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. 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. Of these methods, the oxygen radical absorbance capacity (ORAC) assay is gaining popularity in the agricultural and nutritional sciences. 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). The ORAC assay has several disadvantages, such as the use of a fluorescent dye and that the derived ORAC values are not directly related to second order rate constants for reactions between antioxidants and radicals. 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.

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. 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. The measurement of lipid-soluble compounds has also been attempted using the ORAC-EPR method with cyclodextrin. 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 Q10 (CoQ10), and oils.

For determinations of reactivity against free radicals in nonaqueous 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. 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...
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<sub>10</sub>, which is reduced in living cells<sup>22</sup> 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-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) were obtained from Aldrich (St. Louis, Missouri). 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-butoxy 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<sup>2+</sup> in MgO. The BAC assay exploited the fact that the intensity in the presence of antioxidant (<i>I</i>) decreased compared with that in the absence of antioxidant (<i>I</i><sub>0</sub>), which can be formulated with the reaction kinetics of spin trap and antioxidant against the tert-butoxy radical. Fiber 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<sup>2+</sup> standard EPR signals. The arrow indicates a signal used for quantifications.

Fig. 1. EPR spectra for DMPO spin adduct to tert-butoxy 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<sup>2+</sup> standard EPR signals. The arrow indicates a signal used for quantifications.

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

\[
I_0/I - 1 = \frac{k_A [A]_0}{[T]_0} \tag{3}
\]

, where \(k_A\) and \(k_T\) are second order rate constants of reactions (1) and (2), respectively, \([T]_0\) and \([A]_0\) denote the initial concentration 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 \(k_A/k_T\), representing the antioxidant capacity of antioxidant relative to that of spin trap. Since we can measure trolox, a commonly accepted antioxidant standard,<sup>13</sup> to get \(k_A/k_T\), where \(k_T\) is second order rate constant for the standard trolox, a BAC value expressed as a trolox-equivalent antioxidant capacity \((k_A/k_T)\) 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,

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

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

\[
I_0/I - 1 = \frac{\sum k_{i}c_{i} [A]_0}{[T]_0} \tag{5}
\]

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

\[
I_0/I - 1 = \frac{\left(\sum (k_{i}c_{i}/w_{i})\right)}{k_{T}} \tag{6}
\]

, where \(w_i\) is the molecular weight of antioxidant \(A_i\) and \(m_T\) is the
mol amount of spin trap; indicating that the plot of $I_0/I - 1$ versus $M/m_1$ should also show a line with the slope of $[\Sigma(k_{ci}/w_i)/k_i]$ that is characteristic for an antioxidant mixture. By dividing this slope by $k_i/k_T$, discussed above, the BAC value, $[\Sigma(k_{ci}/w_i)/k_i]$ 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 ($a_m = 1.32$ 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,$^{(25)}$ 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 the signal 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., $k_i/k_T$) passed through each point and the origin, were calculated and averaged, and the standard deviation was calculated. The $k_i/k_T$ value was used to calculate trolox-equivalent BAC values. In Fig. 3a, the plots for $\alpha$-tocopherol (Line A) and quercetin (Line C) are also illustrated. The order of the slopes in Fig. 3a is $\alpha$-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.

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 ($[\Sigma(k_{ci}/w_i)/k_i]$) and $k_i/k_T$, the BAC value for oryzanol ($[\Sigma(k_{ci}/w_i)/k_i]$) was calculated to be $111 \pm 10 \mu$mol 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 $0.173 \pm 0.005$ and $0.918 \pm 0.050$ 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,$^{(11)}$ 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

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**Table 1. BAC Values of Various Antioxidants**

| Antioxidant | BAC Value (μmol trolox/g) |
|-------------|------------------------|
| $\alpha$-tocopherol | $111 \pm 10$ |
| Quercetin | $918 \pm 50$ |
| Trolox | $173 \pm 5$ |

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**Fig. 2.** The time course of EPR signal intensity during light illumination. a) Effect of di-tert-buty1 peroxide concentration. DMPO concentration was fixed at 2.5 mM. b) Effect of DMPO concentration. Di-tert-buty1 peroxide concentration was fixed at 250 mM.
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 using 2-methyl-2-nitrosopropane as a spin trap.\(^{(30)}\) 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 a pure and reproducible tert-butoxyl radical adduct of DMPO (Fig. 1). The \(a_0\) and \(a_1\) 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 \((E_T(30) = 38.1)\) are not included. However, the \(a_0\) and \(a_1\) were reported to be 1.342 mT and 0.832 mT, respectively, in 1,2-dimethoxyethane \((E_T(30) = 38.2)\).\(^{(28)}\) 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.

### Table 1. Antioxidant capacities of pure chemicals

| Compounds  | BAC* | ORAC-EPR a | H-ORAC c |
|------------|------|-------------|-----------|
| Trolox     | 1.00 ± 0.01 | 1 | 1 |
| α-Tocopherol | 3.29 ± 0.15 | — | — |
| CoQ<sub>10</sub> | 0.173 ± 0.005 | — | — |
| CoQ<sub>10</sub>H<sub>2</sub> | 0.918 ± 0.050 | — | — |
| Caffeic acid | 0.118 ± 0.012 | 1.6 | 4.37 ± 0.24 |
| Genistein  | n.d. a | 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 | — | — |

*\(k_A/k_T\) described in the Materials and Methods. Data are expressed as mean ± SEM (\(n = 3\)). aFrom Ref. 18. bFrom Ref. 12. cNo detectable antioxidant capacity.

AAPH, a water-soluble azo compound, can consistently produce alkoxyl radical in aqueous solvent and has been employed in ORAC\(^{(15)}\) 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 a pure and reproducible tert-butoxyl radical adduct of DMPO (Fig. 1). The \(a_0\) and \(a_1\) 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 \((E_T(30) = 38.1)\) are not included. However, the \(a_0\) and \(a_1\) were reported to be 1.342 mT and 0.832 mT, respectively, in 1,2-dimethoxyethane \((E_T(30) = 38.2)\).\(^{(28)}\) 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.

\[ I_0 \]
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.\(^{(27,35)}\) Both alkoxyl and peroxyl radicals are oxygen-centered \(\sigma\)-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 easily be 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,\(^{(15)}\) where polyphenols are partially ionized around neutral pH and react 10 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 cyclo-\(\sigma\)-dextrin.\(^{(13,19)}\) 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.\(^{(33)}\) 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 \([\Sigma k_i c_i w_i/k_j]\). 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-aminodimopropane) 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.

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