Original Article

Structural specificity of electric potentials in the coulometric-array analysis of catechins and theaflavins

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We have established a novel method to evaluate the redox properties of tea polyphenols by HPLC-coulometric-array analysis. We plotted the quantity of electricity (μC) on the vertical axis and the electric potential (mV), adjusted with the associated palladium reference electrode, on the horizontal axis to provide "quantity versus potential (QP) plot". The patterns of the plots correspond to the derivative of a hydrodynamic voltammogram or a current-voltage curve, with the electric potentials of the peaks in the QP plot corresponding to the half-wave potentials in the current-voltage curve. We confirmed that catechins and theaflavins are oxidized depending on the electric potentials of their partial structures, and found that all compounds showing a peak at 0 mV in the QP plots produce hydrogen peroxide (H_2O_2) during the autoxidation process.

***Key Words*: catechins, coulometric-array analysis, electric potentials, hydrogen peroxide, theaflavins**

Oxidation of biological components in our body by reactive oxygen species and free radicals have been thought to be major or minor factors in various life-style related diseases, such as cardiovascular diseases, diseases of nervous system, diabetes, cancer and aging. To prevent these diseases, plant polyphenols such as flavonoids and hydroxycinnamates have been expected to play important roles as antioxidants.(1) Although the evidence for flavonoids as protective agents against these diseases is accumulating, their mechanisms of antioxidant action still remain to be elucidated. To investigate relationship of redox properties and biological activities of flavonoids it is important to evaluate their redox properties including structural specificity by simple methods with definite results.

Catechins are the main polyphenols contained in green tea. A variety of physiological functions such as antioxidant activity, antibacterial activity and antimutagenic activity have been ascribed to catechins.(2) Epicatechin (EC), epicatechin gallate (ECg), epigallocatechin (EGC) and epigallocatechin gallate (EGCg) are the major catechins. The chemical structures of these compounds differ in the number of hydroxyl groups in the B ring and the presence or absence of a galloyl group (Fig. 1A). It has been reported that these differences affect their physicochemical properties and physiological activities.(3) The free radical scavenging ability of these catechins can likely be ascribed to the hydroxyl groups on the B ring and a galloyl group bound to the 3-position of the C ring. The O-H bond dissociation enthalpies (BDEs) of the pyrogallol structure possessing three hydroxyl groups on the B ring are lower than those of the catechol structure possessing two hydroxyl groups at the ortho position of the B ring.(3) The resorcinol structure possessing two hydroxyl groups in the meta position of the A ring shows higher BDEs than does the catechol structure.(3) In fact, EGC and EGCg are unstable under neutral and alkaline conditions as compared with EC and ECg, owing to their pyrogallol structure, which is termed as a gallyl structure. Consequently, the stability of these structures increases in the order: pyrogallol, catechol, and resorcinol. In human serum or in buffers containing human serum albumin (HSA), EGCg is more stable than EGC because of the stabilizing interaction of HSA with EGCg.(4) Although it has been reported in these studies that various factors such as the chemical structure, pH, concentration of oxygen, and the presence of antioxidants such as HSA and ascorbic acid govern the stability of catechins, the effects of the number and the position of the hydroxy groups of catechins on their stability or antioxidant activity have not been comprehensively investigated using an established methodology.

Theaflavins, a series of red pigments in black tea, are formed by oxidative dimerization of two catechin molecules during the "fermentation" of tea leaves. Theaflavin (TF1), theaflavin-3-*O*-gallate (TF2A), theaflavin-3'-*O*-gallate (TF2B) and theaflavin-3,3'-di-*O*-gallate (TF3) are the major compounds of theaflavins (Fig. 1B). The number and the position of the galloyl groups are governed by the precursor catechin and determine the structure of the corresponding theaflavin. Theaflavins have been reported to show antioxidant activity,(5,6) antimutagenic activity,(6) and cholesterol absorption inhibitory activity.(7) Some of these physiological functions are reported to be equivalent or superior to those of catechins. In addition to catechins and theaflavins, there are many natural and synthetic methylated catechins which differ in the position of the methoxy group. The redox properties of these methylated catechins (Fig. 2) and theaflavins have not been fully investigated.

Coulometric-array-HPLC uses several electrodes for electrochemical detection to provide a series of electric potentials in the detector, allowing the quantity of electricity generated by the redox reaction at the electrode surface to be measured. HPLC with electrochemical detection is valuable for the highly selective and sensitive analysis of redox-active compounds, such as determining the concentration of catechins in the blood after intake of green teas.(8,9) In this study, we developed a simple method to evaluate the redox properties of catechins, theaflavins and methylated catechins by coulometric-array-HPLC. A "quantity versus potential (QP) plot" was constructed by plotting quantity of electricity generated (μC) on the vertical axis and electric potential (mV), adjusted with the associated palladium reference electrode, on the horizontal axis. The electric potential of the peaks in a QP plot broadly reflect the redox potentials of the compound. This approach was used to clarify the structure-activity relationship

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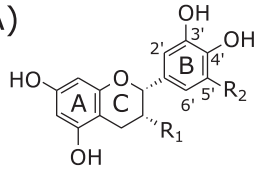
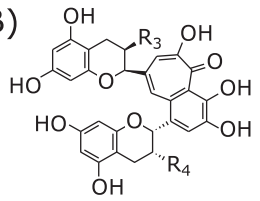
(A)



Catechins	R_1	R_2	t_R (min)
(–)-Epicatechin (EC)	OH	H	13.9
(–)-Epigallocatechin (EGC)	OH	OH	11.6
(–)-Epicatechin gallate (ECg)	gallate	H	16.4
(–)-Epigallocatechin gallate (EGCg)	gallate	OH	14.0

(B)



