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Preparation and Antioxidant Activity of Green Tea Extract Enriched in Epigallocatechin (EGC) and Epigallocatechin Gallate (EGCG)

JIANHUI HU,† DANRONG ZHOU,† AND YUQIONG CHEN*

College of Horticulture and Forestry Science, Huazhong Agricultural University, Key Laboratory of Horticultural Plant Biology, Ministry of Education, No. 1 Shizishan Street, Hongshan District, Wuhan City, Hubei Province, China 430070

The present study investigated effects of solvents, temperature, and duration on extracting efficiency of catechins from green tea by an orthogonal test. The results suggested that extraction of epigallocatechin gallate (EGCG) was highly dependent on these factors, whereas that of epigallocatechin (EGC) was significantly affected by duration and solvent ($P < 0.05$). Effects of these factors on epicatechin (EC), catechin (C), and epicatechin gallate (ECG) were not significant. A two-step preparation was adopted to produce green tea extract enriched in EGC (GTE-EGC) and green tea extract enriched in EGCG (GTE-EGCG). The optimum conditions for GTE-EGC were that green tea was brewed in 75% ethanol at 30 °C for 10 min, whereas for GTE-EGCG the same tea was brewed in 35% ethanol at 90 °C for 60 min. Compared with GTE-EGC, GTE-EGCG had EGCG increased by 110.42%, whereas EGC decreased by 40.38% with EC and ECG being unchanged. Most importantly, GTE-EGCG possessed greater antioxidant activity (in vitro) than GTE-EGC.

KEYWORDS: Green tea; extract; EGCG; EGC; antioxidant activity

INTRODUCTION

Green tea is one of the most widely consumed beverages in the world. Numerous in vitro and in vivo studies have suggested the beneficial health properties of green tea and tea polyphenols including antioxidation, antitumor, hypertension and hyperlipidemia reduction, and antimicrobial activity (*1, 2*). The major polyphenolic compounds in green tea are catechins, which mainly include (−)-epigallocatechin gallate (EGCG), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epicatechin (EC) (*3*).

Due to the beneficial effects of green tea and catechins, green tea extracts have been widely applied to different fields, particularly in food and beverage industries as additives (*4, 5*). Therefore, detailed studies of the production of green tea extracts enriched in active components are of great interest. Researchers have done much work on factors affecting the extraction of catechins and caffeine in past years (*6*−*9*). Recently, the work of Labbe et al. (*10*) has shown that there was a variable interdependence between the brewing duration and the brewing temperature on catechin and caffeine concentrations. On the basis of these observations, production of green tea extracts enriched in EGC and EGCG is feasible by modifying its parameters of duration and temperature. However, little information is available regarding the effects of multiextraction factors on the production of catechins-enriched green tea extracts and evaluation of bioactivities of the final products. In the present study, an orthogonal experiment was used to optimize the three major extraction factors, namely, solvents, temperature, and duration, for the extraction of catechins from green tea. On the basis of the optimized conditions, a two-step extraction procedure was used to prepare EGC-enriched green tea extract (GTE-EGC) and EGCG-enriched green tea extracts (GTE-EGCG). In addition, the antioxidant abilities of GTE-EGC and GTE-EGCG were evaluated by the in vitro assays for hydroxyl and superoxide radicals scavenging activities.

MATERIALS AND METHODS

Materials. Green tea was purchased from Baokang County Tea Factory, Hubei Province, China. It was identified as grade particle green tea according to the Green Tea Quality Standard (GH016-84) of China. HPLC grade standards of EC, EGC, ECG, EGCG, (+)-catechin (C), and caffeine were purchased from Fisher Chemical Reagent Co., Ltd. Methanol (chromatographic grade) was purchased from Fisher ChemAlert Guide. 2-Deoxyribose (DR) and thiobarbituric acid (TBA) were purchased from Sigma Co. Superoxide dismutase (SOD) reagent kit was purchased from Nanjing Jiancheng Bioengineering Institute, China. The analytical grade of ethanol (95%) and other chemical reagents were from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. HPLC grade water was produced by a purification system (Millipore Direct-Q).

