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REVIEWS

The Chemistry behind Antioxidant Capacity Assays

DEJIAN HUANG,*,† BOXIN OU,§ AND RONALD L. PRIOR#

Food Science and Technology Program, Department of Chemistry, National University of Singapore, Singapore 117543, Singapore; Brunswick Laboratories, 6 Thatcher Lane, Wareham, Massachusetts 02571; and Arkansas Children's Nutrition Center, Agricultural Research Service, U.S. Department of Agriculture, 1120 Marshall Street, Little Rock, Arkansas 72202

This review summarizes the multifaceted aspects of antioxidants and the basic kinetic models of inhibited autoxidation and analyzes the chemical principles of antioxidant capacity assays. Depending upon the reactions involved, these assays can roughly be classified into two types: assays based on hydrogen atom transfer (HAT) reactions and assays based on electron transfer (ET). The majority of HAT-based assays apply a competitive reaction scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. These assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays. ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes color when reduced. The degree of color change is correlated with the sample's antioxidant concentrations. ET-based assays include the total phenols assay by Folin−Ciocalteu reagent (FCR), Trolox equivalence antioxidant capacity (TEAC), ferric ion reducing antioxidant power (FRAP), "total antioxidant potential" assay using a Cu(II) complex as an oxidant, and DPPH. In addition, other assays intended to measure a sample's scavenging capacity of biologically relevant oxidants such as singlet oxygen, superoxide anion, peroxynitrite, and hydroxyl radical are also summarized. On the basis of this analysis, it is suggested that the total phenols assay by FCR be used to quantify an antioxidant's reducing capacity and the ORAC assay to quantify peroxyl radical scavenging capacity. To comprehensively study different aspects of antioxidants, validated and specific assays are needed in addition to these two commonly accepted assays.

Keywords: Antioxidants; assay; hydrogen atom transfer reaction; electron-transfer reaction; free radicals; oxidants

1. INTRODUCTION

Clinical trials and epidemiological studies have established an inverse correlation between the intake of fruits and vegetables and the occurrence of diseases such as inflammation, cardiovascular disease, cancer, and aging-related disorders (*1*). Dietary antioxidants, including polyphenolic compounds, vitamins E and C, and carotenoids, are believed to be the effective nutrients in the prevention of these oxidative stress related diseases (*2*). Antioxidants have thus become a topic of increasing interest recently. A literature search revealed that the number of publications on antioxidants and oxidative stress has nearly quadrupled in the past decade (1684 in 1993; 6510 in 2003) (*3*). It is of great interest to the general public, medical and nutritional experts, and health and food science researchers to know the antioxidant capacity and constituents in the foods we consume. Due to the complexity of the composition of foods, separating each antioxidant compound and studying it individually is costly and inefficient, notwithstanding the possible synergistic interactions among the antioxidant compounds in a food mixture. Therefore, it is very appealing to researchers to have a convenient method for the quick quantitation of antioxidant effectiveness in preventing diseases. However, such methods are yet to be developed. A total antioxidant capacity assay using one chemical reaction seems to be rather unrealistic and not easy to come by, yet there are numerous published methods claiming to measure total antioxidant capacity in vitro.

Ironically, the biggest problem is the lack of a validated assay that can reliably measure the antioxidant capacity of foods and biological samples. Several reviews have been published, and the opinions vary considerably. There seems to be no consensus of opinions, most probably due to the fact that the area of antioxidants is such a complex topic. In a review by Frankel

* Author to whom correspondence should be addressed (e-mail chmhdj@nus.edu.sg; telephone 65-6874-8821; fax 65-6775-7895).
† National University of Singapore.
§ Brunswick Laboratories.
# Arkansas Children's Nutrition Center.

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Table 1. In Vitro Antioxidant Capacity Assays

assays involving hydrogen atom transfer reactions	ORAC (oxygen radical absorbance capacity)
$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$	TRAP (total radical trapping antioxidant parameter)
$ROO^{\bullet} + LH \rightarrow ROOH + L^{\bullet}$	Crocin bleaching assay
	IOU (inhibited oxygen uptake)
	inhibition of linoleic acid oxidation
	inhibition of LDL oxidation
assays by electron-transfer reaction	TEAC (Trolox equivalent antioxidant capacity)
$M(n) + e$ (from AH) $\rightarrow AH^{\bullet+} + M(n-1)$	FRAP (ferric ion reducing antioxidant parameter)
	DPPH (diphenyl-1-picrylhydrazyl)
	copper(II) reduction capacity
	total phenols assay by Folin–Ciocalteu reagent
other assays	TOSC (total oxidant scavenging capacity) (*90*)
	inhibition of Briggs–Rauscher oscillation reaction (*91*)
	chemiluminescence (*92*)
	electrochemiluminescence (*93*)

and Meyer, the authors pointed out that it is problematic to use one-dimensional methods to evaluate multifunctional food and biological antioxidants (*4*). The authors suggested that a general testing protocol should properly (a) choose a biologically relevant substrate, (b) test various oxidation conditions, (c) measure both initial and secondary oxidation products, (d) compare antioxidants at the same molar concentrations of active components, and (e) quantify on the basis of induction period, percent inhibition, or rates of hydroperoxide formation or decomposition, or IC_{50} (antioxidant concentration to achieve 50% inhibition). Rice-Evans and co-workers developed the Trolox equivalent antioxidant capacity (TEAC) assay, which has been broadly applied in assaying food samples (*5*). In her review article, Sanchez-Moreno suggested that the 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) assay was an easy and accurate method with regard to measuring the antioxidant capacity of fruit and vegetable juices or extracts (*6*). The oxygen radical absorbance capacity (ORAC) assay has found even broader application for measuring the antioxidant capacity of botanical samples (*7*) and biological samples (*8*). The total radical-trapping antioxidant parameter (TRAP) assay has also been widely used (*9*). These assays differ from each other in terms of substrates, probes, reaction conditions, and quantitation methods. It is extremely difficult to compare the results from different assays as Frankel and co-workers have already concluded (*4*). In the meantime, new assays claiming to measure antioxidant capacity continue to be reported (*10*, *11*). **Table 1** lists the major antioxidant capacity assays.

The complexity of the topic of antioxidants plus the confusion introduced by improper use of questionable methods leads to the disarray of the antioxidant research community and industry. Due to the lack of a standard assay, it is difficult to compare the results reported from different research groups, and the food and nutraceutical industry cannot perform strict quality control for antioxidant products. An open discussion of the pros and cons of various antioxidant capacity assays is needed so that validated benchmark methods can be identified for further development to a standard method broadly applicable by antioxidant researchers. To achieve this goal, we review herein the chemistry behind the common antioxidant capacity assays. We do not intend to be comprehensive to cover all reported assays; instead, we focus on the ones with certain degrees of influence and applications.

2. MULTIFACETED NATURE OF ANTIOXIDANTS

The word "antioxidant" is increasingly popular in modern society as it gains publicity through mass media coverage of its health benefits. The dictionary definition of antioxidant is rather straightforward but with a traditional annotation (*12*): "a substance that opposes oxidation or inhibits reactions promoted by oxygen or peroxides, many of these substances (as the tocopherols) being used as preservatives in various products (as in fats, oils, food products, and soaps for retarding the development of rancidity, in gasoline and other petroleum products for retarding gum formation and other undesirable changes, and in rubber for retarding aging)". A more biologically relevant definition of antioxidants is "synthetic or natural substances added to products to prevent or delay their deterioration by action of oxygen in air. In biochemistry and medicine, antioxidants are enzymes or other organic substances, such as vitamin E or β-carotene, that are capable of counteracting the damaging effects of oxidation in animal tissues" (*13*). The biologically relevant definition fits better to the concept of antioxidants known to the general public as people are more aware of their health than prevention of rubber autoxidation.

Depending on the scientific discipline, the scope and protection targets are significantly different. In the chemical industry, antioxidants often refer to compounds that retard autoxidation of a chemical product such as rubber and plastics. The autoxidation is caused primarily by radical chain reactions between oxygen and the substrates. Effective antioxidants are radical scavengers that break down radical chain reactions. Sterically hindered phenols and amines are often used as antioxidants in the rubber and plastic industries. In food science, antioxidants have a broader scope, in that they include components that prevent fats in food from becoming rancid as well as dietary antioxidants—"a substance in foods that significantly decreases the adverse effects of reactive species, such as reactive oxygen and nitrogen species, on normal physiological function in humans" (*14*), as defined by the Institute of Medicine. Like the other definitions, this definition does not provide limitation on the mechanism(s) of antioxidant action. Therefore, a dietary antioxidant can (sacrificially) scavenge reactive oxygen/nitrogen species (ROS/RNS) to stop radical chain reactions, or it can inhibit the reactive oxidants from being formed in the first place (preventive). Dietary antioxidants often broadly include radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors, and antioxidant enzyme cofactors. Selenium is a cofactor of selenoproteins (e.g., glutathione peroxidase), which reduce peroxides to alcohols and water. Selenium per se does

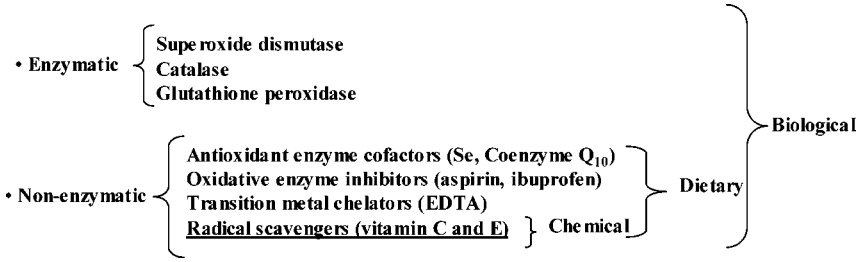


Figure 1. Broad scope of antioxidants.

not directly function as a ROS/RNS scavenger; therefore, the in vitro antioxidant capacity reported on selenium compounds is totally irrelevant to the role of selenium in a biological system. Whereas autoxidation of a lifeless matter occurs by radical chain reactions, oxidation in a biological system is primarily mediated by a host of redox enzymes. Nonetheless, nonenzymatic lipid autoxidation by radical chain reaction may still occur and lead to oxidative stress. Consequently, biological antioxidants include enzymatic antioxidants (e.g., superoxide dismutase, catalase, and glutathione peroxidase) and nonenzymatic antioxidants such as oxidative enzyme (e.g., cyclooxygenase) inhibitors, antioxidant enzyme cofactors, ROS/RNS scavengers, and transition metal chelators. Halliwell defined biological antioxidants as "molecules which, when present in small concentrations compared to the biomolecules they are supposed to protect, can prevent or reduce the extent of oxidative destruction of biomolecules" (*15*). **Figure 1** outlines the scope of antioxidants in three fields. Despite the difference in scope, a radical chain reaction inhibitor is commonly regarded as an antioxidant and also the most extensively studied.