Theaflavins	R_3	R_4	t_R (min)
Theaflavin (TF1)	OH	OH	20.8
Theaflavin-3-*O*-gallate (TF2A)	gallate	OH	21.4
Theaflavin-3'-*O*-gallate (TF2B)	OH	gallate	21.8
Theaflavin-3,3'-di-*O*-gallate (TF3)	gallate	gallate	21.6

(C)

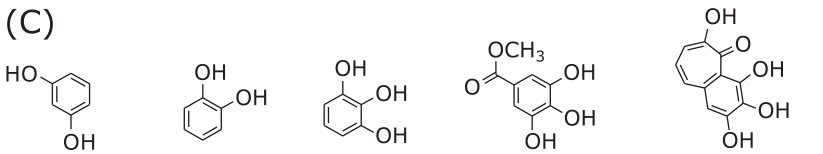


Fig. 1. Structures of tea polyphenols and their model compounds. (A) catechins, (B) theaflavins, (C) model compounds. The retention times of these compounds under the HPLC conditions of the present study were also listed.

of the redox properties of these compounds and to evaluate the stability of catechins and theaflavins, some of which are oxidized with concomitant formation of hydrogen peroxide (H_2O_2).

Materials and Methods

Materials. EC, EGC, ECg and EGCg were provided by Mitsui Norin Co., Ltd. (Shizuoka, Japan). Catechol, methyl gallate, purpurogallin, and pyrogallol were obtained from Kanto Chemical Co. (Tokyo, Japan). H_2O_2, sorbitol and xylenol orange were obtained from Wako Pure Chem. Ind., Ltd. (Osaka, Japan). Resorcinol was obtained from Sigma-Aldrich Co. (St. Louis, MO). TF1, TF2A, TF2B and TF3 were isolated and purified, as follows. A crude theaflavin mixture (crude TF) prepared by incubating tea catechins in the presence of polyphenol peroxidases was purchased from Yaizu Suisankagaku-Industry Co., Ltd. (Shizuoka, Japan). The crude TF was solubilized in water. Then, the catechins and caffeine in the solution were removed by medium pressure column chromatography using a reverse-phase preparative column (Ultra Pack 50 mm i.d. × 300 mm; Yamazen, Osaka, Japan) and a gradient mobile phase containing 10 to 80% acetonitrile (MeCN). The resulting mixture was composed of TF1, TF2A, TF2B and TF3. These theaflavins were further purified by preparative HPLC on a CAPCELL PAK C18 column (20 mm i.d. × 250 mm; Shiseido, Tokyo, Japan) with a mobile phase containing 22% MeCN and 0.05% phosphoric acid. The aqueous solutions containing the respective theaflavins were purified on a Sep-PakC18 cartridge, eluted with ethanol, and lyophilized. The structures of the purified theaflavins were confirmed by NMR.[10]

(–)-Epicatechin-3'-*O*-methylether (EC-3'-*O*-Me), (–)-epicatechin-4'-*O*-methylether (EC-4'-*O*-Me), (–)-epicatechin-3'-*O*-methylether gallate (ECg-3'-*O*-Me), (–)-epicatechin-4'-*O*-methylether gallate (ECg-4'-*O*-Me), (–)-epicatechin 3''-*O*-methyl-gallate (ECg-3''-*O*-Me), (–)-epicatechin 4''-*O*-methyl-gallate (ECg-4''-*O*-Me), (–)-epigallocatechin-3'-*O*-methylether (EGC-3'-*O*-Me), (–)-epigallocatechin-4'-*O*-methylether (EGC-4'-*O*-Me), (–)-epigallocatechin-3'-*O*-methylether gallate (EGCg-3'-*O*-Me) and (–)-epigallocatechin-4'-*O*-methylether gallate (EGCg-4'-*O*-Me) were purchased from Nagara Science Co., Ltd. (Gifu, Japan) (Fig. 2). (–)-Epigallocatechin 3''-*O*-methyl-gallate (EGCg-3''-*O*-Me) and (–)-epigallocatechin 4''-*O*-methyl-gallate (EGCg-4''-*O*-Me) were synthesized according to the method described previously.[11] All

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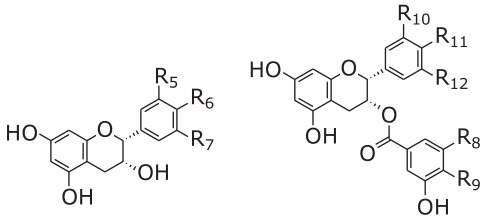

Methylated catechins	R_5	R_6	R_7	R_8	R_9	R_{10}	R_{11}	R_{12}	t_R (min)
EC-3'-*O*-Me	OCH_3	OH	H	—	—	—	—	—	16.1
EC-4'-*O*-Me	OH	OCH_3	H	—	—	—	—	—	16.3
EGC-3'-*O*-Me	OCH_3	OH	OH	—	—	—	—	—	12.8
EGC-4'-*O*-Me	OH	OCH_3	OH	—	—	—	—	—	13.9
ECg-3'-*O*-Me	—	—	—	OH	OH	OCH_3	OH	H	18.4
ECg-4'-*O*-Me	—	—	—	OH	OH	OH	OCH_3	H	19.3
ECg-3"-*O*-Me	—	—	—	OCH_3	OH	OH	OH	H	18.0
ECg-4"-*O*-Me	—	—	—	OH	OCH_3	OH	OH	H	17.9
EGCg-3'-*O*-Me	—	—	—	OH	OH	OCH_3	OH	OH	16.1
EGCg-4'-*O*-Me	—	—	—	OH	OH	OH	OCH_3	OH	16.3
EGCg-3"-*O*-Me	—	—	—	OCH_3	OH	OH	OH	OH	15.8
EGCg-4"-*O*-Me	—	—	—	OH	OCH_3	OH	OH	OH	15.7

Fig. 2. Structures of methylated catechins. The retention times of these compounds under the HPLC conditions of the present study were also listed.

other chemicals were of reagent grade and were used without further purification.

