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Assessment of radical scavenging capacity of antioxidants contained in foods and beverages in plasma solution

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The assessment of the radical scavenging capacity of antioxidants has been the subject of extensive studies and controversy. The aim of this study is to develop a simple and inexpensive method for the assessment of the radical scavenging capacity of antioxidants contained in foods and beverages in plasma solution, a biologically relevant heterogeneous medium. Three types of probes, hydrophilic pyranine, with low reactivity, hydrophilic pyrogallol red (PGR), with high reactivity, and lipophilic BODIPY, with moderate reactivity, were separately used to measure the amount and rate of peroxyl radical scavenging. The amount of radicals scavenged by antioxidants was assessed from the lag phase produced by antioxidants in the decay of pyranine and BODIPY, while the reactivity of the antioxidants was assessed from their effect on the decay rate of PGR. Two liquid and two solid samples were tested. Commercial bottled green tea and vegetable juice were found to scavenge 15.6 and 3.45 mmol radicals L^{-1} and the former scavenged peroxyl radicals 81 times faster than the latter. As for the solid samples, instant coffee powder was found to scavenge several times more radicals and more rapidly than green tea powder. This method may be applied to the assessment of the radical scavenging capacity of antioxidants contained in foods, beverages, and supplements in biologically relevant heterogeneous media.

Introduction

Aerobic organisms are protected from oxidative stress induced by reactive oxygen and related species by an efficient defense network system, in which various antioxidants with multiple functions play their respective roles.[1] The accumulating evidence for the involvement of oxidative stress in the pathogenesis of various diseases has attracted much attention from the public as well as scientists to the putative beneficial effects of antioxidants.[1–6] Many epidemiological studies support the beneficial effects of antioxidants.[7] However, large-scale double-blind, randomized, controlled clinical intervention studies and their meta-analyses have not confirmed the beneficial effects of supplementation with antioxidants such as vitamin E against diseases such as cardiovascular diseases and neurological disorders.[8] On the other hand, recent studies show that antioxidants are effective for patients under high oxidative stress.[9–12] In order to assess the role and effects of antioxidants in the maintenance of human health, it is important to understand the mechanisms and dynamics of antioxidant action.

Some antioxidants are proteins and enzymes, which prevent the formation of reactive species by, for example, the reduction of peroxides and sequestration of metal ions, while others are small molecules that scavenge and remove reactive oxidants. Radical scavenging is one of the important functions of antioxidants in the defense network. Some radical scavenging antioxidants are synthesized *in vivo*, while many others are taken from foods, beverages, and supplements. Furthermore, some antioxidants act as signaling messengers to induce antioxidant compounds and/or enzymes and a battery of cytoprotective proteins.[13]

Physiologically essential polyunsaturated fatty acids and their esters are vulnerable to oxidation, and lipid peroxidation mediated by free radicals exerts deleterious effects by inducing membrane damage and producing reactive products that are cytotoxic *per se* and capable of modifying proteins and DNA bases. Considering the physiological concentrations and reactivity of antioxidants, the role of radical scavenging antioxidants is thought to primarily comprise scavenging peroxyl radicals to prevent chain propagation and inhibit lipid peroxidation.[14] Hydroxyl radicals and even alkoxyl radicals react with biological molecules too rapidly for antioxidants to scavenge efficiently *in vivo*.[14]

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The assessment of radical scavenging capacity has been the subject of extensive studies and argument.[1,14–20] The radical scavenging capacity of specific antioxidant compounds and mixtures of antioxidants contained in foods, beverages, and natural products has been assessed by numerous methods. The reactivity of antioxidants toward free radicals has often been assessed by reaction with stable free radicals such as 1,1-diphenyl-2-picrylhydrazyl (DPPH)[21] and galvinoxyl,[22,23] which are commercially available nitrogen- and oxygen-centered radicals, respectively. Their reaction with antioxidants can be measured easily by a visible absorption spectrophotometer. Different types of aryloxyl radicals have also been used.[24]

Furthermore, a competition method has been widely used. For example, radical scavenging capacity was assessed from the effect of an antioxidant on the decay of probes such as fluorescein,[25] pyranine,[26] and pyrogallol red (PGR)[27] induced by free radicals. However, each of these assays has limitations and it is difficult to compare data from different laboratories partly because the capacity has been assessed under different reaction conditions such as probes, reaction media, solvents, and pH.

