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Simultaneous determination of catechins and procyanidins in bottled tea drinks by LC/MS

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ABSTRACT

Catechins, major polyphenol constituents of green tea, are well known because of their antioxidative activity and chemopreventive effects against cancers. Bottled tea drinks contain not only green tea epicatechins, namely (−)−epigallocatechin gallate (EGCG), (−)−epicatechin gallate (ECG), (−)−epigallocatechin (EGC) and (−)−epicatechin (EC), but also four tea catechin epimers, namely (−)−gallocatechin gallate (GCG), (−)−catechin gallate (CG), (−)−gallocatechin (GC) and (+)−catechin (C). These catechin epimers in bottled tea drinks are produced during the sterilization step in their manufacture. In the present study, we determined the amounts of the eight tea catechins and two procyanidins, including heat−epimerized catechins in bottled oolong tea drinks by liquid chromatography/ mass spectrometry (LC/MS). Oolong tea is a traditional Chinese tea that has long been believed to be beneficial to health, such as by decreasing body fat. We investigated the antioxidative activity of heat−epimerized catechins compared with their corresponding precursors. The antioxidative activity of the heat−epimerized catechins were greater than the parent tea catechins and procyanidins.

Key words: LC/MS; catechins; procyanidins; bottled tea drinks; heat−epimerized catechin; oolong tea; epigallocatechin gallate (EGCG); gallocatechin gallate (GCG); antioxidative activity.

INTRODUCTION

Recently, bottled tea drinks are becoming increasingly popular, and the consumption of bottled tea drinks is increasing in Japan. It is generally believed that polyphenols are active components responsible for beneficial effects associated with drinking tea. Green tea leaves (*Camellia sinensis L.*) contain low−molecular −weight polyphenols consisting mainly of flavan−3−ol monomers, which are referred to as catechins. As shown in Fig. 1, there are several isomers of this compound: (+)−catechin (C), (−)−epicatechin (EC), (−)−epigallocatechin (EGC), (−)−epicatechin gallate (ECG), and (−)−epigallocatechin gallate (EGCG). These tea catechins have been reported to have various physiological functions, such as antiviral [1], antioxidative [2, 3], antimutagenic [4, 5], anticarcinogenic [6], and antiobesity [7] activities. A hypocholesterolemic activity of catechins has also been reported in experimental animals [8−11]. Consequently, interest in the therapeutic applications of tea catechins for the prevention of disease has been increasing.

Although the total amounts of catechins in green tea and oolong tea have been reported, information on the compositions of catechins in bottled tea drinks has been limited [12]. Oolong tea and green tea are popular beverages in Japan and China. These teas are manufactured from the same plant species, *Camellia sinesis L.*, and are produced through different processing methods. Oolong tea

