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Simple Assessment of Radical Scavenging Capacity of Beverages

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The radical-scavenging antioxidants play an important role against oxidative stress in the defense system in vivo. The beneficial effects of antioxidants contained in foods and beverages have been well-accepted, and their antioxidant capacity has been assessed by various methods. In the present study, a simple method is proposed in which the total radical scavenging capacity is assessed from the bleaching of pyranine and pyrogallol red induced by free radicals generated from azo initiator. The total content of antioxidants contained in red wine, green tea, and cassis drink and their reactivities toward peroxyl radicals were measured from the lag phase and rate of bleaching using pyranine and pyrogallol red as a probe, respectively. It was found that this method to follow the bleaching of two probes by visible light spectrophotometer is convenient and applicable for assessment of total radical scavenging capacity of both content and activity of the antioxidants contained in beverages.

KEYWORDS: Antioxidant capacity assessment; beverage; pyranine; pyrogallol red; radical scavenging

INTRODUCTION

The aerobic organisms are protected from oxidative stress by an efficient defense network system in which various antioxidants with different functions play important roles. The radical scavenging antioxidants inhibit free radical-mediated oxidation of lipids, proteins, and DNA, which is implicated in the pathogenesis of various disorders and diseases. Natural antioxidants contained in fruits, vegetables, and beverages received much attention (*1*). The antioxidant capacity of pure compounds and commercial products has been assessed by several methods (*2*−*4*). Each method measures a specific factor and has its own merits and demerits. It is often argued that each component contained in foods and beverages should be identified and measured, but it is difficult to determine the numerous compounds and their concentrations. Furthermore, the antioxidants often act cooperatively or synergistically together with other antioxidants in vivo, and it is also necessary to measure the total antioxidant capacity of the mixtures of antioxidants.

It is usually easier and more accurate to measure *relative* antioxidant capacity than the absolute capacity. For this purpose, competitive techniques have been employed widely to compare the antioxidant compound or mixtures with a reference probe. Various reference compounds have been used such as crocin (*5*), 2,2′-azinobis(3-ethylbenzothiazoline-6- sulfonic acid (ABTS) (*6*), β-phycoerythin (*7*), fluorescein (*8*), 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3*a*,4*a*-diaza-*S*-indacene-3-undecanoic acid (BODIPY) (*9*), pyrogallolsulfonphthalein (pyrogallol red, PGR) (*10*), pyranine (8-hydroxy-1,3,6-pyrenetrisulfonic acid, trisodium salt) (*11*), β-carotene (*12*), and *N*,*N*-diphenyl-*p*-phenylenediamine (*13*).

The competition kinetics has been well-understood. Assuming a simple competition between the probe, PGR in this study, and antioxidant (IH) for scavenging peroxyl radicals derived from the azo initiator (A—N═N—A), the reactions are thought to proceed as shown below.

$$\mathrm{A{-}N{=}N{-}A} \rightarrow \mathrm{N_2} + 2\mathrm{A}^{\bullet} \xrightarrow{\mathrm{O_2}} 2\mathrm{AO_2}^{\bullet} \quad (1)$$

$$2\mathrm{AO_2}^{\bullet} + \mathrm{PGR} \xrightarrow{k_{\mathrm{PGR}}} \text{bleaching} \quad (2)$$

$$2\mathrm{AO_2}^{\bullet} + \mathrm{IH} \xrightarrow{k_{\mathrm{IH}}} \mathrm{AOOH} + \mathrm{I}^{\bullet} \quad (3)$$

$$2\mathrm{AO_2}^{\bullet} \rightarrow \text{nonradical products} \quad (4)$$

When the concentration of PGR is sufficiently high to scavenge all $AO_2^{\bullet}$ radicals, the self-interactions between $AO_2^{\bullet}$ radicals (reaction 4) are not important (*14*). Under these circumstances, the ratio of the rate of consumption of PGR in the absence of antioxidant (R_0) to that in its presence (R_{IH}) is given by the following equation:

$$R_0/R_{\mathrm{IH}} = 1 + k_{\mathrm{IH}}[\mathrm{IH}]/k_{\mathrm{PGR}}[\mathrm{PGR}] \quad (5)$$

In the present study, the free radical scavenging capacity of commercial beverage Cassis-I A50 prepared from black

