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DNA Breakage by Flavan-3-ols and Procyanidins in the Presence of Cupric Ion

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DNA breakage by flavan-3-ols and procyanidins in the presence of cupric ion was investigated by gel electrophoretic and computer analysis. Flavan-3-ols and procyanidins cleaved λ-DNA in a concentration-dependent manner. The DNA-breaking activity was remarkably enhanced approximately in connection with the increase of the number of dihydroxy or trihydroxy groups in a molecule. Although the presence of cupric ions was indispensable for DNA cleavage by (−)-epicatechin (EPI), excess amounts of cupric ions inhibited the activity. A number of single-strand breaks occurred prior to double-strand breaks. A maximum rate of DNA cleavage by EPI occurred at pH 7.0. A correlation was not observed between the oxidation rate of EPI and the cleavage activity. DNA breakage by EPI was strongly inhibited by the addition of catalase or various kinds of radical scavengers. From the results obtained, a breaking mechanism was proposed that DNA chains were broken by oxygen radicals generated by a local oxidation of EPI in the vicinity of DNA via DNA–Cu^{2+}–EPI complexes.

Polyphenols including hydrolyzable, condensed, and other tannins are widely distributed in the plant kingdom and consumed daily by humans in milligram to gram quantities (Brown, 1980). It has been reported that tannic acid has antimutagenic effects (Gichner et al., 1987), and hydrolyzable tannins have antitumor activities (Miyamoto et al., 1987). Contrary to these reports, tannic acid is known to be a naturally occurring hepatoxin (Handler and Baker, 1944) and to induce nucleolar changes in hepacytes (Rao et al., 1987) and liver cancer (Korpâssy, 1959).

Such in vitro genotoxic assays as the DNA breakage test will be helpful to assess the potential genetic hazard by food components to humans. In the previous paper (Shirahata et al., 1985), we reported that hydrolyzable tannins can cleave DNA chains in the presence of Cu^{2+}. In order to clarify the relationship between the structures of hydrolyzable and condensed tannins and DNA breaking activity, we developed a rapid and exact method for quantitative determination of nucleic acid breaking activity with a combination of gel electrophoresis and microcomputer analysis.

Flavan-3-ols are constituent units of condensed tannins, and procyanidins belong to condensed tannins. This paper describes the DNA-breaking activity of flavan-3-ols and procyanidins in the presence of Cu^{2+} analyzed by this new method.

MATERIALS AND METHODS

DNA and Reagents. Double-stranded DNA from λ-phage was purchased from Biotech Co. Plasmid pBR322 DNA was prepared as described before (Shirahata et al., 1985). Catalase was obtained from Sigma Chemical Co. Superoxide dismutase was purchased from Toyobo Co. Various radical scavengers were all commercial grade. All flavan-3-ols and procyanidins were isolated from various kinds of plants, and their chemical structures were determined as reported previously. Flavan-3-ols: (+)-catechin (CAT) (Nonaka and Nishioka, 1982), (−)-epicatechin (EPI) (Nonaka et al., 1983), (+)-gallocatechin

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(GAL) (Nonaka et al., 1983), (−)-epigallocatechin 3-*O*-gallate (EGG) (Nonaka et al., 1983). Procyanidins: procyanidin B-1 (B-1) ((−)-epicatechin(4β→8)-(+)-catechin) (Nonaka et al., 1981), procyanidin C-1 (C-1) ((−)-epicatechin(4β→8)-(−)-epicatechin(4β→8)-(−)-epicatechin) (Nonaka et al., 1984).

DNA Breakage Test and Gel Electrophoresis. A mixture of 1 μg of λ-DNA, flavan-3-ols or procyanidins of varying concentrations from 1 to 100 μM, and metallic sulfate was incubated in 30 μL of 8 mM phosphate buffer (pH 7.0) at 37 °C for 2 h. The reaction was stopped by adding 20 μL of 15 mM EDTA (pH 7) containing 0.05% bromophenol blue and 15% sucrose. The sample was analyzed by gel electrophoresis. The pH dependence was tested in 50 mM buffer. We used acetate buffer for pH 4.0, 5.0, and 5.9, phosphate buffer for pH 7.0 and 8.0, Tris-HCl buffer for pH 9.0, and carbonate–bicarbonate buffer for pH 10.0. The oxidation of EPI in these buffers was followed by measuring the visible spectrum with a Shimazu UV-240 spectrophotometer equipped with a constant-temperature cell holder. A reaction mixture containing 150 μM EPI, 200 μM Cu^{2+}, and 8 mM buffer was incubated at 37 °C in a quartz microcell, and the change of the visible spectrum accompanying the oxidation of EPI was examined.