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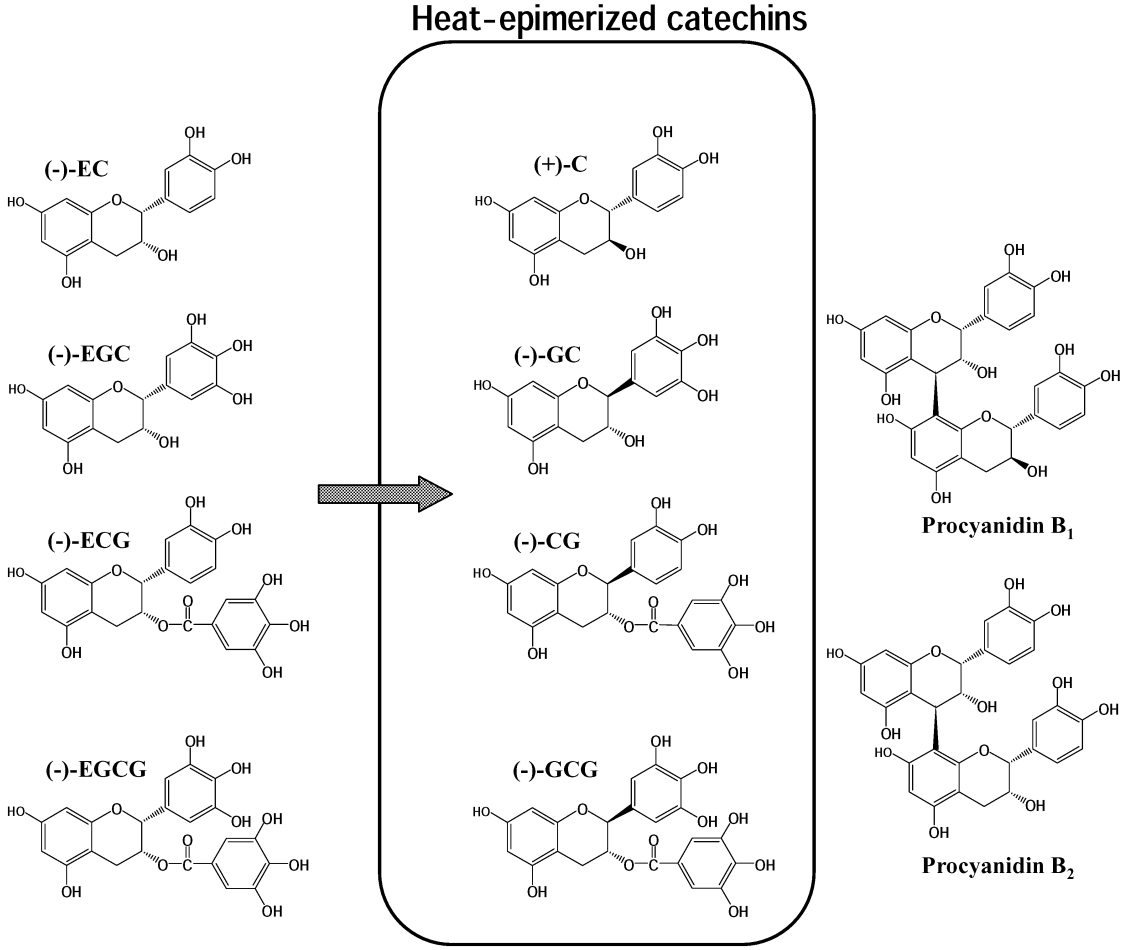

Figure 1. Structures of catechins.
(+)− C, (+)− catechin; (−)−EC, (−)−epicatechin; (−)−EGC, (−)−epigallocatechin; (−)−EGCG, (−)−epigallocatechin gallate; (−)−ECG, (−)−epicatechin gallate; (−)−GC, (−)−gallocatechin; (−)−CG, (−)−catechin gallate; (−)−GCG, (−)−gallocatechin gallate; Procyanidin B_1, epicatechin−(4 β→8)−catechin; Procyanidin B_2, epicatechin−(4 β→8)−epicatechin.

is semi−fermented, and green tea is not fermented. Oolong tea has long been believed to be beneficial to health, such as by decreasing body fat. In the present, we study measured the amount of tea catechins in bottled oolong tea drinks that are commonly consumed in Japan, using liquid chromatography/ mass spectrometry (LC/MS). The heat−epimerized catechins present in bottled tea drinks are produced during the sterilization step while the drink is being manufactured. These products are autoclaved for pasteurization, generally at 120℃ for several minutes. During pasteurization, considerable amounts of catechins are epimerized at the 2−position. EC, EGC, ECG, and EGCG are epimerized to C, (−)−gallocatechin (GC), (−)−catechin gallate (CG), and (−)−gallocatechin gallate (GCG), respectively [13].

In this study, the effect of heat−epimerized catechins on the antioxidative activities and radical scavenging activity was compared with tea catechins *in vitro*.