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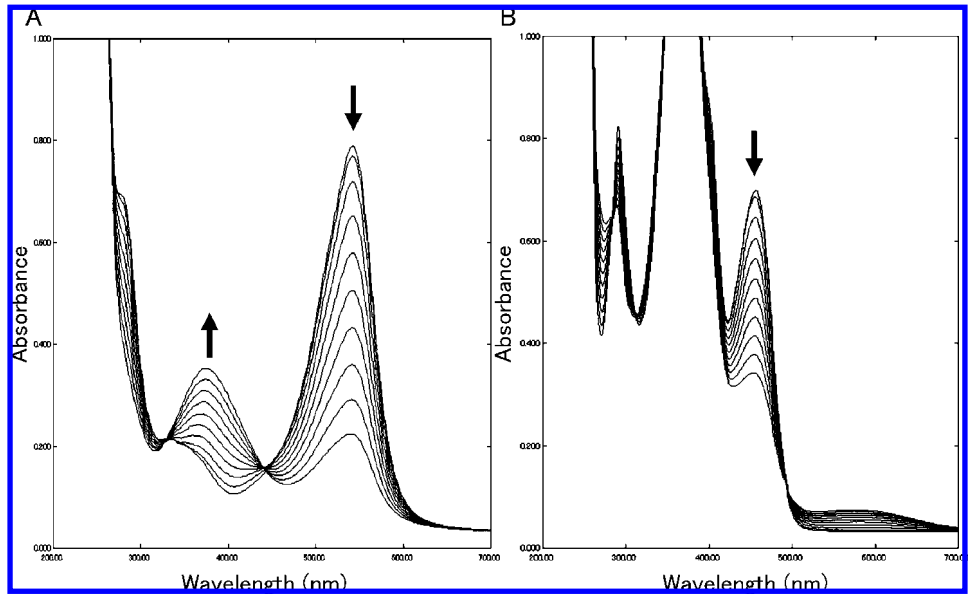

Figure 1. Change in absorption spectroscopy of (**A**) pyrogallol red (30 μM) and (**B**) pyranine (50 μM) induced by free radicals in SDS (0.5 M) micelle solution containing methyl palmitate and methyl linoleate (0.5 vol % each) at 37 °C: (**A**) 6 mM AIPH; (**B**) 50 mM AAPH. The absorption spectra were recorded every 3 min. The upward and downward arrows denote increase and decrease in absorbance, respectively.

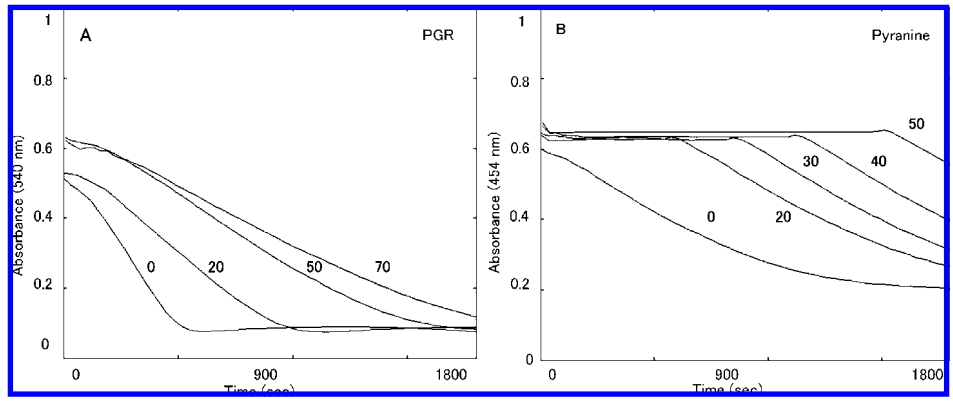

Figure 2. Effect of Trolox on the consumption of (**A**) pyrogallol red (30 μM) and (**B**) pyranine (50 μM) induced by free radicals. The reaction was induced by the addition of 30 mM AIPH to the SDS (0.5 M) micelle solution containing methyl palmitate and methyl linoleate (0.5 vol % each) at 37 °C. Numbers in the panels show the concentration of Trolox in μM.