3. ANTIOXIDANT CAPACITY ASSAYS

If one peruses the scientific papers on antioxidants, one will find many terms used by different researchers to describe antioxidant capacity. Terms one can find include total antioxidant "capacity" (or efficiency, power, parameter, potential, potency, and activity). The "activity" of a chemical would be meaningless without the context of specific reaction conditions such as pressure, temperature, reaction media, coreactants, and reference points. Because the "antioxidant activity" measured by an individual assay reflects only the chemical reactivity under the specific conditions applied in that assay, it is inappropriate and misleading to generalize the data as indicators of "total antioxidant activity". The other terms listed above are more independent of specific reactions and have similar chemical meanings. To be consistent in the review, we use "capacity" to refer to the results obtained by different assays. Oxidant-specific terms such as "peroxyl radical scavenging capacity", "superoxide scavenging capacity", "ferric ion reducing capacity" and the like would be more appropriate to describe the results from specific assays than the loosely defined terms "total antioxidant capacity" and the like.

On the basis of the chemical reactions involved, major antioxidant capacity assays can be roughly divided into two categories: (1) hydrogen atom transfer (HAT) reaction based assays and (2) single electron transfer (ET) reaction based assays. The ET-based assays involve one redox reaction with the oxidant (also as the probe for monitoring the reaction) as an indicator of the reaction endpoint. Most HAT-based assays monitor competitive reaction kinetics, and the quantitation is derived from the kinetic curves. HAT-based methods generally are composed of a synthetic free radical generator, an oxidizable molecular probe, and an antioxidant. HAT- and ET-based assays are intended to measure the radical (or oxidant) scavenging capacity, instead of the preventive antioxidant capacity of a sample. Because the relative reaction rates of antioxidants (or substrates) against oxidants, particularly peroxyl radicals, are the key parameters for sacrificial antioxidant capacity, we will analyze autoxidation and its inhibition kinetics before in-depth analysis of the individual assays.

3.1. Basic Kinetics of Autoxidation and Its Inhibition. Ingold and Denisov have independently and extensively analyzed the chemistry and kinetics of inhibited autoxidation of hydrocarbons (*16*). A typical autoxidation, initiated by an azo compound, and the action of its inhibitors include the following elementary steps (assuming one antioxidant scavenges two radicals and oxygen is in large excess; R_2N_2 = azo compound; LH = substrate; AH = antioxidant):

initiation

$$R_2N_2 \rightarrow 2R^\bullet + N_2 \quad (1)$$

$$R^\bullet + O_2 \rightarrow ROO^\bullet \quad (2)$$

$$ROO^\bullet + LH \rightarrow ROOH + L^\bullet \quad (3)$$

propagation

$$L^\bullet + O_2 \rightarrow LOO^\bullet \quad (4)$$

$$LOO^\bullet + LH \rightarrow LOOH + L^\bullet \quad (5)$$

inhibition

$$LOO^\bullet + AH \rightarrow LOOH + A^\bullet \quad (6)$$

termination

$$A^\bullet + (n-1)LOO^\bullet \rightarrow \text{nonradical products} \quad (7)$$

$$LOO^\bullet + LOO^\bullet \rightarrow \text{nonradical products} \quad (8)$$

Under steady-state conditions, the rate of uninhibited (R_{un}) and inhibited (R_{inh}) peroxide formation (or oxygen consumption) can be expressed by the equations

$$R_{un} = \{k_3/(2k_8)^{1/2}\}[LH]R_i^{1/2} \quad (9)$$

$$R_{inh} = \{k_3[LH]R_i\}/nk_6[AH] \quad (10)$$

where k_3, k_8, and k_6 denote the rate constants for propagation, termination, and inhibition, respectively. A good radical chain breaker should (a) react much more quickly with radicals ($k_6 \gg k_3$), wheres (b) the antioxidant radical, $A^\bullet$, does not react or reacts only very slowly (rate constant $\ll k_3$) with LH. Antioxidants can also scavenge alkoxyl radicals, which can be formed by the decomposition of peroxides through metal-catalyzed Fenton-type reactions. Scavenging of alkoxide radicals can be significant as this prevents the formation of cytotoxic compounds (such as 4-hydroxy-2-nonenal from linoleic acid lipid peroxide) (*17*). In the presence of antioxidants, lipid peroxide accumulation should be minimal until all of the antioxidants are sacrificed. In this sense, the reaction between antioxidants and the radicals from decomposed lipid peroxide would be insignificant. Hence, prevention of the primary oxidation is the key function of sacrificial antioxidants.

Abuja and Esterbauer simulated the kinetics of peroxidation of low-density lipoproteins (LDL) in the presence or absence of α-tocopherol using the following parameters:

constant radical flux rate ($ROO^\bullet$), $R_i = 2 \times 10^{-6}$ $(M\cdot s)^{-1}$

rate constant of hydrogen atom abstraction from LDL by $ROO^\bullet$, $k_3 = 3$ $(M\cdot s)^{-1}$

rate constant of hydrogen abstraction from tocopherol (TocOH), $k_6 = 10^6$ $(M\cdot s)^{-1}$

rate constant of radical coupling between $ROO^\bullet$ and $TocO^\bullet$, $k_7 = 2.5 \times 10^6$ $(M\cdot s)^{-1}$

rate constant of reaction between $TocO^\bullet$ and LDL, 0.07 $(M\cdot s)^{-1}$

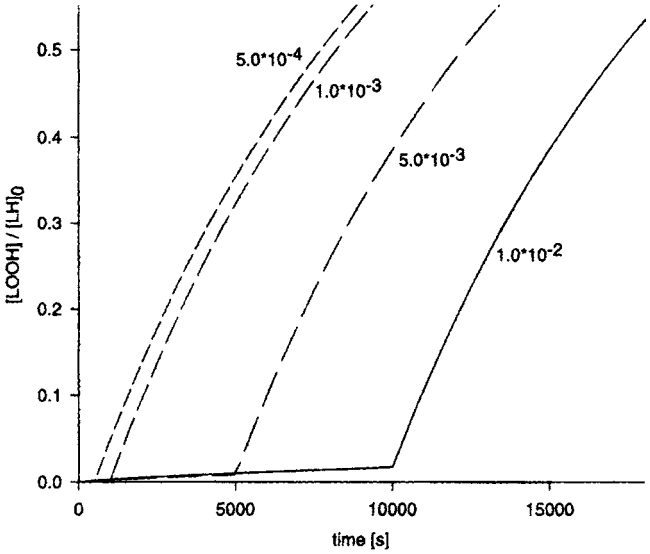


Figure 2. Simulated kinetic curves of LDL autoxidation in the presence of different tocopherol concentrations. (Reprinted with permission from *Chem. Res. Toxicol.* **1995**, *8*, 753–763. Copyright 1995 American Chemical Society.)

The simulated kinetic curves of tocopherol-inhibited autoxidation of LDL show distinct lag phases (**Figure 2**), and the length of the lag phase is linearly directly proportional to the concentration of tocopherol. For a pure antioxidant compound, the most important parameter is the rate constant with peroxyl radicals (k_6).

3.2. Inhibited Oxygen Uptake (IOU) Method. Experimentally, the measurement of the rate of oxygen uptake or conjugated diene peroxide formation was applied to derive the k_6 values of a pure antioxidant compound (*18*). Using styrene as a substrate and azoisobutrylnitrile (AIBN) as a radical initiator, Ingold and co-workers measured the oxygen consumption rates in the presence or absence of tocopherols in chlorobenzene using a pressure transducer system under one atmospheric pressure of oxygen. The k_6 value was calculated by applying the equation

$$[O_2]_0 - [O_2]_t = -(k_3[RH]/k_6[\ln(1 - t/\tau)]$$

where τ is the induction period, or lag time, and t is any time point before the acceleration phase. The k_6 values of the tocopherols were 2.35×10^6 (α), 1.66×10^6 (β), 1.59×10^6 (γ), and 6.5×10^5 (δ) $M^{-1}\cdot s^{-1}$. The IOU method has not found broad usage. The reason could be that (1) the experimental data were collected under unrealistically high oxygen pressure; (2) accurate measurement of oxygen uptake rates may be difficult, especially at the inhibition period when the uptake rate is slow; (3) food samples normally have lower antioxidant concentrations, and the sensitivity of this method may not be sufficient; and (4) phase transition between inhibited oxidation and uninhibited oxidation may not be as distinct as that of the tocopherols and may lead to ambiguous τ values (*19*).

3.3. Inhibition of Induced Lipid Autoxidation. This method artificially induces autoxidation of linoleic acid or LDL by either Cu(II) or an azo initiator as reported by Pryor and co-workers (*20*). The progress of autoxidation is monitored by UV absorbance at 234 nm (A_{max} of conjugated diene peroxides from linoleic acid oxidation) (*21*). This method is more sensitive than the IOU method mentioned above as it uses 10 times less initiator and substrate. The reaction can be carried out in micelles or in organic solvents. In micelles, reaction progress cannot be

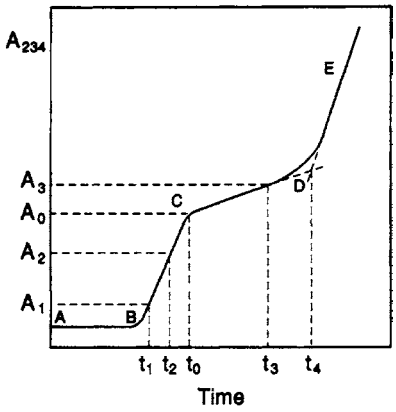


Figure 3. Representative UV trace of the inhibited autoxidation of linoleic acid measured at 234 nm in 0.10 M SDA/0.05 M phosphate buffer (pH 7.4): line AB, spontaneous autoxidation without initiator; B, initiator (ABAP) added; C, α-tocopherol (at 10^{-4} M) added; D, cross-point for the inhibited and uninhibited lines; ta, time when the antioxidant is added; t4, time corresponding to point D. The data of $d[CD]_{uninh}/dt$, $d[CD]_{inh}/d_t$, and T are derived from this trace. (Reprinted with permission from ref *20*. Copyright 1993 American Chemical Society.)