EXPERIMENTAL

Materials. Bottled oolong teas were purchased from the market. All of the following chemicals were obtained from Sigma−Aldrich Inc. (Germany): CG (M.W.:442.4), GC (M.W.:306.3), C (M.W.: 290.3), EC (M.W.:290.3), EGC (M.W.:306.3), ECG (M.W.:442.4), EGCG (M.W.:458.4), GCG (M.W.:458.4), procyanidin B_1 (M.W.: 578.5), procyanidin B_2 (M.W.:578.5), 5,5−dimethyl−1−pyrroline−*N*−oxide (DMPO), hypoxanthine (HPX). All of the chemicals listed below were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan): 4−hydroxybenzoic acid, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, acetonitrile, acetic acid, methanol, ethanol, linoleic acid, Tween 40, chloroform, β−carotene, acetylthiocholine iodide, diethylenetriamine−*N, N, N', N'', N''*− pentaacetic acid (DTPA), xanthine oxidase (XOD), superoxide dismutase (SOD) from bovine erythrocyte, thiobarbituric acid (TBA), 2,6−di−*t*−butyl−4−methylphenol (BHT), *n*−butyl alcohol, pyridine.

LC/MS conditions. The assay of catechins in bottled oolong tea drinks was developed using a Model M−8000 LC/MS system (HITACHI, Tokyo, JAPAN) with SSI (sonic spray ionization) by a negative mode. The analytical column was an Inertsil ODS−3 (150 x 2.1 mm I.D., GL Science, Tokyo, JAPAN) operated at 40℃. For the quantitative analysis of catechins included in bottled tea drinks, 1% CH_3COOH:CH_3OH (95:5) was used as mobile phase A, and the ratio in mobile phase B was 5:95; the gradient ran from 10% B up to 55% B over 30 min, at a flow rate of 0.2 mL/min. Nitrogen gas was used as a carrier gas with an output pressure of 0.50 MPa and an ion source inlet pressure at 0.39 MPa. The drift voltage was 40 V. The sampling aperture was heated at 120℃ and the shield temperature was 230℃.

The individual catechin derivatives were quantified and calibrated using 4−hydroxybenzoic acid as an internal standard (I.S.). The linear regression of the peak area ratios of catechins/I.S. vs. concentration was used to obtain a calibration curve, and the regression equation was then used to calculate the concentrations of catechins. The calibration curve ranged from 3.125 μg/mL to 50 μg /mL for GC, EGC, C, EGCG, EC, GCG, EC and CG, and from 0.100 μg/mL to 3.125 μg/mL for procyanidin B_1 and procyanidin B_2, respectively. The linear regression equations and the correlation coefficients from the calibration curves were $y = 0.0463 x + 0.1104$ ($r^2 = 0.9966$) for GC, $y = 0.0924 x - 0.0140$ ($r^2 = 0.9954$) for procyanidin B_1, $y = 0.0434 x + 0.0115$ ($r^2 = 0.9985$) for EGC, $y = 0.0560 x + 0.0988$ ($r^2 = 0.9955$) for C, $y = 0.0396 x - 0.0066$ ($r^2 = 0.9969$) for procyanidin B_2, $y = 0.0547 x - 0.0926$ ($r^2 = 0.9990$) for EGCG, $y = 0.0579 x + 0.0967$ ($r^2 = 0.9981$) for EC, $y = 0.0995 x - 0.2788$ ($r^2 = 0.9965$) for GCG, $y = 0.1018 x - 0.0432$ ($r^2 = 0.9993$) for ECG, $y = 0.1539 x + 0.0253$ ($r^2 = 0.9987$) for CG, respectively.

Sample preparation of bottled tea drinks. Ten brands of bottled oolong tea drinks were purchased from several local supermarkets in Japan. After the drinks were vigorously shaken, 1.4 mL aliquots of each were mixed with 0.1 mL of a 4−hydroxybenzoic acid internal standard solution (2 mg/mL). The mixture was then filtered with a 0.45 μm membrane filter (DISMIC−13HP, PTFE, poly−tetrafluoroethylene, Advantec Co., Ltd. Japan) and a 10 μL aliquot of the sample was subjected to HPLC analysis.