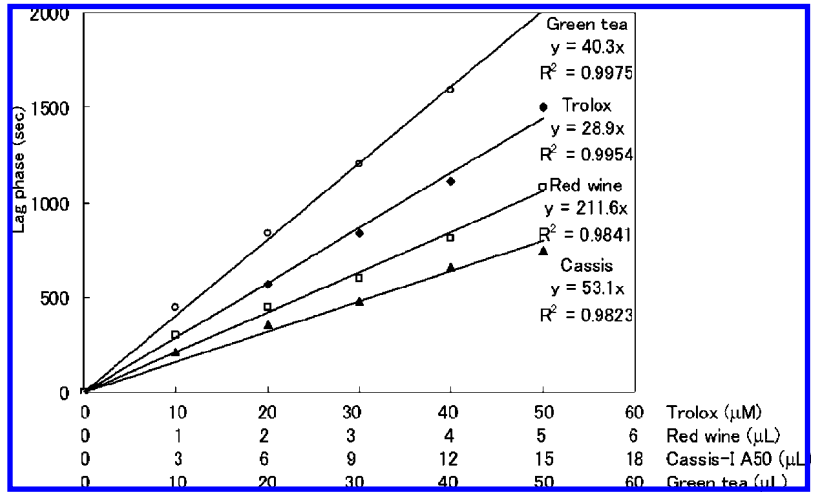

Figure 3. Lag phase for pyranine consumption produced by Trolox, red wine, green tea, and Cassis-I A50. The reaction was induced by the addition of 30 mM AIPH to the SDS (0.5 M) micelle solution containing methyl palmitate and methyl linoleate (0.5 vol % each) and 50 μM pyranine at 37 °C.

currant, green tea, and red wine was assessed by using PGR and pyranine as a reference compound. Both probes have characteristic absorption in the visible light region and react with free radicals with different reactivity. Trolox (6-hydroxy-

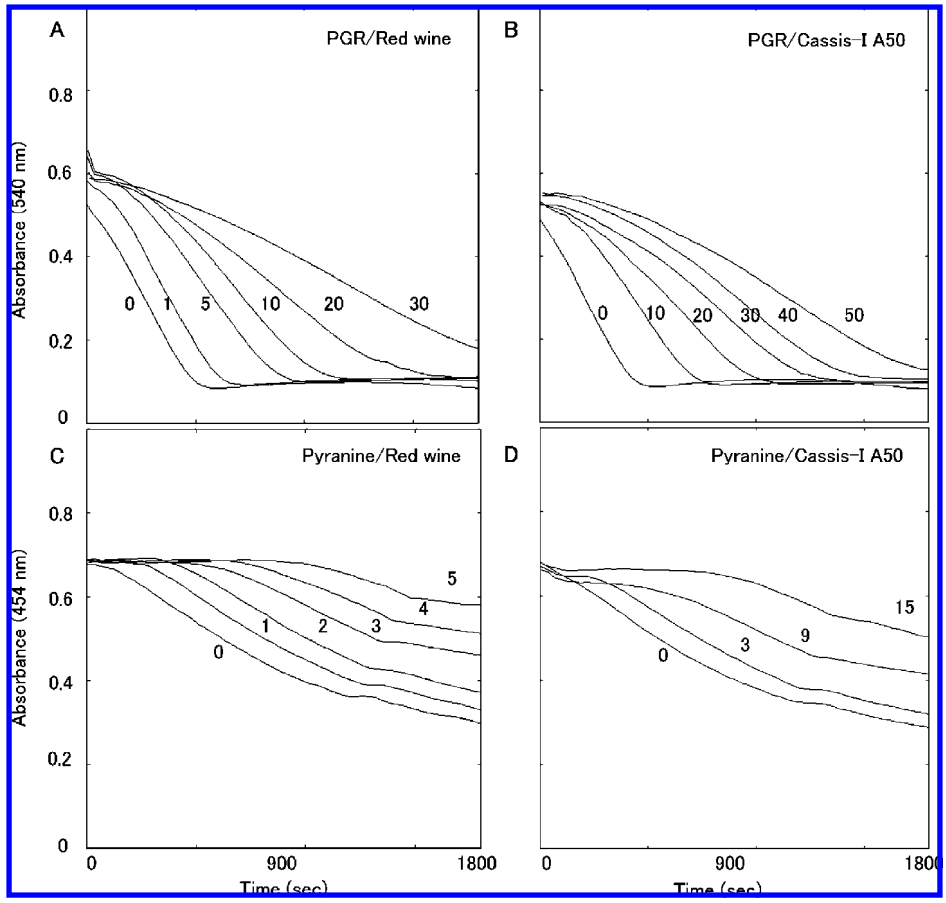

Figure 4. Effects of red wine and Cassis-I A50 on the consumption of PGR and pyranine. The reaction was induced by the addition of 30 mM AIPH to the SDS (0.5 M) micelle solution containing methyl palmitate and methyl linoleate (0.5 vol % each) and (**A**, **B**) 30 μM pyrogallol red or (**C**, **D**) 50 μM pyranine at 37 °C. Numbers in the panels show the volume of red wine and Cassis-I A50 added in μL.

