the lamp was calculated; the results are listed in **Table 3**. The slope of the peak area ratio vs the mass ratio for all of the components was observed to be stable for a run time of approximately 2000 h.

Quantitative Results. The consumption of bottled green tea has dramatically increased because of the ease of preparation and health benefits. Although the contents of EC, EGCg, C, and GCg in both bottled and brewed tea have been reported (*32*, *36*), the difference in the contents of EGC, ECg, GC, and Cg with regard to the content of total catechins remains unclear. By using the method that we developed, we analyzed nine catechins, GA, and ST as well as CAF and TB in commercial bottled tea and brewed tea (**Table 4**). The average, maximum, and minimum values in **Table 4** were calculated based on the data for 20 samples. The original catechins, their epimers, and CAF were detected in all of the bottled tea samples. The Cg content was less than the DL in all of the brewed tea samples. Furthermore, while ST was detected in all of the brewed tea samples, its concentration in the bottled tea samples was less than the DL. The concentrations of the original tea catechins were substantially higher in the brewed tea samples than in the bottled tea samples. However, the bottled tea contained similar concentrations of the original catechins (EGC, EGCg, EC, and ECg) and their epimers (GC, GCg, C, and Cg, respectively). EGCg3″Me was not detected in most of the samples. In a previous report, EGCg3″Me was detected in most of the tea leaves plucked from tea gardens in Taiwan by using an electrochemical detector (*16*). The electrochemical detector is more sensitive than the UV detector. However, the amount of EGCg3″Me ranged from 0.1 to 1.4 mg/g in tea leaves when 0.5 g of dry tea leaves was steeped in boiling distilled water (50 mL) for 3 min at 90 °C. Thus, the amount of 0.1 mg/g of EGCg3″Me in tea leaves could be calculated from 1 μg/mL of the injected sample. Because the developed method using the UV detector showed that the QL of EGCg3″Me was 0.63 μg/mL, we could quantify approximately 0.06 mg/g of EGCg3″Me in tea leaves. The EGCg3″Me content in Benifuuki, Benifuji, and Benifomare cultivars, which are classified as Assam hybrids, was higher than those of the other tea cultivars (*10*, *29*). Because most of the cultivars classified as Chinese hybrids were planted in Japan, EGCg″3Me could not be detected in most of the bottled tea and brewed tea samples. The quantitative data revealed that the consumption of only 200 mL of brewed tea contributes approximately 220−420 mg to the total dietary catechins. In contrast, the consumption of 500 mL of bottled tea contributes approximately 170−900 mg to the total dietary catechins.

Table 4. Concentrations of Tea Catechins, GA, ST, TB, and CAF in Brewed Tea and Commercial Bottled Tea Measured by HPLC Using Catechol as an Internal Standard

	concentration (μg/mL)					
	brewed tea[a]			bottled tea		
compound	avg[b]	max[c]	min[d]	avg	max	min
EGC	706	866	521	119	218	49
EGCg	599	881	272	150	331	63
EC	167	241	111	28	59	11
EGCg3″Me	79 (3)[e]	94	ND[f]	44 (1)	44	ND
EGC	75	127	28	32	93	13
GC	53	113	24	195	469	85
GCg	13	21	5	162	458	56
C	21	46	9	43	122	19
Cg	ND	ND	ND	37	138	11
GA	8 (6)	9	tr[g]	13 (16)	29	ND
ST	78	128	18	6 (1)	6	ND
TB	13	19	7	9 (4)	10	ND
CAF	388	501	108	165	240	97

[a] Hot water (430 mL) at 90 °C was added to green tea leaves (10 g), and the mixture was allowed to stand at 90 °C for 1 min. This treatment was conducted in triplicate, and the average value was treated as a single data point. [b] Average value calculated from 20 samples. [c] Maximum value in the 20 samples. [d] Minimum value in the 20 samples. [e] Number of quantitative samples. The average value was obtained from the quantitative samples. [f] Not detected because the levels were less than the corresponding DL. [g] Trace level, DL ≤ tr < QL.

In conclusion, we developed an HPLC-UV-based method for simultaneous analysis of catechins, GA, and ST as well as TB and CAF in tea by using catechol as an internal standard. This method yielded good repeatability, reproducibility, recovery rates, and component resolution. Furthermore, the addition of ascorbic acid preserved the stability of the expensive catechin reference standards in the stock solution when stored at −30 °C. In addition, the slope of the peak area ratio vs the mass ratio was stable for a run time of 2000 h. The quantitative data reported in this study revealed that the consumption of only 200 mL of brewed tea contributes approximately 220−420 mg to the total dietary catechins, while the consumption of 500 mL of bottled tea contributes approximately 170−900 mg to the total dietary catechins.