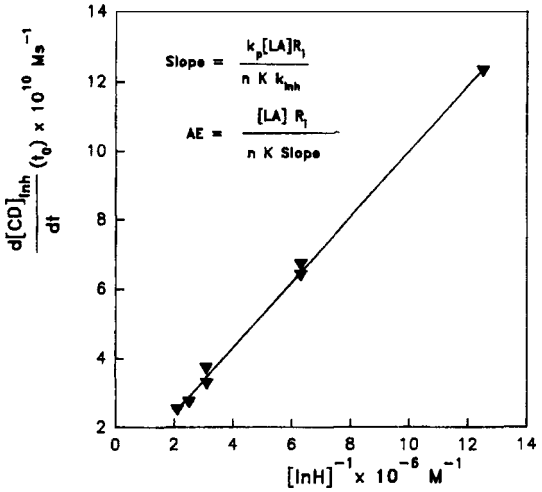


Figure 4. Calculation of the *AE* value for α-tocopherol in 0.10 M SDS/0.05 M phosphate buffer (pH 7.4); $d[CD]_{inh}/dt(t_0)$ is the rate of conjugated diene formation at the time ($t = t_0$) when an antioxidant is added. (Reprinted with permission from ref *20*. Copyright 1993 American Chemical Society.)

followed directly by a UV spectrometer and sample workup is necessary; this limits the efficiency of the method. Typically, the assay solution contains free radical initiator [2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 4 mM), substrate (linoleic acid, 2.5 mM), antioxidant, and dissolved oxygen (air saturated media). A typical kinetic curve of conjugated diene formation is depicted in **Figure 3**. In the absence of an initiator, negligible reaction occurs (curve AB). In the presence of a radical initiator (AAPH in this case), the reaction starts and conjugated diene oxides accumulate rapidly (curve BC). When an antioxidant is added, the reaction slows (curve CD) until the antioxidant is consumed, and the reaction rate increases to the uninhibited level (DE). The duration of the phase CD, or lag time, is dependent on the concentration and capacity of the antioxidant. The slopes of the curve CD (rate of oxidation) are inversely proportional to the concentration of antioxidants. The antioxidant efficiency, as defined by the authors, of a sample is obtained from the slope (*S*) of the curve (**Figure 4**).

Alternatively, relative antioxidant efficiency (RAE) is defined as the ratio of the slope of α-tocopherol to the slope of the sample.

$$\mathrm{RAE} = \frac{S_{\alpha-\mathrm{tocopherol}}}{S_{\mathrm{AH}}}$$

For linoleic acid, k_3 (3 $M^{-1}\cdot s^{-1}$) is much smaller than k_6 (10^6 $M^{-1}\cdot s^{-1}$) of tocopherol. According to the literature, the major parameters of the reactions are $[LH]_0 = 2.6$ mM and $[AAPH]_0 = 1$ mM. Therefore, $R_i = 2\,ek_d[AAPH] = 2 \times 0.5 \times 3.72 \times 10^{-7} \times 10^{-3} = 3.72 \times 10^{-10}$ M/s. Thus, the calculated slope for tocopherol is

$$S_{(\alpha-\mathrm{tocopherol})} = \frac{k_3\,[\mathrm{LH}]R_i}{nk_6} = \frac{3 \times 2.6 \times 10^{-3} \times 3.72 \times 10^{-10}}{2 \times 10^6} = 1.45 = 10^{-18}\ (M^2\cdot s^{-1})$$

Apparently, the slope is extremely small, and thus a lag phase would appear, which is consistent with the kinetic simulations as discussed earlier. Experimentally, it could be difficult to accurately measure such a small change in the rate of conjugated diene oxide formation rates in the inhibited phase. In addition, many organic compounds found in food absorb light at 234 nm. This was demonstrated by Ruberto (*17*), who found that catechol and hydroquinone reacted with peroxyl radicals to form *o*-quinone and *p*-quinone, which absorb light at 234 nm. The use of linoleic acid as a lipid source of course bears little similarity to the lipids in a biological system. In the presence of water, linoleic acid will form micelles, which further complicate the assay as the antioxidant distribution between two phases can be critical. Using linoleic acid or its methyl ester as an oxidation substrate does, however, simplify the assay, rendering it more reproducible than the assays in which lipids from a biological source are used. Those lipids, although they have biological similarity, suffer from lot-to-lot variability, which is not desired in a chemical quantitation method. It should also be noted that conjugated dienes can be formed by only polyunsaturated fatty acids and linoleic acid can lead to only one type of conjugated dienes.

Frankel and co-workers applied Cu(II) as the initiator to induce the oxidation of LDL. The oxidation progress was monitored (gas chromatograph) by the concentrations of hexanal formed in the headspace of the reaction vessels (*22*). Hexanal was chosen because it was suggested to be a major secondary decomposition product of *n*−6 fatty acid peroxide. Percent inhibition (%In) of the formation of hexanal was used as a parameter to compare antioxidant capacity. It is calculated according to the equation

$$\%\mathrm{In} = [(C - S)/C] \times 100$$

where C is the amount of hexanal formed in the control (no antioxidant added) and S is the amount of hexanal formed when antioxidant was present. The sample concentration that led to 50% inhibition, IC_{50}, is used to compare the capacities of different antioxidants. Because hexanal is only the secondary oxidation product and is only one of many other products from LDL lipid peroxide decompositions, it is not clear if the hexanal concentration can be an unbiased marker for the degree of LDL oxidation. In addition, hexanal has a rather high boiling point (131 °C), and under ambient temperature, the vapor pressure is low and the majority of the formed hexanal will be in the liquid phase and not be measured using this method. It was found that Cu(II) alone does not induce the autoxidation of lipids. Instead, the reaction was initiated by antioxidants (e.g., tocopherols) present in the LDL. The tocopherol was first converted to free radical by donating an electron to Cu(II) [generating Cu(I)]. The tocopherol radical then slowly induces the autoxidation of LDL (eqs 21−25) (*23*). In agreement with this finding, Ingold and co-workers observed that, in the absence of antioxidants, Cu(II) failed to trigger LDL peroxidation (*24*). Moreover, the generated Cu(I) can decompose peroxides (LOOH) by a Fenton-type reaction (eq 15) and initiate more radical chain reactions. Overall, Cu(II) may act as a catalyst in the presence of excessive antioxidants, and the antioxidants may act as pro-oxidants. Thus, Cu(II) is a questionable initiator for assaying the radical chain-breaking capacity of antioxidants.

$$\mathrm{Cu(II)} + \mathrm{AOH} \rightarrow \mathrm{Cu(I)} + \mathrm{AO^\bullet} + \mathrm{H^+} \quad (11)$$

$$\mathrm{AO^\bullet} + \mathrm{L{-}H} \rightarrow \mathrm{AOH} + \mathrm{L^\bullet} \quad (12)$$

$$\mathrm{L^\bullet} + \mathrm{O_2} \rightarrow \mathrm{LOO^\bullet} \quad (13)$$

$$\mathrm{LOO^\bullet} + \mathrm{L{-}H} \rightarrow \mathrm{LOOH} + \mathrm{L^\bullet} \quad (14)$$

$$\mathrm{Cu(I)} + \mathrm{LOOH} \rightarrow \mathrm{Cu(II)} + \mathrm{LO^\bullet} + \mathrm{HO^-} \quad (15)$$

3.4. Assays Using Molecular Probes. The experimental complexity and the limitations of directly monitoring reaction kinetics of the inhibited autoxidation of lipids have led to the development of more convenient methods in assessing the antioxidant capacity of a sample. Several colorimetric and fluorometric antioxidant capacity assays apply a radical reaction but without a chain propagation step, an essential step in lipid autoxidation. It is thus debatable as to the relevance of these approaches to radical chain-breaking antioxidant capacity (*25*). In general, these assays apply a thermal radical generator to give a steady flux of peroxyl radicals in air-saturated solution. Added antioxidant competes with probes (substrates in this case) for the radicals and inhibits or retards the probe oxidation. Assays with this feature include total radical trapping antioxidant parameter (TRAP) assay, oxygen radical absorbance capacity (ORAC) assay, and crocin bleaching assay. These assays have the following components: (a) an azo radical initiator, normally AAPH; (b) a molecular probe (UV or fluorescence) for monitoring reaction progress; (c) antioxidant; and (d) reaction kinetic parameters collected for antioxidant capacity quantitation.

3.4.1. Basic Kinetic Considerations. Generally, if a probe (PH) competes with an antioxidant (AH) for constant flux of peroxyl radicals ($ROO^\bullet$) generated from thermal decomposition of an azo compound, the elementary reactions are as follows [assuming one PH (or AH) scavenges two $ROO^\bullet$]

$$\mathrm{ROO^\bullet} + \mathrm{PH} \rightarrow \mathrm{ROOH} + \mathrm{P^\bullet} \quad (16)$$

$$\mathrm{P^\bullet} + \mathrm{ROO^\bullet} \rightarrow \mathrm{ROOP} \quad (17)$$

$$\mathrm{ROO^\bullet} + \mathrm{AH} \rightarrow \mathrm{ROOH} + \mathrm{A^\bullet} \quad (18)$$

$$\mathrm{A^\bullet} + \mathrm{ROO^\bullet} \rightarrow \mathrm{ROOA} \quad (19)$$

assuming the reaction is under steady state. The rate of probe oxidation can be expressed by the following equation:

$$V = -\frac{\mathrm{d[PH]}}{\mathrm{d}t} = \frac{k_{16}R_i[\mathrm{PH}]}{2k_{16}[\mathrm{PH}] + 2k_{18}[\mathrm{AH}]}$$

When there is no antioxidant ([AH] = 0), the uninhibited reaction rate $V_0 = 0.5R_i$. Therefore

$$\frac{V_0}{V} = 1 + \frac{k_{18}[\mathrm{AH}]}{k_{16}[\mathrm{PH}]} \tag{20}$$

On the basis of eq 20, three scenarios would arise during the course of inhibited reactions:

(a) If $100k_{16}[\mathrm{PH}] < k_{18}[\mathrm{AH}]$, $V_0/V > 100$ (normally in the beginning of the reaction, then insignificant spectroscopic changes for the probe would be observed (lag phase). This would occur if either $k_{18} \gg k_{16}$ (AH is an antioxidant) or k_{18} is comparable to or less than k_{16}, but [AH] is much larger than [PH] (AH is a retardant).

(b) As the reaction proceeds, antioxidant is consumed by the constant flux of peroxyl radicals. The oxidation of the probe would progress significantly but at a slower speed than V_0. Or if PH itself is such a potent antioxidant that $k_{16}[\mathrm{PH}]$ can no longer be neglected in comparison with $k_{18}[\mathrm{AH}]$, then no lag phase will occur. The spectroscopic change is significant but at $V < V_0$.

(c) When AH is depleted, the reaction rate is V_0.

