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Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements

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Methods available for the measurement of antioxidant capacity are reviewed, presenting the general chemistry underlying the assays, the types of molecules detected, and the most important advantages and shortcomings of each method. This overview provides a basis and rationale for developing standardized antioxidant capacity methods for the food, nutraceutical, and dietary supplement industries. From evaluation of data presented at the First International Congress on Antioxidant Methods in 2004 and in the literature, as well as consideration of potential end uses of antioxidants, it is proposed that procedures and applications for three assays be considered for standardization: the oxygen radical absorbance capacity (ORAC) assay, the Folin−Ciocalteu method, and possibly the Trolox equivalent antioxidant capacity (TEAC) assay. ORAC represent a hydrogen atom transfer (HAT) reaction mechanism, which is most relevant to human biology. The Folin−Ciocalteu method is an electron transfer (ET) based assay and gives reducing capacity, which has normally been expressed as phenolic contents. The TEAC assay represents a second ET-based method. Other assays may need to be considered in the future as more is learned about some of the other radical sources and their importance to human biology.

KEYWORDS: Standardized methods; antioxidant capacity; foods, dietary supplements; nutraceuticals; ORAC; Folin−Ciocalteu method; TEAC

INTRODUCTION

The First International Congress on Antioxidant Methods was convened in Orlando, FL, in June 2004 for the express purpose of dealing with analytical issues relative to assessing antioxidant capacity (AOC) in foods, botanicals, nutraceuticals, and other dietary supplements and proposing one or more analytical methods that could be standardized for routine assessment of AOC. Highlights from this Congress, dealing with the chemistry of antioxidant analytical methods will be summarized. Research on antioxidants has increased considerably during the past 10 years. On the basis of information in the Medline database alone, manuscripts mentioning "antioxidant" increased 340% while the number of manuscripts in the plant, animal, and human area increased only 39%.The number of methods and variations in methods to measure antioxidants in botanicals that have been proposed has also increased considerably. Reviews of some of the methods have been published recently (*1*−*5*). In this paper we consider several of the more commonly used methods for measuring AOC, outlining the reaction mechanisms and major advantages and disadvantages of each.

A factor that provides a distinct challenge in the assay of antioxidant capacity is that within biological systems, there are at least four general sources of antioxidants: (1) enzymes, for example, superoxide dismutase, glutathione peroxidase, and catalase; (2) large molecules (albumin, ceruloplasmin, ferritin, other proteins); (3) small molecules [ascorbic acid, glutathione, uric acid, tocopherol, carotenoids, (poly)phenols]; and (4) some hormones (estrogen, angiotensin, melatonin, etc.). On the other hand, there are multiple free radical and oxidant sources [e.g., $O_2^{\bullet -}$, $^1O_2$, $HO^{\bullet}$, $NO^{\bullet}$, $ONOO^-$, HOCl, $RO(O)^{\bullet}$, $LO(O)^{\bullet}$], and both oxidants and antioxidants have different chemical and physical characteristics. Individual antioxidants may, in some cases, act by multiple mechanisms in a single system (*6*) or by a different single mechanism depending on the reaction system. Furthermore, antioxidants may respond in a different manner to different radical or oxidant sources. For example, carotenoids are not particularly good quenchers of peroxyl radicals relative to phenolics and other antioxidants but are exceptional in quenching singlet oxygen, at which most other phenolics and antioxidants are relatively ineffective. However, singlet oxygen is not a radical and does not react via radical mechanisms but reacts mostly by the addition to double bonds, forming endoperoxides that can be reduced to alkoxyl radicals that initiate radical chain reactions. Because multiple reaction characteristics and mechanisms as well as different phase localizations are usually involved, no single assay will accurately reflect all of the radical sources or all antioxidants in a mixed or complex system. Clearly, matching radical source and system charac-

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teristics to antioxidant reaction mechanisms is critical in the selection of appropriate AOC assay methods, as is consideration of the end use of the results. It must be appreciated at the outset that there is no simple universal method by which AOC can be measured accurately and quantitatively.