The objective of the present study is to develop a simple and inexpensive method to assess the radical scavenging capacity of antioxidant compounds and their mixtures contained in foods, beverages, supplements and natural products in a biologically relevant *in vitro* system. Admittedly, it is important to isolate, identify, and measure the radical scavenging capacity of a specific antioxidant compound contained in foods and natural products, but it is also necessary to assess the total antioxidant capacity of mixtures of various antioxidants, since they may interact with each other to exert synergistic or antagonistic effects.

In the present study, three kinds of probes, hydrophilic pyranine, with low reactivity, hydrophilic pyrogallol red (PGR), with high reactivity, and lipophilic 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid (BODIPY), with moderate reactivity, were used to measure both the amount and rate of peroxyl radical scavenging separately. This method of assessing the amount and rate of radical scavenging separately using multiple probes in a biologically relevant heterogeneous medium has not been applied before.

Various reaction media have been applied for the assessment of radical scavenging capacity, including homogeneous aqueous or organic solutions and heterogeneous aqueous suspensions of micelles, liposomal membranes, low-density lipoproteins, erythrocytes, and cultured cells. In the present study, antioxidant capacity was assessed in an aqueous solution of plasma, which has the advantage of providing both hydrophilic and lipophilic domains in a biologically relevant medium. This is important as there are both water-soluble and lipid-soluble antioxidants. It may be noteworthy that artificial micelles and liposomal membranes are not always the same as biological membranes or lipoproteins. In some cases, cyclodextrin was used to dissolve lipophilic antioxidants.[28] It is also important to note that the rate of incorporation of antioxidants into erythrocytes and cultured cells, which determines their apparent antioxidant capacity, is markedly dependent on the type of antioxidants and reaction conditions.[29]

Furthermore, in the present study, the peroxyl radical was chosen as a target radical for scavenging, since the peroxyl radical plays an important role as a chain-carrying species in lipid peroxidation and, as mentioned above, is the primary target radical for antioxidants to scavenge *in vivo*.[14] Peroxyl radicals were generated by thermal decomposition of 2,2′-azobis-(2-amidinopropane) dihydrochloride (AAPH). This decomposes thermally to yield carbon-centered radicals, which react rapidly with molecular oxygen to produce peroxyl radicals in the aqueous phase. The half-life of AAPH is 175 hours at 37 °C and hence it is assumed that AAPH produces peroxyl radicals at a virtually constant rate for the first several hours.[30] This is critically important for quantitative assessment.

Materials and methods

Materials

2-Carboxy-2,5,7,8-tetramethyl-6-hydroxychromane (Trolox) was purchased from Cayman Chemical Company (Michigan, USA). Several commercial products were tested, including bottled green tea, vegetable juice, green tea powder "maccha," and instant coffee powder.

AAPH obtained from Wako Pure Chemical Industries (Osaka, Japan) was used as received to generate free radicals at a constant and controlled rate. Three kinds of probes were used. PGR and pyranine were purchased from Sigma Aldrich (Tokyo, Japan). A lipophilic probe, BODIPY, was purchased from Molecular Probes (Invitrogen, Oregon, USA). PGR and pyranine were added to the reaction mixture as a PBS solution, while BODIPY was added as a DMSO solution. The concentration of DMSO was kept below 1 vol% of the total solution.

Plasma preparation

The animal experiments and care were approved by the Institutional Animal Care and Use Committee of Kyoto Prefectural University of Medicine. Wild-type male C57BL/6J mice were purchased from Shimizu Laboratory Supplies Co. Ltd (Kyoto, Japan) and maintained under standardized conditions of a 12 hour/12 hour light/dark schedule. Blood was collected in heparin-containing tubes from mice. Plasma was obtained by centrifugation at 3500 rpm for 15 min at 4 °C, frozen on ice immediately and stored until analysis.

Spectrophotometric measurement of probe decay

The rates of reaction of pyranine, PGR, and BODIPY with peroxyl radicals were followed in PBS containing mouse plasma by measuring the decay of the probe at 454 nm, 540 nm, and 586 nm, respectively, with a microplate reader SpectraMax M2 (Molecular Devices, Sunnyvale, CA) equipped with a thermostatted cell maintained at 37 °C under air. The concentrations of pyranine, PGR, and BODIPY were selected as 50, 30, and 10 μM, respectively, to obtain a maximum absorbance around 0.5 at the abovementioned wavelengths.