The appearance and duration of three phases is dependent on (1) the nature of the antioxidant and its concentration relative to the probe and (2) the probe's reactivity to the radicals. The kinetic curves of ORAC, TRAP, and crocin bleaching assays bear some similarity to the above kinetic model. The major difference among these assays is the quantitation approaches. The ORAC assay applies the area under the kinetic curve (AUC) approach, the TRAP assay relies on lag time, and the crocin bleaching assay utilizes initial reaction rate.

3.4.2. Oxygen Radical Absorbance Capacity Assay. Originally developed by Cutler and Cao, the first version of the ORAC assay employed B-phycoerythrin (*26*) (B-PE, a fluorescent protein) as the probe. The fluorescence decay of B-PE is an indication of damage from its reaction with the peroxyl radical. Later, Ou and co-workers found that B-PE suffered several disadvantages: (1) B-PE, a protein product isolated from *Porphyridium cruentum*, has a large lot-to-lot variability (*27*); (2) B-PE is photobleached under plate-reader conditions; and (3) B-PE interacts with polyphenols due to the nonspecific protein binding and loses fluorescence even without added radical generator (**Figure 5**).

To solve these problems, Ou replaced B-PE with fluorescein (FL) (3′,6′-dihydroxyspiro[isobenzofuran-1[3*H*], 9′[9*H*]-xanthen]-3-one) (*28*). FL is a synthetic nonprotein probe and overcomes the limitations of B-PE. In addition, the reaction products of FL with peroxyl radical have been characterized, and the product pattern was consistent with a classic HAT reaction mechanism. The improved ORAC assay provides a direct measure of the hydrophilic and lipophilic chain-breaking antioxidant capacity versus peroxyl radicals (*29*).

The detailed procedures of the high-throughput ORAC assay operating on a 96-well plate fluorescence reader are described by Huang et al. (*30*). In general, samples, controls, and standard (Trolox of four or five different concentrations for construction of a standard curve) are mixed with fluorescein solution and incubated at constant temperature (37 °C) before AAPH solution is then added to initiate the reaction. The fluorescence intensity [485 nm (ex)/525 nm (em)] is measured every minute for 35 min at ambient conditions (pH 7.4, 37 °C). As the reaction progresses, fluorescein is consumed and FL intensity decreases. In the presence of antioxidant, the FL decay is inhibited. A typical ORAC assay kinetic curve is shown in **Figure 6**.

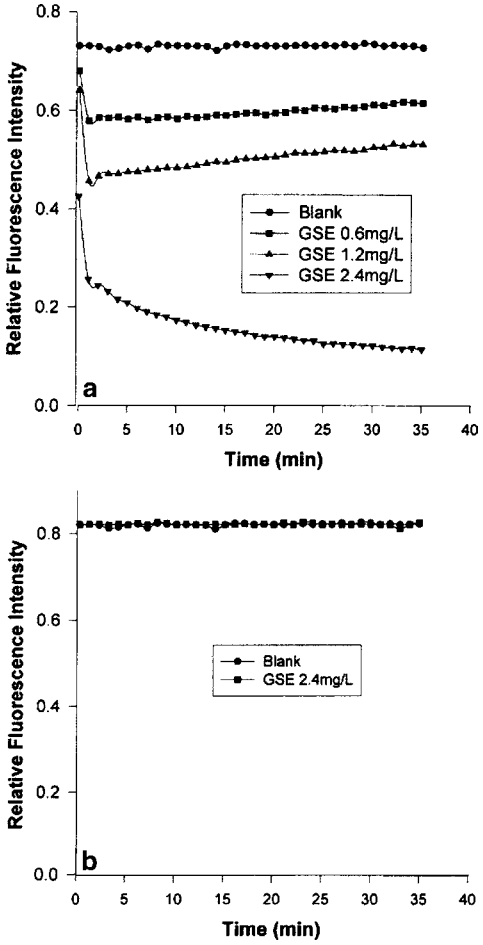


Figure 5. (**a**) Relative fluorescence versus time (minutes) of reaction: blank and grape seed extracts (GSE) at various concentrations using B-PE as the fluorescent probe. (**b**) Blank and GSE at 2.4 mg/L using FL as the fluorescent probe. (Reprinted with permission from *J. Agric. Food Chem.* **2001**, *49*, 4619–4626. Copyright 2001 American Chemical Society.)

Data reduction from the ORAC assay is achieved by (1) calculating of the area under the kinetic curve (AUC) and net AUC ($\mathrm{AUC_{sample}} - \mathrm{AUC_{blank}}$), (2) obtaining a standard curve by plotting the concentration of Trolox and the AUC (linear or quadratic fit between 0.78 and 12.6 μM Trolox), and (3) calculating the Trolox equivalents of a sample using the standard curve. These steps can be performed automatically on an Excel or similar data processing program. The high-throughput assay can analyze 16 samples at once and has the capacity of testing several hundred samples daily by just using one plate-reader coupled with an eight-channel automatic liquid handling system.

It should be noted that in some cases, antioxidant samples have different curves of concentration versus AUC from that of Trolox standard. Forcing the AUC of the sample to the standard equation of Trolox would lead to scattered ORAC values of the sample. This is normally due to sample matrix interference, which is amplified when the antioxidant activity of a sample is low and a large concentration of samples is needed in order to give measurable AUC.

The advantage of the AUC approach is that it applies equally well for both antioxidants that exhibit distinct lag phases and those samples that have no lag phases. This approach unifies the lag time method and initial rate method, and it is particularly useful for food samples, which often contain multiple ingredients and have complex reaction kinetics. There is a direct linear correlation of AUC and a broad range of sample types, including

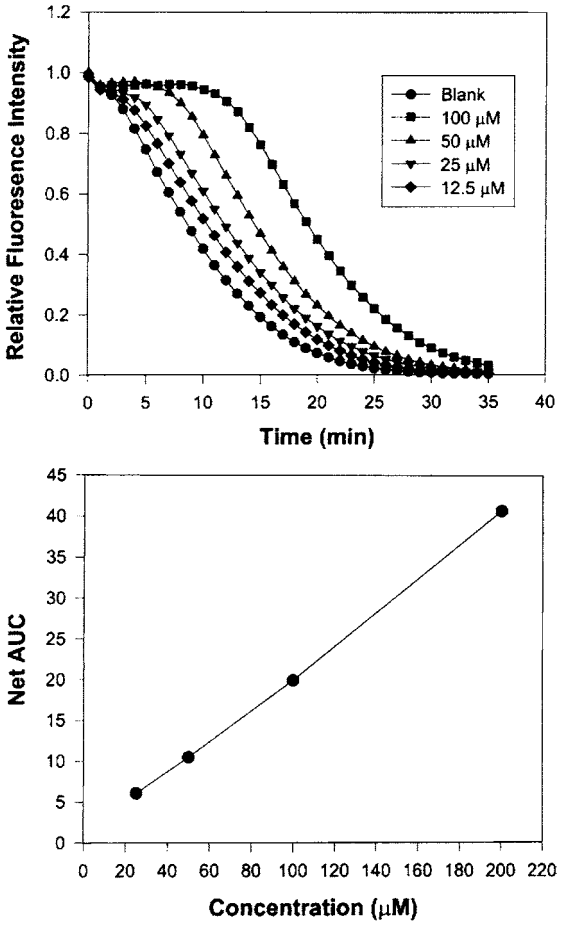


Figure 6. (**a**) Fluorescence decay curve of fluorescein in the presence of α-tocopherol and AAPH. (**b**) Linear plot of the net AUC versus α-tocopherol concentration. (Reprinted with permission from ref *29*. Copyright 2002 American Chemical Society.)

raw fruit and vegetable extracts, plasma, and pure phytochemicals (*26*). Therefore, the ORAC assay has been broadly applied in academics and the food and supplement industry as a method of choice to quantify antioxidant capacity. In fact, an antioxidant database has been generated applying the ORAC assay in combination with the total phenols assay (*31*, *32*).

Many antioxidants are lipophilic, and it is also known that the antioxidant capacity of a compound is dependent upon reaction media (*33*−*35*). Therefore, an organic solvent based ORAC assay would be particularly useful for lipophilic samples. However, fluorescein is not sufficiently lipid soluble, and its fluorescence intensity in nonpolar organic solvent is rather low. To overcome this problem, Naguib applied 4,4-difluoro-3,5-bis(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-*s*-indacene (BODIPY 665/676) as a fluorescent probe and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) as a peroxyl radical generator. The reaction could be carried out either in liposome or on an octane and butyronitrile mixture (*36*). By applying this assay, the antioxidant capacity of various carotenoids was quantified. However, this assay is 100 times less sensitive than the ORAC assay, probably due to the low efficiency of the radical generator, AMVN. In addition, the fluorescent quenching mechanism of BODIPY by peroxy radical remains to be investigated.

3.4.3. Crocin Bleaching Assay. This assay measures the inhibition capacity of antioxidants in protecting the bleaching of crocin, a naturally occurring carotenoid derivative, by the free radical generator AAPH (*37*). Ursini and co-workers later applied this method to the analysis of plasma antioxidant capacity (*38*). Experimentally, the reaction was carried out by first preparing a 2.0 mL phosphate buffer (0.1 M, pH 7.0) containing 10 μM crocin and certain amounts of antioxidants. Next the radical initiator AAPH (50 μL, 0.5 M) was added to initiate the reaction. The progress of the reaction was monitored by a UV−vis spectrometer at a wavelength of 443 nm, the absorption maximum of crocin ($\epsilon = 1.33 \times 10^5$ M^{-1} cm^{-1}). The bleaching rate becomes linear ~1 min after the addition of AAPH and was monitored for 10 min. To eliminate possible interferences from the sample itself, blanks without crocin were also monitored under the same wavelength. The initial crocin bleaching rates were obtained from the kinetic curves in the presence (V) or absence (V_0) of antioxidants. The relationship between V and V_0 obeyed the equation (similar to eq 16)

$$V_0/V = 1 + (k_a/k_c) \times [\mathrm{AH}]/[\mathrm{C}] \qquad (21)$$

where k_a is the rate constant for the reaction of antioxidants with ROO•, k_c is the rate constant for the reaction between ROO• and crocin, [C] is the concentration of crocin, and [AH] is the concentration of antioxidant. A plot of [AH]/[C] versus V_0/V should give a linear line curve with a slope of k_a/k_c, which indicates the relative peroxyl radical scavenging capacity. For a given antioxidant, the k_a/k_c value was divided by the k_a/k_c value for the sample. For plasma, a linearity curve was obtained with a slope of 0.79. The antioxidant capacity of vitamin C was ranked surprisingly high at 7.7 Trolox equivalence (for comparison, the ORAC value of vitamin C is 0.95) (*24*).