ESR assay

Evaluation of the $O_2^{\cdot-}$ scavenging activity: Using the Electron spin resonance (ESR) spectroscopic spin−trapping technique with an hypoxanthine−xanthine oxidase (HPX−XOD) reaction system, the $O_2^{\cdot-}$ scavenging activity was estimated, as reported method [17] with a slight modification. All of the chemicals were dissolved in a 0.1 M phosphate buffer (pH 7.8). A 15 μL amount of 9.2 M DMPO, 35 μL of 9.5 mM DTPA, 50 μL of 2 mM HPX, 50 μL of catechin solution and 50 μL of 0.4 unit/mL XOD were used. A SOD solution (0 − 50 unit/mL) instead of the catechin solution was used for preparing a calibration curve. The $O_2^{\cdot-}$ scavenging activity of the catechin was expressed in terms of SOD−like activity (unit/mg).

ESR spectroscopy conditions: The ESR spectra were recorded on a computerized JES−RE 1 X spectrometer (JEOL, Tokyo, Japan). The ESR spectroscopic conditions were set as follows: magnetic field, 335.2 ± 5 mT; power, 8 mW; sweep time, 2 min; modulation, 79 μT; amplitude, 200; time constant, 0.1 s.

TBA assay

The antioxidative activity was measured by a slightly modified TBA method using linoleic acid [18]. One mL of a linoleic acid solution (125 mg of linoleic acid was dissolved in 100 mL of a 1% SDS solution) and different concentrations of a catechin 80%−ethanol solution in test tubes were incubated for 18 h at 60℃, and cooled on ice. Then, 250 μL of a 0.5% BHT ethanol solution and 1 mL of a 0.4% TBA solution in phosphate buffer (pH 3) were added, and the mixture was kept for 30 min at 5℃ with an air−tight cap. It was shaken, and heated for 1 h at 95℃ with a loose cap. After a 2.5 mL amount of distilled water was added, the reaction solution was shaken with an extraction solution (1−butanol and pyridine in the ratio 15:1), and centrifuged at 1600 × g for 20 min. The absorbance of the supernatant was measured at 532 nm, and the antioxidative activity of catechin was expressed in 50% inhibitory concentration (IC_{50}). The IC_{50} values of catechins were determined graphically.

β −carotene bleaching assay

According to the method [19], with a slight modification, the antioxidative activity of catechin was determined. A 0.25 mL amount of 0.1 mg/mL β−carotene in chloroform, 0.1 mL of 0.1 g/mL linoleic acid in chloroform, and 0.5 mL of 20% w/v Tween 40 in chloroform were mixed in a vessel, and the chloroform was removed by N_2 gas. Emulsion carotene−linoleate had been prepared by adding 45 mL of distilled water and 5 mL of a 0.2 M phosphate buffer (pH 6.8) to the mixture. A 0.1 mL amount of different concentrations of catechin 80%−ethanol in test tube was mixed vigorously with 4.9 mL of the emulsion carotene−linoleate. Immediately, the absorbance of the mixture was measured at 470 nm. After standing the mixture at 50℃ for 60 min, the absorbance of the mixture was measured again. Using the degradation rate of β−carotene, the antioxidative activity of catechin was expressed in IC_{50}. The IC_{50} values of catechins were determined graphically.

RESULTS AND DISCUSSION

Composition of tea catechins in bottled tea drinks.

