HPLC Method for Evaluation of the Free Radical-scavenging Activity of Foods by Using 1,1-Diphenyl-2-picrylhydrazyl

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An HPLC method for evaluation of the free radical-scavenging activity of foods by using 1,1-diphenyl-2-picrylhydrazyl (DPPH) is reported. The activity was evaluated by measuring the decrease of DPPH detected at 517 nm. By using this novel method, we determined the free radical-scavenging activity of several antioxidants: ascorbic acid, α-tocopherol, Trolox, and cysteine. The results gave good correlation between the radical-scavenging activity determined by HPLC and by conventional colorimetry. This methodology was applied to determine the free radical-scavenging activity of 8 beverages. The activity of coffee was the highest, followed by red wine, green tea, oolong tea, black tea, rosé wine, white wine, and orange juice. The results well agree with those of previous reports. This method is expected to be useful for a simple and rapid determination of free radical-scavenging activity in colored foods, because coloring substances in foods do not interfere with the measurement.

Key words: radical-scavenging activity; 1,1-diphenyl-2-picrylhydrazyl; antioxidant; beverage

There is considerable recent evidence that free radicals induce oxidative damage to biomolecules. This damage causes atherosclerosis, aging, cancer, and several other diseases.1) Antioxidants, which scavenge free radicals, are known to possess an important role in preventing these free radical-induced diseases. Dietary foods contain a wide variety of free radical-scavenging antioxidants; for example, flavonoids and antioxidative vitamins such as ascorbic acid and α-tocopherol.2) These compounds are particularly rich in vegetables, fruits, tea, and wine. Epidemiological studies have shown that higher intake of fresh vegetables, fruits, tea, and wine is associated with lower risk of mortality from cancer and coronary heart disease.3-5) There is currently strong interest in natural antioxidants and their role in human health and nutrition.6)

In consideration of these points, the free radical-scavenging activity of antioxidants in foods, and their structure-activity relationship have been substantially investigated and reported. ESR and chemiluminescence methods have been frequently employed for measuring the radical-scavenging activity of antioxidants against 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anion radical (O2⁻), hydroxyl radical (OH·), and peroxyl radical (ROO·).7-12) In recent years, the oxygen radical absorbance capacity assay and the enhanced chemiluminescence assay have been developed for evaluation of the antioxidative activity of antioxidants in foods, serum and other biological fluids.13-17) However, these methods not only need special equipment and technical skill for the analysis, but also give complicated signals from the measurements. On the other hand, colorimetry with DPPH, a stable free radical, has been reported as a simple method for evaluation of the free radical-scavenging activity.18,19) However, this method is not applicable to colored foods due to interference by food pigments.

In this study, we have established a DPPH-HPLC method that is simple and rapid for determining the free radical-scavenging activity in colored foods. This method was applied to evaluate the free radical-scavenging activity of selected commercial beverages.

Materials and Methods

Materials. DPPH, L-ascorbic acid, DL-α-tocopherol, L-cysteine hydrochloride monohydrate, tris(hydroxymethyl) aminomethane (Tris), and methanol (HPLC grade) were obtained from Nacalai Tesque (Kyoto, Japan). Ethanol was obtained from Wako Pure Chemical Industries (Osaka, Japan), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). The water used in this experiment was purified with Milli-Q Labo equipment (Millipore Japan, Tokyo, Japan). Coffee, black tea, green tea, oolong tea, red wine, rosé wine, white wine, and orange juice were purchased at local markets. These were all canned beverages, except for the wines.

Measurement of radical-scavenging activity. Ascorbic acid and cysteine were dissolved in Tris-HCl buffer (100 mM, pH 7.4). α-Tocopherol and Trolox were dissolved

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8 Part of this study was presented at the 213th ACS National Meeting in April, 1997 at San Francisco, CA, U.S.A.
9 Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
in ethanol. Beverages were diluted with water of an appropriate volume.

(1) **Colorimetric analysis.** An aliquot of antioxidant solution or beverage (200 µl) was mixed with the 100 mM Tris-HCl buffer (pH 7.4, 800 µl) and then added to 1 ml of 500 µM DPPH in ethanol (final concentration of 250 µM). The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance at 517 nm by DPPH was measured by a UV-2100PC UV-VIS spectrophotometer (Shimadzu, Kyoto, Japan).