Why Do We Need a Standardized AOC Method? Although it may seem intuitive, one might question why we need standardized analytical methods of AOC. Agreement on standardized test methods allows for (1) guidance for appropriate application of assays, (2) meaningful comparisons of foods or commercial products, (3) a means to control variation within or between products, and (4) provision of quality standards for regulatory issues and health claims. Too many analytical methods result in inconsistent results, inappropriate application and interpretation of assays, and improper specification of AOC. Without some agreement on standards for quantities and units, marketing of botanicals and associated trade becomes haphazard, science becomes "unscientific", and technological development of nutraceuticals is handicapped.

Factors for Consideration in Method Selection and Development. In the selection of any method for standardization, a first consideration is that the method has been used for a sufficient amount of time and in a number of different laboratories such that the strengths and weaknesses of the assay have become apparent and some time has been spent in dealing with these issues. This is not to say that newer methods may not potentially be as good or better, but use over time will generally point this out. A standardized method for AOC should meet the following "ideal" requirements: (1) measures chemistry actually occurring in potential application(s); (2) utilizes a biologically relevant radical source; (3) simple; (4) uses a method with a defined endpoint and chemical mechanism; (5) instrumentation is readily available; (6) good within-run and between-day reproducibility; (7) adaptable for assay of both hydrophilic and lipophilic antioxidants and use of different radical sources; (8) adaptable to "high-throughput" analysis for routine quality control analyses.

Performance characteristics that should be considered in the standardization of an assay include (a) analytical range, (b) recovery, (c) repeatability, (d) reproducibility, and (e) recognition of interfering substances.

REACTION MECHANISMS

Differentiation between Hydrogen Atom Transfer (HAT) and Single Electron Transfer (SET). Antioxidants can deactivate radicals by two major mechanisms, HAT and SET. The end result is the same, regardless of mechanism, but kinetics and potential for side reactions differ. Proton-coupled electron transfer and HAT reactions may occur in parallel, and the mechanism dominating in a given system will be determined by antioxidant structure and properties, solubility and partition coefficient, and system solvent. Bond dissociation energy (BDE) and ionization potential (IP) are two major factors that determine the mechanism and the efficacy of antioxidants (*7*). There is often confusion in the literature and mistaken attribution of reaction mechanisms. Thus, along with specific procedures, there must be definitive recognition of mechanisms and identification of appropriate applications. Indeed, a protocol is needed that involves measurement of more than one property because polyphenols have multiple activities, and the dominant activity depends on the medium and substrate of testing.

HAT-based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation (AH = any H donor)

$$X^{\bullet} + AH \rightarrow XH + A^{\bullet} \quad (1)$$

Hence, many scientists feel these are most relevant to reactions where antioxidants typically act. Relative reactivity in HAT methods is determined by the BDE of the H-donating group in the potential antioxidant, dominating for compounds with ΔBDE of ~ -10 kcal/mol and ionization potential (ΔIP) of < -36 kcal/mol (*7*). Antioxidant reactivity or capacity measurements are based on competition kinetics. HAT reactions are solvent and pH independent and are usually quite rapid, typically completed in seconds to minutes. The presence of reducing agents, including metals, is a complication in HAT assays and can lead to erroneously high apparent reactivity.

SET-based methods detect the ability of a potential antioxidant to transfer one electron to reduce any compound, including metals, carbonyls, and radicals (*7*):

$$X^{\bullet} + AH \rightarrow X^{-} + AH^{\bullet +} \quad (1)$$

$$AH^{\bullet +} \xleftrightarrow{H_2O} A^{\bullet} + H_3O^{+} \quad (2)$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O \quad (3)$$

$$M(III) + AH \rightarrow AH^{+} + M(II) \quad (4)$$

SET and HAT mechanisms almost always occur together in all samples, with the balance determined by antioxidant structure and pH. Relative reactivity in SET methods is based primarily on deprotonation (*8*) and IP (*7*) of the reactive functional group, so SET reactions are pH dependent. In general, IP values decrease with increasing pH, reflecting increased electron-donating capacity with deprotonation. The antioxidant mechanism is predominantly SET for compounds with a ΔIP of > -45 kcal/mol. A correlation between redox potential and SET methods has been suggested (*2*) but not consistently demonstrated.

SET reactions are usually slow and can require long times to reach completion, so antioxidant capacity calculations are based on percent decrease in product rather than kinetics. When $AH^{\bullet +}$ has a sufficient lifetime, secondary reactions become a significant interference in assays and can even lead to toxicity or mutagenicity in vivo (*9*). SET methods are very sensitive to ascorbic acid and uric acid, which are important in maintaining plasma redox tone, and reducing polyphenols are also detected. Importantly, trace components and contaminants (particularly metals) interfere with SET methods and can account for high variability and poor reproducibility and consistency of results.

