Original Article

Novel antioxidant capacity assay for lipophilic compounds using electron paramagnetic resonance spectroscopy

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A novel antioxidant capacity assay for lipophilic compounds was developed using electron paramagnetic resonance (EPR) spectroscopy. The assay is based on antioxidant's scavenging ability against the *tert*-butoxyl radical generated photolytically from di-*tert*-butyl peroxide in ethyl acetate, and named the *tert*-butoxyl-based antioxidant capacity (BAC) assay. The radical was trapped by spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide, and EPR signal intensity of the spin adduct was used as a quantitative marker of radical levels. Signal intensity decreased in a dose-dependent manner in the presence of an antioxidant that competitively reacts with the radical, which was utilized to evaluate BAC values. The BAC method enabled the accurate estimation of antioxidant capacity for lipophilic materials that may counteract lipid peroxidation in biological membranes. The BAC values for quercetin and caffeic acid are 0.639 ± 0.020 and 0.118 ± 0.012 trolox equivalents, respectively, which are much smaller than values obtained by other aqueous methods such as H-ORAC and ORAC-EPR. Thus, antioxidants present in a non-aqueous environment should be evaluated using a non-aqueous system. In combination with *in situ* ascorbate reduction, the BAC method was capable of accurately determining the antioxidant capacity of water-insoluble materials that may be reduced in living cells.

***Key Words*:** ***tert*-butoxyl, BAC, spin trapping, ORAC**

There is recent abundant evidence that oxidative stress is involved in the pathogenesis of various diseases; thus, antioxidants have garnered considerable attention for their protective role against oxidative stress.[1,2] In the search for novel antioxidants and/or to perform mechanistic investigation of antioxidant actions, the antioxidant capacity of various foods and supplements has been extensively evaluated using a variety of methods.[3–11] Of these methods, the oxygen radical absorbance capacity (ORAC) assay is gaining popularity in the agricultural and nutritional sciences.[12,13] The ORAC assay is a method to determine antioxidant capacity against free radicals that are generated from the azo radical initiator AAPH (2,2'-azobis-(2-amidinopropane) dihydrochloride).[7] The ORAC assay has several disadvantages, such as the use of a fluorescent dye[14–16] and that the derived ORAC values are not directly related to second order rate constants for reactions between antioxidants and radicals.[17] In contrast, the ORAC-EPR method was developed to overcome these disadvantages by quantifying AAPH-derived radicals by electron paramagnetic resonance (EPR) spectroscopy with the spin-trapping technique.[18]

Most antioxidant capacity assays measure reactivity against oxidants in an aqueous environment. However, biological systems possess lipid-soluble antioxidants (i.e., α-tocopherol) that protect various membranes from oxidative damage, and their reactivity in the lipid phase is not always coincident with that in aqueous phase.[1] Thus, it is also important to independently measure antioxidant capacity in non-aqueous environments. The original ORAC method measures the antioxidant capacity in aqueous solutions and is called hydrophilic ORAC (H-ORAC) as AAPH is insoluble in a non-aqueous solvent. Application of the ORAC method has been extended to lipophilic compounds (lipophilic ORAC, L-ORAC) by employing a solvent mixture of water/acetone and cyclodextrin to suspend water-insoluble materials.[7] The measurement of lipid-soluble compounds has also been attempted using the ORAC-EPR method with cyclodextrin.[19] However, these methods evaluate antioxidant capacity in aqueous environments, and do not necessarily reflect protective capacity in biomembranes. Moreover, these techniques are not sufficiently effective to solubilize major lipid-soluble antioxidants such as α-tocopherol, coenzyme Q_{10} (CoQ_{10}), and oils.

For determinations of reactivity against free radicals in non-aqueous environments, we developed a novel antioxidant capacity assay for lipophilic compounds. The assay utilizes photo-cleavage of di-*tert*-butyl peroxide (DBP) to generate the *tert*-butoxyl radical in a constant and reproducible manner, and was termed the *tert*-butoxyl-based antioxidant capacity (BAC) assay. This method employs the electron paramagnetic resonance (EPR) spin trapping method, similar to ORAC-EPR. The spin trapping method is an established technique for free radical identification and quantification, where unstable free radicals are trapped and stabilized with trapping compounds.[20,21] The BAC method stabilizes *tert*-butoxyl radical by spin trap, 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), and the resultant spin adduct is identified and quantified with EPR spectrometry. In the presence of an antioxidant, spin adduct level decreases depending on the amount of antioxidant because the antioxidant scavenges *tert*-butoxyl radicals. The BAC value can be calculated based on competitive kinetics describing dose-dependent decreases. Since the BAC assay is carried out with a non-aqueous solvent and oxygen-centered free radical, the BAC value is an optimal index of the reactivity of lipid-soluble compounds toward oxygen-centered free radical in a non-aqueous environment, and thus can represent the protective capability against lipid peroxidation in biological membranes.