(2) **HPLC analysis.** An antioxidant or beverage was reacted with 500 µM DPPH in ethanol by the same procedure of colorimetric analysis as described above, and then subjected to a reversed-phase HPLC analysis. The HPLC equipment consisted of a Shimadzu LC-6A pump, a Rheodyne injector fitted with a 20 µl loop and a Shimadzu SPD-10AV UV-VIS detector set at 517 nm (0.064 aufs). Analyses were performed in a TSKgel Octyl-80Ts column (4.6 × 150 mm, Tosoh, Tokyo, Japan) at ambient temperature with a mobile phase of methanol/water (70:30, v/v) at a flow rate of 1 ml/min.

The DPPH radical-scavenging activity was evaluated from the difference in peak area decrease of the DPPH radical detected at 517 nm between a blank and a sample. Trolox was used as a control standard. The data are expressed as µmol of Trolox equivalent per 100 ml of each beverage, because Trolox is a stable antioxidant and is widely used as an index of antioxidative activity.

**Results and Discussion**

**Establishment of the DPPH-HPLC method**

In order to evaluate the free radical-scavenging activity of colored foods, we developed the DPPH-HPLC method. This method is based on the reduction of DPPH, a stable free radical, the structure of DPPH and its reduction by an antioxidant is shown in Fig. 1. Because of its odd electron, DPPH gives a strong absorption maximum at 517 nm by visible spectroscopy (purple color). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical-scavenging antioxidant, the absorption strength is decreased, and the resulting decolorization is stoichiometric with respect to the number of electrons captured.\(^{18}\) This reaction has been widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate the antioxidative activity of foods and plant extracts.\(^{20-23}\)

In order to determine the optimum condition for HPLC, we tested octyl and octadecyl columns and various compositions of methanol or acetonitrile in water as the mobile phase. The optimum condition for HPLC analysis was achieved in an octyl column with methanol/water (70:30, v/v) as the mobile phase. Figure 2(a) shows an HPLC chromatogram of the DPPH radical detected by the absorbance at 517 nm. The retention time of the DPPH radical was 9.0 min. A typical chromatogram of the DPPH radical after the addition of an antioxidant is shown in Fig. 2(b). As indicated, the peak of the DPPH radical is decreased in the presence of an antioxidant. Therefore, the free radical-scavenging activity can be evaluated by the decrease in peak area of the DPPH radical detected at 517 nm.

**Measurement of radical-scavenging activity by DPPH-HPLC and colorimetry**

The radical-scavenging activity of ascorbic acid, α-tocopherol, Trolox, and cysteine as antioxidants was measured by both DPPH-HPLC and colorimetry by using the DPPH radical (250 µM in the reaction mixture). When the DPPH radical was scavenged by an antioxidant through donation of hydrogen to form the reduced DPPH-H, the molar absorptivity at 517 nm changed from 9660 to 1640 and the color turned from...
purple to yellow.

Figure 3 shows the dose-response curve for the radical-scavenging activity of those antioxidants determined by the DPPH-HPLC method. The radical-scavenging activity was increased with increasing concentration of all the antioxidants. A liner response curve for radical-scavenging activity was obtained up to 80 μM of Trolox. The concentrations of ascorbic acid, α-tocopherol, Trolox, and cysteine to give 100% radical-scavenging activity were 92, 125, 117, and 248 μM, respectively. That is, 1 mol of ascorbic acid, α-tocopherol, Trolox, and cysteine trapped approximately 2.7, 2.0, 2.1, and 1.0 mol of DPPH, respectively. It is assumed that DPPH was trapped molecule-for-molecule by cysteine and in the ratio of two for one by α-tocopherol. In our results, the radical-scavenging activity of α-tocopherol and cysteine agree with that obtained by Blois,¹⁸ that is, one molecule of cysteine reacted with one molecule of DPPH to produce 0.5 molecule of cystine. Cysteine thus interacted with DPPH in the ratio of 1:1 after all. The reaction mechanism of DPPH with α-tocopherol can be explained by a two-step-reaction: in the first step, one molecule of DPPH reacts with one molecule of α-tocopherol to produce the α-tocopheroxy radical; then, the α-tocopheroxy radical reacts with another molecule of DPPH to form α-tocopheroquinone. Hence, two molecules of DPPH are reduced by one molecule of α-tocopherol.

We determined the radical-scavenging activity of low concentrations of Trolox (0–50 μM) to identify the detection limit by the DPPH-HPLC method. Figure 4 shows the relationship between the decrease in peak area and the Trolox concentration. The obtained values were the same as the expected values and produced little standard deviation at 25 and 50 μM Trolox. Therefore, as low as 25 μM Trolox in the reaction mixture could be precisely determined. From these results, it is possible to determine the radical-scavenging activity in a concentration range of 25 to 80 μM Trolox.