CHARACTERISTICS OF CANDIDATE AOC METHODS

AOC Methods Utilizing HAT Reaction Mechanisms. A number of assays have been developed for the detection of both general and specific antioxidant action. Of these, oxygen radical absorbance capacity (ORAC), and total radical-trapping antioxidant parameter (TRAP) (and some of its variants) meet the most requirements for screening assays outlined above and may merit standardization. The other methods noted below are more appropriate for individual applications.

ORAC: General Chemistry. The ORAC assay is based upon the early work of Ghiselli et al. (*10*) and Glazer (*11*), as developed further by Cao et al. (*12*). ORAC measures antioxidant inhibition of peroxyl radical induced oxidations and thus reflects classical radical chain breaking antioxidant activity by H atom transfer (*13*). In the basic assay, the peroxyl radical reacts with a fluorescent probe to form a nonfluorescent product,

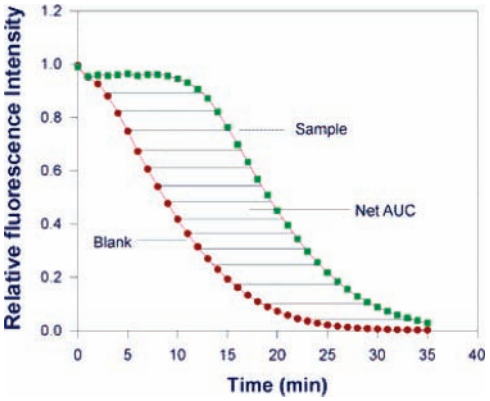

Figure 1. ORAC antioxidant activity of tested sample expressed as the net area under the curve (AUC). From Brunswick Laboratories (2003), used with permission.

which can be quantitated easily by fluorescence. Antioxidant capacity is determined by a decreased rate and amount of product formed over time:

$$R{-}N{=}N{-}R \xrightarrow{O_2} N_2 + 2ROO^{\bullet}\ (14)$$

$$ROO^{\bullet} + \text{probe (fluorescent)} \rightarrow ROOH + \text{oxidized probe (loss of fluorescence)}$$

$$ROO^{\bullet} + AH \rightarrow ROOH + A^{\bullet}$$

$$ROO^{\bullet} + A^{\bullet} \xrightarrow{\text{fast}} ROOA$$

B-phycoerythrin (B-PE), a protein isolated from *Porphyridium cruentum*, was used as the fluorescent probe in the early studies (*12*). However, use of B-PE in antioxidant assays has shortcomings in that (1) B-PE has lot-to-lot variability in reactivity to peroxyl radicals, leading to inconsistency in assay results (*15*); (2) B-PE becomes photobleached after exposure to excitation light; and (3) polyphenols, particularly proanthocyanidins, bind to B-PE via nonspecific protein binding. Both of these latter factors cause false low ORAC values. The fluorescent probes that are currently preferred—fluorescein (FL; 3′,6′-dihydroxyspiro[isobenzofuran-1[3H],9′[9H]-xanthen]-3-one) (*13*) or dichlorofluorescein (H_2DCF-dA; 2′,7′-dichlorodihydrofluorescein diacetate)—are more stable and less reactive (*6*). The oxidized products of FL induced by peroxyl radicals have been identified by LC-MS, and the reaction mechanism has been verified as a classic HAT mechanism (*13*).

Probe reaction with peroxyl radicals is followed by loss of fluorescence over time. Traditional antioxidant analyses followed extension of the lag phase only, but antioxidant effects often extend well beyond early stages of oxidation (*2*, *3*). To avoid underestimation of antioxidant activity and to account for potential effects of secondary antioxidant products, the ORAC assay follows the reaction for extended periods, for example, ≥30 min. Calculation of protective effects of an antioxidant (AOX) is from the net integrated areas under the fluorescence decay curves (AUC) [$AUC_{AOX} - AUC_{no\ AOX}$], as shown in **Figure 1**, and accounts for lag time, initial rate, and total extent of inhibition in a single value.