In this study, we demonstrated that BAC values are derived from the reaction between DBP and antioxidants, and established the optimum assay conditions. We determined the BAC values

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for several antioxidants and compared these with ORAC and ORAC-EPR values. The BAC assay showed that antioxidant capacity in the non-aqueous phase differed substantially from that in the aqueous phase, which proved important for measuring non-aqueous antioxidant capacity. In combination with ascorbate treatment, the BAC method was also effective in estimating the biological antioxidant capacity of CoQ_{10}, which is reduced in living cells[22] and was applicable for evaluating the true antioxidant capacity of lipophilic materials that are reduced in biological systems.

Materials and Methods

DMPO was purchased from Dojindo (Kumamoto, Japan). DBP and 6-hydroxyl-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were obtained from Aldrich (St. Louis, Mississippi). Doubly distilled Milli-Q water (Millipore, Billerica, Massachusetts) was used. All other reagents were purchased from Wako Pure Chem., Ind., Ltd. (Osaka, Japan). All reagents were used without further purifications.

Light from a 200 W mercury-xenon UV-visible arc lamp (L9588-06A-02, Hamamatsu Photonics, Hamamatsu, Japan) was guided through a flexible quartz fiber tube into the cavity of an EPR spectrometer (JES-MA10 or JES-FA100, JEOL RESONANCE, Akishima, Japan). Unless otherwise stated, the light wavelength was limited from 400 to 730 nm with the combination of a long-pass filter and a cold filter (LU0400 and SC1601, respectively, Asahi Spectra, Tokyo, Japan). The EPR spectrometer and the lamp were simultaneously controlled using a PC with software that enables automatic EPR measurement and data analysis for the BAC assay (JEOL RESONANCE, Akishima, Japan).

Fifty microliter each of DBP and DMPO solutions in ethyl acetate was mixed with 100 µl of sample solution in ethyl acetate, and loaded into a quartz flat sample cell (JEOL RESONANCE, Akishima, Japan). The final concentrations of DBP and DMPO were 250 mM and 5 mM, respectively, unless otherwise stated. Quercetin (0.5 M) was first dissolved in dimethylsulfoxide (DMSO) and diluted with ethyl acetate. The maximum DMSO concentration in the quercetin sample solutions was 0.5%, an amount producing negligible effects on EPR signals. The sample cell was placed inside the EPR cavity and illuminated *in situ*, followed by immediate recording of EPR spectra. Typical spectrometer settings were as follows: field modulation width, 0.16 mT; microwave power, 8 mW; field scan width/rate, ±5 mT/2 min: time constant 0.1 s. The illumination time was 30 s unless otherwise stated.

DMPO trapped photolytically generated *tert*-butoxyl radicals as spin adducts that exhibited six EPR signals (Fig. 1). The peak-to-peak intensity of the signal at the lowest field position was used to quantify the spin adduct and was normalized with a standard EPR signal by Mn^{2+} in MgO. The BAC assay exploited the fact that the intensity in the presence of antioxidant (I) decreased compared with that in the absence of antioxidant (I_0), which can be formulated with the reaction kinetics of spin trap and antioxidant against the *tert*-butoxyl radical.[23] In the presence of spin trap (T) and antioxidant (A), both react with the radical (R) competitively:

$$R + T \rightarrow \text{spin aduct} \quad (1)$$

$$R + A \rightarrow \text{product(s)} \quad (2)$$

If [T] and [A] exist large-excess over [R], the following equation can be derived:

$$I_0/I - 1 = (k_A/k_T)([A]_0/[T]_0) \quad (3)$$

, where k_T and k_A are second order rate constants of reactions (1) and (2), respectively. $[T]_0$ and $[A]_0$ denote the initial concentration

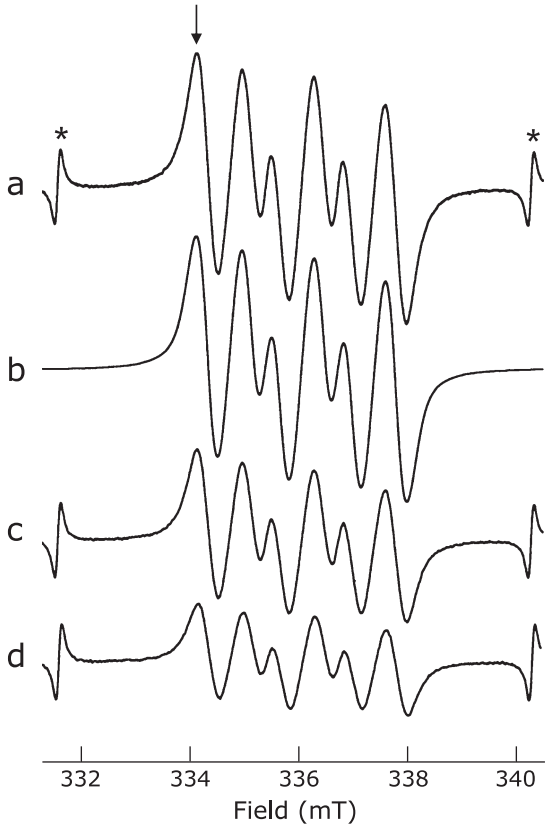

Fig. 1. EPR spectra for DMPO spin adduct to *tert*-butoxyl radical in ethyl acetate. a) Spectrum obtained using visible-light illumination of the mixture of di-*tert*-butyl peroxide (250 mM) and DMPO (5 mM). b) Simulation of a). c) and d) a) plus 1 mM and 2 mM of trolox, respectively. Signals with an asterisk are due to Mn^{2+} standard EPR signals. The arrow indicates a signal used for quantifications.