2,5,7,8-tetramethylchromane-2-carboxylic acid) was also tested for comparison.

MATERIALS AND METHODS

Materials. Azo initiator was used to generate free radicals at a constant rate. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2′-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (AIPH) were obtained from WAKO Pure Chemical Industries (Osaka, Japan). Methyl palmitate and methyl linoleate were obtained from Tokyo Kasei Kogyo Co. (Tokyo, Japan) and used as received. Trolox, PGR, and pyranine purchased from Sigma Aldrich Japan K. K. (Tokyo, Japan) were used without purification. Commercial Cassis-I A50 (Meiji Seika Kaisha, Ltd., Tokyo), green tea (ITO EN, Ltd., Tokyo), and red wine were purchased for test.

Methods. The micelles were prepared by vigorously mixing methyl palmitate and methyl linoleate (1 vol % each of the total solution) in phosphate buffered saline (PBS, pH 7.4) containing 0.5 M sodium dodecyl sulfate (SDS) with a vortex mixer for 2 min. The consumption of PGR and pyranine was measured by following the decrease in absorbance at 540 and 454 nm, respectively, with a UV−visible light spectrophotometer (Shimadzu UV-2450) equipped with a thermostatted cell. The reaction was started by an addition of either AAPH or AIPH as an aqueous solution into the reaction mixture thermostatted at 37 °C.

RESULTS

The bleaching of PGR and pyranine induced by the reaction with the free radicals can be easily followed with spectrophotometer as shown in **Figure 1**. Either AAPH (*15*) or AIPH (*16*−*20*) was used to generate free radicals at a constant rate, which is essential for kinetic study. The two azo initiators were used to confirm that the bleaching of probes was dependent on the flux of free radicals, not on the type of azo compound. AIPH decomposes 3.8 times faster than AAPH (*20*). Both AAPH and AIPH induced a consumption of PGR and pyranine at a constant rate. The consumption of PGR and pyranine induced by free radicals was suppressed by the antioxidant which scavenges free radicals in competition with PGR or pyranine. The effect of Trolox is shown as an example in **Figure 2**. Trolox suppressed the consumption of PGR in a dose-dependent manner, whereas Trolox inhibited the consumption of pyranine completely at the same concentration range. The different effect is due to the higher reactivity of PGR toward peroxyl radicals than pyranine. PGR scavenges peroxyl radicals in competition with Trolox, but pyranine reacts with peroxyl radicals too slowly to compete with Trolox. Trolox produced a clear lag phase for the consumption of pyranine, and when Trolox was consumed completely, pyranine decreased at the same rate as that without Trolox.

The length of the lag phase was directly proportional to the concentration of Trolox (**Figure 3**). The lag phase t_{lag} is expressed by the following equation:

$$t_{lag} = n[\mathrm{IH}]/R_i \qquad (6)$$

where R_i is the rate of free radical generation from the azo initiator and n a stoichiometric number for the antioxidant IH, that is, the number of free radical molecules scavenged by each antioxidant molecule. Since the n for Trolox is known as 2, R_i is calculated from the slope of the plot in **Figure 3** as $R_i = 2/(\text{slope}) = 2/28.9 \times 10^6 = 6.92 \times 10^{-8}$ M/s.

The effects of commercial beverage Cassis-I A50, green tea, and red wine on the bleaching of PGR and pyranine were then