The concentrations of AAPH and plasma were varied between 0 and 100 mM and between 0 and 20 vol%, respectively, to confirm the concentration dependence, while those of the test samples were chosen to obtain an appropriate lag phase (between 20 and 120 min) and rate of probe decay, as described later in the Results section. Unless otherwise noted, the effects of test samples were measured at an initial concentration of 50 mM AAPH and 10 vol% plasma in PBS. It should be noted that the concentration of total plasma lipids was higher than 1 mM, *i.e.*, 100 times that of BODIPY.

The reaction was started by the addition of AAPH dissolved in PBS and the absorbance change was recorded continuously over time. The lag phase was determined graphically by extrapolating the slope of maximum probe decay to its intersection with the slope of minimum probe decay at the initial stage, as reported previously.[31–33] The consumption rate of PGR was measured from the slope of the decay curve against time at the initial stage.

The experiments were repeated several times for each system. Typical examples of probe decay are shown in the figures and the results are given in the tables by average and SD.

Results

Amount of free-radical scavenging

In the absence of AAPH, pyranine was stable in PBS and no significant change in its absorption at 454 nm was observed, but the addition of AAPH immediately induced consumption of pyranine at a constant rate without a lag phase (Fig. 1). The rate of pyranine decay was directly proportional to the AAPH concentration (data not shown). The addition of mouse plasma inhibited the decay of pyranine and produced a distinct lag phase (Fig. 1). It is assumed that this lag phase was produced by endogenous antioxidants in the plasma such as ascorbate, uric acid, and proteins.

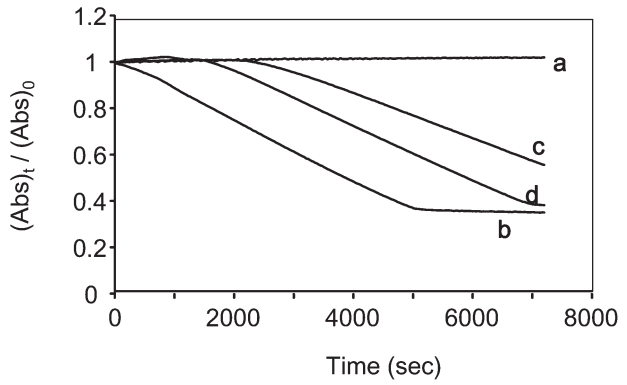

Fig. 1 Decay of pyranine (454 nm) induced by peroxyl radicals generated from AAPH at 37 °C in aqueous PBS solution in the absence and presence of 10 vol % mouse plasma at 37 °C. [AAPH] = 50 mM. [Pyranine] = 50 microM. Line a: without AAPH; b: without plasma; c: with plasma; d: with 0.5 % bottled green tea without plasma.

As observed with hydrophilic pyranine, lipophilic BODIPY added to a PBS solution of plasma was similarly consumed in the presence of AAPH and its decay could be followed from its visible absorption at 540 nm. BODIPY is assumed to be distributed and consumed by radicals within lipoproteins in plasma suspensions. BODIPY consumption is inhibited by scavenging radicals in either the aqueous phase or the lipophilic domain of lipoproteins.

The lag phase was measured at different plasma concentrations, ranging from 0 to 20 vol% (Fig. 2). The lag phase was directly proportional to the plasma concentration and a plot of the lag phase against the plasma concentration gave a straight line. The slopes obtained by linear regression were 217 and 283 s per plasma volume% for pyranine and BODIPY, respectively (Fig. 2C and 2D).

The rate of free-radical flux R_i from AAPH under the reaction conditions employed here was calculated from the lag phase for pyranine produced by Trolox, a water-soluble analogue of α-tocopherol. The lag phase is given by eqn (1):[32]

$$\text{Lag phase} = n[\text{IH}]/R_i \quad (1)$$

where n, IH, and R_i are stoichiometric numbers, *i.e.*, the number of free-radical molecules scavenged by each antioxidant molecule, the antioxidant concentration, and the rate of free-radical flux from AAPH, respectively. Trolox added to an aqueous solution containing 10 vol% plasma increased the lag phase for the consumption of pyranine and BODIPY in a concentration-dependent manner (Fig. 3A and 3B). A plot of the lag phase as a function of Trolox concentration gave a straight line, as shown in Fig. 3C and 3D for pyranine and BODIPY, respectively. It is known that each Trolox molecule scavenges two molecules of free radicals,[32] *i.e.*, n = 2.0. Several series of experiments were performed and an average slope was obtained of 45.9 s μM^{-1}, from which the rate of free-radical flux from 50 mM AAPH in plasma solution was calculated, as shown in eqn (2):

$$R_i = n/\text{slope} = 2/45.9 = 4.4 \times 10^{-2}\ \mu\text{M s}^{-1} = 4.4 \times 10^{-8}\ \text{M s}^{-1} \quad (2)$$