of spin trap and antioxidant, respectively. Thus, plot of $I_0/I - 1$ against $[A]_0/[T]_0$ gives a line with the slope of k_A/k_T, representings the antioxidant capacity of antioxidant relative to that of spin trap. Since we can measure trolox, a commonly accepted antioxidant standard,[13] to get k_S/k_T, where k_S is second order rate constant for the standard trolox, a BAC value expressed as a trolox-equivalent antioxidant capacity (k_A/k_S) can be obtained.

While the above-mentioned BAC value is derived for pure substances, we can also formulate the BAC value for mixtures as follows. Assuming that a sample contains antioxidants A_i (i = 1, 2, ..., n) that react with radical R independently and competitively,

$$R + A_i \rightarrow \text{product(s)} \ (i = 1, 2, ..., n) \quad (4)$$

we can derive an equation similar to Eq. (3) from reactions (1) and (4):

$$I_0/I - 1 = (\Sigma k_{Ai}[A_i]_0)/(k_T[T]_0) \ (i = 1, 2, ..., n) \quad (5)$$

, where k_{Ai} is second order rate constants of i-th reaction in Eq. (4) and $[A_i]_0$ is the initial concentration of the antioxidant A_i. If the sample weight is M and the weight of antioxidant A_i is c_iM, where c_i is the weight ratio of antioxidant A_i and $\Sigma c_i = 1$, Eq. (5) can be converted to:

$$I_0/I - 1 = ([\Sigma(k_{Ai}c_i/w_i)]/k_T)(M/m_T) \ (i = 1, 2, ..., n) \quad (6)$$

, where w_i is the molecular weight of antioxidant A_i and m_T is the

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mol amount of spin trap; indicating that the plot of $I_0/I - 1$ versus M/m_T should also show a line with the slope of $[\Sigma(k_{Ai}c_i/w_i)]/k_T$ that is characteristic for an antioxidant mixture. By dividing this slop by k_S/k_T, discussed above, the BAC value, $[\Sigma(k_{Ai}c_i/w_i)]/k_S$, for a mixture sample can be obtained that indicates antioxidant capacity mol-equivalent to trolox for the unit weight of a mixture sample.

To compare the BAC value of oxidized CoQ_{10} with that of its reduced form, CoQ_{10} (6 mM) (Japanese Pharmacopoeia Reference Standards Laboratory) was dissolved in ethyl acetate and was vigorously shaken with aqueous potassium ferricyanide (0.3 M) or aqueous ascorbic acid (0.3 M) to make fully oxidized or reduced CoQ_{10} solutions, respectively. To confirm the effect of these redox reagents alone, ethyl acetate shaken with aqueous potassium ferricyanide or aqueous ascorbate was also made. All ethyl acetate layers were collected and their BAC values were measured.

Results

EPR spectra for the DMPO spin adduct of *tert*-butoxyl radical. Fig. 1a shows an EPR spectrum obtained by illuminating a mixed solution of DBP (250 mM) and DMPO (5 mM). The spectrum was simulated by assuming a single paramagnetic species with a nitrogen nucleus ($a_N = 1.32$ mT) and a hydrogen nucleus ($a_H = 0.81$ mT) (Fig. 1b). These hyperfine coupling constants are comparable with those for the *tert*-butoxyl radical spin adduct of DMPO reported elsewhere,[24] indicating that the *tert*-butoxyl radical was generated and trapped by DMPO. In the presence of the common antioxidant trolox, a vitamin E analogue, the signal intensity decreased in a dose-dependent manner, as shown in Fig. 1c and d, without change in the spectral shape.

Establishment of assay conditions. To establish optimal assay conditions, we monitored the intensity of thesignal marked with an arrow in Fig. 1a with changing concentrations of DBP or DMPO during light illumination. Fig. 2a shows the effect of DBP concentration and indicates a dose-dependent increase in EPR signal intensity. We selected 250 mM DBP for the highest EPR signal intensity. As shown in Fig. 2b, the EPR signal increased linearly with illumination time using DMPO concentrations above 5 mM, showing that the spin adduct is sufficiently stable for quantification under these conditions. Increasing DMPO concentration enhances the EPR signal intensity; however, but the effect is saturated above 5 mM of DMPO. The larger EPR signal with more concentrated DMPO is better for precise analysis. However, since the BAC assay is based on kinetic competition between DMPO and antioxidant, a more concentrated DMPO requires a more concentrated antioxidant to detect decreases in EPR signal, making it challenging to evaluate samples with low antioxidant capacity. Thus, 5 mM of DMPO with an illumination time from 15 to 30 s was selected for the assay. Under these conditions, the spin adduct showed an EPR signal with a signal-to-noise ratio larger than 100. We also examined the effect of light wavelength using different longpass filters. Although the absorption maximum of DBP is below 300 nm, the tail of absorption extends above 400 nm. To prevent unexpected photoreactions by high-energy light, we used the light above 400 nm that was able to produce sufficient radicals for this assay as shown in Fig. 1 and 2. For prompter analysis, one can use light with shorter wavelength since light above 300 nm can generate radicals 26 times faster than light above 400 nm.