The total ion (TIC) and mass chromatogram of eight catechins and two procyanidins under the negative ion mode are shown in Fig.2. The retention times of GC, procyanidin B_1, EGC, C, 4−hydroxybenzoic acid, procyanidin B_2, EGCG, EC, GCG, ECG, and CG were 6.02 min, 9.63 min, 12.67 min, 13.23 min, 14.61 min, 15.33 min, 18.14 min, 19.37 min, 21.11 min, 23.73 min, and 25.71 min, respectively. The deprotonated ions $[M-H]^-$ of catechins and procyanidins were clearly observed as a base peak, in each spectrum. Many bottled tea drinks are available at local markets in Japan. Fig. 3 A shows the TIC of catechins in bottled oolong tea drinks. The contents of catechins in different brands of bottled oolong tea drinks are given in Table 1. The precision of the method was established from 3 assays. The coefficient of variation (C.V.) values for the intra− and inter−day assay for the peak areas of catechins on mass chromatograms were less than 5% and 7%, respectively. As can be seen in Table 1, the contents of catechins in these tea drinks differ significantly. The percentage composition of individual catechins in these tea drinks is different from that in green tea. Normally, 10−20% of the catechins in green tea leaves are EGC and EGCG [21]. The relative percentage of heat−epimerized catechins accounted for only 0.84−1.43% of the total catechins in green tea. In contrast, the oolong tea drinks contained mostly EGCG (30−47%), followed by GCG (21−34%), GC (14−27%), and EGC (9−17%). The relatively high heat−epimerized catechins level in bottled tea drinks is derived from the thermal conversion of tea catechins. This indicates that EGCG, ECG, EGC, and EC were