Orthogonal Experimental Design for the Extraction of Catechins. To optimize the extraction of catechins from green tea, an orthogonal

* Author to whom correspondence should be addressed (telephone +86-27-8728-1741; fax +86-27-8728-0781; e-mail chenyq@mail.hzau.edu.cn).

† These authors contributed equally to this work.

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Table 1. Factors and Their Levels of the Orthogonal Experiment L_{16} (4^5)

	factors		
level	*A* (temperature, °C)	*B* (duration, min)	*C* (solvent)
1	30	10	water
2	50	30	35% ethanol
3	70	60	55% ethanol
4	90	90	75% ethanol

design L_{16} (4^5) was conducted using three factors (extraction solvent, temperature, and duration) and four levels as shown in **Table 1**. One gram of the green tea was brewed in 60 mL of the solvent under the indicated condition (**Table 1**). The infusion was filtered by "double-ring" no. 102 filter paper (Xinhua Paper Industry Co. Ltd., Hangzhou, China), and then the filtrate was diluted to 100 mL with ultrapure water for the determination of catechins and caffeine by high-performance liquid chromatography (HPLC).

Preparation of GTE-EGC and GTE-EGCG. *Preparation of GTE-EGC.* Ten grams of green tea sample was brewed in 75% ethanol at 30 °C for 10 min in a thermostated water bath (solvent/tea = 60:1, v/w). The infusion was then filtered. The filtrate was concentrated by a rotary evaporator under reduced pressure and finally dried by using vacuum concentration (N1001, EYELA) at 55 ± 2 °C.

Preparation of GTE-EGCG. Infused leaves after the extraction of EGC were brewed in 35% ethanol at 90 °C for 60 min (solvent/tea = 60:1, v/w). The infusion was then filtered, concentrated, and finally dried as above.

HPLC Analysis of Catechins and Caffeine. Catechins and caffeine in the extracts were determined according to the method of Zhou et al. (*11*). Each sample of green tea extract collected during brewing in the different treatments was filtered through a 0.25 μm filter to be analyzed. Standard curves were calculated from individual catechins and caffeine at different concentrations. Correlations obtained ranged from 0.990 to 0.999. For the HPLC detection (Varian; column, Agilent TC-C 18, 4.6 × 150 mm), wavelength was 280 nm. Two mobile phases were used for gradient elution. Mobile phase A contained 0.1% formic acid in methanol, and mobile phase B contained 0.1% formic acid in water. The following elution gradient plan was adopted: 0−17 min, 25% B; 17−20 min, 25−35% B; 20−25 min, 35% B; 25−27 min, 35−20% B; 27−30 min, 20% B. Injection volume was 20 μL. Flow rate was 1.0 mL min^{-1}.

Antioxidant Activity of GTE-EGC and GTE-EGCG. Scavenging effects of extracts on hydroxyl radicals (·OH) were performed, as described by Halliwell et al. (*12*). Reaction mixtures in a final volume of 1.0 mL contained DR (60 mM), KH_2PO_4/KOH buffer (pH 7.4, 50 mM), $FeCl_3$ (1 mM), EDTA (l.04 mM), GTEs (20.0 mg mL^{-1}, 0.1 mL), H_2O_2 (10 mM), and ascorbic acid (2 mM). Solutions of $FeCl_3$ and ascorbic acid were made up immediately before use. After incubation at 37 °C for 1 h, the color was developed by adding 1 mL of TBA (1%, w/v) and 1 mL of HCl (25%, v/v); the reaction mixtures were then heated in boiling water for 15 min. The absorbance of the resulting solution was measured at 532 nm.

Scavenging effects of extracts on superoxide radicals ($O_2^{\cdot -}$) were assayed by using the xanthine oxidase method (*13*). The measurement was finished following the reagent kit.

Statistical Analysis. Statistical comparisons were performed using the statistical software (SAS Institute, Cary, NC) with analysis of variance (ANOVA). All data were expressed as mean ± standard deviation (SD) of three replicates; means for significant differences were compared by using Duncan's test.