Measurement of BAC values. Line B in Fig. 3a shows the change in EPR signal intensity when the trolox concentration was increased stepwise. The intensity I_0 or I of the signal was plotted according to Eq. (3). The slope of the lines (i.e., k_S/k_T) passed through each point and the origin, were calculated and averaged, and the standard deviation was calculated. The k_S/k_T value was used to calculate trolox-equivalent BAC values. In Fig. 3a, the plots for α-tocopherol (Line A) and quercetin (Line C) are also illustrated. The order of the slopes in Fig. 3a is α-tocopherol>trolox>quercetin, indicating that the BAC values are in this order. Table 1 lists the BAC values of various antioxidants. ORAC-EPR and H-ORAC values are also listed in Table 1 for comparison.

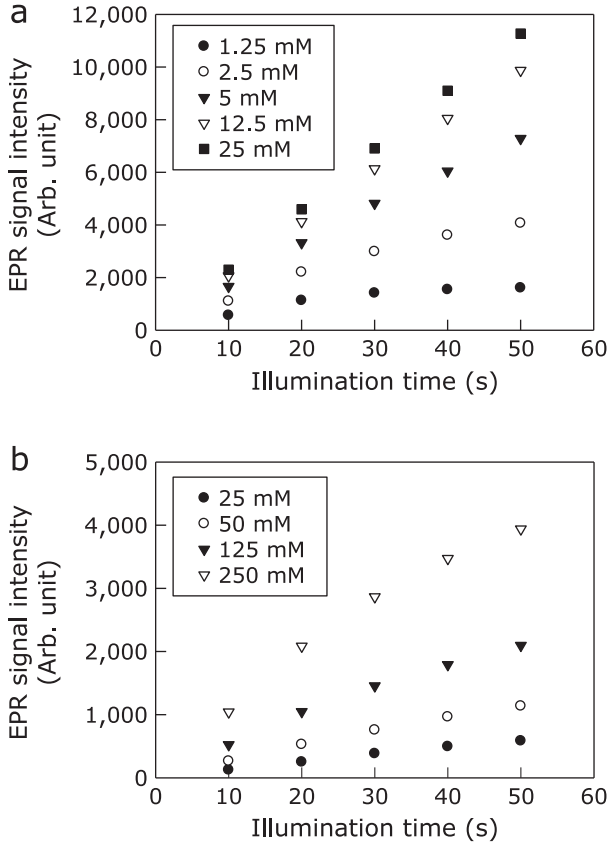

Fig. 2. The time course of EPR signal intensity during light illumination. a) Effect of di-*tert*-butyl peroxide concentration. DMPO concentration was fixed at 2.5 mM. b) Effect of DMPO concentration. Di-*tert*-butyl peroxide concentration was fixed at 250 mM.

The plot of $I_0/I - 1$ against the sample weight of oryzanol, a rice bran oil containing a mixture of fatty acids and antioxidants, shows a line crossing the origin as expected from Eq. (6) for mixed samples (Fig. 3b). The result demonstrates that the BAC method can be applied to a variety of mixtures. From the slope of the line ($[\Sigma(k_{Ai}c_i/w_i)]/k_T$) and k_S/k_T, the BAC value for oryzanol ($[\Sigma(k_{Ai}c_i/w_i)]/k_S$) was calculated to be 111 ± 10 μmol trolox/g (mean ± SEM, $n = 3$).

Difference between oxidized and reduced CoQ_{10}. Fig. 4 shows the $I_0/I - 1$ plot for oxidized and reduced CoQ_{10}, producing the BAC values of 0.173 ± 0.005 and 0.918 ± 0.050 trolox equivalents, respectively, indicating that only reduced CoQ_{10} shows strong antioxidant capacity. Since potassium ferricyanide or ascorbic acid cannot be dissolved in ethyl acetate, ethyl acetate shaken with an aqueous solution of these redox reagents did not alter the EPR signal.