ORAC values are usually reported as Trolox equivalents. A standard curve is generated using the AUC for five standard concentrations of Trolox, and the Trolox equivalents of the sample are calculated using the following linear or quadratic relationships ($Y = a + bX$, linear; or $Y = a + bX + cX^2$, quadratic) between Trolox concentration (Y) (μM) and the net area under the FL decay curve (X) ($AUC_{sample} - AUC_{blank}$). A linear regression was used in the range of 6.25−50 μM Trolox, although use of a quadratic regression extends slightly the dynamic range of the assay (Wu et al., unpublished data). Data are expressed as micromoles of Trolox equivalents (TE) per liter or per gram of sample (μmol of TE/g or μmol of TE/L) (*13*, *16*).

As originally configured, the $ORAC_{FL}$ assay is limited to measurement of hydrophilic chain breaking antioxidant capacity against only peroxyl radicals. This ignores lipophilic antioxidants that are particularly important against lipid oxidation in all systems as well as other radicals ($HO^{\bullet}$, $HOO^{\bullet}$, $ONOO^{\bullet}$, $O_2^{\bullet-}$, etc.) that are very reactive physiologically. To be made more broadly applicable, the ORAC assay has been adapted to measure lipophilic as well as hydrophilic antioxidants using a solution of 50% acetone/50% water (v/v) containing 7% randomly methylated β-cyclodextrin (RMCD) to solubilize the antioxidants (*17*, *18*). The lipophilic and hydrophilic components are selectively extracted before assay (*16*). The ORAC assay has been used to study the AOC of many compounds and food samples (*16*, *18*−*25*). Industry has accepted the method to the point that some nutraceutical manufacturers are beginning to include ORAC values on product labels (*26*, *27*).

Advantages/Disadvantages of ORAC. The ORAC assay provides a controllable source of peroxyl radicals that model reactions of antioxidants with lipids in both food and physiological systems, and it can be adapted to detect both hydrophilic and hydrophobic antioxidants by altering the radical source and solvent (*2*, *16*, *28*, *29*). Frankel and Meyer (*5*) have criticized ORAC (and the same for TRAP) in that it is assumed that the antioxidant mechanism and protection of B-PE by antioxidants mimics critical biological substrates. Although detailed mechanistic studies were not completed using B-PE, they have been done with fluorescein (*13*), and the reaction has been determined to be a HAT mechanism. The principles of the ORAC method can be adapted to utilize other radical sources (*28*).

The ORAC method is readily automated. The method was first automated on the COBAS FARA II (*30*) and more recently has undergone additional improvements in instrumentation and fluorescent probe (*13*, *16*). Excellent results have been obtained using a multichannel liquid handling system coupled with a fluorescence microplate reader in either a 96- or 48-well format (*13*, *29*), although the assay coefficient of variation is slightly lower in the 48-well format (4−5%, compared to 4−10% with a 96-well format) (*16*). Because the ORAC reaction is temperature sensitive, close temperature control throughout the plate is essential. Incubation of the reaction buffer at 37 °C prior to the AAPH being dissolved decreased the intra-assay variability (*16*). Small temperature differences in the external wells of the microplate can decrease the reproducibility of the assay (*31*). This is not unique to the ORAC assay, but will be true for any assay that is highly temperature sensitive that uses microplates and microplate readers in the assay.

Fluorescent markers, although sensitive, require detection by fluorometers, which may not be routinely available in analytical laboratories, although this instrument is used routinely in many cell culture laboratories. The long analysis time (∼1 h) has also been a major criticism, but this limitation has been partially overcome by development of high-throughput assays (*29*).

TRAP: General Chemistry. This method monitors the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals generated by AAPH or ABAP [2,2′-azobis(2-amidinopropane) dihydrochloride] and a target probe (*10*, *14*,

32). Different variations of the method have used oxygen uptake (*32*), fluorescence of R-phycoerythrin (*10*, *33*), or absorbance of 2,2′-azinobis(3-ethylbenzothiazoline-6-suslfonic acid (ABTS) (*34*) as the reaction probe. The basic reactions of the assay are similar to those of ORAC. Requirements for the assay are that the probe must be reactive with ROO$^{\bullet}$ at low concentrations, there must be a dramatic spectroscopic change between the native and oxidized probe (to maximize sensitivity), and no radical chain reaction beyond probe oxidation should occur. Typically, oxidation of the probe is followed optically (*34*) or by fluorescence (*10*). Antioxidant activity has been determined as time to consume all of the antioxidant, by extension of the lag time for appearance of the oxidized probe when antioxidants are present, and by percent reduction of a reaction. TRAP values are usually expressed as a lag time or reaction time of the sample compared to corresponding times for Trolox.