Table 3. Multivariate Analysis of Orthogonal Experiment[a]

	F					
factor	EGCG	EGC	C	EC	ECG	caffeine
A	3.90a	1.68	2.99	2.08	1.44	24.13b
B	3.31a	3.49a	0.88	1.68	1.19	0.71
C	14.27b	3.42a	1.7	2.82	2.14	14.07b

[a] Letters following entries indicate significant effect on the result at (a) $P < 0.05$ and (b) $P < 0.01$. $F(3, 22)_{0.05} = 3.05$, $F(3, 22)_{0.01} = 4.82$.

Table 4. Duncan's Test (LSR) for Variable Levels of Catechins and Caffeine[a]

		mean (μg mL^{-1})		
factor	level	EGC	EGCG	caffeine
A	1	386.4A	277.1B	136.1B
	2	368.2A	265.6B	136.4B
	3	338.9A	337.3B	136.8B
	4	301.3A	437.5A	215.1A
B	1	351.0A	252.0B	161.1A
	2	337.3B	276.0B	162.8A
	3	309.5B	406.3A	150.9A
	4	225.6B	363.3AB	149.8A
C	1	318.8B	208.5C	113.6B
	2	343.4B	386.3A	157.4A
	3	331.7B	314.2B	181.9A
	4	371.0A	342.5A	182.6A

[a] Data are expressed as means of $n = 3$ treatments. Different letters express significant difference among levels of a single factor for catechins by Duncan's test (LSR) ($P < 0.05$).

Table 2. Results of Catechins and Caffeine Content in the Orthogonal Experiment L_{16} (4^5)[a]

	factors									
treatment	A	B	C	EGCG	EGC	C	EC	ECG	caffeine	total catechins
1	1	1	1	126.7 ± 3.6	358.1 ± 0.2	187.4 ± 0.2	49.6 ± 1.5	88.6 ± 6.8	107.0 ± 8.9	810.3 ± 11.4
2	1	2	2	308.8 ± 1.6	403.3 ± 9.4	188.6 ± 1.5	57.6 ± 0.1	141.0 ± 0.6	129.4 ± 4.1	1094.3 ± 3.9
3	1	3	3	368.3 ± 0.4	401.7 ± 5.8	190.3 ± 0.3	61.2 ± 1.0	189.8 ± 30.3	157.0 ± 2.0	1211.3 ± 25.4
4	1	4	4	304.5 ± 3.4	382.7 ± 16.0	188.7 ± 0.6	56.2 ± 1.5	179.7 ± 14.2	151.2 ± 3.1	1108.2 ± 36.2
5	2	1	2	312.8 ± 0.1	375.0 ± 9.1	190.0 ± 0.1	55.1 ± 0.7	176.8 ± 27.0	127.2 ± 5.5	1109.8 ± 35.3
6	2	2	1	199.3 ± 3.7	388.0 ± 13.0	190.0 ± 0.4	56.7 ± 1.6	113.4 ± 0.3	122.1 ± 28.2	947.6 ± 11.1
7	2	3	4	320.4 ± 2.7	377.9 ± 8.6	187.4 ± 0.3	58.2 ± 1.3	165.6 ± 1.3	129.5 ± 15.0	1109.5 ± 3.2
8	2	4	3	230.0 ± 1.8	331.7 ± 6.8	190.0 ± 0.3	54.1 ± 5.8	113.5 ± 16.0	166.6 ± 2.4	916.8 ± 24.6
9	3	1	3	228.7 ± 9.6	380.6 ± 7.3	190.3 ± 0.2	57.1 ± 0.7	120.8 ± 1.5	164.2 ± 0.9	977.5 ± 0.1
10	3	2	4	269.9 ± 7.6	345.2 ± 4.7	188.8 ± 0.7	55.8 ± 0.2	169.7 ± 26.6	159.9 ± 0.2	1024.9 ± 29.1
11	3	3	1	213.8 ± 0.1	298.7 ± 0.1	190.2 ± 0.6	47.3 ± 0.1	112.0 ± 0.1	83.7 ± 0.1	861.9 ± 0.6
12	3	4	2	372.9 ± 6.1	371.3 ± 16.4	190.5 ± 0.4	57.8 ± 1.9	209.5 ± 36.5	139.5 ± 6.9	1201.9 ± 11.7
13	4	1	4	435.3 ± 6.2	578.1 ± 17.4	192.4 ± 0.8	82.5 ± 5.2	194.8 ± 6.3	245.8 ± 20.4	1476.9 ± 30.0
14	4	2	3	229.8 ± 4.5	212.7 ± 10.3	190.6 ± 0.1	68.5 ± 7.7	221.3 ± 47.5	239.6 ± 16.9	862.9 ± 1.1
15	4	3	2	390.7 ± 13.4	223.9 ± 28.7	188.6 ± 0.1	65.4 ± 10.5	219.1 ± 84.6	233.5 ± 21.0	1000.1 ± 49.7
16	4	4	1	294.3 ± 16.7	230.5 ± 28.7	190.9 ± 0.3	56.5 ± 3.0	212.3 ± 43.3	141.7 ± 18.1	980.0 ± 87.5