Discussion

Since peroxyl radicals play a pivotal role in the lipid peroxidation of biomembranes,[1] we first attempted to establish a stable peroxyl radical generating system in non-aqueous media. The attempt to detect pure peroxyl radical adduct was successful using azobis(isobutyronitrile) as a radical precursor in benzene.[25] However, we ultimately found that only non-polar solvents were able to stabilize the peroxyl spin adduct. Using more polar solvents such as ethyl acetate that are suitable for dissolving or

Table 1. Antioxidant capacities of pure chemicals

Compounds	BAC[a]	Antioxidant Capacities ORAC-EPR[b]	H-ORAC[c]
Trolox	1.00 ± 0.01	1	1
α-Tocopherol	3.29 ± 0.15	—	—
CoQ_{10}	0.173 ± 0.005	—	—
$CoQ_{10}H_2$	0.918 ± 0.050	—	—
Caffeic acid	0.118 ± 0.012	1.6	4.37 ± 0.24
Genistein	n.d.[d]	0.43	5.93 ± 0.45
Quercetin	0.639 ± 0.020	7.1	7.28 ± 0.22
Catechin	0.240 ± 0.039	0.71	6.76 ± 0.22
N-Acetylcysteine	0.0290 ± 0.0021	0.4	—

[a]k_A/k_S described in the Materals and Methods. Data are expressed as mean ± SEM (n = 3). [b]From Ref. 18. [c]From Ref. 12. [d]No detectable antioxidant capacity.

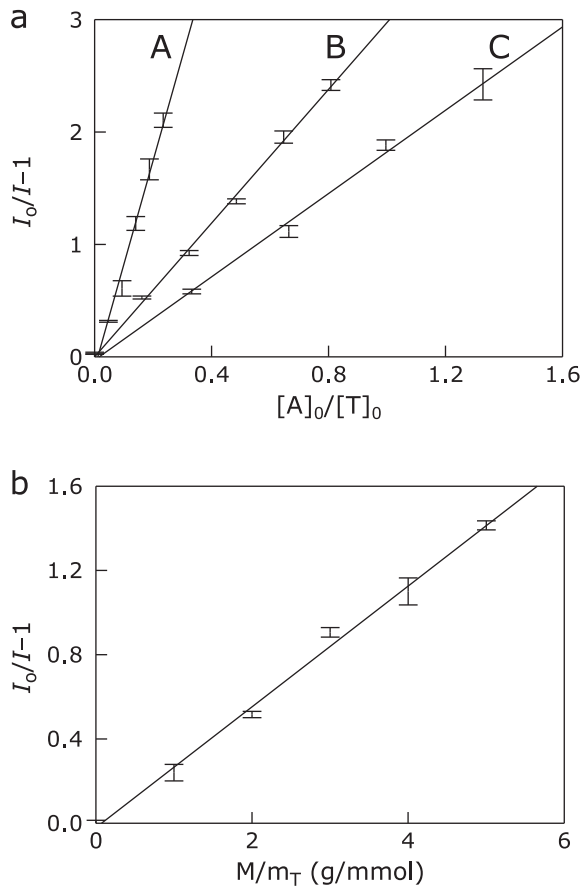

Fig. 3. a) Plot of $I_0/I - 1$ against $[A]_0/[T]_0$, where I_0 and I denote EPR signal intensities in the absence and presence of antioxidant, respectively. Line A, α-tocopherol; Line B, trolox; Line C, quercetin. $[A]_0$ and $[T]_0$ represent the initial concentrations of antioxidant and DMPO, respectively. The slope of regression lines represents k_A/k_T for antioxidants. b) Plot of $I_0/I - 1$ against M/m_T for oryzanol, where M and m_T are sample weight and mol amount of spin trap, respectively. The slope of the regression line corresponds to $[\Sigma(k_{Ai}c_i/w_i)]/k_T$ explained in Materials and Methods. Data points in both figures are expressed as mean ± SEM (n = 3).

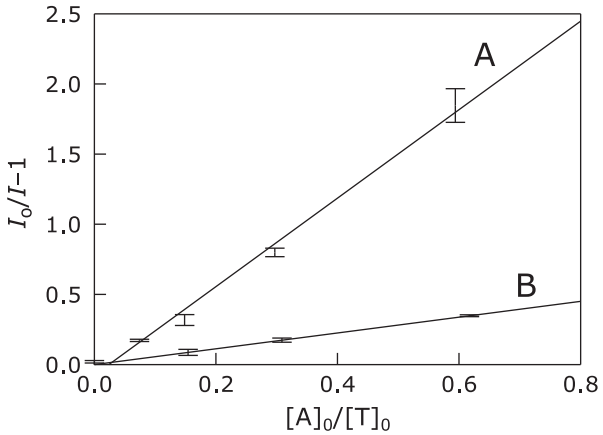

Fig. 4. Plot of $I_0/I - 1$ against $[A]_0/[T]_0$ for oxidized and reduced CoQ_{10}. Line A, reduced form; Line B, oxidized form.

extracting a variety of samples, the peroxyl radical adduct was easily decomposed into the alkoxyl radical adduct. Since other peroxyl radical adducts are also promptly converted to alkoxyl radical adducts,[26] we next attempted to create a stable alkoxyl radical generating system.