The crocin bleaching assay has found limited applications in food samples so far. Reaction rate constants between ROO• and phytochemicals may vary greatly, and some of them have rates comparable to that of crocin (thus, no lag phase), whereas others will give a lag phase. In this case, the inhibited bleaching rates are very small and are not sensitive to the concentration changes of antioxidants. This could be the reason vitamin C has an unusually large antioxidant capacity value. Crocin absorbs at a rather short wavelength (450 nm), and many food pigments, such as carotenoids, absorb light at the same wavelength. To avoid the interference for each sample, a sample blank (a mixture containing only AAPH and food sample) must be tested at the same time. Finally, crocin is a mixture of natural pigments extracted from saffron and is subject to lot-to-lot variability, which limits its industrial application in a quantitative procedure.

3.4.4. Total Peroxyl Radical-Trapping Antioxidant Parameter Assay (39). Detailed accounts on the history and current state of the TRAP assay can be found in a review by Ghiselli and co-workers (*40*). The TRAP assay uses R-phycoerythrin (R-PE) as a fluorescent probe (*41*). The reaction progress of R-PE with AAPH was monitored fluorometrically ($\lambda_{ex} = 495$ nm and $\lambda_{em} = 575$ nm). A typical kinetic curve of fluorescence decay is shown in **Figure 7**.

The antioxidant capacity of an unknown sample was expressed as Trolox equivalence (X) by the equation

$$C_{\mathrm{Trolox}}/T_{\mathrm{Trolox}} = X/T_{\mathrm{plasma}} \qquad (22)$$

where C_{Trolox} is Trolox concentration, T_{Trolox} is the lag time of the kinetic curve of R-PE in the presence of Trolox, X is the antioxidant capacity of plasma, and T_{plasma} is the lag time of the kinetic curve in the presence of plasma. X is then multiplied by 2.0 (the stoichiometric factor of Trolox) and by the dilution factor of the sample to give the TRAP value (μmol/L). To obtain the T_{Trolox} from the same kinetic curve of the sample, Trolox was added to the reaction mixture when R-PE fluorescence was

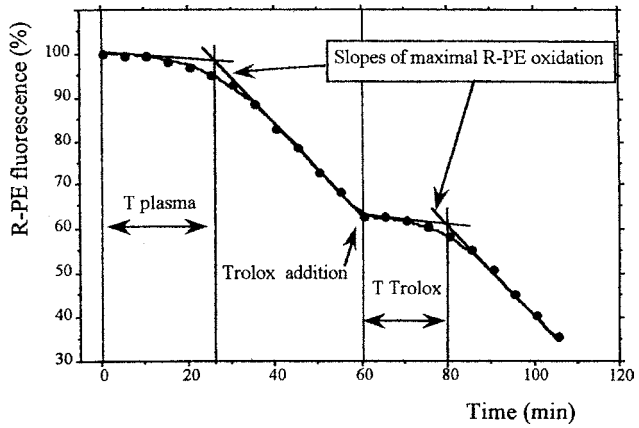


Figure 7. Kinetics of R-PE oxidation initiated by 5 mM AAPH in the presence of plasma (8 μL) before and after Trolox addition (1.8 μM final solution). The antioxidant capacity of each plasma sample is calculated by comparing the two lag phases obtained in the presence and in the absence of trolox. (Reprinted with permission from ref *38*. Copyright 1998 Elsevier.)

~50% of the initial value. The reaction was followed until the fluorescence decay rate resumed to the level before the Trolox addition. The lag phase was then calculated by extrapolating the curves of maximal R-PE oxidation before and after Trolox addition (**Figure 7**). Not all of the samples will yield a lag phase. Ursini and co-workers simulated effects of antioxidants on the lag phase of peroxidation and found that lag time-based measurements of antioxidant capacity overestimated the antioxidant capacity of weaker antioxidants (*34*). Valkonene and co-workers modified the TRAP assay by applying dichlorofluorescin diacetate (DCFH-DA) as the molecular probe (*42*). In the presence of AAPH, DCFH-DA was oxidized and also (somehow) hydrolyzed in the process, to produce highly fluorescent dichlorofluorescein (DCF). The increase of fluorescence signal is an indication of oxidation progress.

4. ET-BASED ASSAYS

These assays include perhaps the most popular, but often misunderstood by its name, total phenols assay by Folin−Ciocalteu reagent (FCR). In addition, also grouped into this category are the Trolox equivalent antioxidant capacity (TEAC) assay, the ferric ion reducing antioxidant power (FRAP) assay, the *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD) assay, and the Cu(II) reduction capacity assay. These methods involve two components in the reaction mixture, antioxidants and oxidant (also the probe). They are based on the following electron-transfer reaction:

$$\text{probe (oxidant)} + \text{e (from antioxidant)} \rightarrow \text{reduced probe} + \text{oxidized antioxidant}$$

The probe itself is an oxidant that abstracts an electron from the antioxidant, causing color changes of the probe. The degree of the color change is proportional to the antioxidant concentrations. The reaction end point is reached when color change stops. The change of absorbance (ΔA) is plotted against the antioxidant concentration to give a linear curve. The slope of the curve reflects the antioxidant's reducing capacity, which is expressed as Trolox equivalence (TE) or gallic acid equivalent (GAE). These assays resemble the redox titration in classical chemical analysis. Because there is not a competitive reaction involved and there is no oxygen radical in the assays, it is questionable how the assay results relate to the antioxidant capacity of a sample. To make the correlation, it is assumed that antioxidant capacity is equal to reducing capacity (*43*).

4.1. Total Phenols Assay by Folin−Ciocalteu Reagent. FCR was initially intended for the analysis of proteins taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue (*44*). Many years later, Singleton and co-workers extended this assay to the analysis of total phenols in wine; since then the assay has found many applications (*45*). The FCR-based assay gained popularity and is commonly known as the total phenols (or phenolic) assay. The FCR actually measures a sample's reducing capacity, but this is not reflected in the name "total phenolic assay". Numerous publications applied the total phenols assay by FCR and an ET-based antioxidant capacity assay (e.g., FRAP, TEAC, etc.) and often found excellent linear correlations between the "total phenolic profiles" and "the antioxidant activity". This is not surprising if one considers the similarity of chemistry between the two assays. One of the assays may just be redundant. A recent report of using polyphenol oxidase for assaying total phenols in tea may be more specific to phenolic compounds (*46*).

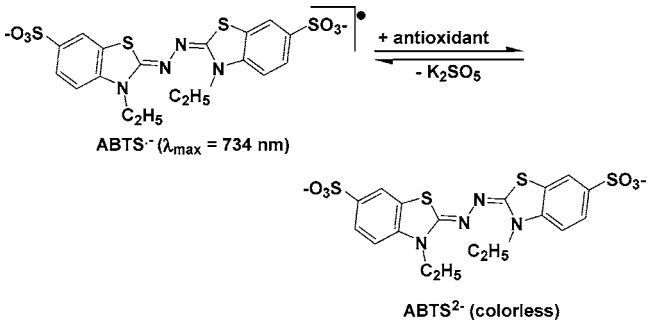
The FCR is typically made by first boiling (for 10 h) the mixture of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$, 100 g), sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$, 25 g), concentrated hydrochloric acid (100 mL), 85% phosphoric acid (50 mL), and water (700 mL). After boiling, lithium sulfate ($Li_2SO_4 \cdot 4H_2O$, 150 g) is added to the mixture to give an intense yellow solution—the FC reagent. Contamination of reductants leads to a green color, and the addition of oxidants such as bromine can restore the desired yellow color. The exact chemical nature of the FC reagent is not known, but it is believed to contain heteropolyphosphotunstates−molybdates. Sequences of reversible one- or two-electron reduction reactions lead to blue species, possibly $(PMoW_{11}O_{40})^{4-}$. In essence, it is believed that the molybdenum is easier to be reduced in the complex and electron-transfer reaction occurs between reductants and Mo(VI):

$$\text{Mo(VI)} + \text{e} \rightarrow \text{Mo(V)}$$

Obviously, the FC reagent is nonspecific to phenolic compounds as it can be reduced by many nonphenolic compounds [e.g., vitamin C, Cu(I), etc.]. Phenolic compounds react with FCR only under basic conditions (adjusted by a sodium carbonate solution to pH ~10). Dissociation of a phenolic proton leads to a phenolate anion, which is capable of reducing FCR. This supports the notion that the reaction occurs through electron-transfer mechanism. The blue compounds formed between phenolate and FCR are independent of the structure of phenolic compounds, therefore ruling out the possibility of coordination complexes formed between the metal center and the phenolic compounds.

Despite the undefined chemical nature of FCR, the total phenols assay by FCR is convenient, simple, and reproducible. As a result, a large body of data has been accumulated, and it has become a routine assay in studying phenolic antioxidants.

4.1. Trolox Equivalent Antioxidant Capacity Assay. The TEAC assay was first reported by Miller and Rice-Evans in 1993 (*47*) and later improved (*48*). In the improved version, $ABTS^{\bullet -}$, the oxidant, was generated by persulfate oxidation of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{2-}$). Specifically, 7 mmol of ABTS ammonium was dissolved in water and treated with 2.45 mmol of potassium persulfate, and the mixture was then allowed to stand at room temperature for 12−16 h to give a dark blue solution. This solution was diluted

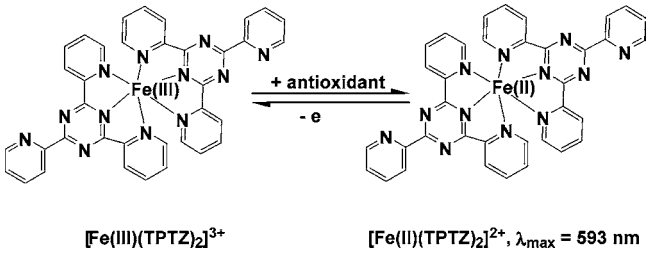


with ethanol or buffer (pH 7.4) until the absorbance reached 0.7 at 734 nm. One milliliter of the resulting solution was mixed with 10 μL of sample. The absorbance was read at 30 °C, 1, 4, and 6 min after mixing at 30 °C. The difference of the absorbance reading is plotted versus the antioxidant concentrations to give a straight line. The concentration of antioxidants giving the same percentage change of absorbance of the ABTS$^{\bullet-}$ as that of 1 mM Trolox was regarded as TEAC.