Coulometric-array-HPLC. The HPLC equipment supplied by ESA (Chelmsford, MA) consisted of a Model 540 autosampler (set to a temperature of 4°C, injection volume 10 μl), a Model 582 solvent delivery system, a 502 column oven (set to a temperature of 35°C), and a CoulArray Model 5600A eight-channel coulometric array detector. A series of working potentials of –400, –220, –40, +140, +320, +500, +680 and +860 mV, which were adjusted with the associated palladium reference electrode, were applied to the eight electrochemical cells of the detector. Alternatively, a series of working potentials of –150, –100, –50, 0, +50, +100, +150 and +200 mV were applied. The compounds were solubilized in mobile phase A (10 μM) and separated on a CAPCELL PAK C18 UG120 column (4.6 mm i.d. × 250 mm; Shiseido). The mobile phase was a gradient mixture of phosphate buffer and organic solvent. Mobile phase A was 50 mM NaH_2PO_4 adjusted to pH 3.0 by adding phosphoric acid and methanol (MeOH) (99:1, v/v). Mobile phase B was 50 mM NaH_2PO_4 adjusted to pH 3.45 by adding phosphoric acid, MeCN and MeOH (3:6:1, v/v). The gradient elution used was as follows: 0% B in 0–0.2 min, 0–57% B in 0.2–20 min, 57–100% B in 20–25 min. The flow rate was set to 1 ml/min. The retention times of catechins, methylated catechins and theaflavins under these conditions were listed in Fig. 1 and 2. Coulometric-array analysis of each polyphenol was repeated three times. The mean value of the quantity of electricity (μC) produced, with its standard deviation at respective electric potentials, was plotted in a QP plot.

Measurement of H_2O_2. The amount of H_2O_2 formed during the autoxidation of a catechin or a theaflavin was measured using the FOX assay, which is based on the oxidation of Fe^{2+} to Fe^{3+} by H_2O_2.[12] The resulting Fe^{3+} quantitatively forms a complex with xylenol orange which absorbs between 540 and 580 nm. FOX reagent comprised 250 μM $Fe(NH_4)_2(SO_4)_2$, 25 mM H_2SO_4, 100 μM xylenol orange and 100 mM sorbitol. Each catechin or theaflavin sample was dissolved in MeOH to a final concentration of 100 mM and then diluted to 500 μM with 10 mM phosphate buffer (PB, pH 6.0). The samples were incubated by standing at 37°C for 0, 30, 60, 90 and 120 min, then the sample (15 μl) and FOX reagent (135 μl) were mixed and incubated for 30 min. The absorbance of each sample mixture was measured at 550 nm. A sample solution in the presence of catalase was used as a control containing no H_2O_2.

Results

A typical intact printout of the coulometric-array HPLC chromatograms for EGCg analyzed is shown in Fig. 3. The chromatograms depict electric current (μA) on the vertical axis and retention time (min) on the horizontal axis at the indicated electric potentials (mV) measured using a palladium reference electrode. The quantity of electricity produced (μC), which is proportional to the area under each curve (AUC), is recorded by the coulmetric-array-HPLC system. In the case of EGCg, AUC of the peak at around 14 min in the chromatogram increased in the electric potential from –50 mV, showed two peaks at 0 and 100 mV, and then decreased. Plots of the quantity of electricity on the vertical axis and electric potential on the horizontal axis are termed "QP plots" in this study. Fig. 4 and 5 show typical QP plots of the four catechins and indicate the relationship between the quantity of electricity produced and the electric potential applied to the electrochemical cells in the HPLC system. When the measurements were carried out between –400–+860 mV with 180 mV gaps (Fig. 4), all catechins showed similar patterns, with two peaks in the QP plots. When the measurements were carried out between –150–+200 mV with 50 mV gaps (Fig. 5), EGC and EGCg oxidized at 0 mV, EC oxidized at 50 mV, and ECg and EGCg oxidized at 100 mV. This result indicates that the superim-

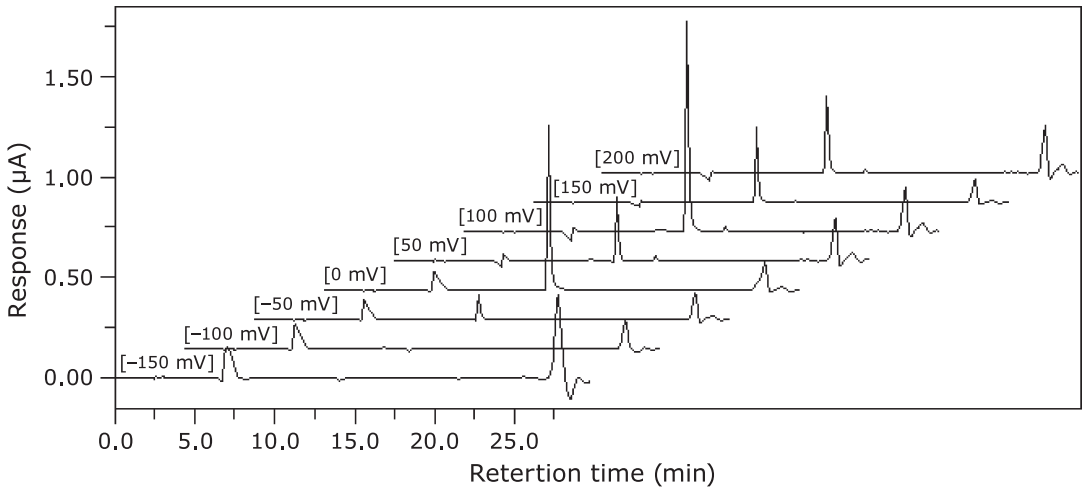

Fig. 3. Chromatograms of EGCg analyzed by coulometric-array HPLC. EGCg solution (10 μM) in mobile phase A (50 mM phosphate buffer:MeOH = 99:1, pH 3) was measured from –150 mV to +200 mV by coulometric-array-HPLC.