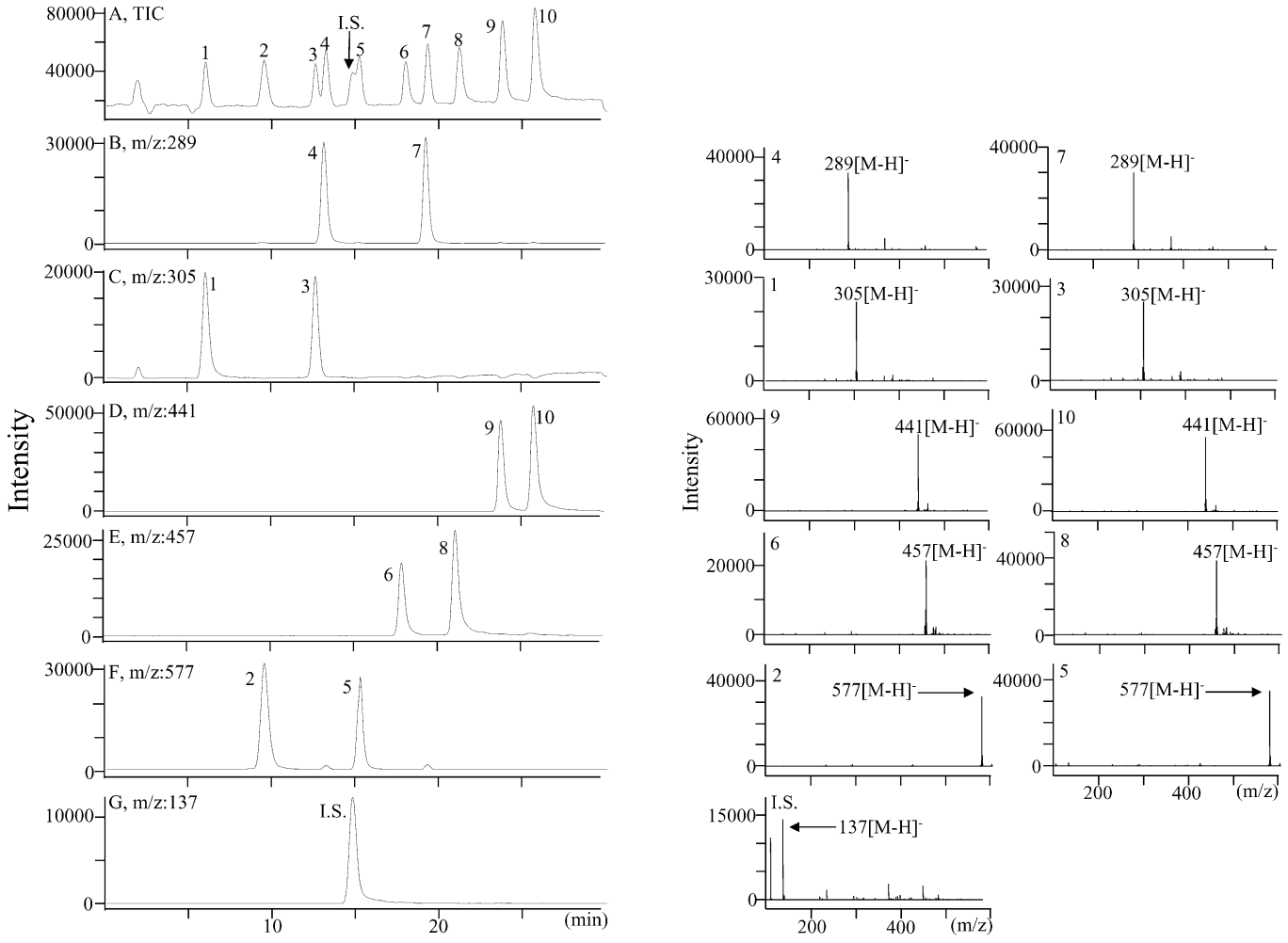

Figure 2. LC/MS analysis of standard catechins.

Total ion chromatogram (TIC) (A), Mass chromatogram at m/z:289 (B), at m/z:305 (C), at m/z:441 (D), at m/z:457 (E), at m/z:577 (F), at m/z:137 (G). Mass spectrum of catechins and procyanidins (1−10). Peak numbers are 1, GC; 2, Procyanidin B_1; 3, EGC; 4, C; 5, Procyanidin B_2; 6, EGCG; 7, EC; 8, GCG; 9, ECG; 10, CG; I.S., 4−hydroxybenzoic acid. LC/SSI−MS conditions: Column, Inertsil ODS−3 (4.6×150 mm I.D.) ; Column temperature , 40℃ ; Injection volume, 5 μL ; Eluent (A), 1% CH_3COOH : MeOH = 95 : 5; Eluent (B), 1% CH_3COOH : MeOH = 5:95 ; gradient condition 10−55% B (30 min); Flow rate, 0.2 mL/min ; MS−detector, Ion source, SSI (Sonic Spray Ionization) negative mode ; Shield temperature, 230℃; Sampling aperture, 120℃; Drift voltage, 40 V.