Table 1. 50% Radical-scavenging Activity Concentration of Antioxidants by the DPPH-HPLC Method and Colorimetry

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>DPPH-HPLC</th>
<th>Colorimetry</th>
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<tbody>
<tr>
<td>Ascorbic acid</td>
<td>47.6 ± 2.0</td>
<td>45.8 ± 2.3</td>
</tr>
<tr>
<td>Cysteine</td>
<td>101.2 ± 6.6</td>
<td>106.3 ± 8.2</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>58.8 ± 3.7</td>
<td>60.0 ± 3.6</td>
</tr>
<tr>
<td>Trolox</td>
<td>53.0 ± 1.5</td>
<td>53.3 ± 2.6</td>
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* Each value is the mean ± SD of 4–6 replicate assays.

Application of the DPPH-HPLC method to beverages
To evaluate the applicability of the DPPH-HPLC method to colored foods, the radical-scavenging activities of some commercial beverages were determined (Table II). The radical-scavenging activities are expressed as the Trolox equivalent. Among 8 commercial beverages, the radical-scavenging activity (μmol of Trolox equivalent/100 ml) of coffee was the highest (965), followed by red wine (944), green tea (746), and black tea (678), black tea (584), rose wine (480), white wine (322), and orange juice (216).

In a comparison of the 3 wines, the radical-scavenging activity decreased in the order of red wine > rose wine > white wine (Table II). Our result agrees with those of previous reports which described that red wines contained higher radical-scavenging activity than white wines due to the higher amount of phenolic compounds. 24–27 Phenolic compounds in wine have been shown to inhibit in vitro oxidation of human low-density lipoprotein, and drinking red wine decreases the risk of mortality from coronary heart disease. 26–28 This effect has been described as the ‘French paradox’. 2,29

Tea is widely consumed as a daily beverage. The most significant compounds in tea leaf are catechins which constitute the major component and seem to be responsible for the antioxidative activity. In general, the content of catechins in tea is related to the degree of fermentation during manufacture. Green tea, oolong tea, and black tea are classified according to their manufacturing process as non-fermented, semi-fermented, and fermented teas, respectively. Green tea is manufactured from fresh leaves without fermentation, making the loss of catechins by oxidation lower than that of black tea and oolong tea. Therefore, the content of catechins in the 3 teas was in the order green tea > oolong tea > black tea. Our result gave the highest radical-scavenging activity in green tea, followed by oolong tea and black tea (Table II), and this may be correlated with their content of catechins. In addition, our result agrees with that obtained by Yen and Chen, 30 who investigated the radical-scavenging effect of tea extracts on DPPH by spectrophotometric measurements at 517 nm.

Therefore, the majority of the free radical-scavenging activity of these beverages seems to have originated from such phenolic compounds as flavonoids and phenolic acids, and their antioxidative activities have been reported by many researchers. 7,26–28,30 Further study is needed to know about individual antioxidants and their contribution to free radical-scavenging activity.

In summary, these results indicate that the simple and rapid DPPH-HPLC method can be successfully applied to measure the free radical-scavenging activity of commercial beverages. It seems to be advantageous to measure the total activity of the beverages by this technique. The DPPH-HPLC method is superior to colorimetry because there is no interference from food pigments. It is anticipated that this method will be applicable to a wide variety of vegetables and other food products.

References

<table>
<thead>
<tr>
<th>Beverage</th>
<th>Radical-scavenging activity, (μmol Trolox equivalent/100 ml)</th>
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<tbody>
<tr>
<td>Red wine</td>
<td>944 ± 25</td>
</tr>
<tr>
<td>Rosé wine</td>
<td>480 ± 27</td>
</tr>
<tr>
<td>White wine</td>
<td>322 ± 36</td>
</tr>
<tr>
<td>Green tea</td>
<td>746 ± 73</td>
</tr>
<tr>
<td>Oolong tea</td>
<td>678 ± 6</td>
</tr>
<tr>
<td>Black tea</td>
<td>584 ± 44</td>
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<tr>
<td>Coffee</td>
<td>965 ± 71</td>
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<tr>
<td>Orange juice</td>
<td>216 ± 36</td>
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</tbody>
</table>

* Radical-scavenging activity was measured by the DPPH-HPLC method. Data are expressed as μmol of Trolox equivalent/100 ml of each beverage. Each value is the mean ± SD of 3 samples.