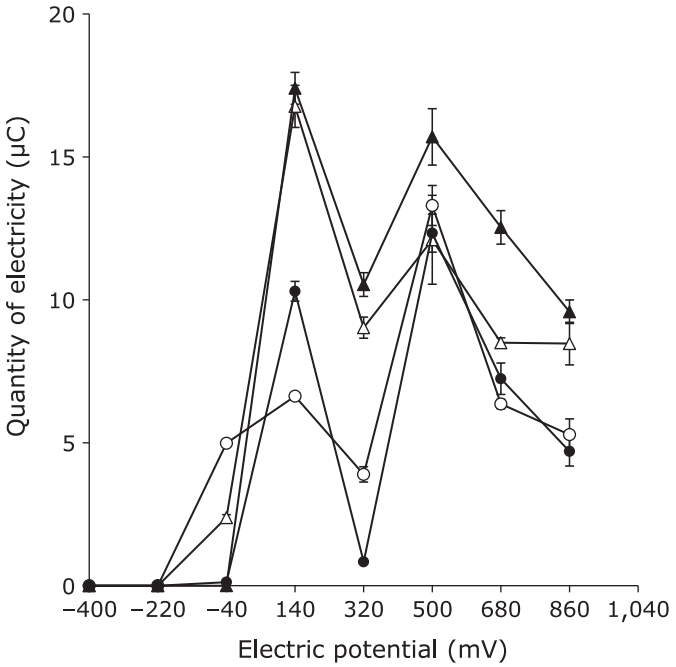

Fig. 4. QP plot of catechins from –400 mV to 860 mV with 180 mV gaps. A catechin solution (10 μM) in mobile phase A (50 mM phosphate buffer:MeOH = 99:1, pH 3) was measured from –400 mV to +860 mV by coulometric-array-HPLC. ●: EC, ▲: ECg, ○: EGC, △: EGCg.

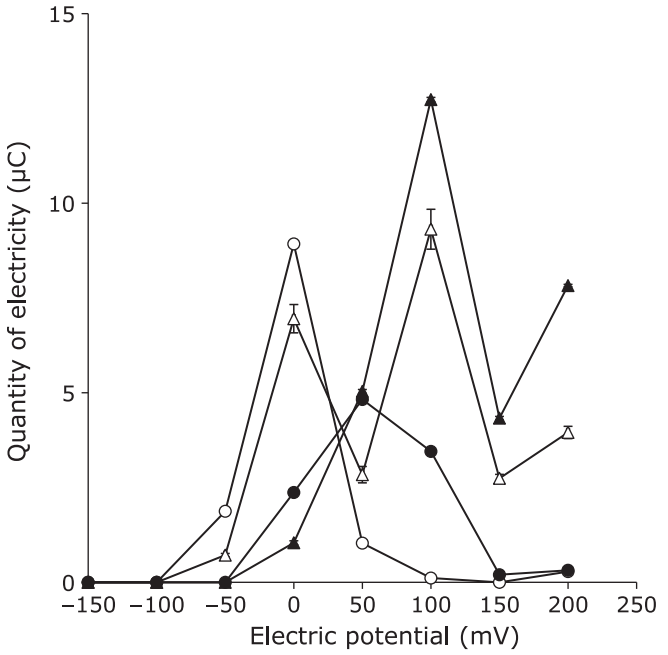

Fig. 5. QP plot of catechins from –150 mV to 200 mV with 50 mV gaps. A catechin solution (10 μM) in mobile phase A (50 mM phosphate buffer:MeOH = 99:1, pH 3) was measured from –150 mV to +200 mV by coulometric-array-HPLC. ●: EC, ▲: ECg, ○: EGC, △: EGCg.

posed broad peaks of EGCg at around 140 mV obtained by application of 180 mV gaps in Fig. 4 were separated into different peaks by application of 50 mV gaps.

When the four theaflavins were analyzed between –400–+860 mV (Fig. 6), they oxidized at 140 and 500 mV. Setting the applied potentials to –150–+200 mV with 50 mV gaps (Fig. 7) resulted in TF1, TF2A and TF2B oxidizing at 0 mV, and TF2A and TF2B oxidizing at a potential of 100 mV. For TF3, a large peak at 50 mV was observed. This peak was separated into two peaks by setting the applied potentials to –10–+95 mV with 15 mV gaps (data not shown).

Resorcinol, catechol, pyrogallol, methyl gallate and purpurogallin are simple compounds possessing partial structures of tea catechins and theaflavins (Fig. 1C). Measurement of these compounds between –400–+860 mV (Table 1) resulted in the oxidation of catechol, pyrogallol, methyl gallate and purpurogallin at +140 mV. On the other hand, the peak in the case of resorcinol was obseved at +680 mV. When the measurements were conducted between –150–+200 mV with 50 mV gaps (Table 1), the peaks for catechol, pyrogallol, methyl gallate and purpurogallin were observed at 50, 0, 100 and 0 mV, respectively. No peak was observed for resorcinol in this potential range.