[a] Data are expressed as means ± SD of $n = 3$ treatments.

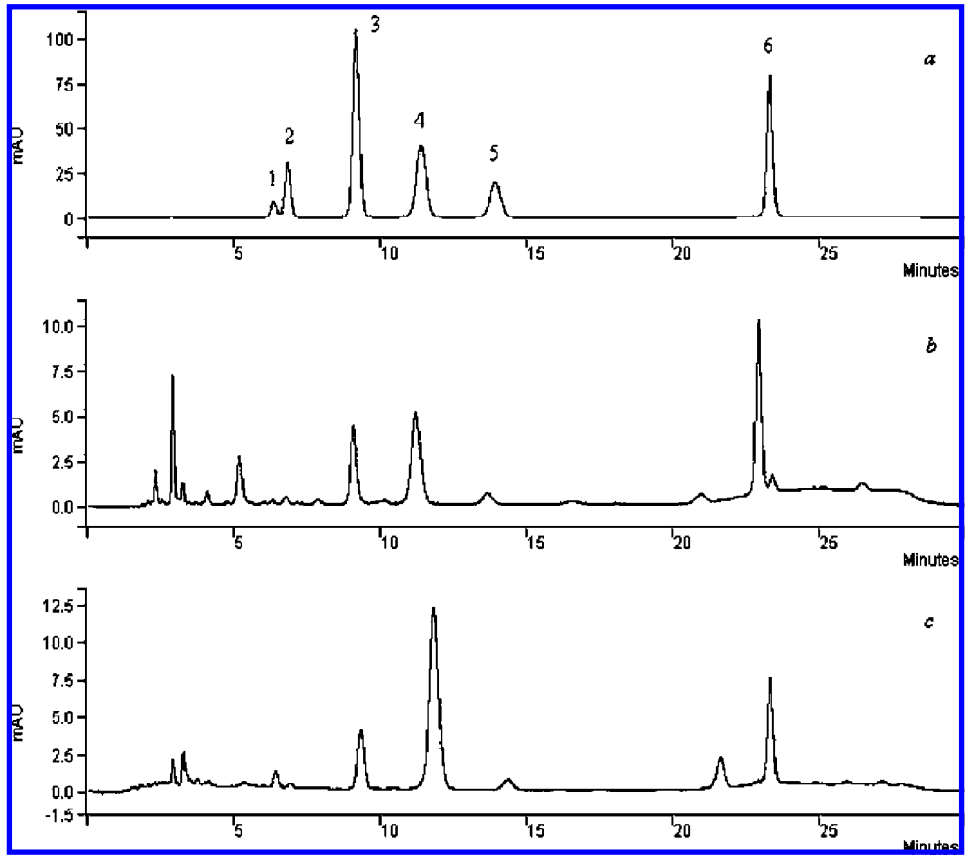

Figure 1. Chromatograms of HPLC analysis: (**a**) standard of catechins and caffeine; (**b**) GTE-EGC; (**c**) GTE-EGCG. Peaks: 1, EGC; 2, C; 3, caffeine; 4, EGCG; 5, EC; 6, ECG.