Due to its operational simplicity, the TEAC assay has been used in many research laboratories for studying antioxidant capacity, and TEAC values of many compounds and food samples are reported. The TEAC values for pure antioxidant compounds do not show clear correlation between TEAC values and the number of electrons an antioxidant can give away. The TEAC values of ascorbic acid (1.05), α-tocopherol (0.97), glutathione (1.28), and uric acid (1.01) are almost the same, although glutathione can normally donate one electron (to form oxidized glutathione) whereas the others are two-electron reductants. Ferulic acid (1.90) and *p*-coumaric acid (2.00) have comparable TEAC values. However, caffeic acid has a TEAC value of 1.00 even though its structure is similar to that of ferulic acid. The TEAC value difference between quercetin (3.00) and kaempferol (1.00) is also rather surprising as they have similar chemical structures (*14*).

Apparently, the reaction rate differences between antioxidants and oxidants are not reflected in the TEAC values because the TEAC assay is an end-point assay.

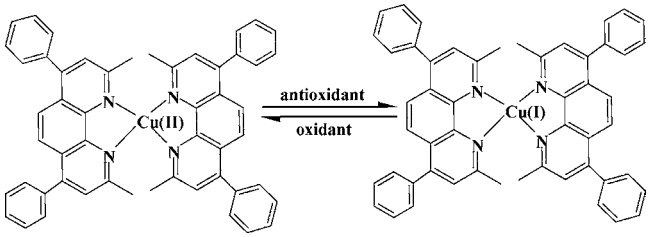
4.2. Ferric Ion Reducing Antioxidant Power Assay. The FRAP assay also takes advantage of electron-transfer reactions. Herein a ferric salt, Fe(III)(TPTZ)$_2$Cl$_3$ (TPTZ = 2,4,6-tripyridyl-*s*-triazine), is used as an oxidant (*49*). The redox potential of



Fe(III) salt (~0.70 V) is comparable to that of ABTS$^{\bullet-}$ (0.68 V). Therefore, essentially, there is not much difference between TEAC assay and the FRAP assay except TEAC is carried out at neutral pH and FRAP assay under acidic (pH 3.6) conditions. The FRAP assay involves the following procedures: The oxidant in the FRAP assay is prepared by mixing TPTZ (2.5 mL, 10 mM in 40 mM HCl), 25 mL of acetate buffer, and 2.5 mL of $FeCl_3 \cdot H_2O$ (20 mM). The conglomerate is referred to as "FRAP reagent". The final solution has Fe(III) of 1.67 mM and TPTZ of 0.83 mM. Therefore, the TPTZ is deficient as the ideal reaction stoichiometry between Fe(III) and TPTZ is 1 to 2. The oxidant is not just Fe(III)(TPTZ)$_2$, it also contains other Fe(III) species which can lead to potential problems as many metal chelators in food extract could bind Fe(III) and form complexes that are also capable of reacting with antioxidants. To measure FRAP value, 300 μL of freshly prepared FRAP reagent is warmed to 37 °C and a reagent blank reading is taken at 593 nm; then 10 μL of sample and 30 μL of water are added. Absorbance readings are taken after 0.5 s and every 15 s until 4 min. The change of absorbance ($\Delta A = A_{4min} - A_{0min}$) is calculated and related to ΔA of an Fe(II) standard solution. ΔA is linearly proportional to the concentration of antioxidant. One FRAP unit is arbitrarily defined as the reduction of 1 mol of Fe(III) to Fe(II). The FRAP values for ascorbic acid, α-tocopherol, and uric acid are identical (2.0). The FRAP value of bilirubin is 1-fold higher than that of ascorbic acid. These results suggest that 1 mol of vitamin C can reduce 2 mol of Fe(III) and that 1 mol of bilirubin can reduce 4 mol of Fe(III). This is in conflict with the fact that both vitamin C and bilirubin are two-electron reductants. It is known that when bilirubin is oxidized, it is transformed to beliverdin (by losing two hydrogen atoms, not just electrons), which happens to have an absorption at 593 nm with coeffiicency ($\epsilon_{593} = 1 \times 10^4$) comparable with that of Fe(II)(TPTZ)$_2$.[50]

Pulido and co-workers (*51*) measured the FRAP values of several polyphenols in water and methanol. However, the absorption (A_{593}) does not stop at 4 min; instead, it slowly increased even after several hours. Polyphenols with such behaviors include caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin. The FRAP values of these compounds cannot be obtained accurately if 4 min reaction time was followed.

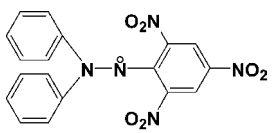
4.3. Total Antioxidant Potential Assay Using Cu(II) as an Oxidant. There is little published information on this assay. However, an industrial laboratory is providing service using it as a measure of total antioxidant potential (*52*). The method is based on reduction of Cu(II) to Cu(I) by reductants (antioxi-



dants) present in a sample. A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), forms a 2:1 complex with Cu(I), which has a maximum absorbance at 490 nm (*53*). It was found that 1 mol of α-tocopherol can reduce 2 mol of Cu(II) to Cu(I) (*54*).

More recently, Zaporozhets et al. reported a method for measuring the antioxidant power of herbal products based on solid-phase spectrophotometry using tetrabenzo[*b*,*f*,*j*,*n*][1,5,9,-13]tetraazacyclohexadecine−Cu(II) complex immobilized on silica gel. The absorbance of the modified sorbent (712 nm) increases when the Cu(II) is reduced (*55*).

4.4. 2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay. DPPH is one of a few stable and commercially



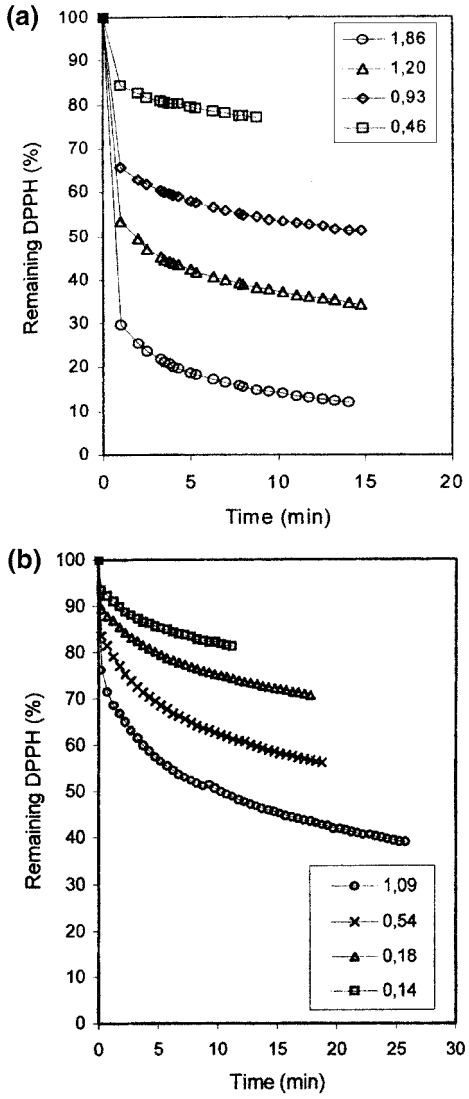


Figure 8. DPPH bleaching kinetics in the presence of different concentrations of α-carotene (**a**) and β-xanthophylls (**b**). [Reprinted with permission from ref *56* (Wiley). Copyright 2000 Society of Chemical Industry.]

available organic nitrogen radicals and has a UV−vis absorption maximum at 515 nm. Upon reduction, the solution color fades; the reaction progress is conveniently monitored by a spectrophotometer.

The DPPH assay is typically run by the following procedure: DPPH solution (3.9 mL, 25 mg/L) in methanol is mixed with sample solution (0.1 mL). The reaction progress absorbance of the mixture is monitored at 515 nm for 30 min or until the absorbance is stable. Upon reduction, the color of the solution fades. The percentage of the DPPH remaining is calculated as

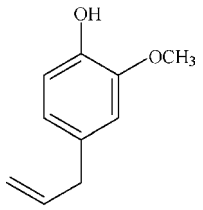
$$\%\mathrm{DPPH}_{\mathrm{rem}} = 100 \times [\mathrm{DPPH}]_{\mathrm{rem}}/[\mathrm{DPPH}]_{T=0} \qquad (23)$$

$\%\mathrm{DPPH}_{\mathrm{rem}}$ is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} concentration is calculated from the kinetic curve and defined as T_{EC50}. A representative kinetic curve of a DPPH assay is shown in **Figure 8**.

Sanchez-Moreno and co-workers classified the kinetic behavior of the antioxidant compound as follows: <5 min (rapid), 5−30 min (intermediate), and >30 min (slow). They further proposed a parameter, called "antiradical efficiency (AE)" (*56*), to express the antioxidant capacity of a certain antioxidant. AE is calculated as

$$\mathrm{AE} = (1/\mathrm{EC}_{50})T_{\mathrm{EC50}} \qquad (24)$$

The DPPH assay is technically simple, but some disadvantages limit its applications. Besides the mechanistic difference from the HAT reaction that normally occurs between antioxidants and peroxyl radicals, DPPH is a long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. This is evident from the T_{EC50} values ranging from 1.15 min (ascorbic acid) to 103 min (rutin). Consequently, the antioxidant capacity is not properly rated. The reaction kinetics between DPPH and antioxidants are not linear to DPPH concentrations (**Figure 8**). It is thus rather arbitrary to express antioxidant capacity using EC_{50}. Finally, it was reported that the reaction of DPPH with eugenol was reversible (*57*). This would result in falsely low readings for antioxidant capacity of samples containing eugenol and other phenols bearing a similar structure type (*o*-methoxyphenol).



Eugenol

The DPPH assay was believed to involve hydrogen atom transfer reaction, but a recent paper suggested otherwise. On the basis of the kinetic analysis of the reaction between phenols and DPPH (*58*), Foti and co-workers suggested that the reaction in fact behaves like an ET reaction. The authors found that the rate-determining step for this reaction consists of a *fast* electron-transfer process from the phenoxide anions to DPPH. The hydrogen atom abstraction from the neutral ArOH by DPPH becomes a marginal reaction path, because it occurs *very* slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol. In addition, the author found that adventitious acids or bases present in the solvent may dramatically influence the ionization equilibrium of phenols and cause a reduction or an enhancement, respectively, of the measured rate constants. This renders the DPPH assay much less chemically sound as a valid assay for antiradical activity of measurement.

5. OVERALL CONSIDERATIONS OF ET- AND HAT-BASED ASSAYS

ET-based assays measure an antioxidant's reducing capacity, and the HAT-based assays quantify hydrogen atom donating capacity. It is apparent that the hydrogen atom transfer reaction is a key step in the radical chain reaction. Therefore, the HAT-based method is more relevant to the radical chain-breaking antioxidant capacity. Pedulli and co-workers studied the antioxidant capacity of phenothiazine and related compounds. These aromatic amines exhibit antioxidant capacity because of their low N−H bond dissociation energies (77−80 kcal/mol). If the hydrogen atom is replaced with a methyl group, the peroxyl radical scavenging capacity is lost, despite the fact that the methylated analogue has a redox potential similar to that of the parent amine. Therefore, radical trapping capacity directly relates

to the hydrogen atom donating ability of a compound and is not correlated to the redox potentials alone (*59*). Although the reducing capacity of a sample is not directly related to its radical scavenging capacity, it is an important parameter of antioxidants. Some water soluble oxidants such as peroxynitrite and hypochlorite can be readily reduced to harmless species. In this regard, a chemically valid ET-based assay will provide useful information.