In the range –400–+860 mV with 180 mV gaps, the peaks for methylated EGCgs, methylated ECgs and EGC-3'-*O*-Me in the QP plots were observed both at +140 and +500–+680 mV (Table 1), whereas methylated EC and EGC-4'-*O*-Me oxidized only in the range +500–+680 mV. Between –150–+200 mV with 50 mV gaps, the following peaks were observed: EGCg-3"-*O*-Me

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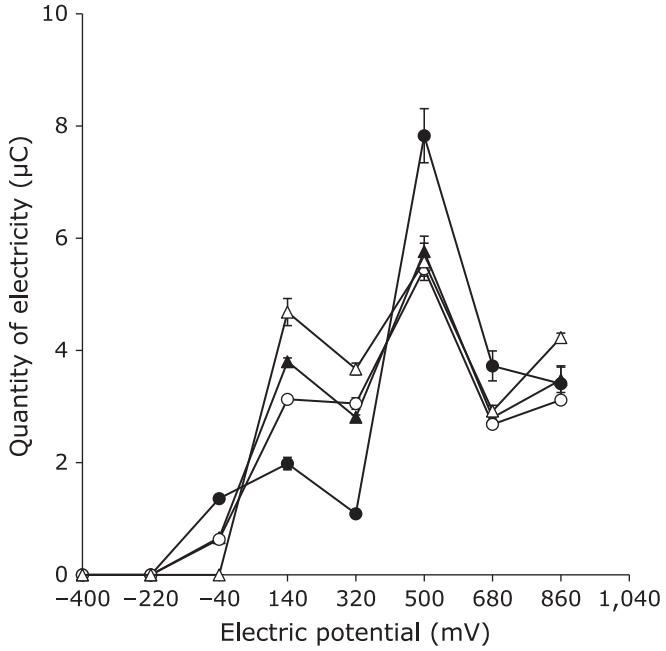

Fig. 6. QP plot of theaflavins from –400 mV to 860 mV with 180 mV gaps. A theaflavin solution (10 μM) in mobile phase A (50 mM phosphate buffer:MeOH = 99:1, pH 3) was measured from –400 mV to +860 mV by coulometric-array-HPLC. ●: TF1, ▲: TF2A, ○: TF2B, △: TF3.

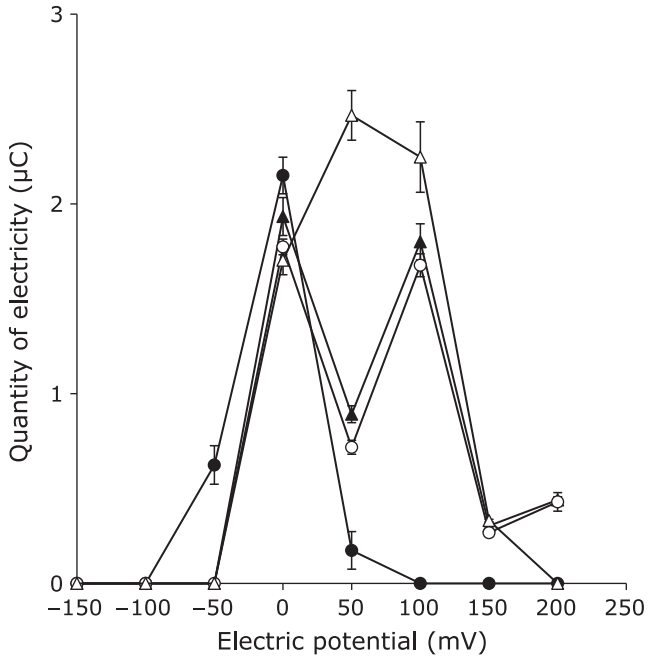

Fig. 7. QP plot of theaflavins from –150 mV to 200 mV with 50 mV gaps. A theaflavin solution (10 μM) in mobile phase A (50 mM phosphate buffer:MeOH = 99:1, pH 3) were measured in –150 mV to +200 mV by coulometric-array-HPLC. ●: TF1, ▲: TF2A, ○: TF2B, △: TF3.

and EGCg-4''-*O*-Me at 0 mV, EGC-3'-*O*-Me at +50 mV, and ECg-3'-*O*-Me, ECg-4'-*O*-Me, ECg-3''-*O*-Me, ECg-4''-*O*-Me EGCg-3'-*O*-Me, EGCg-3''-*O*-Me and EGCg-4'-*O*-Me at +100 mV. For EC-3'-*O*-Me, EC-4'-*O*-Me and EGC-4'-*O*-Me, no peak was observed in the range –150–+200 mV.

Table 1. The electric potentials of peaks in the QP plots of model compounds and methylated catechins

Compounds	–150–+200 mV	–400–+860 mV
Resorcinol	—	680 mV
Catechol	50 mV	140 mV
Pyrogallol	0 mV	140, 500 mV
Methyl gallate	100 mV	140, 500 mV
Purpurogallin	0 mV	140, 500 mV
EC-3'-*O*-Me	—	680 mV
EC-4'-*O*-Me	—	500 mV
EGC-3'-*O*-Me	50 mV	140, 680 mV
EGC-4'-*O*-Me	—	500 mV
ECg-3'-*O*-Me	100 mV	140, 500 mV
ECg-4'-*O*-Me	100 mV	140, 500 mV
ECg-3''-*O*-Me	100 mV	140, 680 mV
ECg-4''-*O*-Me	100 mV	140, 500 mV
EGCg-3'-*O*-Me	100 mV	140, 500 mV
EGCg-4'-*O*-Me	100 mV	140, 500 mV
EGCg-3''-*O*-Me	0, 100 mV	140, 680 mV
EGCg-4''-*O*-Me	0 mV	140, 500 mV

The electric potentials of peaks in the QP plots of each compound were analyzed using a coulometric-array-HPLC system in the range –150–+200 mV with 50 mV gaps, or in the range –400–+860 mV with 180 mV gaps. "—" means no peak was observed in the range.