Table 5. Concentrations of Constituents in the Solutions of GTE-EGC and GTE-EGCG[a]

	GTE-EGC (μg mL^{-1})	GTE-EGCG (μg mL^{-1})
EGC	195.9a ± 0.0	127.9b ± 0.0
EGCG	134.4b ± 0.0	282.8a ± 0.0
EC	123.5 ± 0.0	118.0 ± 0.0
C	31.2a ± 0.0	22.9b ± 0.0
ECG	222.3 ± 0.0	222.9 ± 0.0
caffeine	62.4a ± 0.0	31.6b ± 0.0
total catechins	707.3 ± 2.8	774.5 ± 5.5

[a] Data are expressed as means ± SD of $n = 3$ treatments. Different letters following means in the same row express significant difference between two extracts by Duncan's test (LSR) ($P < 0.01$).

Table 6. Scavenging Rate of $^{\bullet}OH$ and $O_2^{\bullet-}$ by GTE-EGC and GTE-EGCG[a]

GTE	scavenging rate of $^{\bullet}OH$ (%)	scavenging rate of $O_2^{\bullet-}$ (units mL^{-1})
EGC	28.4a ± 4.0	5.50b ± 1.0
EGCG	72.2a ± 2.2	24.3b ± 0.7

[a] Data are expressed as means ± SD of $n = 3$ treatments. Different letters following means in the same row express significant difference between two extracts by Duncan's test (LSR) ($P < 0.01$).

RESULTS

Results of Orthogonal Experimental of Catechins and Caffeine Contents. The results of the orthogonal experiment and the ANOVA are presented in **Tables 2** and **3**. Mean contents of EGC, EGCG, and caffeine at different levels and factors are shown in **Table 4**.

The solubilization of EGC depended significantly only on the solvent and duration ($P < 0.05$, **Table 3**). The maximum content of EGC (371.0 μg mL^{-1}) was created by extraction with 75% ethanol, and the maximum concentration was immediately reached at 10 min (**Table 4**).

The solubilization of EGCG highly depended on the three factors ($P < 0.05$, **Table 3**), and the influence intensity was in the order of extracting solvent > temperature > duration. The content of EGCG sharply enhanced and reached peak value (386.3 μg mL^{-1}) when extracted with 35% ethanol. When extracted at 90 °C, the concentration increased to 437.5 μg mL^{-1}; it was statistically higher than other temperature treatments ($P < 0.05$). The content affected by duration was increased first and decreased afterward, and its maximum concentration reached 406.3 μg mL^{-1} at 60 min (**Table 4**).

However, EC, C, and ECG were not significantly affected by the involved factors. Meanwhile, the content of caffeine in the samples was obviously influenced by solvent and temperature ($P < 0.05$, **Table 3**).

Through the statistical analysis mentioned above, the optimized conditions for extracting EGC and EGCG were as follows: green tea brewed with 75% ethanol at 30 °C for 10 min to prepare GTE-EGC, and the residue brewed with 35% ethanol at 90 °C for 60 min to prepare GTE-EGCG.

Constituent Concentrations of GTE-EGC and GTE-EGCG. According to the HPLC data (**Figure 1** and **Table 5**), it appeared that the GTE-EGC and GTE-EGCG were very different in catechin components ($P < 0.05$). In the first GTEs, EGC accounted for 27.69% of total catechins, whereas in the second GTEs, EGG accounted for 16.51% of total catechins, decreased by 40.38%. Meanwhile, in the first GTEs, EGCG accounted for 19.00% of total catechins, whereas in second GTEs, EGCG accounted for 36.51% of total catechins, increased by 110.42%. There were few changes in the contents of EC

and ECG of the two GTEs. The concentrations of C and caffeine in both extracts were also discrepant ($P < 0.01$), and they were reduced by 26.60 and 49.36% in the second extract, respectively. These results confirm the fact that it is possible to produce GTEs enriched in EGCG and EGC by brewing tea in a two-step extraction. GTE-EGC could be prepared in the first extraction and GTE-EGCG obtained by the extraction of infused leaves after the first brewing step.

Effects of GTE-EGC and GTE-EGCG on Antioxidant Activity. The antioxidant activities of GTE-EGC and GTE-EGCG at the same concentration are shown in **Table 6**. Comparatively, GTE-EGCG, which had a scavenging rate of 72.2% of $^{\bullet}OH$, was the more effective one; it had a better scavenging effect (24.3 units mL^{-1}) on $O_2^{\bullet -}$. The scavenging rate of GTE-EGCG was higher than that of GTE-EGC (28.4% and 5.50 units mL^{-1}), increased by 154.23 and 342.82%, respectively.