Gel electrophoresis was done in 0.7, 1.2, or 2.5% agarose slab gel or in 5 or 8% polyacrylamide gel (Shirahata et al., 1985). DNA fragments larger than 3×10^5 Da were analyzed by agarose gel electrophoresis and smaller DNA fragments by polyacrylamide gel electrophoresis. Single-strand breaks were detected by alkaline gel electrophoresis (Shirahata et al., 1985).

Estimation of Mean Molecular Weights of DNA Fragments. DNA fragments after electrophoresis were visualized under irradiation with a short-wave ultraviolet light source, and photographs were taken on type 667 nega/posi film with a Polaroid camera with UV and red filters. Negatives were traced on a Shimazu UV-240 spectrophotometer equipped with a gel scanner.

A calibration curve of molecular weight was made of λ-DNA-Hind III or pBR322 DNA-Hae III fragments. The molecular weights and migration distances of marker DNA were input to the computer by using desitizer and a calibration curve most fitting the input data was determined among approximated curves from 0 to 20 orders of magnitude by the least-squares method using computer graphics. Each coefficient of the curve was calculated by solving a matrix with the Gauss–Jordan method.

Next, densitometric data of DNA fragments treated with tannins were input, and a distribution pattern of molecular weights of DNA fragments was calculated by the use of the calibration curve of molecular weights. If the exposure and development are suitable, the optical density of the developed film is known to be proportional to the amount of DNA (Pulleyblank et al., 1977).

Since the correspondence between molecular weights of DNA fragments and distances migrated is logarithmic on gel electrophoresis, DNA fragments having larger molecular weights tend to be overestimated (Ordahal et al., 1976). Therefore, the relative molecular numbers of DNA fragments were calculated by dividing the optical density on the film by the molecular weights, and the mean molecular weights of DNA fragments and mean breakage numbers were computed according to

$$M_n = \sum n_i M_i / \sum n_i \qquad \mathrm{MBN} = M/M_n - 1$$

where M_n = mean molecular weight of DNA fragments, n_i = relative molecular number of DNA fragments having molecular weight of M_i, M_i = molecular weight of DNA fragments at each measurement, MBN = mean breakage number, M = molecular weight of native DNA molecules.

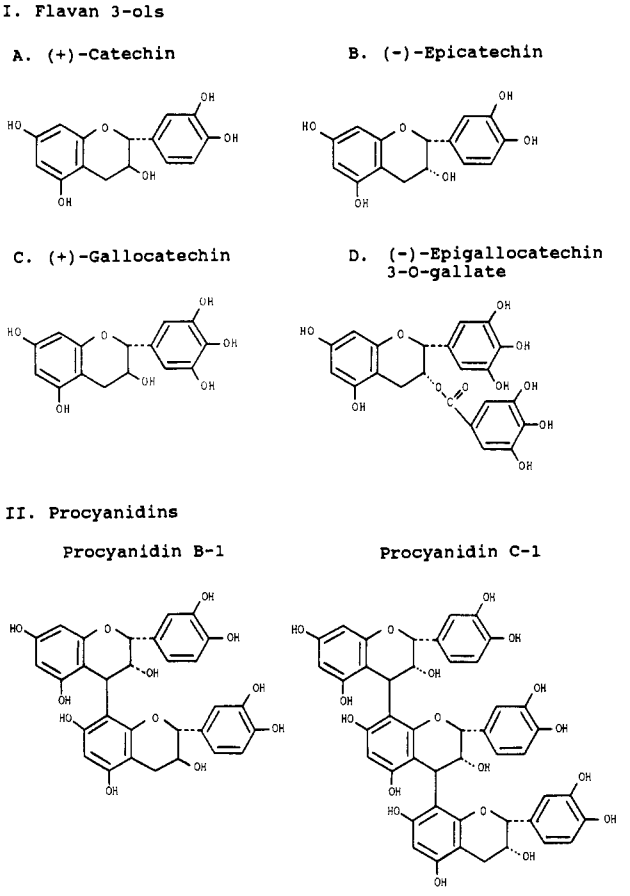

Figure 1. Structures of flavan-3-ols and procyanidins utilized in these studies.