Table 1. Composition of oolong tea catechins in bottled drinks

Sample	catechins (μg/ml)									
	EGC	GC	EC	C	EGGG	GGG	ECG	CG	Procyanidin B_1	Procyanidin B_2
A	254.21 ±4.36	280.01 ±4.11	66.05 ±0.71	81.42 ±2.60	587.24 ±4.97	432.19 ±2.58	169.78 ±2.33	90.83 ±0.56	3.49 ±0.11	4.41 ±0.02
B	29.08 ±1.24	43.14 ±1.85	7.44 ±0.09	10.91 ±0.23	77.64 ±1.19	57.15 ±2.37	20.10 ±0.50	11.15 ±0.21	0.71 ±0.03	0.52 ±0.02
C	39.16 ±0.85	55.36 ±1.64	7.86 ±0.21	15.05 ±0.06	95.80 ±3.89	66.01 ±1.27	19.67 ±0.11	13.06 ±0.26	1.13 ±0.04	0.42 ±0.01
D	16.15 ±0.18	25.77 ±0.05	5.04 ±0.07	8.34 ±0.23	55.01 ±0.81	46.23 ±0.54	12.64 ±0.28	10.35 ±0.16	0.65 ±0.03	0.22 ±0.01
E	31.89 ±0.41	41.01 ±1.93	7.26 ±0.34	9.92 ±0.40	91.48 ±2.67	63.53 ±1.48	23.26 ±0.61	11.69 ±0.19	0.69 ±0.03	0.58 ±0.02
F	57.18 ±0.41	71.55 ±2.23	13.42 ±0.35	23.18 ±46.00	131.12 ±3.34	104.28 ±1.72	30.75 ±0.83	22.92 ±0.78	2.13 ±0.05	0.72 ±0.03
G	33.72 ±1.54	43.66 ±1.22	6.55 ±0.12	8.24 ±0.35	92.61 ±3.36	66.52 ±0.51	18.24 ±0.84	11.03 ±0.47	0.83 ±0.02	0.51 ±0.02
H	70.85 ±1.91	110.75 ±3.85	12.40 ±0.59	23.75 ±1.14	191.73 ±7.40	140.52 ±5.41	40.65 ±1.90	27.80 ±0.72	0.49 ±0.02	0.24 ±0.01
I	35.02 ±0.76	50.08 ±2.24	6.75 ±0.16	10.24 ±0.29	109.40 ±1.87	83.98 ±0.79	24.97 ±1.15	17.21 ±0.75	0.47 ±0.02	0.29 ±0.01
J	18.43 ±0.84	37.87 ±1.23	8.96 ±0.34	17.83 ±0.48	74.49 ±2.15	54.62 ±1.02	14.15 ±0.50	9.97 ±0.02	1.67 ±0.03	0.28 ±0.01

Values are expressed as mean ± SD. n = 3 observations per sample. The C.V. % values are between 0.2 and 4.8.

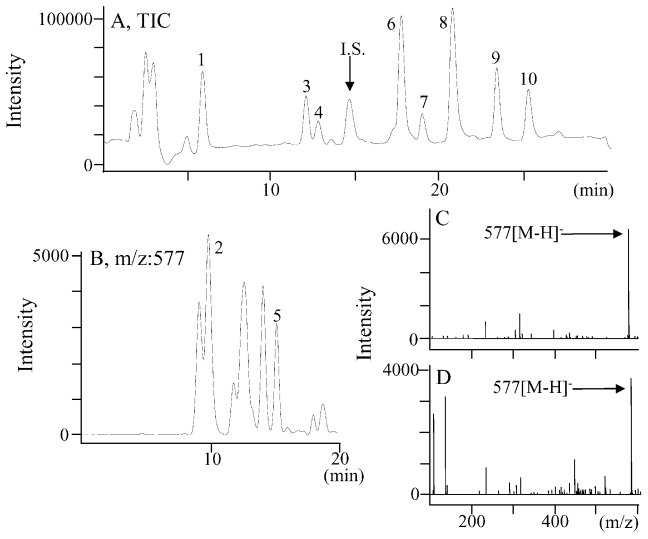

Figure 3. LC/MS analysis of catechins in bottled oolong tea drink. Sample, oolong tea drink (sample A). Total ion chromatogram (TIC) (A), mass chromatogram at m/z 577(B), mass spectrum of the peak with retention time 9.63 min (C) , mass spectrum of the peak with retention time 15.33 min (D). LC/MS conditions and peak numbers are the same as Fig. 2.

susceptible to epimerization and converted to their corresponding C−2 epimers, namely GCG, CG, GC, and C at 95℃ for 30 min. The result was in agreement with previous reports [12, 22] that considered the epimerization reactions of catechins. It was known that heat−epimerized catechins present in bottled tea drinks are produced during the sterilization step in their manufacture.