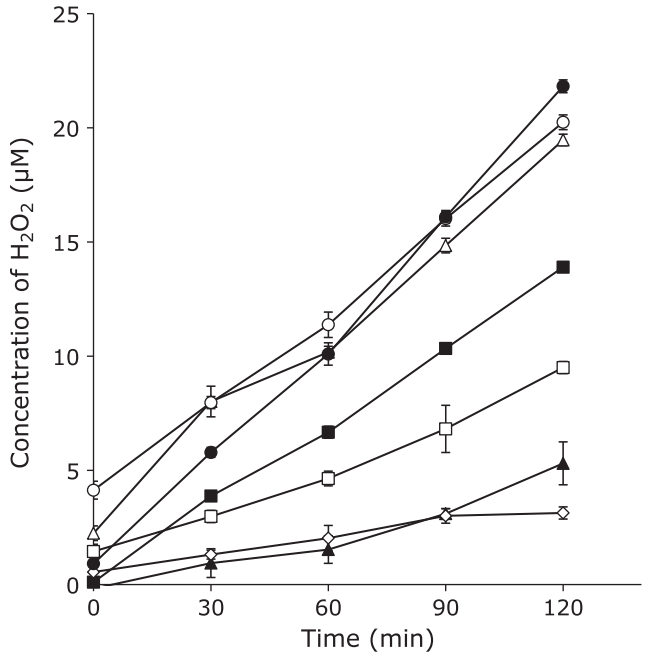

Fig. 8. Time dependence of H_2O_2 formation from EGC, EGCg, theaflavins and pyrogallol. Aqueous solutions of the compounds (500 μM) were prepared in 10 mM phosphate buffer (pH 6.0). After incubating each solution at 37°C for 120 min, FOX reagent was added and the mixture was incubated at 37°C for 30 min. Absorbance was monitored at 550 nm. ■: EGC, ▲: EGCg, ◇:TF1, △: TF2A, ○: TF2B, □: TF3, ●: pyrogallol.

The time dependencies of the concentration of H_2O_2 formed from catechins, theaflavins and related model compounds due to autoxidation processes are shown in Fig. 8. The pyrogallol-type catechins, EGC and EGCg, produced substantial amount of H_2O_2. TF2A and TF2B produced more H_2O_2 than TF1 and TF3. Among the model compounds, pyrogallol produced H_2O_2, whereas resorcinol, catechol, EC, ECg and methyl gallate produced no detectable amount of H_2O_2 under these conditions (data not shown).

Discussion

The plots in the conventional voltammetric analyses, such as cyclic voltammetry and differential pulse voltammetry and the conventional plots in the coulometric-array analysis show the relationship between electric current (μA) and electric potentials (mV). A typical example of the conventional plots in the coulometric-array analysis was shown in Fig. 3. Compared with Fig. 3, it is easy to identify the peak position of ECCg in QP plots in Fig. 5, because the vertical axis is not for the current (μA) but for the quantity of electricity (μC), which is total amount of electricity produced in the electrode and precisely calculated by the apparatus. Consequently QP plots allowed the redox properties of the compounds to be simply evaluated by comparing the electric potentials (mV) of the peaks. Tea catechins have previously been analyzed by the conventional coulometric-array method.[8,9,13–15] These studies reported the voltage dependency of peak heights of the four tea catechins at +50–+500 or +350–+700 mV,[9,14] and showed typical chromatograms for catechins at −10, +70, and +150 mV.[15] However, comprehensive research into the structure-potential relationship of tea polyphenols using coulometric-array HPLC analysis has not been reported. Aaby *et al.*[15] presented a hydrodynamic voltammogram (HDV) of (+)-catechin at +100–+800 mV with 100 mV gaps obtained by coulometric-array analysis, implying that the derivative of this voltammogram showed two peaks at around +150 and +600 mV. Our present study of four catechins showed two peaks at +140 and +500 mV in the QP plot with 180 mV gaps (Fig. 4). This indicates that the pattern in a QP plot corresponds to the derivative of a hydrodynamic voltammogram or a current-voltage curve, and that peaks in a QP plot correspond to the half-wave potentials in a current-voltage curve in the conventional coulometric-array analysis. Since the QP plots (Fig. 4) of all four catechins show two peaks at the same electric potentials (+140 and +500–+680 mV), it was difficult to compare the redox properties of these catechins in detail under these conditions. When the measurement was carried out at −150–+200 mV with 50 mV gaps (Fig. 5), EGC and EGCg were oxidized at 0 mV, EC was oxidized at +50 mV, and ECg and EGCg oxidized at +100 mV. These results reveal that the peaks observed as superimposed broad peaks upon the application of 180 mV gaps were separated by the application of 50 mV gaps. Since the phenolic groups (A and B rings and galloyl moiety) are not conjugated each other, it is supposed that the oxidation reactions in the respective phenolic groups independently occur and the patterns in the QP plots simply reflect the superimposed patterns of all groups. The peaks at 500 or 680 mV correspond to A ring for the four catechins. The pattern of ECg in Fig. 5 should correspond to the superimposed pattern of EC with a peak at 50 mV and methyl gallate with a peak at 100 mV. In the same manner, the pattern of EGCg in Fig. 5 corresponds the superimposed pattern of EGC with a peak at 0 mV and methyl gallate with a peak at 100 mV. When the four theaflavins and the model compounds (catechol, pyrogallol, methyl gallate, purpurogallin) were measured between −400–+860 mV with 180 mV gaps, these compounds were oxidized at +140 and +500–+680 mV (Fig. 6 and Table I). When the applied potentials were set with 50 mV gaps in the range −150–+200 mV, the superimposed broad peaks at +140 mV were separated (Fig. 7). TF2A and TF2B showed a similar pattern, with peaks at 0 and 100 mV, whereas TF1 showed only one peak at 0 mV, allowing the peaks at +100 mV to be ascribed to the galloyl moieties of TF2A and TF2B. Comparison of these QP plots set with 50 mV gaps in Fig. 5, Fig. 7 and Table 1 allowed the following structure-electric potential relationships of catechins and theaflavins to be elucidated:

1. The pyrogallol type catechins, EGC and EGCg, and pyrogallol show a peak at 0 mV in the QP plots.
2. A catechol type catechin, EC, and catechol show a peak at +50 mV.
3. The galloyl type catechins, ECg and EGCg, the galloyl type theaflavins,TF2A, TF2B, and methyl gallate show a peak at +100 mV.
4. TF1, TF2A, TF2B and purpurogallin show a peak at 0 mV.
5. The peak at 50 mV in the QP plot of TF3 with two galloyl moieties is likely due to the superimposition of two peaks.