The amount of peroxyl radicals scavenged by the antioxidants in the plasma was calculated from eqn (3):

$$n[\text{IH}] = \text{lag phase (tlag)} \times R_i \quad (3)$$

When V vol% plasma gives a lag phase tlag (in seconds), the amount of radicals scavenged is given by $100 \times V \times \text{tlag} \times R_i$ mol L^{-1}. The lag phase was measured at different plasma concentrations for pyranine and BODIPY (Fig. 2) and the amount of radicals scavenged by the plasma was calculated as $(9.74 \pm 0.53) \times 10^{-4}$ and $(1.32 \pm 0.042) \times 10^{-3}$ M, respectively.

The capacity for scavenging radicals was assessed for four commercial samples: bottled green tea, vegetable juice, "maccha" green tea powder, and instant coffee powder. These inhibited the consumption of pyranine and BODIPY and produced a clear lag phase in a concentration-dependent manner. The results for vegetable juice are shown in Fig. 4 as an example.

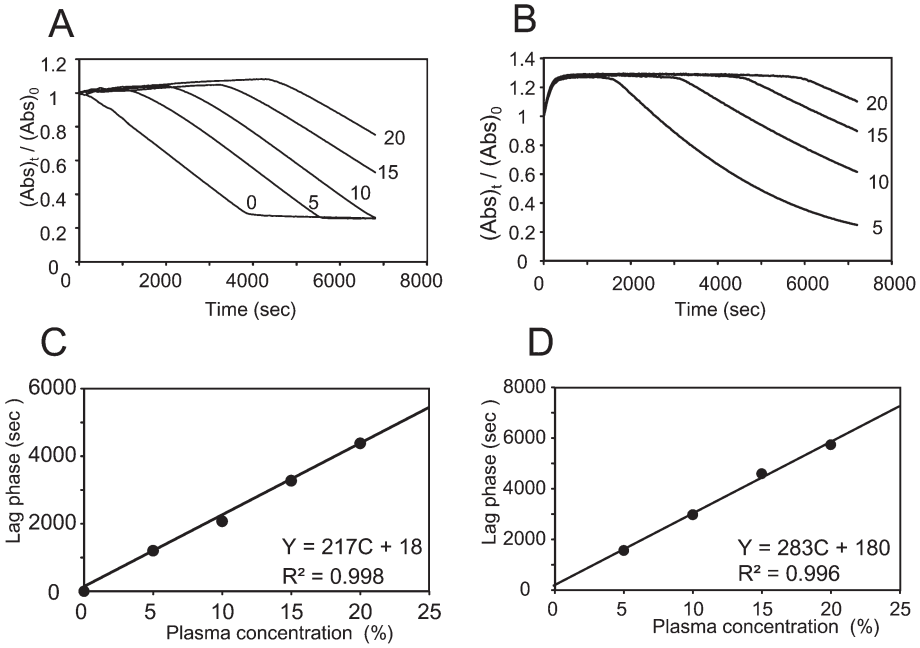

Fig. 2 Effect of plasma concentration on the decay of pyranine and BODIPY induced by AAPH at 37 °C. (A, B) decay of pyranine and BODIPY, respectively. The numbers on the graph show the plasma concentration in vol%. (C, D) plot of lag phase for pyranine and BODIPY, respectively, against plasma vol%. [AAPH] = 50 mM, [pyranine] = 50 μM, [BODIPY] = 10 μM.