Computer programming was done with Basic language. Since there was a possibility that DNA chains were broken by Cu^{2+} alone, DNAs treated with Cu^{2+} alone were used as control DNAs in all experiments. Mean breakage numbers of DNA by flavan-3-ols or procyanidins were estimated by subtracting mean breakage numbers with Cu^{2+} alone from mean breakage numbers with the flavan-3-ols or procyanidin–Cu^{2+} mixture. Breakage numbers with Cu^{2+} alone were a few percent of breakage numbers with the flavan-3-ols or procyanidin–Cu^{2+} mixture. Standard errors of duplicate experiments were within 15%, and standard errors of the repeated measurements of the same sample on densitometric and computer analysis were within 5%.

RESULTS AND DISCUSSION

Double-Stranded DNA Breakage by Flavan-3-ols and Procyanidins. The chemical structures of flavan 3-ols and procyanidins used are shown in Figure 1. First, double-stranded DNA breakage by these compounds was investigated. λ-DNAs were treated with these compounds (1–100 μM) in the presence of Cu^{2+} (200 μM) at 37 °C for 2 h. Double-stranded DNA-breaking activities of flavan-3-ols and procyanidins are shown in Figure 2. The DNA-breaking activities were remarkably enhanced with increased concentrations of these compounds. This tendency was greatly different from that of hydrolyzable tannins, of which DNA-breaking activities were weak and suppressed at tannin concentrations more than 100 μM (Shirahata et al., 1985).

The DNA-breaking activity was remarkably enhanced in connection with the increase in number of dihydroxy or trihydroxy groups per molecule in the order CAT, EPI,

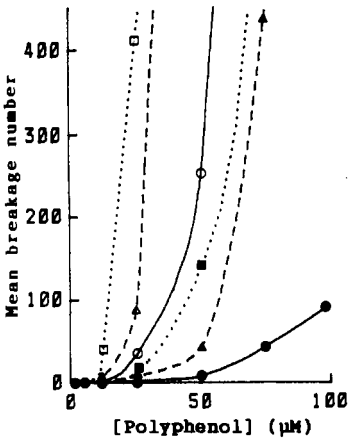

Figure 2. Double-strand breaks per DNA molecule caused by the treatment with flavan-3-ols and procyanidins at various concentrations in the presence of 200 μM Cu^{2+} in 8 mM phosphate buffer (pH 7.0) for 2 h: (●) (+)-catechin; (▲) (−)-epicatechin; (■) (+)-gallocatechin; (○) (−)-epigallocatechin 3-*O*-gallate; (△) procyanidin B-1; (□) procyanidin C-1.

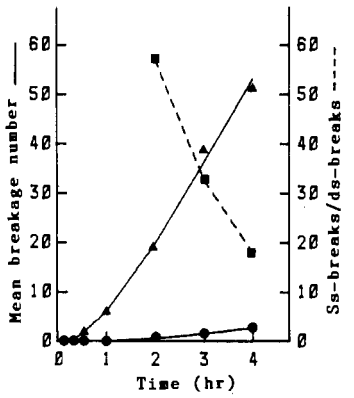

Figure 3. Double- and single-strand breaks per DNA molecule caused by the treatment with the mixture 25 μM (−)-epicatechin and 200 μM Cu^{2+} for various periods of time: (●) double-strand breaks; (▲) single-strand breaks; (■) ratio of single-strand (ss) breaks against double-strand (ds) breaks.

GAL, EGG, B-1, and C-1. EPI had stronger DNA-breaking activity than CAT, a stereoisomer, suggesting that the 2,3-cis stereoisomer might be more favorable for DNA breakage than 2,3-trans one. GAL having a trihydroxy group in the molecule exhibited stronger activity than EPI having a dihydroxy group, suggesting that trihydroxy groups might be more favorable for DNA breakage than dihydroxy groups, whereas B-1, which have only two dihydroxy groups in the molecule, exhibited stronger DNA-breaking activity than EGG, a flavan-3-ol having two trihydroxy groups. C-1 exhibited the strongest DNA-breaking activity among the compounds examined, suggesting that higher tannin structure might be more favorable for DNA breakage.