ABBREVIATIONS USED

EGCg, epigallocatechin gallate; ECg, epicatechin gallate; EGC, epigallocatechin; EC, epicatechin; GCg, gallocatechin gallate; Cg, catechin gallate; GC, gallocatechin; C, catechin; EGCg3″Me, epigallocatechin-3-*O*-(3-*O*-methyl)gallate; GCg3″Me, gallocatechin-3-*O*-(3-*O*-methyl)gallate; GA, gallic acid; ST, strictinin; CAF, caffeine; TB, theobromine; HPLC, high-performance liquid chromatography; *r*, correlation coefficient; RSD, relative standard deviation; DL, detection limit; QL, quantification limit.

LITERATURE CITED

(1) Del Rio, D.; Stewart, A. J.; Mullen, W.; Burns, J.; Lean, M. E.; Brighenti, F.; Crozier, A. HPLC-MS analysis of phenolic compounds and purine alkaloids in green and black tea. *J. Agric. Food Chem.* **2004**, *52*, 2807−2815.

(2) Dufrese, C. J.; Farnworth, E. R. A review of latest research findings on the health promotion properties of tea. *J. Nutr. Biochem.* **2001**, *12*, 404−421.

(3) Seto, R.; Nakamura, H.; Nanjo, F.; Hara, Y. Preparation of epimers of tea catechins by heat treatment. *Biosci., Biotechnol., Biochem.* **1997**, *61*, 1434−1439.

(4) Guo, Q.; Zhao, B.; Shen, S.; Hou, J.; Hu, J.; Xin, W. ESR study on the structure-antioxidant activity relationship of tea catechins and their epimers. *Biochim. Biophys. Acta* **1999**, *1427*, 13−23.

(5) Ikeda, I.; Kobayashi, M.; Hamada, T.; Tsuda, K.; Goto, H.; Imaizumi, K.; Nozawa, A.; Sugimoto, A.; Kakuda, T. Heat-epimerized tea catechins rich in gallocatechin gallate and catechin gallate are more effective to inhibit cholesterol absorption than tea catechins rich in epigallocatechin gallate and epicatechin gallate. *J. Agric. Food Chem.* **2003**, *51*, 7303−7307.

(6) Nonaka, G.; Kawahara, O.; Nishioka, I. Tannins and related compounds. XV. A new class of dimeric flavan-3-ol gallates, theasinensins A and B, and proanthocyanidin gallates from green tea leaf. *Chem. Pharm. Bull.* **1983**, *31*, 3906−3914.

(7) Zhou, B.; Yang, L.; Liu, Z-L. Strictinin as an efficient antioxidant in lipid peroxidation. *Chem. Phys. Lipids* **2004**, *131*, 15−25.

(8) Tachibana, H.; Kubo, T.; Miyase, T.; Tanino, S.; Yoshimoto, M.; Sano, M.; Maeda-Yamamoto, M.; Yamada, K. Identification of an inhibitor for interleukin 4-induced epsilon germline transcription and antigen-specific IgE production in vivo. *Biochem. Biophys. Res. Commun.* **2001**, *280*, 53−60.

(9) 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.

(10) Maeda-Yamamoto, M.; Nagai, H.; Suzuki, Y.; Ema, K.; Kanda, E.; Mitsuda, H. Changes in *O*-methylated catechin and chemical component contents of 'Benifuuki' green tea (*Camellia sinensis L.*) beverage under various extraction conditions. *Food Sci. Technol. Res.* **2005**, *11*, 248−253.

(11) Horie, H.; Kohata, K. Analysis of tea components by high-performance liquid chromatography and high-performance capillary electrophoresis. *J. Chromatogr. A* **2000**, *881*, 425−438.

(12) Dalluge, J. J.; Nelson, B. C. Determination of tea catechins. *J. Chromatogr. A* **2000**, *881*, 411−424.

(13) Merken, H. M.; Beecher, G. R. Measurement of food flavonoids by high-performance liquid chromatography: A review. *J. Agric. Food Chem.* **2000**, *48*, 577−599.

(14) Yang, X. R.; Ye, C. X.; Xu, J. K.; Jiang, Y. M. Simultaneous analysis of purine alkaloids and catechins in *Camellia sinensis*, *Camellia ptilophylla* and *Camellia assamica* var. kucha by HPLC. *Food Chem.* **2007**, *100*, 1132−1136.