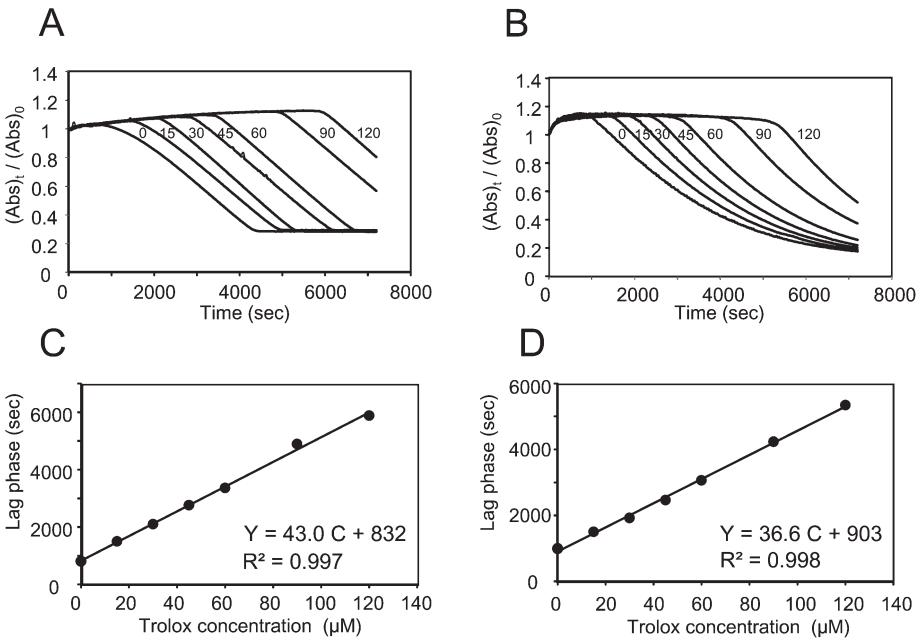

Fig. 3 Lag phase produced by Trolox for the decay of pyranine and BODIPY. (A, B) effect of Trolox concentration on the decay of pyranine and BODIPY, respectively. (C, D) plot of lag phase against Trolox concentration. Initial concentrations: plasma 10 vol%, AAPH 50 mM, pyranine 50 μM, BODIPY 10 μM. Numbers on the graph show the Trolox concentration in μM.

The amount of radicals scavenged by the antioxidants in the test samples, *i.e.*, n[IH], was calculated from eqn (4):

$$n[\mathrm{IH}] = (\mathrm{tlag\ (sample)} - \mathrm{tlag\ (plasma)}) \times R_i \qquad (4)$$

where tlag (sample) and tlag (plasma) denote the lag phases produced by the test samples and plasma alone, respectively. The tlag (plasma) value was dependent on the individual mouse, but the capacity for radical scavenging, as calculated

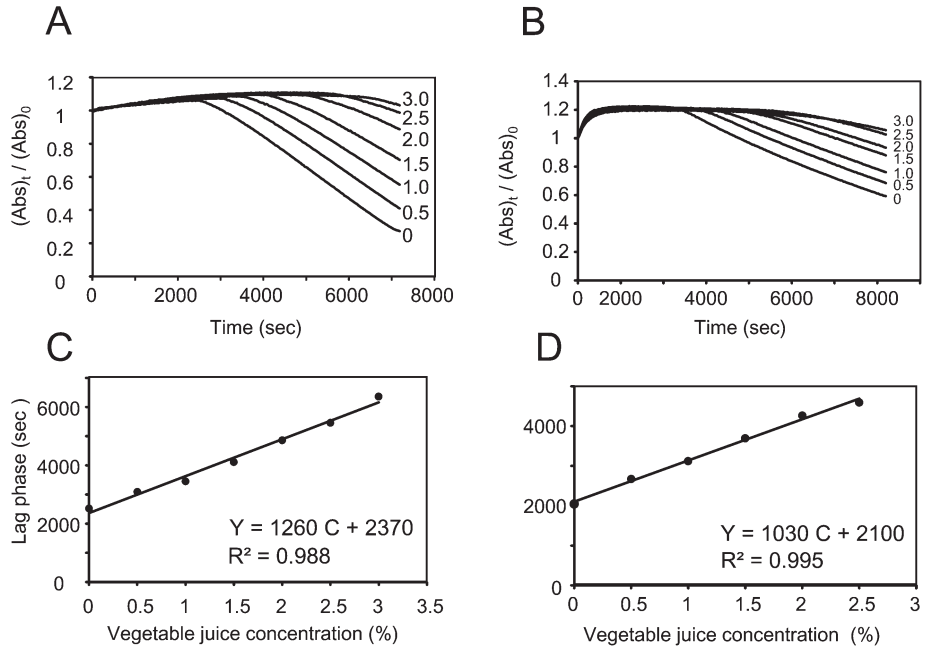

Fig. 4 Effect of vegetable juice on the decay of pyranine (A) and BODIPY (B) induced by AAPH at 37 °C and plot of lag phase produced for decay of pyranine (C) and BODIPY (D) against vegetable juice concentration. AAPH 50 mM, pyranine 50 μM, BODIPY 10 μM.

from eqn (4), was independent of the plasma used, as long as the same plasma was used for all test samples.

All four kinds of test samples displayed a good correlation between the lag phase and the amount of test sample added to the plasma solution. The plot of lag phase against test sample concentration gave a satisfactory straight line, and the slope and the intercept at the *Y*-axis obtained by linear regression are summarized in Table 1. This shows that the slopes obtained for pyranine and BODIPY are similar for each sample. It should be noted that the units of the slope are s per vol% and s mg^{-1} mL^{-1} for liquid and solid samples, respectively.