Course of DNA Breakage by EPI. In order to clarify the characteristics of DNA breakage by flavan-3-ols and procyanidins in more detail, experiments were done with a mixture of EPI (25 μM) and Cu^{2+} (200 μM). After DNAs were treated with the EPI–Cu^{2+} mixture for various periods of time, a part of the reaction mixture was analyzed by neutral gel electrophoresis to detect double-strand breaks. The remainder was applied to alkali gel electrophoresis to detect single-strand breaks. As shown in Figure 3, double-strand breaks were first detected after 2 h and the mean breakage number was increased to 3.0 after 4 h. On the other hand, single-strand breaks were first detected after 20 min and the mean breakage number of single-strand breaks reached 52 after 4 h. The ratio of number of single-strand breaks against the number of double-strand breaks decreased with the increase of the number of double-strand breaks. These data suggested that single-strand breaks and double-strand breaks were not independent phenomena with each other, and EPI might first break single-strand and the accumulation of single-strand breaks might result in double-strand breaks (Freifelder and Trumbo, 1969).

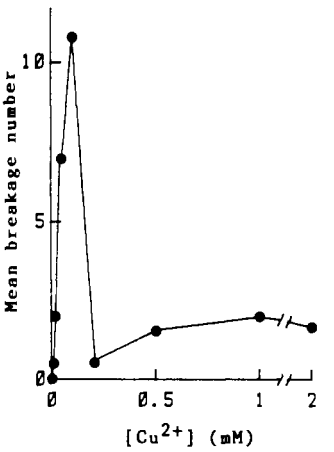

Figure 4. Effect of Cu^{2+} concentrations on the double-strand breaks of DNA by the mixture of (−)-epicatechin and Cu^{2+}: [(−)-epicatechin] = 25 μM; [Cu^{2+}] = 0, 6, 12, 25, 50, 100, 200, 500, 1000, 2000 μM; reaction time 2 h.

Requirement of Cu^{2+} for DNA Breakage by EPI. The effects of various concentrations of Cu^{2+} on DNA breakage by EPI were examined. As is evident from Figure 4, EPI could not break DNA in the absence of Cu^{2+}. DNA-breaking activity of EPI increased with Cu^{2+} concentration to 100 μM. However, concentration of Cu^{2+} higher than 200 μM inhibited the breaking activity of EPI.

Next, effects of metallic ions other than Cu^{2+} on the DNA breaking activity of EPI were examined. λ-DNAs were treated with a mixture of 50 μM EPI and 0.5 mM metallic ions (as sulfates) at 37 °C for 2 h, and the mean numbers of double-strand breaks were measured. Cu^{2+} exhibited an overwhelmingly strong effect and caused about 86.2 double-strand breaks per DNA molecule, whereas only Fe^{2+} and Fe^{3+} had extremely weak activity and caused 0.9 and 0.4 double-strand breaks per DNA molecule, respectively. But, Ni^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and Zn^{2+} had no effect on the breaking reaction.

Effect of pH on the DNA-Breaking Activity and Oxidation of EPI. Double-strand breaks caused by the mixture of EPI and Cu^{2+} were examined in the range pH 4.0–10.0 (Figure 5). Double-strand breaks occurred predominately at pH 7.0. The breaking reaction was weakened in both acidic and alkaline pH regions. Since a possibility that DNA was broken by Cu^{2+} alone at higher pH was considered, DNA was treated with Cu^{2+} alone at higher pH. However, no double-strand break was detected by the treatment with Cu^{2+} alone in the pH range examined.

Next, the oxidation rate of EPI was examined in order to investigate the relationship between the breaking reaction and the oxidation rate of EPI. Spectral changes of EPI–Cu^{2+} mixture accompanied by the oxidation of EPI were examined at various pH values. Since the oxidation of EPI catalyzed by Cu^{2+} accompanied the increase of absorption in the visible region having a peak at 435 nm, the oxidation rate was represented by the increase of absorption at 435 nm for 20 min. As is evident from a broken line in Figure 5, the oxidation rate of EPI increased with