Particularly, one brand of bottled tea drink (sample A) contained relatively greater amounts of each catechin as compared with other tea drinks. It has been known that high concentrations of catechins showed antiobesity activities [23, 24]. A recent report shows that the ingestion of a tea rich in catechins leads to a reduction in body fat and malondialdhyde−modified LDL in men [23]. Ikeda et al. reported that heat−epimerized tea catechins rich in GCG and CG are more effective to inhibit cholesterol absorption than tea catechins rich in EGCG and ECG [14]. These reports suggest that tea catechins (also in bottled tea drinks) might be useful in the prevention and improvement of obesity and lifestyle−related diseases through improving lipid metabolism. Recently, tea beverages including enriched catechins for body fat reduction, such as sample A, have been commercialized in Japan.

Bottled oolong tea drinks contain procyanidins, which are

known as condensed catechins. Compared with green tea, oolong tea contains approximately half the EGCG, while polymerized polyphenols are double. The polymerized polyphenols of oolong tea, such as procyanidins, are produced by its unique fermentation. The effect of oolong tea on nutrient absorption may be an important factor on weight reduction. There are some studies that showed the inhibition of fat [25]. A recent study showed that oolong tea increases energy expenditure by its polymerized polyphenols [26]. The contents of procyanidin B_1 [epicatechin−(4 β→8)−catechin] and procyanidin B_2 [epicatechin−(4 β→8)−epicatechin] in bottled oolong tea drinks were found to be 0.47−3.49 μg/mL and 0.22−4.41 μg/mL, respectively. Mass chromatogram of oolong tea drink (sample A) at m/z 577 under the negative ion mode is shown in Fig. 3 B. Figs. 3 C, D show the mass spectrum of the peak of procyanidin B_1 with a retention time of 9.63 min, and that of the peak of B_2 with a retention time of 15.33 min, respectively. The deprotonated ion $[M-H]^-$ of procyanidin B_1 and B_2 was clearly observed at m/z 577 as a base peak, in each spectrum.

Anti−oxidative Effects and Radical Scavenging Activity of Tea Catechins.

The anti−oxidative effects of catechins, found by using the β−carotene discolor method and the TBA assay, are listed in Table 2. EGCG and GCG have antioxidative activities on the auto−oxidation of linoleic acid. Although procyanidins have been reported to have powerful antioxidative activity [27, 28] , procyanidins show less antioxidative activity than EGCG and GCG in our present results. Interestingly, GCG shows more antioxidative activity than that of EGCG based on the TBA assay. Chen et al. [12] reported that the stability of catechin is pH−dependent. It was found that the higher is the pH value of the medium, the greater is the percentage of degraded catechin. These results indicate that GCG might have more stable antioxidative activity than that of EGCG.

Table 2. Anti−oxidative effects of tea catechins

	IC_{50} (μM)			
	EGCG	GCG	Procyanidin B_1	Procyanidin B_2
The discoloration of β−carotene (pH 6.8)	57.0 ±12.1	66.9 ±11.2	937.0 ±33.1	922.4 ±13.0
The thiobarbituric acid assay (pH 3.0)	138.7 ±11.9	60.7 ±7.8	190.5 ±23.9	194.8 ±20.0

Table 3. Radical scavenging activity of tea catechins

	EGCG	GCG	Procyanidin B_1	Procyanidin B_2
SOD−like activity (Unit/mg)	588 ±21.2	655 ±23.6	124 ±4.5	123 ±3.7

The scavenging activities of catechins on superoxide anions generated by the HPX−XOD system using ESR, are listed in Table 3. The superoxide anions ($O_2^{\cdot-}$) scavenging potentials of heat−epimerized catechins *in vitro* were comparable to those of tea catechins. This agrees with Unno et al. [29], who reported on the scavenging activity of tea catechins on superoxide radicals generated by a phenazine methosulfate and the NADH system.

These results suggest that heat−epimerized catechins have the same, or greater, antioxidative activities compared to tea catechins. It has also been suggested that catechins can reduce oxidative stress by their antioxidative properties.

There are considerable amounts of heat−epimerized catechins, such as GCG and CG, in bottled tea drinks. The present study showed a possibility that heat−epimerized catechins may be more effective for antioxidative activity than the parent tea catechins and procyanidins.

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