Table 1 Linear correlation between the lag phase for the consumption of pyranine or BODIPY and the test sample concentration[a]

	Probe	
Test sample	Pyranine	BODIPY
Liquid sample, in s per vol%		
Bottled green tea	$3.54 \times 10^3\ C + 1.00 \times 10^3$	$3.48 \times 10^3\ C + 1.25 \times 10^3$
Vegetable juice	$1.26 \times 10^3\ C + 2.37 \times 10^3$	$1.03 \times 10^3\ C + 2.10 \times 10^3$
Solid sample, in s mg^{-1} mL^{-1}		
Green tea powder (DMSO)	$1.46 \times 10^3\ C + 1.93 \times 10^3$	$2.69 \times 10^3\ C + 1.03 \times 10^3$
Green tea powder (MeOH)	$1.10 \times 10^3\ C + 1.44 \times 10^3$	$2.25 \times 10^3\ C + 1.09 \times 10^3$
Green tea powder (water)	$1.70 \times 10^3\ C + 2.31 \times 10^3$	$1.12 \times 10^3\ C + 1.45 \times 10^3$
Instant coffee powder	$4.71 \times 10^4\ C + 0.08 \times 10^4$	$4.42 \times 10^4\ C + 0.11 \times 10^4$

[a] Solvent used for extraction from solid samples is shown in parentheses. DMSO: dimethylsulfoxide, MeOH: methanol.

The amounts of radicals scavenged by the test samples, calculated by eqn (4) from the lag phases for pyranine and BODIPY, are summarized in Table 2. They can also be calculated from the slope of the correlation between the lag phase (in seconds) and the sample concentration *C* by eqn (5):

$$\text{Lag phase} = \text{slope} \times C + a \quad (5)$$

Table 2 Amount of free radicals scavenged by test samples[a]

	Pyranine	BODIPY
Liquid sample, mol L^{-1}		
Bottled green tea	$(1.42 \pm 0.16) \times 10^{-2}$ $[1.56 \times 10^{-2}]$	$(1.92 \pm 0.51) \times 10^{-2}$ $[1.53 \times 10^{-2}]$
Vegetable juice	$(4.95 \pm 0.48) \times 10^{-3}$ $[5.55 \times 10^{-3}]$	$(4.03 \pm 0.84) \times 10^{-3}$ $[4.55 \times 10^{-3}]$
Solid sample, mmol g^{-1}		
Green tea powder (DMSO)	$(6.64 \pm 0.20) \times 10^{-2}$ $[6.44 \times 10^{-2}]$	$(1.30 \pm 0.24) \times 10^{-1}$ $[1.18 \times 10^{-1}]$
Green tea powder (MeOH)	$(5.38 \pm 0.71) \times 10^{-2}$ $[4.83 \times 10^{-2}]$	$(9.86 \pm 0.76) \times 10^{-2}$ $[9.90 \times 10^{-2}]$
Green tea powder (water)	$(9.06 \pm 1.36) \times 10^{-2}$ $[7.48 \times 10^{-2}]$	$(4.75 \pm 0.53) \times 10^{-2}$ $[4.93 \times 10^{-2}]$
Instant coffee powder	2.17 ± 0.12 [2.07]	2.11 ± 0.18 [1.94]

[a] The numbers in brackets show the amount calculated from the slope of the plot by eqn (5). The solvents used for extraction from green tea powder are shown in parentheses. DMSO: dimethylsulfoxide; MeOH: methanol.

where C is the concentration of the test sample in vol% or mg mL^{-1} for liquid or solid samples, respectively, and a is the lag phase produced by plasma alone without added antioxidant. The amount of free radicals scavenged by the test sample is given by 100 × slope × R_i mol L^{-1} for liquid samples and by slope × R_i mol g^{-1} for solid samples. The results thus obtained from the slope are also included, in brackets, in Table 2.

The results in Table 2 show that the radical scavenging capacity of green tea powder varied considerably with the solvent used for extraction. Higher amounts were observed for BODIPY than for pyranine when DMSO and methanol were used, whereas extraction by water gave reverse results. This may be ascribed to the different amounts of lipophilic and hydrophilic antioxidants in the extracts. Interestingly, instant coffee powder contained more antioxidants than green tea powder.