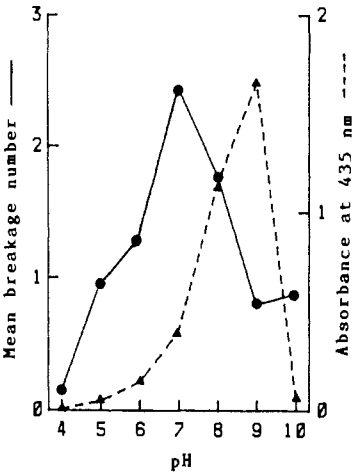

Figure 5. Effect of pH on the DNA-breaking activity and the oxidation rate of (−)-epicatechin. Double-strand breaks were examined after treatment of DNA with the mixture of 25 μM (−)-epicatechin and 25 μM Cu^{2+} for 2 h. The oxidation rate of (−)-epicatechin was represented by the increase of the absorption at 435 nm after 20 min as described in Materials and Methods. Key: (●) mean number of double-strand breaks per DNA molecule; (▲) absorbance at 435 nm.

Table I. Effect of Superoxide Dismutase, Catalase, and Various Radical Scavengers on DNA Breakage by the (−)-Epicatechin–Cu^{2+} Mixture

addition	concn	specificity	inhibn, %
SOD	30 units/mL	$O_2^{\cdot-}$	8
catalase	100 units/mL	H_2O_2	86
AET[a]	1×10^{-3} M	general	100
KBr	1×10^{-1} M	·OH	98
KI	1×10^{-2} M	·OH	100
HCOONa	1×10^{-1} M	·OH	89
KSCN	1×10^{-2} M	·OH	100
NaN_3	1×10^{-2} M	$^1O_2$	100
DABCO[b]	1×10^{-1} M	$^1O_2$	89

[a] AET = 2-(aminoethyl)isothiuronium. [b] DABCO = 1,4-diazabicyclo[2.2.2]octane.

pH to pH 9. The oxidation rate appeared to decrease at pH 10. However, it was suggested that oxidation products formed at this pH range differed from those in lower pH range because the peak of the spectrum shifted from 430 to 385 nm. A correlation between oxidation rate and breaking activity of EPI was not found because the DNA-breaking activity decreased in spite of the increase in oxidation rate with pH in the range 7–9.

Effect of Catalase, Superoxide Dismutase, and Various Kinds of Radical Scavengers on DNA Breakage by the EPI–Cu^{2+} Mixture. A possibility was presumed that various kinds of oxygen radicals generated by the aerobic oxidation of EPI via one-electron-transfer oxidation catalyzed by Cu^{2+} might be responsible for DNA breakage by EPI. Therefore, effects of various kinds of enzymes and radical scavengers were examined on the DNA-breaking reaction with a 25 μM EPI and 100 μM Cu^{2+} mixture for 2 h (Table I).

A double-strand break value of about 10.3 was caused by the EPI–Cu^{2+} mixture. Superoxide dismutase, which changes superoxide anion radicals $O_2^{\cdot-}$ to O_2 and H_2O_2, inhibited the breaking reaction by only 8%. On the other hand, catalase showed a strong inhibition of 86%. The breaking reaction was completely suppressed by 2-(aminoethyl)isothiuronium (AET), a general radical scavenger. However, 2-mercaptoethylamine (MEA), the same general radical scavenger as AET, remarkably accelerated the breaking reaction and broke DNA chains at about 130 sites.

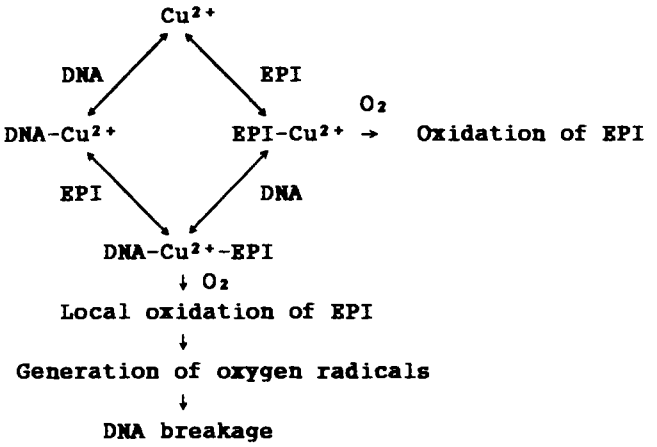

Figure 6. Proposed breaking mechanism of DNA by (−)-epicatechin in the presence of Cu^{2+}.