AAPH, a water-soluble azo compound, can consistently produce alkoxyl radical in aqueous solvent and has been employed in ORAC[13] and ORAC-EPR[18] methods. Although a short-lived peroxyl radical adduct was observed,[27] it was confirmed that the alkoxyl radical adduct is the sole stable product of AAPH decomposition, where rapid conversion of peroxyl radical to alkoxyl radical takes place.[18,28,29] Thus, we tested lipid-soluble counterpart of AAPH, 2,2'-azobis-(2,4-dimethylvaleronitrile) (AMVN), in non-aqueous solvents. However, AMVN gave a mixture of alkoxyl and peroxyl radical adducts, as reported elsewhere,[28] and the ratio varied depending on the solvent used. Therefore, we concluded that a pure and stable radical generating system cannot be established with these azo compounds in non-aqueous solvents.

It was reported that the photo-cleavage of DBP gives the *tert*-butoxyl radical[30] using 2-methyl-2-nitrosopropane as a spin trap. The DMPO spin adduct of the *tert*-butoxyl radical, generated with the thermolysis of di-*tert*-butylperoxyoxalate, has been extensively characterized.[24] We found that strict control of light illumination of the mixture of DMPO and DBP in a non-aqueous solvent gave s pure and reproducible *tert*-butoxyl radical adduct of DMPO (Fig. 1). The a_N and a_H of the adduct have been tabulated for a variety of solvents and have shown prominent dependence on solvent polarity,[24] where the values for ethyl acetate ($E_T(30) = 38.1$)[31] are not included. However, the a_N and a_H were reported to be 1.342 mT and 0.832 mT, respectively, in 1,2-dimethoxyethane ($E_T(30) = 38.2$),[24] comparable to the values of the adduct obtained in ethyl acetate. Thus, there is no doubt that the adduct in Fig. 1 was derived from the *tert*-butoxyl radical.

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Antioxidants competitively suppressed adduct formation, which could be utilized for the analysis of antioxidant capacity. Using a relatively high concentration of DBP, this system can generate sufficient alkoxyl radical to perform consistent quantification using the EPR signal intensity (Fig, 2). On the other hand, the DMPO concentration should be suppressed for the estimation of weak antioxidant capacity whereas it should be maintained to obtain sufficient EPR signal intensity. Thus, we selected 250 mM of DBP and 5 mM of DMPO as the fixed assay condition.

The use of alkoxyl radical instead of peroxyl radical for the estimation of antioxidant capacity toward lipid peroxidation might be criticized because the alkoxyl radical reacts much faster than the peroxyl radical.[32] However, it has been shown that the relative reactivity of antioxidants against the peroxyl radical agrees well with that against other oxygen-centered radicals under a similar reaction mechanism and environment.[27,33] Both alkoxyl and peroxyl radicals are oxygen-centered σ-radicals that react with antioxidants by the same mechanism, i.e., hydrogen abstraction and radical addition to unsaturated bonds. Thus, BAC values can be a reasonable measure of antioxidant capacity against lipid peroxidation.

The linear plots shown in Fig. 3 demonstrate that this assay system is based on the simple competitive reaction scheme expressed by Eqs. (3) and (6). The BAC values can be easily obtained from the slope of the plotted line. By using trolox as a common standard, the values can be directly compared with those obtained using ORAC[13] and ORAC-EPR.[18,34] The BAC values for quercetin and caffeic acid are lower than unity, whereas corresponding ORAC/ORAC-EPR values are much higher than unity (Table 1), suggesting that the reactivity of these polyphenols in a non-aqueous environment is completely different from that in an aqueous environment. It has been shown that the reactivity of polyphenols has a large pH dependence in aqueous solutions,[35] where polyophenols are partially ionized around neutral pH and react *ca* ten times faster than unionized forms at low pH. Since phenolic hydroxyl groups remain unionized in non-aqueous solvents, it is natural that these polyphenols have much lower antioxidant capacities in non-aqueous environments than those in an aqueous solvent at neutral pH. In regards to biological relevance, lipid-soluble antioxidants may function in biomembranes where entire reactions occur under a non-aqueous environment. Therefore, establishment of a method using a non-aqueous reaction system is necessary for the estimation of lipid-soluble antioxidants. In aqueous methods using AAPH, lipid-soluble samples have been suspended in mixed solvents and/or cyclodextrin.[13,19] However, the reaction media in these methods are still under an aqueous environment where an antioxidant's reactivity in non-aqueous media cannot be estimated well. Moreover, vitamin E, CoQ_{10}, and the various oils that are the major lipid-soluble antioxidants in foods or supplements do not have adequate solubility in aqueous media regardless of these special solubilization techniques. Therefore, these aqueous systems cannot measure or tend to underestimate the antioxidant capacity of water-insoluble materials. As a result, the antioxidant capacity of lipid-soluble materials should be estimated using a non-aqueous system like the BAC method.

The result for oryzanol demonstrates the applicability of the BAC method to mixtures as well as pure substances (Fig. 3b). To date, a variety of foods, supplements, and nutrients (i.e., mixed samples) have been estimated using the ORAC method.[13] On the other hand, only pure substances have been tested using the ORAC-EPR method[18,19,27,34] because no formula for antioxidant capacity has been derived for mixed samples assuming competitive kinetics. In this paper, we obtained an equation for mixed samples [Eq. (6)] that can be successfully applied to the plot for oryzanol and defined the parameter expressing antioxidant capacity of mixture samples ($[\Sigma(k_{Ai}c_i/w_i)]/k_S$). This parameter can also be applied to all methods utilizing competitive reaction kinetics such as the ORAC-EPR method.