Thus, the total amount of free radicals that can be scavenged by plasma, biological samples, and natural and commercial products may be assessed by this method. This may be applied to examine the effect of antioxidant supplementation on the free-radical scavenging capacity of biological fluids and tissues.

Rate of radical scavenging

The rate of peroxyl radical scavenging was assessed from the effect of the test samples on the decay rate of PGR induced by AAPH.[27,32,34] This demonstrates the reactivity of the antioxidant toward peroxyl radicals. PGR is water-soluble and much more reactive than pyranine and competes well with antioxidants for scavenging peroxyl radicals. Many antioxidants did not inhibit PGR consumption completely, but rather retarded the decay of PGR by scavenging peroxyl radicals in competition with PGR. The effects of instant coffee powder on the decay of PGR are shown in Fig. 5A as an example. PGR was not consumed in the absence of AAPH. A short lag phase was produced by vitamin C, which can inhibit the decay of PGR completely.[27,32] The antioxidants contained in instant coffee powder suppressed the decay of PGR in a concentration-dependent manner.

The rates of PGR decay in the absence and presence of an antioxidant, denoted R_0 and R_{IH}, respectively, are given by eqn (6) and (7):

$$R_0 = k_{PGR} \times [\text{radical}] \times [\text{PGR}] \quad (6)$$

$$R_{IH} = R_0 \times (k_{PGR} \times [\text{radical}] \times [\text{PGR}])/(k_{PGR} \times [\text{radical}] \times [\text{PGR}] + k_{IH} \times [\text{radical}] \times [\text{IH}]) \quad (7)$$

where k_{PGR} and k_{IH} are the rate constants for the reactions of peroxyl radicals with PGR and antioxidant, respectively. The ratio of the rates of PGR decay in the absence and presence of an antioxidant is given by eqn (8):

$$R_0/R_{IH} = (k_{IH}[\text{IH}]/k_{PGR}[\text{PGR}]) + 1 \quad (8)$$

A plot of R_0/R_{IH} as a function of [IH]/[PGR] should give a straight line, the slope being k_{IH}/k_{PGR}, which represents the relative reactivity of the antioxidant toward peroxyl radicals. The results for instant coffee powder included in Fig. 5B as an example show a linear correlation with an intercept at the Y-axis of 1, as expected from eqn (8). Similarly, all the samples tested gave a straight line with an intercept at the Y-axis close to 1 (data not shown). The calculated slopes for each sample are summarized in Table 3. This shows that the antioxidants contained in the bottled green tea used in this study were about ten times more reactive than those in vegetable juice,

Table 3 Ratios of the rate constants for scavenging peroxyl radicals by test sample and PGR, k_{IH}/k_{PGR}

Antioxidant compound: k_{IH}/k_{PGR} in (1/Ms)/(1/Ms)	
Trolox	0.17 ± 0.03
Liquid sample: k_{IH}/k_{PGR} in μM per vol%	
Bottled green tea	33.0 ± 18.3
Vegetable juice	3.35 ± 1.81
Solid sample: k_{IH}/k_{PGR} in μM mg^{-1} mL^{-1}	
Green tea powder (DMSO)	20.3 ± 2.75
Green tea powder (MeOH)	9.24 ± 4.06
Green tea powder (water)	10.7 ± 0.9
Instant coffee powder	67.9 ± 2.1

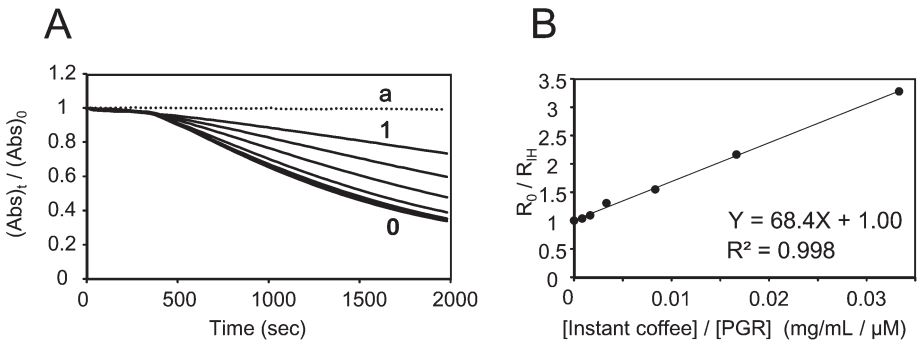

Fig. 5 Effect of instant coffee powder on the decay of pyrogallol red (PGR) induced by AAPH in 10 vol% plasma. (A) The broken line "a" shows the result without AAPH. The solid lines correspond to the results for 1, 0.5, 0.25, 0.1, 0.05, 0.025, and 0 mg mL^{-1} from top to bottom. (B) Plot of R_0/R_{IH} against [Instant coffee]/[PGR] mg mL^{-1} μM^{-1}. The initial concentrations of AAPH and PGR were 50 mM and 30 μM, respectively.

the antioxidants extracted from green tea powder by DMSO were about twice as reactive toward peroxyl radicals than those extracted by methanol or water, and the antioxidants contained in instant coffee powder were several times more reactive than those in green tea powder.