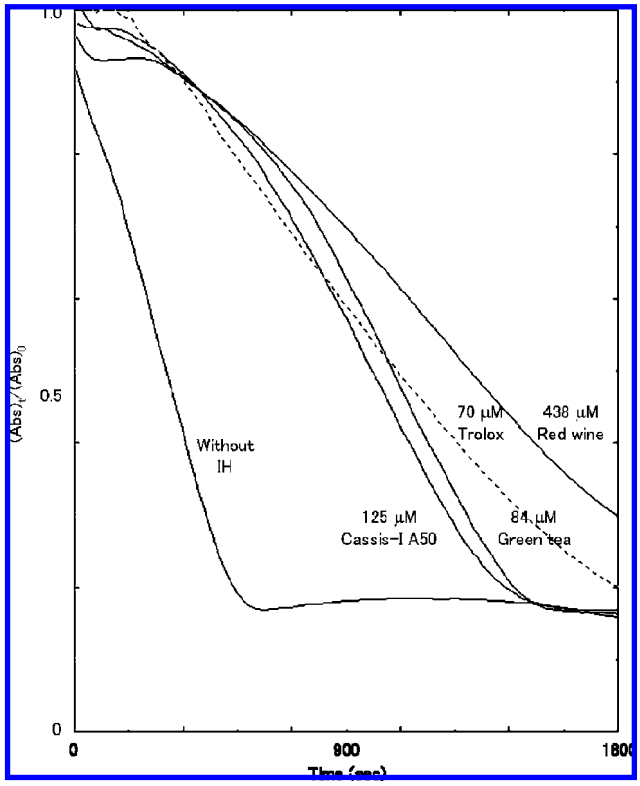

Figure 5. Effects of beverages and Trolox on bleaching of PGR. The reaction was initiated by the addition of 30 mM AIPH to the SDS micelle solution containing 1 vol % of beverage or 70 μM Trolox (dotted line).

studied. Numerous studies have been performed on red wine and green tea. On the other hand, the studies on cassis or black currant are scarce. They all suppressed the bleaching of PGR in a concentration-dependent manner, while they inhibited pyranine consumption completely (**Figure 4**; the data for green tea are not shown). As observed for Trolox, the lag phase produced by red wine, green tea, and Cassis-I A50 was directly proportional to their amount added (**Figure 3**).

The concentrations of antioxidants contained in cassis, green tea, and red wine are not known, and therefore in **Figure 3** the lag phase was plotted as a function of the actual volume in μL in total solution, 3 mL. If we denote the total concentration of antioxidants in the beverage as C (μM), the lag phase is given by $10^{-3}nCv/3R_i$, where v (μL) is the volume of beverage in a 3 mL reaction mixture. From the slope of the plot in **Figure 3** and R_i determined above, nC which is the apparent moles of free radicals that can be scavenged by antioxidant molecule is calculated by $(3 \times 10^3)(6.92 \times 10^{-8})$(slope) as 11.0, 8.4, and 43.8 mM respectively for cassis, green tea, and red wine. These concentrations were calculated assuming n to be 1. However, these beverages contain polyphenolic compounds whose n is much larger than 1. Therefore, the actual concentrations of antioxidants must be smaller than those shown above.

In **Figure 5** are shown the effects of beverages (1 vol %) on the rate of bleaching of PGR. The concentrations of antioxidants in the reaction mixture are calculated as 110, 84, and 438 μM for cassis, green tea, and red wine, respectively. The result of 70 μM Trolox was included in **Figure 5** for comparison. PGR decreased at a constant rate in the presence of Trolox after 150 s. This lag phase may be ascribed to the time to reach equilibrium after addition of the AIPH solution. On the other hand, the rate of bleaching of PGR in the presence of beverages increased with time. This is because these beverages contain many kinds of antioxidants with different reactivities toward peroxyl radicals. The highly reactive antioxidant inhibited PGR bleaching almost completely at the beginning, but the rate increased with time as the reactive antioxidants were depleted.

The rates of PGR bleaching in the absence or presence of Trolox, red wine, green tea, or Cassis-I A50 in the steady state after the initial stage were measured from the slope, and the plot of eq 5 is shown in **Figure 6**. The ratio of the rate of PGR bleaching in the absence of antioxidant (R_0) to that in the presence of antioxidant (R_{IH}) was plotted against the antioxidant concentrations in μM, not volume. The concentrations contained in cassis, green tea, and red wine were calculated from the above data. A good linear relationship was obtained for every beverage and Trolox. From the slope of the linear regression, the ratio of the rate constants for Trolox relative to PGR was obtained as $k_{Trolox}/k_{PGR} = 0.82$. This value is in reasonable agreement with 0.71 reported previously in phosphate buffer (pH 7.0)/ethanol (70/30) at 37 °C (*14*). The ratios of the rate constants k_{IH}/k_{PGR} for three kinds of beverages are included in **Figure 6**. The smaller ratios than that for Trolox suggest that the remaining antioxidants in the beverages have smaller reactivities than Trolox. Apparently, the true antioxidant capacity should be assessed at the initial stage where potent antioxidants functions.