CoQ_{10} exists primarily in the reduced form in living cells which may accumulate to millimolar levels of ascorbate to maintain reductive environment.[22,36] However, commercially available CoQ_{10} is oxidized since the oxidized form is more stable in air. As shown in Fig. 4, CoQ_{10} only in the reduced form acts as an effective antioxidant. Thus, if a sample contains an ascorbate-reducible substance like CoQ_{10}, its antioxidant capacity does not necessarily express the value in living cells unless the sample is treated with ascorbate. Whereas methods using an aqueous system, i.e., ORAC or ORAC-EPR, cannot be easily combined with ascorbate treatment, the sample solution for the BAC method can be reduced *in situ* by shaking with aqueous ascorbate that is immiscible with the sample solution. Therefore, the BAC method provides a tool for estimating the true antioxidant capacity of water-insoluble materials that are reduced in biological system.

In conclusion, the BAC assay provides a novel, precise, and easily assessable method for estimating the antioxidant capacity of lipid-soluble materials functioning in non-aqueous compartments of biological system. It was demonstrated that antioxidant capacity in a non-aqueous environment may be accurately estimated using a non-aqueous evaluation system like the BAC method.

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Abbreviations

AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
AMVN	2,2'-azobis(2,4-dimethylvaleronitrile)
BAC	*tert*-butoxyl-based antioxidant capacity
CoQ_{10}	coenzyme Q_{10}
DBP	di-*tert*-butyl peroxide
DMPO	5,5-dimethyl-1-pyrroline-*N*-oxide
DMSO	dimethylsulfoxide
EPR	electron paramagnetic resonance
H-ORAC	hydrophilic ORAC
L-ORAC	lipophilic ORAC
ORAC	oxygen radical absorbance capacity

Conflict of Interest

No potential conflicts of interest were disclosed.

References

1 Halliwell B, Gutteridge JMC. *Free Radicals in Biology and Medicine (4th ed.)*, Clarendon: Oxford University press, 2007.
2 Papas AM. *Antioxidant Status, Diet, Nutrition, and Health*, Boca Laton: CRC Press, 1999.
3 Benzie IF, Strain JJ. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": the FRAP assay. *Ana Biochem* 1996; **239**: 70–76.
4 Ghiselli A, Serafini M, Maiani G, Azzini E, Ferro-Luzzi A. A fluorescence-based method for measuring total plasma antioxidant capability. *Free Rad Biol Med* 1994; **18**: 29–36.
5 Glazer AN. Fluorescence-based assay for reactive oxygen species: a protective role for creatinine. *FASEB J* 1988; **2**: 2487–2491.
6 Miller NJ, Rice-Evans C, Davies MJ, Gopinathan V, Milner A. A novel method for measuring antioxidant capacity and its application to monitoring