These results indicate that catechins and theaflavins are oxidized during HPLC-coulometric-array analysis depending on the electric potential of the respective partial structures. The sequence of the electric potentials (mV) of the partial structures of catechins was determined to be:

pyrogallol structure (0 mV)<catechol structure (+50 mV)<
gallate structure (+100 mV)<resorcinol structure (+500 mV).

When the methylated catechins were measured between −400–+860 mV with 180 mV gaps, all compounds with a galloyl moiety investigated in this study (ECg-3'-*O*-Me, ECg-4'-*O*-Me, ECg-3"-*O*-Me, ECg-4"-*O*-Me, EGCg-3'-*O*-Me, EGCg-4'-*O*-Me, EGCg-3"-*O*-Me and EGCg-4"-*O*-Me) were oxidized at potentials of +140 and +500–+680 mV (Table 1). These results are quite similar to those for ECg and EGCg (Fig. 4). When the measurements of these methylated catechins were carried out between −150–+200 mV with 50 mV gaps, the patterns in the QP plots were different from those of the parent unmethylated compounds. For example, the peak position of EGCg-3'-*O*-Me resembles that of ECg (Fig. 5), showing that the redox character of pyrogallol is changed to that of catechol by methylation of the 3'-position of OH. This indicates that methylation of the 3'-position of OH in the B-ring of EGCg changes the unstable character of EGCg to a stable one. The pattern of EGCg-4'-*O*-Me resembles that of methyl gallate (Table 1), showing that methylation of the 4'-position of OH in the B-ring of EGCg abolishes the redox character of the B-ring of pyrogallol. Similarly, methylation of the 4"-position of OH in the galloyl moiety of EGCg abolishes or weakens the redox character of the galloyl moiety. These results strongly suggest that methylation of a specific phenolic OH generally changes the redox character of the compound as if that OH was substituted with a hydrogen atom. The effect of methylation on the redox character of EC in the cases of EC-3'-*O*-Me and EC-4'-*O*-Me was obvious. When the measurement was carried out between −400–+860 mV with 180 mV gaps, no peak at around +140 mV was observed, and when the measurement was carried out between −150–+200 mV with 50 mV gaps, again no peak was observed in this range. The patterns in the QP plots of EGC-3'-*O*-Me and EGC-4'-*O*-Me resemble those of EC and resorcinol, respectively. These results were additional examples indicating that methylation of a specific phenolic OH in the structures of catechins changes the QP plots as if that OH were substituted with a hydrogen atom.

As described above, the electric potential of the peaks in the QP plots reflected the structures of the catechins, methylated catechins and theaflavins studied. This indicates that in the coulometric-array analysis the potential at which catechins and theaflavins are oxidized depends on partial structures such as pyrogallol, catechol, resorcinol, galloyl and benzotropolone, and can be ascribed to chemical characteristics of these structures. Since the phenolic groups (the A ring, B ring or galloyl moiety) and benzotropolone ring are not conjugated with each other, the oxidation of a given OH group in a given aromatic ring should not affect the oxidation of OH groups in other rings in the redox processes during the coulometric analysis.

Substantial amounts of H_2O_2 were formed from EGC, EGCg, 4 theaflavins and pyrogallol during autoxidation processes at 37°C for 120 min (Fig. 8). These polyphenols are so unstable that small amounts of H_2O_2 might be produced even prior to incubation. In contrast, resorcinol, catechol and methyl gallate produced little H_2O_2 under these conditions (data not shown). The compounds that produce H_2O_2 commonly show a peak at 0 mV in the QP plots,

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whereas those that do not produce H_2O_2 do not. It has been reported that H_2O_2 is formed during autoxidation of EGC and EGCg at room temperature or at 37°C[12,16] and during oxidation of (+)-catechin at 80°C.[17] The latter study indicates that compounds possessing the catechol or galloyl moiety generate H_2O_2 at temperatures above 37°C. Since pyrogallol-type catechins, theaflavins and pyrogallol react at lower electric potentials (0 mV in the QP-plots) than catechol-type catechins and the galloyl moiety, the former compounds are apt to be oxidized under mild conditions. All theaflavins investigated in this study produced H_2O_2. This suggests that both the oxidation at 0 mV and H_2O_2 production are ascribed to the presence of the benzotropolone ring, not the galloyl moiety. The order of the amount of H_2O_2 production was TF2A, TF2B>TF3>TF1, indicating that the effects of the galloyl moieties in theaflavins on H_2O_2 production are not simple and further studies are needed to clarify the mechanisms.

This study confirmed that the redox properties of catechins, methylated catechins, and theaflavins can be evaluated by the coulometric-array-HPLC using specific protocols, e.g., measurement with 50 mV gaps. Further studies are necessary to apply this method to other polyphenols and to beverages containing various types of polyphenols which are separated by HPLC.