In contrast to a single antioxidant compound, the reactivity of multiple antioxidants contained in foods and beverages is difficult to show in absolute terms, as test samples usually contain mixtures of antioxidants with different reactivities and the apparent k_{IH} changes with the consumption of antioxidants.

Discussion

The assessment of the free-radical scavenging capacity of various antioxidant compounds, and also natural and commercial products, has been the subject of extensive studies and arguments.[14,17,18,20] The ORAC method[35] has been used most frequently, but this method has an inherent drawback: it does not distinguish between the concentration and reactivity of antioxidants.[1] In other words, it is not clear if a higher ORAC value is due to the higher concentration or higher reactivity of an antioxidant or both.

The present study provides a simple method to assess both the concentration and the reactivity of antioxidants capable of scavenging peroxyl radicals: not only single specific antioxidant compounds but also mixtures of antioxidants contained in liquid and solid samples and natural and commercial products without expensive tools such as liquid or gas chromatography and mass spectrometers. Furthermore, both water-soluble and lipid-soluble antioxidants can be assessed in the same biologically relevant medium.

The standard deviation of the relative rate of radical scavenging shown in Table 3 is very large. This may be due to the fact that the test samples contain many kinds of antioxidant compounds with different reactivities and the apparent rate constant k_{IH} varies with time as the more reactive antioxidants are consumed. This may be pointed out as an inherent limitation of this method.

As described above, this method also determines the antioxidant capacity of plasma or other biological fluids for scavenging free radicals and can be applied for assessment of the effects of antioxidant intake from the diet or supplementation. The assessment and interpretation of the antioxidant capacity of plasma have been the subject of extensive studies and arguments.[1,19,36,37] It may be worth noting that the capacity of plasma for scavenging free radicals may be assessed, but that this may not necessarily correlate with its capacity to inhibit plasma oxidation or with the level of oxidative stress *in vivo*. The efficacy of antioxidant intake for inhibition of oxidation *in vivo* may be better assessed from the levels of some specific oxidation products of lipids or proteins in biological samples. Furthermore, it is important to bear in mind that the oxidation of biological molecules *in vivo* may be induced and mediated by multiple oxidants *via* non-radical as well as free-radical mechanisms and that each antioxidant reacts with various oxidants with different selectivity, specificity, and reactivity.

Furthermore, it should be noted that capacity for scavenging free radicals does not always correlate with that for inhibition of the oxidation of biological molecules, as the efficacy of antioxidant action depends on several factors, including the fate of antioxidant-derived radicals and interaction with other antioxidants, as well as its capacity for scavenging radicals. For example, it was found previously that BODIPY was a useful probe for the assessment of radical scavenging capacity, but not for the capacity to inhibit lipid peroxidation.[38]

Antioxidant effects *in vivo* are dependent on the bioavailability of antioxidants as determined by uptake, distribution, metabolism, and excretion, except for their effects in the gastrointestinal tract. It is imperative to choose an appropriate method and conditions to assess "antioxidant capacity," depending on the target to be measured. Biological antioxidant capacity may be assessed from the effects of antioxidant administration on the level of oxidation products *in vivo*, such as hydroxyoctadecadienoic acid, isoprostane, protein carbonyls, and 8-oxoguanine,[39] but the effects of antioxidants against diseases are another issue.

Abbreviations

AAPH	2,2′-Azobis(2-amidinopropane)dihydrochloride
BODIPY	4,4-Difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene-3-undecanoic acid
DMSO	Dimethylsulfoxide
DPPH	1,1-Diphenyl-2-picrylhydrazyl
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffered saline
PGR	Pyrogallol red
Trolox	2-Carboxy-2,5,7,8-tetramethyl-6-hydroxychromane

Conflict of interest

Mayuko Morita and Toshikazu Yoshikawa are affiliated with the collaborative research department (Department of Gastrointestinal Immunology), which receives donations from Nichinichi Pharmaceutical Co. Ltd.

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