the antioxidant status in premature neonates. *Clin Sci (Lond)* 1993; **84**: 407–412.
7 Prior RL, Hoang H, Gu L, *et al*. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL)) of plasma and other biological and food samples. *J Agric Food Chem* 2003; **51**: 3273–3279.
8 Wayner DDM, Burton GW, Ingold KU, Locke S. Quantitative measurement of the total peroxyl radical-trapping antioxidant capacity of human blood plasma by controlled peroxidation. The important contribution made by plasma proteins. *FEBS Lett* 1985; **187**: 33–37.
9 Whitehead TP, Thorpe GHC, Maxwell SRJ. Enhanced chemiluminescent assay for antioxidant capacity in biological fluids. *Anal Chim Acta* 1992; **266**: 265–277.
10 Yamaguchi T, Takamura H, Matoba T, Terao J. HPLC method for evaluation of the free radical-scavenging activity of foods by using 1-1-diphenyl-2-picrylhydrazyl. *Biosci Biotechnol Biochem* 1997; **62**: 1201–1204.
11 Yen GC, Duh PD. Scavenging effect of methanolic extracts of peanut hulls on free-radical and active-oxygen species. *J Agric Food Chem* 1994; **42**: 629–632.
12 Ou B, Hampsch-Woodill M, Prior RL. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J Agric Food Chem* 2001; **49**: 4619–4626.
13 Wu X, Beecher GR, Holden JM, Haytowitz DB, Gebhardt SE, Prior RL. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J Agric Food Chem* 2004; **52**: 4026–4037.
14 Togashi DM, Szczupak B, Ryder AG, Calvet A, O'Loughlin M. Investigating tryptophan quenching of fluorescein fluorescence under protolytic equilibrium. *J Phys Chem A* 2009; **113**: 2757–2767.
15 Posokhov YO, Kyrychenko A, Ladokhin AS. Steady-state and time-resolved fluorescence quenching with transition metal ions as short-distance probes for protein conformation. *Anal Biochem* 2010; **407**: 284–286.
16 Li T, Yang Z, Li Y, Liu Z, Qi G, Wang B. A novel fluorescein derivative as a colorimetric chemosensor for detecting copper(II) ion. *Dyes Pigm* 2011; **88**: 103–108.
17 Niki E. Assessment of antioxidant capacity *in vitro* and *in vivo*. *Free Rad Biol Med* 2010; **49**: 503–515.
18 Kohri S, Fujii H, Oowada S, *et al*. An oxygen radical absorbance capacity-like assay that directly quantifies the antioxidant's scavenging capacity against AAPH-derived free radicals. *Anal Biochem* 2009; **386**: 167–171.
19 Sueishi Y, Ishikawa M, Yoshioka D, *et al*. Oxygen radical absorbance capacity (ORAC) of cyclodextrin-solubilized flavonoids, resveratrol and astaxanthin as measured with the ORAC-EPR method. *J Clin Biochem Nutr* 2012; **50**: 127–132.
20 Janzen EG. Spin trapping. *Methods Enzymol* 1984; **105**: 188–198.
21 Buettner GR. Spin trapping: ESR parameters of spin adducts. *Free Rad Biol Med* 1987; **3**: 259–303.
22 Bhagavan HN, Chopra RK. Coenzyme Q10: absorption, tissue uptake, metabolism and pharmacokinetics. *Free Rad Res* 2006; **40**: 445–453.
23 Buettner G.R, Mason RP. Spin-trapping methods for detecting superoxide and hydroxyl free radicals *in vivo* and *in vitro*. *Methods Enzymol* 1990; **186**: 127–133.
24 Janzen EG, Coulter GA, Oehler UM, Bergsma JP. Solvent effects on the nitrogen and β-hydrogen hyperfine splitting constants of aminoxyl radicals obtained in spin trapping experiments. *Can J Chem* 1982; **60**: 2725–2733.
25 Sueishi Y, Yoshioka D, Oowada S, *et al*. Is the oxygen radical absorbance capacity (ORAC) method a peroxyl-radical scavenging assay?. *Z Phys Chem* 2010; **224**: 921–928.
26 Reszka K, Bilski P, Sik RH, Chignell CF. Photosensitized generation of superoxide radical in aprotic solvents: an EPR and spin trapping study. *Free Rad Res Comms* 1993; **19 Suppl 1**: S33–S44.
27 Nakajima A, Masuda Y, Matsuda E, Tajima K, Sameshima H, Ikenoue Y. Abilities of antioxidants to eliminate the peroxyl radical derived from 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). *Appl Magn Reson* 2013; **44**: 997–1005.
28 Krainev AG. Bigelow DJ. Comparison of 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN) as free radical initiators: a spin-trapping study. *J Chem Soc Parkin Trans* 1996; **2**: 747–754.
29 Rojas Wahl RU, Zeng L, Madison SA, DePinto RL, Shay BJ. Mechanistic studies on the decomposition of water soluble azo-radical-initiators. *J Chem Soc Parkin Trans* 1998; **2**: 2009–2017.
30 Parkins MJ, Roberts BP. A probe for homolytic reactions in solution. Part VIII. An electron spin resonance study of the rates of fragmentation and spin trapping of t-butoxycarbonyl radicals and acyl radicals. *J Chem Soc Parkin Trans* 1974; **2**: 297–304.
31 Rasid Gazi HA, Biswas R. Excited state charge transfer reaction in (mixed solvent + electrolyte) systems: role of reactant.solvent and reactant.ion interactions. *J Chem Sci* 2011; **123**: 265–277.
32 Howard JA, Scaiano JC. Radical reaction rates in liquids. Oxyl-, peroxyl-, and related radicals. In: Hellwege KH, Madelung O, eds. *Landolt-Bornstein*. Berlin: Springer-Verlag, 1984; **13d**: 12–26, 298–317.
33 Mukai K, Daifuku K, Okabe K, Tanigaki T, Inoue K. Structure-activity relationship in the quenching reaction of singlet oxygen by tocopherol (vitamin E) derivatives and related phenols. Finding of linear correlation between the rates of quenching of singlet oxygen and scavenging of peroxyl and phenoxyl radicals in solution. *J Org Chem* 1991; **56**: 4188–4192.
34 Nakajima A, Matsuda E, Masuda Y, Sameshima H, Ikenoue T. Characteristics of the spin-trapping reaction of a free radical derived from AAPH: further development of the ORAC-EPR assay. *Anal Bioanal Chem* 2012; **403**: 1961–1970.
35 Mitani S, Ouchi A, Watanabe E, Kanesaki Y, Nagaoka S, Mukai K. Stopped-flow kinetic study of the aroxyl radical-scavenging action of catechins and vitamin C in ethanol and micellar solutions. *J Agric Food Chem* 2008; **56**: 4406–4417.
36 Bergsten P, Amitai G, Kehrl J, Dhariwal KR, Klein HG, Levine M. Millimolar concentrations of ascorbic acid in purified human mononuclear leukocytes. Depletion and reaccumulation. *J Biol Chem* 1990; **265**: 2584–2587.

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