DISCUSSION

Researchers usually use boiling water or organic solvents such as acetonitrile, methanol, ethyl acetate, and ethanol to extract catechins. Although water extraction is favorable for its cost and safety, the yield is lower compared with organic solvent extraction. Earlier in 1995, Suematsu et al. (*14*) probed the effect of solvents on catechin extraction and considered that 50% acetonitrile was the most suitable solvent at room temperature, as did Shishikura et al. (*15*). Methanol was also proved to be good for catechin extraction by Yao et al. (*16*). However, these solvents are harmful to human bodies, and the products could not be used in medicine, cosmetics, and food. Ethanol, which is harmless to humans, is more suitable to extract catechins (*17*). Rusak et al. (*18*) studied the effects of different extraction solvents on the quantitative and qualitative contents of phenolics in tea infusions and concluded that 40% ethanol was the most effective in the prolonged extraction of catechins, especially EGCG. The 35% ethanol used for the preparation of GTE-EGCG in our results shows considerable agreement with the above studies. Ethanol is effective in catechin extraction, mainly due to the existence of mass phenolic hydroxyl and benzenoid in catechin molecules, an association reaction that takes place among catechin molecules under the hydrogen bond and hydrophobic group, and the fact that ethanol can destroy hydrogen bonds and prevent association reactions among catechin molecules, thereby favoring the extraction of tea catechins and improving extraction efficiency (*19*). Meanwhile, ethanol has protective effects on catechins and can prevent epimerization among catechin molecules during extraction and separation (*20*). Thus, catechins cannot be easily oxidated.

The study also showed that EGC was highly dependent on duration but independent of extraction temperature, whereas EGCG was not only very dependent on duration but also extraction temperature. These results are in agreement with Bazinet et al. (*21*) and Labbe et al. (*10*). Labbe et al. divided catechins into two groups: the time-dependent compounds (EGC and EC) and the time/temperature-dependent compounds (C, EGCG, GCG, and ECG). However, in this study, the solubilization of EC and ECG, which were independent of temperature and duration, opposed their conclusion. Price et al. (*22*) suggested that the ungallated epicatechin and epigallocatechin infused more quickly than the gallated flavanols epicatechin gallate and epigallocatechin gallate through detecting rate constant. They further proposed that the infusion rate should be related to the inverse of the square root of the mass of the molecule. Moreover, the stability of tea catechins was subject to heating temperature and duration, and high temperature and long duration would accelerate their degradation during solubilization in aqueous systems (*23*, *24*). As shown in the results of Su et al. (*24*), infusion heating at 100 °C for 30 min degraded only 5% of the green tea catechins, and EC, EGC, EGCG, and ECG had similar rates of degradation in the boiling water. On the basis of this work, as well as previous studies, we reduced the extraction temperature in the first step to protect EGC and prolonged the duration in the second step for more EGCG solubilization.

Catechins are the main active components in green tea. It has been found that green tea polyphenol extract as well as individual catechins could activate antioxidant-responsive element (ARE)-dependent genes in transfected HepG2 cells (*25*). Studies also suggested that green tea extracts have strong capability of scavenging free radicals and anticancer activity (*11*, *26*). In the present research, GTE-EGCG displayed greater antioxidant activity, which might be caused by its high total catechins and EGCG content, especially EGCG. Generally, the antioxidative activity of tea catechins is structure dependent (*27*). The three adjacent hydroxyl (OH) groups at positions C-3′, -4′, and -5′ on the B rings of (−)-EGCG, (−)-EGC, (−)- GCG, and (−)-GC are more effective in scavenging free radicals than the two adjacent OH groups at C-3′ and -4′ in (−)-ECG, (−)-CG, (−)-EC, and (−)C, respectively. Moreover, catechins with an additional gallate moiety at C-3 generally hold stronger scavenging effects than non-gallate catechins, that is, (−)-ECG > (−)-EC, and (−)-EGCG > (−)-EGC (*28*, *29*).

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