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Abbreviations

AUC	area under curve
BDE	bond dissociation enthalpy
EC	(−)-epicatechin
ECg	(−)-epicatechin gallate
ECg-3'-*O*-Me	(−)-epicatechin-3'-*O*-methylether gallate
ECg-3"-*O*-Me	(−)-epicatechin 3"-*O*-methyl-gallate
ECg-4'-*O*-Me	(−)-epicatechin-4'-*O*-methylether gallate
ECg-4"-*O*-Me	(−)-epicatechin 4"-*O*-methyl-gallate
EC-3'-*O*-Me	(−)-epicatechin-3'-*O*-methylether
EC-4'-*O*-Me	(−)-epicatechin-4'-*O*-methylether
EGC	(−)-epigallocatechin
EGCg	(−)-epigallocatechin gallate
EGCg-3'-*O*-Me	(−)-epigallocatechin-3'-*O*-methylether gallate
EGCg-3"-*O*-Me	(−)-epigallocatechin 3"-*O*-methyl-gallate
EGCg-4'-*O*-Me	(−)-epigallocatechin-4'-*O*-methylether gallate
EGCg-4"-*O*-Me	(−)-epigallocatechin 4"-*O*-methyl-gallate
EGC-3'-*O*-Me	(−)-epigallocatechin-3'-*O*-methylether
EGC-4'-*O*-Me	(−)-epigallocatechin-4'-*O*-methylether
FOX	ferrous ion oxidation-xylenol orange
HSA	human serum albumin
HDV	hydrodynamic voltammogram
MeCN	acetonitrile
MeOH	methanol
TF1	theaflavin
TF2A	theaflavin-3-*O*-gallate
TF2B	theaflavin-3'-*O*-gallate
TF3	theaflavin-3,3'-di-*O*-gallate
QP plot	quantity versus potential plot

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Rice-Evans CA, Packer L. *Flavonoids in Health and Disease, Second Edition Revised and Expanded*. New York: Marcel Dekker, Inc., 2003; 97–347.
2 Kuroda Y, Hara Y. Preventive effects of tea against human diseases. Age-related diseases and tea. Infectious diseases and tea. Antibacterial and antioxidative activity of tea. In: Kuroda Y, Hara Y, eds. *Health Effects of Tea and Its Catechins*. New York: Kluwer Academic/Plenum Publishers, 2004; 11–85.
3 Thavasi V, Leong LP, Bettens RP. Investigation of the influence of hydroxy groups on the radical scavenging ability of polyphenols. *J Phys Chem A* 2006; **110**: 4918–4923.
4 Ishii T, Ichikawa T, Minoda K, *et al*. Human serum albumin as an antioxidant in the oxidation of (–)-epigallocatechin gallate: participation of reversible covalent binding for interaction and stabilization. *Biosci Biotechnol Biochem* 2011; **75**: 100–106.
5 Leung LK, Su Y, Chen R, Zhang Z, Huang Y, Chen ZY. Theaflavins in black tea and catechins in green tea are equally effective antioxidants. *J Nutr* 2001; **131**: 2248–2251.
6 Shiraki M, Hara Y, Osawa T, Kumon H, Nakayama T, Kawakishi S. Antioxidative and antimutagenic effects of theaflavins from black tea. *Mutat Res* 1994; **323**: 29–34.
7 Ikeda I, Yamahira T, Kato M, Ishikawa A. Black-tea polyphenols decrease micellar solubility of cholesterol *in vitro* and intestinal absorption of cholesterol in rats. *J Agric Food Chem* 2010; **58**: 8591–8595.
8 Lee MJ, Prabhu S, Meng X, Li C, Yang CS. An improved method for the determination of green and black tea polyphenols in biomatrices by high-performance liquid chromatography with coulometric array detection. *Anal Biochem* 2000; **279**: 164–169.
9 Umegaki K, Sugisawa A, Yamada K, Higuchi M. Analytical method of measuring tea catechins in human plasma by solid-phase extraction and HPLC with electrochemical detection. *J Nutr Sci Vitaminol (Tokyo)* 2001; **47**: 402–408.
10 Davis AL, Cai Y, Davies AP. ^{1}H and ^{13}C NMR assignment of theaflavin, theaflavin monogallate and theaflavin digallate. *Magn Reson Chem* 1995; **33**: 549–552.
11 Aihara Y, Yoshida A, Furuta T, *et al*. Regioselective synthesis of methylated epigallocatechin gallate via nitrobenzensulfonyl (Ns) protecting group. *Bioorg Med Chem Lett* 2009; **19**: 4171–4174.
12 Long LH, Clement MV, Halliwell B. Artifacts in cell culture: rapid generation of hydrogen peroxide on addition of (–)-epigallocatechin, (–)-epigallocatechin gallate, (+)-catechin, and quercetin to commonly used cell culture media. *Biochem Biophys Res Commun* 2000; **273**: 50–53.
13 Wu PS, Yasuda S, Tachibana H, Yamada K. Voltage dependency in coulometric analysis of tea polyphenols and isoflavones in foodstuffs. *Food Sci Technol Res* 2003; **9**: 180–184.
14 Chu KO, Wang CC, Chu CY, Rogers MS, Choy KW, Pang CP. Determination of catechins and catechin gallates in tissues by liquid chromatography with coulometric array detection and selective solid phase extraction. *J Chromatogr B Analyt Technol Life Sci* 2004; **810**: 187–195.
15 Aaby K, Hvattum E, Skrede G. Analysis of flavonoids and other phenolic compounds using high-performance liquid chromatography with coulometric array detection: relationship to antioxidant activity. *J Agric Food Chem* 2004; **52**: 4595–4603.
16 Nakayama T, Ichiba M, Kuwabara M, Kajiya K, Kumazawa S. Mechanisms and structural specificity of hydrogen peroxide formation during oxidation of catechins. *Food Sci Technol Res* 2002; **8**: 261–267.
17 Nakayama T, Enoki Y, Hashimoto K. Hydrogen peroxide formation during catechin oxidation is inhibited by superoxide dismutase. *Food Sci Technol Int* 1995; **1**: 65–69.