The HAT-based assay using fluorescent probes has a mechanistic similarity to lipid peroxidation, but under the assay conditions, the concentration of the substrate (in this case the probe) is often smaller than the concentration of antioxidants. This is in contradiction with real situations. In food systems the antioxidant concentration is much smaller than that of the substrate (e.g., lipid). It remains to be seen if the antioxidant capacity measured using the HAT-based assay using a molecular probe can be translated to applications in a real food system, which is also often heterogeneous and under different oxidative stress conditions. It is known that the distribution of antioxidant in two phases has great impact on its effectiveness (*60*).

Mechanistically, electron transfer and hydrogen atom transfer reaction can be difficult to distinguish. Apparent hydrogen atom transfer reaction can be the result of proton-coupled electron transfer (PCET). The detailed mechanism of the reaction is beyond the scope of this review, but experimental (*61*) and theoretical studies (*62*) have shown that tocopherol reaction with oxyradicals predominantly undergoes hydrogen atom transfer reaction.

6. ASSAYS MEASURING OTHER ROS SCAVENGING CAPACITY

Experimental evidence has directly or indirectly suggested that there are six major reactive oxygen species causing oxidative damage in the human body. These species are superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\bullet}$), hydroxyl radical ($HO^{\bullet}$), singlet oxygen ($^1O_2$), and peroxynitrite ($ONOO^-$). To counteract the assault of these ROS, living cells have a biological defense system composed of enzymatic antioxidants that convert ROS/RNS to harmless species. For example, $O_2^{\bullet -}$ is converted to oxygen and hydrogen peroxide by superoxide dismutase (SOD) or reacts with nitric oxide ($NO^{\bullet}$) to form peroxynitrite. H_2O_2 can be converted to water and oxygen by catalase. In contrast, no enzymatic action is known to scavenge $ROO^{\bullet}$, $HO^{\bullet}$, $^1O_2$, and $ONOO^-$. Therefore, the burden of defense relies on a variety of nonenzymatic antioxidants such as vitamins C and E and many phytochemicals that have the property of scavenging oxidants and free radicals. To comprehensively evaluate the oxidant-scavenging capacity of a food sample, assays have to be designed to include these ROS. However, so far the majority of assays are designed to measure a sample's capacity to react with one oxidant (either organic radical or redox active compounds). The peroxyl radical has been the most frequently used ROS in the assays because it is a key radical in autoxidation and it can be generated conveniently from the thermal decomposition of azo compounds. There are also limited numbers of papers describing assays for the scavenging capacity of other ROS. In this section, we briefly describe some of the assays.

6.1. $O_2^{\bullet -}$ Scavenging Capacity Assay. Classically, the SOD activity assay uses the competition kinetics of $O_2^{\bullet -}$ reduction of cytochrome *c* (probe) and $O_2^{\bullet -}$ scavenger (sample). More recently, the method has been adapted to a microplate format (*63*). Cytochrome *c* can be reduced directly by antioxidants, which can also inhibit the xanthine oxidase. Therefore, this method is not suitable for quantifying nonenzymatic antioxidant (*54*). Ewing and Janero reported a high-throughput assay using a nonenzymatic (phenazine methosulfate/NADH/O_2) $O_2^{\bullet -}$ generator and nitroblue tetrazolium (NBT) as a probe (*64*). This assay takes advantage of the reducing property of $O_2^{\bullet -}$. The redox potential of $O_2/O_2^{\bullet -}$ is -0.2 to -0.5 V depending on the medium (vs NHE) (*65*). Because many dietary antioxidants can also exhibit reducing capacity, as demonstrated in the TEAC and FRAP assays, this improved method cannot be applied to nonenzymatic samples. More recently, hydroethidine has been used as the probe in measuring $O_2^{\bullet -}$ scavenging capacity (*66*). Nonfluorescent hydroethidine is oxidized by $O_2^{\bullet -}$ (generated from xanthine oxidase and xanthine mixture) to form a species of unknown structure that exhibits a strong fluorescence signal at 586 nm. Addition of SOD inhibits the hydroethidine oxidation. This approach can avoid the problem of direct reduction of the probe by antioxidant, but possible inhibition of xanthine oxidase by antioxidants remains an issue.

6.2. H_2O_2 Scavenging Capacity Assay. H_2O_2 is rather inert at low concentrations. Under physiological conditions, H_2O_2 oxidation power is believed to be observed in combination with Fe(II) (Fenton reaction). Biologically, H_2O_2 is converted to oxygen and water by catalase. A common assay that claims to measure H_2O_2 scavenging capacity of dietary antioxidants uses horseradish peroxidase to oxidize scopoletin to a nonfluorescent product. In the presence of antioxidants the oxidation is inhibited. The nature of the inhibition is ambiguous because there are several potential inhibition pathways. The antioxidants can inhibit the reaction by (a) reacting directly with H_2O_2, (b) reacting with intermediates formed from enzyme and H_2O_2, or (c) inhibiting the horseradish peroxidase from binding H_2O_2. Therefore, it is difficult to explain the actual chemical meaning of the data (*67*).

6.3. Hydroxyl Radical (HO) Scavenging Assay. Biologically, the hydroxyl radical is widely believed to be generated when hydrogen peroxide reacts with Fe(II) (Fenton reaction). However, the Fe(II)/H_2O_2 mixture has disadvantages in a scavenging assay because many antioxidants are also metal chelators. When the sample is mixed with Fe(II), it may alter the activity of Fe(II) by chelation. As a result, it is impossible to distinguish if the antioxidants are simply good metal chelators or $HO^{\bullet}$ scavengers. Antioxidants in food (such as vitamin C) may act as pro-oxidants by reducing Fe(III) to Fe(II) and make the $HO^{\bullet}$ generation catalytic. In fact, ascorbic acid has been used in combination with catalytic Fe(II) and excess H_2O_2 to generate a constant flux of $HO^{\bullet}$ radicals. Recently, Zhu and co-workers have reported an organic Fenton reaction (*68*). Zhu found that a mixture of tetrachlorohydroquinone (TCHQ, a major metabolite of the widely used biocide pentachlorophenol) and H_2O_2 hydroxylates salicylic acid to yield both 2,3- and 2,5-dihydroxybenzoic acid (DHBA). The hydroxylation is markedly inhibited by hydroxyl radical scavenging agents such as dimethyl sulfoxide and ethanol. The inhibited reaction was not affected by iron chelators, such as diethylenetriaminepentaacetic acid (DTPA), bathophenanthroline disulfonic acid, phytic acid, and bathocuprione disulfonic acid. A comparison of product type and distribution from the TCHQ/H_2O_2 system with that of the Fe(II)/H_2O_2 system suggests that hydroxyl radicals are involved in the organic Fenton reaction system. It would be interesting to see if this metal-free TCHQ/H_2O_2 mixture can be a hydroxyl radical source for assaying the hydroxyl radical scavenging capacity of antioxidants.

The putative hydroxyl radical is an extremely reactive and short-lived species that can hydroxylate DNA, proteins, and

lipids. Therefore, the direct scavenging of the hydroxyl radical by dietary antioxidants in a biological system is unrealistic as the cellular concentration of dietary antioxidants is negligible compared with other biological molecules. The rate constants for $HO^{\bullet}$ reactions have been determined by pulse radiolysis through the deoxyribose method (*69*). The second-order rate constants are $>10^8$ $M^{-1}\cdot s^{-1}$ for many compounds, including unreactive compounds such as benzene (3.2×10^9 $M^{-1}\cdot s^{-1}$) and glucose (1×10^9 $M^{-1}\cdot s^{-1}$). Therefore, the ability of antioxidants to scavenge the $HO^{\bullet}$ radical is not unlikely to provide any protection to biological molecules as the opportunity for $HO^{\bullet}$ and antioxidants to react is extremely small. On the other hand, it is possible to prevent the formation of hydroxyl radicals by either deactivating free metal ions [e.g., Fe(II)] through chelation or converting H_2O_2 to other harmless compounds (such as water and oxygen). Catalase converts H_2O_2 to O_2, and H_2O and metal chelators bind metal ions so that they become inert toward H_2O_2. Thus, dietary nutrients containing metal chelators may act as preventive antioxidants. Quantifying the capacity of the phytochemicals in preventing hydroxyl radical formation in vitro would be more relevant and a valuable guide to antioxidant clinical research. Recently, Ou and co-workers have developed a fluorometric assay for screening the metal [Co(II)] chelating capacity of dietary antioxidants (*70*). The method, christened HORAC [hydroxyl (HO) radicals averting capacity], employs a Co(II) complex mediated Fenton-like reaction. The hydroxyl radical formation under the experimental conditions is indirectly confirmed by the hydroxylation of *p*-hydroxybenzoic acid. Fluorescein (FL) was used as the probe. The fluorescence decay curve of FL is monitored in the absence or presence of antioxidants, the area under the fluorescence decay curve (AUC) is then integrated, and the net AUC is calculated by subtracting the AUC of the blank from that of the sample antioxidant. The quantitation method is the same as that of the ORAC assay except gallic acid is used as the standard. This method has been rigorously validated for linearity, precision, accuracy, and ruggedness. A wide range of phenolic antioxidants can be analyzed. The hydroxyl radical prevention capacity is mainly due to their metal-chelating capability.

6.4. Singlet Oxygen Scavenging Capacity Assay. Singlet oxygen is normally generated in the presence of light and photosensitizers. It is believed that $^1O_2$ is often responsible for UV light-dependent damage to skin (*71*), cataract formation in the lens of the eyes (*72*), macular degeneration (*73*), and photosensitivity resulting from ingestion or absorption of phytochemicals, pharmaceuticals and pesticides that act as photosensitizers (*74*). In the absence of light, $^1O_2$ production can be ambiguous in a biological system. It was suggested that the extracellular $^1O_2$ production by the spontaneous dismutation of superoxide anion has some physiological significance (*75*). On the other hand, chemically, $^1O_2$ can be conveniently generated through non-photochemical decomposition of hydrogen peroxide by metals or hypochlorite (*76*, *77*).