Radical scavengers for ·OH radicals such as KBr, KI, HCOONa, and KSCN strongly inhibited the breaking reaction. But, hydroquinone, a radical scavenger for ·OH radicals, reversed the stimulation of DNA breakage by the EPI–Cu^{2+} mixture and caused a double-strand break value of about 652. Sodium azide and DABCO (1,4-diazabicyclo[2.2.2]octane) as radical scavengers for $^1O_2$ strongly inhibited the DNA-breaking reaction.

These results suggested that several radicals such as H_2O_2, ·OH, or $^1O_2$ produced via Cu^{2+}-catalyzed oxidation of EPI participated in the DNA-breaking reaction. However, since effects of radical scavengers were indirect, the possibility could not be ruled out that addition of radical scavengers affected the oxidation of EPI and changed the kinds of molecules responsible for DNA breakage.

Breaking Mechanism of DNA by the EPI–Cu^{2+} Mixture. Although Cu^{2+} was indispensable for DNA breakage by EPI, excess amounts of Cu^{2+} inhibited the DNA-breaking reaction. The oxidation rate of EPI in the bulky phase was not necessarily correlated with DNA breakage. The DNA-breaking reaction was strongly inhibited by catalase and radical scavengers for ·OH and $^1O_2$, suggesting such oxygen radicals as H_2O_2, ·OH, and $^1O_2$ might take part in DNA breakage by EPI.

Cu^{2+} can be specifically bound to guanine and cytosine or GC pairs in DNA (Richard et al., 1973; Prasal, 1986) and denatures double-stranded DNA (Rhee and Ware, 1983). Aromatic reductones can be also bound to DNA in the presence of Cu^{2+} (Shirahata et al., 1974). DNA or nucleic acid components could regulate the oxidation of aromatic reductones via formation of a mixed complex of DNA (or nucleic acid components)–Cu^{2+}–aromatic reductones (Shirahata et al., 1978). DNA breakage by aromatic reductones could be regulated by adenine or cytosine, which could regulate the oxidation of aromatic reductones in the presence of Cu^{2+} (Shirahata et al., 1982).

Taking into consideration these facts, such a mechanism of DNA breakage by EPI in the presence of Cu^{2+} as shown in Figure 6 can be proposed. That is, Cu^{2+} can bind both DNA and EPI and form DNA–Cu^{2+} and EPI–Cu^{2+} complexes, resulting in the formation of DNA–Cu^{2+}–EPI mixed complex. One-electron transfer in the mixed complex may occur and cause the local oxidation of EPI, which may generate oxygen radicals in the vicinity of DNA chains and be responsible for DNA breakage.

On the other hand, EPI–Cu^{2+} complexes may be unstable and cause the autoxidation of EPI not responsible for DNA degradation. Excess amounts of Cu^{2+} may accelerate the autoxidation of EPI via EPI–Cu^{2+} complexes because of the limited binding sites of DNA with Cu^{2+}, resulting

in a decrease of the DNA breakage number. Higher pH also may make the EPI–Cu^{2+} complex more unstable and accelerate the autoxidation of EPI, resulting in consumption of effective EPI that can bind to the DNA–Cu^{2+} complex and decrease of the DNA breakage number. In this mechanism, DNA breakage by EPI will be greatly influenced by the affinity of DNA or EPI with Cu^{2+} as well as by other coexisting chelating substances.

The rapid and quantitative computer analytical method of the DNA-breaking activities described here will be helpful for assessing the effects of many compounds in foodstuffs that cause DNA lesions. This method can be applied to the detection of single- or double-strand breaks of DNA, RNA, and polynucleotides.

Effects of base components and DNA structures on the DNA-breaking reaction by hydrolyzable and condensed tannins will be published elsewhere.

ABBREVIATIONS USED

AET, 2-(aminoethyl)isothiuronium; AsA, ascorbic acid; B-1, procyanidin B-1; C-1, procyanidin C-1; CAT, (+)-catechin; EGG, (−)-epigalllocatechin 3-*O*-gallate; EPI, (−)-epicatechin; GAL, (+)-gallocatechin; MEA, 2-mercaptoethylamine.

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Registry No. B-1, 20315-25-7; C-1, 37064-30-5; CAT, 154-23-4; EPI, 490-46-0; GAL, 970-73-0; EGG, 989-51-5; Cu, 7440-50-8.

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