DISCUSSION

In the present study, PGR and pyranine were used as a reference compound for assessment of the antioxidant capacity

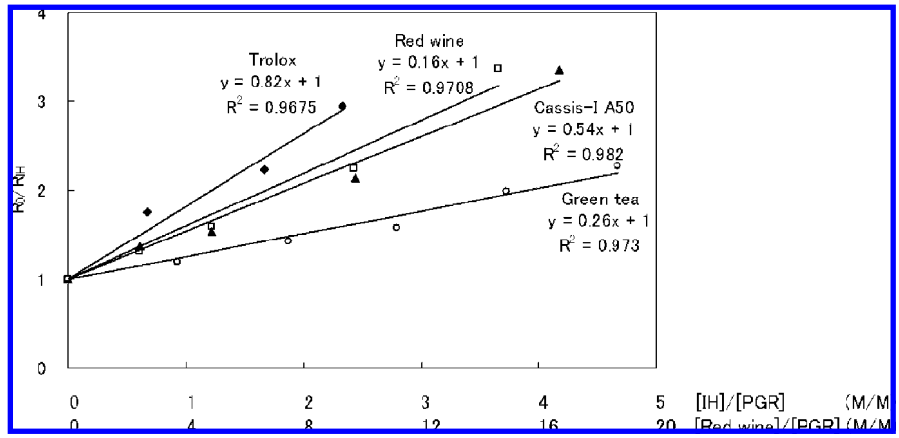

Figure 6. Effects of red wine, green tea, and Cassis-I A50 on the consumption of PGR. The reaction was induced by the addition of 30 mM AIPH to the SDS (0.5 M) micelle solution containing methyl palmitate and methyl linoleate (0.5 vol % each) and 30 μM pyrogallol red at 37 °C. The ratio of the rate of pyrogallol red consumption in the absence (R_0) and presence (R_{IH}) of antioxidant was plotted against antioxidant concentration.

of beverages. They are convenient in that their reaction with free radicals can be followed easily with conventional visible light absorption spectroscopy and that they have different reactivities toward peroxyl radicals. Peroxyl radicals are the most appropriate target free radicals for assessment of antioxidant capacity, because peroxyl radicals are the chain propagating species for lipid peroxidation and their reactivities are not too high for the antioxidants to scavenge in competition with biological molecules. Considering that the molar ratio of physiological concentrations of antioxidants to those of biological molecules to be protected is one to several hundreds and that the antioxidants should scavenge free radicals at least 10 times faster than the substrates, the reactivity (rate constant) of antioxidant to scavenge free radicals should be at least 10^3 times larger than that of substrates. This suggests that the hydroxyl radical and even alkoxyl radicals which react with biological molecules with the rate constant higher than 10^6 $M^{-1}s^{-1}$ are too reactive for the antioxidants to scavenge efficiently. This does not mean that the antioxidants are not effective against hydroxyl radical-induced oxidative damage, since the antioxidants may scavenge the secondary radicals formed by the attack of hydroxyl radical upon biological molecules. In other words, the fact that the antioxidant was effective against hydroxyl radical-induced oxidative damage does not always mean that the antioxidants scavenged hydroxyl radical.

PGR has been used as a probe for assessment of antioxidant capacity (*8, 14, 22*). The kinetics of bleaching of PGR by free radicals generated from AAPH in the absence and presence of antioxidant were studied in detail by Lissi and his colleagues previously (*14*). PGR was also used for measurement of free radical formation from AAPH and AIPH (*20*). PGR has several advantages such as high extinction coefficient at long wavelength and high reactivity toward peroxyl radicals to give stable products, which is suggested by the presence of the isosbestic point at $\lambda = 435$ nm (**Figure 1**). On the other hand, pyranine has much lower reactivity toward peroxyl radicals. Therefore, most of the antioxidants can inhibit pyranine bleaching almost completely to produce a clear lag phase, from which the total amounts of free radicals that can be scavenged by antioxidants may be estimated as described above. Thus, the use of both PGR and pyranine as a probe enables us to assess the antioxidant capacity of mixtures as well as pure antioxidant compound.

It may be noteworthy that the antioxidant efficacy in vivo is determined not only by the radical-scavenging capacity but also by other factors such as bioavailability and localization of antioxidants and the interactions between antioxidants. It is not sound to extrapolate the radical-scavenging capacity measured in the in vitro experiments to the antioxidant efficacy in vivo.

In conclusion, foods and beverages contain many kinds of compounds and their radical scavenging capacity is determined by the concentration and reactivity of the respective component toward free radicals. As shown above, the concentrations can be estimated from the lag phase observed in the bleaching of pyranine, while the reactivity to the free radicals can be assessed by the extent of suppression of PGR bleaching. Thus, a combination of pyranine and PGR as a probe enables us to assess both the total amount and reactivity of antioxidants contained in beverages and foods with a conventional spectrophotometer.

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