Rate constants of singlet oxygen reaction with various compounds have been compiled by Wilkinson and co-workers (*78*). Singlet oxygen can be quenched through physical means by transferring its excitation energy to another molecule (which is excited), or it can add to antioxidants forming endoperoxides. β-Carotene is an excellent physical quencher of $^1O_2$. Singlet oxygen emits characteristic phosphorescence at 1270 nm. The decay rates of the light intensity were used to measure the $^1O_2$ quenching activity of a compound. Foote and co-workers reported a more sensitive method by monitoring the quenching of singlet-oxygen-sensitized (at 703 nm) delayed fluorescence (SOSDF) of tetra-*tert*-butylphthalocyanine (*79*). The authors reported the quenching rates of β-carotene, α-tocopherol, 1,4-diazabicyclo[2.2.2]octane, 2,6-di-*tert*-butyl-4-methylphenol, and lauric acid. The singlet oxygen quenching rates vary by 6 orders of magnitude. Quenching of the visible SOSDF may provide a highly sensitive method for the measurement of $^1O_2$ quenching capacity using commonly available apparatus or in systems where the 1270 nm luminescence is difficult to detect. This method is not yet widely applied.

6.5. Peroxynitrite ($ONOO^-$) Scavenging Capacity Assay. Superoxide and nitric oxide react under diffusion control rate ($k > 10^9$ $M^{-1}\cdot s^{-1}$) to form peroxynitrite (*80*). $O_2^{\bullet -}$ ($E° = -0.33$ V) and NO (0.39 V) are not potent oxidants; its adduct, $ONOO^-$, is not a strong oxidant either. Its protonated form, peroxynitrous acid (ONOOH), is a very strong oxidant ($E° = 2.10$ V). Under physiological pH, ONOOH ($pK_a = 8.0$) rearranges to form much less oxidizing nitrate (*81*). At pH 7.4, the ratio of peroxyntrite and peroxynitrous acid is 4 to 1. $ONOO^-$ and ONOOH often cause the nitration or hydroxylation of aromatic compounds, particularly tyrosine (to nitrotyrosine). Under physiological conditions, peroxynitrite also forms an adduct with carbon dioxide dissolved in body fluid. The adduct is believed to be responsible for the oxidative damage of proteins (*82*).

There are a few papers on the scavenging capacity of antioxidants against $ONOO^-$. Two methods are used for $ONOO^-$ scavenging measurements: (1) inhibition of tyrosine nitration by $ONOO^-$ (*83*) and (2) inhibition of dihydrorhodamine (DHR) 123 oxidation (*84*). Pannala reported the peroxynitrite quenching capacity of catechin and other polyphenols by measuring their inhibition capacity on reaction between peroxynitrite and tyrosine. The method relies on HPLC separation and quantification of nitrotyrosine, and it is thus rather time-consuming. Kooy developed another method based on the inhibition of the oxidation of DHR 123 by peroxynitrite (*85*). The initial rate approach was used to quantify peroxynitrite-scavenging capacity. Using the same method, Chung and co-workers studied the peroxynitrite scavenging and cytoprotective capacity of a marine algae extract (*86*).

7. CONCLUSIONS

ET-Based Assay. Overall, there are a multitude of ET-based assays for measuring the reducing capacity of antioxidants. The assays are carried out at acidic (FRAP), neutral (TEAC), or basic (total phenols assay by FCR) conditions. The pH values have an important effect on the reducing capacity of antioxidants. At acidic conditions, the reducing capacity may be suppressed due to protonation on antioxidant compounds, whereas in basic conditions, proton dissociation of phenolic compounds would enhance a sample's reducing capacity. The oxidant in the FRAP assay has a standard redox potential comparable to that of $ABTS^{2-}$ (~0.7 V), but the redox potential for Mo(VI)/Mo(V) is not known, presumably due to the complex nature of FCR. These oxidants can certainly react with common antioxidants such as vitamins E ($E° = 0.5$ V) and C ($E° = 0.28$ V) and common phenolic compounds. There are many more oxidants to choose from. The question is: what are the criteria of selecting the right oxidant? We do not have a clear guideline, but the selectivity of oxidant should be such that it does not oxidize compounds such as sugar, which is ubiquitous in food but normally not considered to be an antioxidant. Sugar is known to reduce metal ions such as Cu(II) (Fehling's reagent).

Applying multiple ET-based assays to measure the reducing capacity of an antioxidant often leads to excellent linear

correlations between the results. Indeed, we often see, in the research papers on antioxidants, the findings of excellent correlation ($R^2 > 0.99$) between total phenolic contents (measured by FCR) and antioxidant activity (measured by FRAP, TEAC, or DPPH assays) (*87*). Because these assays are based on similar redox reactions, it is therefore somewhat redundant to apply the multitude of assays in quantifying reducing capacity. It is, however, important to use one assay that is commonly accepted and validated. In this regard, the total phenols assay by CFR has the clear advantage over the other ET-based assays. Disregarding the chemistry principles, the total phenols assay by FCR has the following advantages:

(1) The FCR is commercially available, and the procedure is rather standardized.

(2) The long-wavelength (730 nm) absorption of the chromophore minimizes interference from the sample matrix, which is often colored.

(3) It is a commonly accepted assay and routinely practiced in dietary antioxidant research laboratories throughout the world.

(4) A large body of comparable data has been produced (claimed as total phenols content instead of reducing capacity of FCR).

It should be noted that the total phenols assay by FCR is conducted at rather basic conditions (pH 10, necessary for phenols to dissociate protons). Simple phenols (e.g., C_6H_5OH, the phenolic group in tyrosine) react with FCR, although they are not effective radical scavenging antioxidants. Therefore, there may not necessarily be a good correlation between the "total phenols content" and the radical scavenging antioxidant capacity of a sample. To avoid misunderstanding on the actual meaning of "total phenolic contents", we suggest an alternative term, "FCR reducing capacity", be used.

The total phenols assay by FCR is carried out in water, an aqueous phase. For lipophilic antioxidants, this assay in its current form is not applicable. In fact, we have attempted but have been unable to measure the total phenols of the lipid soluble fraction of bee pollen as the sample did not have sufficient water solubility. Therefore, there is an immediate need for a modified FCR for lipophilic samples.

The reducing capacity of a sample is an important parameter reflecting one aspect of the its antioxidation property. However, it is oversimplified to refer to the result as "total antioxidant capacity". The latter encompasses much broader aspects including metal chelating capacity, ROS scavenging capacity, and even oxidative enzyme inhibition capacity (e.g., polyphenol oxidase inhibitors in preventing the browning of fruit). In addition, not all ROS share the same reaction pattern (i.e., electron transfer) toward antioxidants. $ROO^{\bullet}$ abstracts a hydrogen atom from antioxidants, whereas $HO^{\bullet}$ may undergo hydrogen atom abstraction or addition to an unsaturated compound (DNA bases or aromatic amino acid residue). Singlet oxygen can form endoperoxide with dienes or aromatic compounds or be quenched physically through energy transfer. Superoxide anion, on the other hand, is a moderate reductant [reduces Fe(III)]. Therefore, a host of assays measuring individual ROS scavenging capacity are needed to comprehensively evaluate a sample's ROS scavenging capacity.

HAT-Based Assay. Peroxyl radicals play a key role in the unwanted lipid oxidation in food and biological systems. The sacrificial antioxidants, represented by vitamin E, are critical in protecting polyunsaturated fatty acid esters in foods and in cell membranes from autoxidation. Vitamin E functions through a HAT mechanism. A HAT-based assay, represented by the ORAC assay, involves peroxyl radicals as the oxidant and will provide useful information on radical chain-breaking capacity. The ORAC assay has been modified to measure lipophilic antioxidants by using a cyclodextrin derivative as a water solubility enhancer. With interlaboratory (three laboratories) validation and industrial recognition, the ORAC assay has currently emerged as the assay of choice for quantifying the peroxyl radical scavenging capacity of a sample. Broader validations involving at least eight other laboratories are needed for the ORAC assay to become the standard assay adopted by official organizations such as the AOAC International.

Antioxidants in a food system normally refer to substances that can inhibit fatty acid autoxidation. The major antioxidants are metal chelators (e.g., EDTA, preventive) and chain-breaking antioxidants (e.g., BHT, sacrificial) acting as hydrogen atom donors. It is of great importance to know if the reducing capacity of antioxidants measured by FCR and ORAC values can be translated to its inhibition capacity of fatty acid autoxidation in an actual food system. The total phenols assay by FCR and the ORAC assay are carried out in a controlled manner in a homogeneous solution with an artificial oxidant or radical precursor added to initiate the reaction, whereas in a real food lipid system, the reaction occurs without added radical initiator or oxidant. Instead, the reaction is initiated by light, metal ions, or heat during food processing or storage. Moreover, it is often a heterogeneous mixture (as in food emulsions), and the phase distribution of antioxidants will be critical for its effectiveness. Therefore, the antioxidants may behave differently than they do in the antioxidant capacity assays. Hence, more research needs to be carried out to study if ORAC values and (or) FCR reducing capacity can be extended to real food systems. Before then, one should exercise caution when using ORAC values or the total phenols contents as a guideline for optimizing food formulations for extension of oxidative stability.

Application of the in Vitro Assays in Clinical Research. Although the limitations of these assays have been mentioned throughout this paper, it is necessary to emphasize that the assays described herein are strictly based on chemical reactions in vitro. They bear no similarity to biological systems. The validity of the data is limited to a strict chemical sense with context interpretation. Any claims about the bioactivity of a sample based *solely* on these assays such as ORAC, TEAC, and FRAP etc., would be exaggerated, unscientific, and out of context. Moreover, these assays do not measure bioavailability, in vivo stability, retention of antioxidants by tissues, and reactivity in situ.

The role of dietary antioxidants is to help fight excessive ROS in our bodies. By doing so, antioxidants will be sacrificed to protect the biomolecules from being oxidized (as the *antioxidant* definition states), and thus the antioxidant has fulfilled its function. It is of importance to study whether there is a correlation between the intake of high potent antioxidants and the level of oxidative stress. The in vitro nature of these assays should not compromise their value in guiding clinical research. On the contrary, a valid in vitro assay is an invaluable tool for clinical studies if it is combined with bioavailability data and valid oxidative stress biomarker assays. In fact, many studies apply these assays to study the impact of antioxidant consumption on reducing oxidative stress markers (*88*). In addition to the assay validity itself, special attention has to be paid to confounding factors from sample matrix when biological samples are measured, such as blood plasma, tissues, or urine. In this regard, valid sample processing procedure is critical. The synergistic effects of such a combination assays [must be a valid assay or disputable results can be gathered (*89*)] will allow us

to investigate the impact of antioxidant in reducing oxidative stress and thus the implication for disease prevention.

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