(15) 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.

(16) Chiu, F. L.; Lin, J. K. HPLC analysis of naturally occurring methylated catechins, 3″- and 4″-methyl-epigallocatechin gallate, in various fresh tea leaves and commercial teas and their potent inhibitory effect on inducible nitric oxide synthase in macrophages. *J. Agric. Food Chem.* **2005**, *53*, 7035−7042.

(17) 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.

(18) Sharma, V.; Gulati, A.; Ravindranath, S. D.; Kumar, V. A simple and convenient method for analysis of tea biochemicals by reverse phase HPLC. *J. Food Compos. Anal.* **2005**, *18*, 583−594.

(19) Yao, L.; Jiang, Y.; Datta, N.; Singanusong, R.; Liu, X.; Duan, J.; Raymont, K.; Lisle, A.; Xu, Y. HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (*Camellia sinensis*) grown in Australia. *Food Chem.* **2004**, *84*, 253−263.

(20) Nishitani, E.; Sagesaka, Y.-M. Simultaneous determination of catechins, caffeine and other phenolic compounds in tea using new HPLC method. *J. Food Compos. Anal.* **2004**, *17*, 675−685.

(21) Pelillo, M.; Bonoli, M.; Biguzzi, B.; Bendini, A.; Gallina Toschi, T.; Lercker, G. An investigation in the use of HPLC with UV and MS-electrospray detection for the quantification of tea catechins. *Food Chem.* **2004**, *87*, 465−470.

(22) Lin, J. K.; Lin, C. L.; Liang, Y. C.; Lin-Shiau, S. Y.; Juan, I. M. Survey of catechins, gallic acid, and methylxanthines in green, oolong, pu-erh, and black teas. *J. Agric. Food Chem.* **1998**, *46*, 3635−3642.

(23) Unno, T.; Sagesaka, Y. M.; Kakuda, T. Analysis of tea catechins in human plasma by high-performance liquid chromatography with solid-phase extraction. *J. Agric. Food Chem.* **2005**, *53*, 9855−9889.

(24) Wang, H.; Provan, G. J.; Helliwell, K. HPLC determination of catechins in tea leaves and tea extracts using relative response factors. *Food Chem.* **2003**, *81*, 307−312.

(25) Weiss, D. J.; Austria, E. J.; Anderton, C. R.; Hompesch, R.; Jander, A. Analysis of green tea dietary supplements by micellar electrokinetic chromatography. *J. Chromatogr. A* **2006**, *1117*, 103−108.

(26) Stach, D.; Schmitz, O. J. Decrease in concentration of free catechins in tea over time determined by micellar electrokinetic chromatography. *J. Chromatogr. A* **2001**, *924*, 519−522.

(27) Dalluge, J. J.; Nelson, B. C.; Thomas, J. B.; Sander, L. C. Selection of column and gradient elution system for the separation of catechins in green tea using high-performance liquid chromatography. *J. Chromatogr. A* **1998**, *793*, 265−274.

(28) Bronner, W. E.; Beecher, G. R. Method for determining the content of catechins in tea infusions by high-performance liquid chromatography. *J. Chromatogr. A* **1998**, *805*, 137−142.

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

(30) Resources Council, Science and Technology Agency, Japan. *Standard Tables of Food Composition in Japan*, 5th rev. ed. (in Japanese); Japan Science and Technology Agency: Tokyo, Japan, 2000; pp 513−514.

(31) Yamanishi, T.; Hara, Y.; Luo, S.; Wickremasunghe, R. L. Special issue on tea. *Food Rev. Int.* **1995**, *11* (3), 371−546.

(32) 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.

(33) 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.

(34) Wang, H. F.; Tsai, Y. S.; Lin, M. K.; Ou, A. S. Comparison of bioactive components in GABA tea and green tea produced in Taiwan. *Food Chem.* **2006**, *96*, 648−653.

(35) ICH Steering Committee. *ICH Harmonized Tripartite Guideline: Validation of Analytical Procedures: Methodology Q2B*; recommended for adoption at step 4 of the ICH process on Nov 6 1996, pp 1−8.

(36) Ito, R.; Yamamoto, A.; Kodama, S.; Kato, K.; Yoshimura, Y.; Matsunaga, A.; Nakazawa, H. A study on the change of enantiomeric purity of catechins in green tea infusion. *Food Chem.* **2003**, *83*, 563−568.

Received for review February 5, 2007. Revised manuscript received April 19, 2007. Accepted April 23, 2007.

JF070323F