Advantages/Disadvantages of the TRAP Assay. The TRAP assay was designed and is most often used for measurements of in vivo AOC in serum or plasma because it measures nonenzymatic antioxidants, such as glutathione, ascorbic acid, α-tocopherol, and β-carotene (*35*). The method's greatest problem is perhaps its greatest strength; too many different endpoints have been used, so comparisons between laboratories are difficult. However, endpoint and detection method can be tailored to systems and physiological processes of particular interest and readily available instrumentation, respectively. The use of the lag phase is based on the assumption that all of the antioxidants show a lag phase and that the length of the lag phase is proportional to AOC. However, not every antioxidant possesses an obvious lag phase. Moreover, the value obtained from the lag phase alone often underestimates AOC considerably, because the antioxidant value contributed after the lag phase is totally ignored.

The TRAP assay involves the initiation of lipid peroxidation by generating water-soluble peroxyl radicals and is sensitive to all known chain breaking antioxidants, but it is relatively complex and time-consuming to perform, requiring a high degree of expertise and experience. However, the TRAP assay has been criticized as employing an unphysiological oxidative stress (water-soluble peroxyl radicals) (*36*), but the method can be adapted to use lipid-soluble initiators.

Total Oxidant Scavenging Capacity (TOSC): General Chemistry. Developed by Winston et al. (*37*), this method permits quantification of the absorbance capacity of antioxidants specifically toward three potent oxidants, that is, hydroxyl radicals, peroxyl radicals, and peroxynitrite (*38*). This method addresses an important issue in terms of being able to evaluate different antioxidants with different biologically relevant radical sources. The substrate that is oxidized in this assay is α-keto-γ-methiolbutyric acid (KMBA), which forms ethylene. The time course of ethylene formation is followed by headspace analysis of the reaction cell by gas chromatography, and the antioxidant capacity is quantified by the ability of the antioxidant to inhibit ethylene formation relative to a control reaction. The method uses an area under the curve that best defines the experimental points during the reaction time, which can be up to 300 min. Linear dose−response curves for antioxidants can be generated from kinetics of the reaction.

Advantages/Disadvantages of the TOSC Assay. The method has the advantage that it permits the quantification of the antioxidant capacity toward three oxidants, that is, hydroxyl radicals, peroxyl radicals and peroxynitrite. However, the method is not readily adaptable for high-throughput analyses required for quality control in that it requires multiple injections from a single sample into a gas chromatograph to measure the production of ethylene.

The kinetics of the TOSC assay are such that there is not a linear relationship between the percentage inhibition of TOSC by the antioxidant source and antioxidant concentration or dilution (*39*). Thus, calculated dilution factors for 20, 50, and 80% TOSC are determined, and a DT_{50} is calculated, which is the first derivative of the dose−response curve at a TOSC of 50%. Comparison between foods becomes difficult because of these multiple endpoint parameters.

Chemiluminescence (CL): General Chemistry. A high-sensitivity modification of TRAP follows radical reactions with CL. The fundamental chemistry of CL assays is based on the reaction of radical oxidants with marker compounds to produce excited state species that emit chemiluminescence (chemically induced light). Any compounds that react with the initiating radicals inhibit the light production. Oxidant sources of peroxyl radicals include the enzyme horseradish peroxidase (*40*) and H_2O_2-hemin (*41*). By changing the initiator, the reaction can be tailored to differentiate quenching of specific oxidants, for example, $O_2^{\bullet-}$, HO$^{\bullet}$, HOCl, LO(O)$^{\bullet}$, $^{\bullet}$OONO (*42*), and $^1O_2$ (*43*). The most widely used marker compound to trap oxidants and convert weak emissions into intense, prolonged, and stable light emissions is luminol (*40*), although lucigenin and bioluminescent proteins such as Pholasin are becoming more popular (*44*−*48*). Continuous light output depends on constant production of free radical intermediates derived from *p*-iodophenol, luminol, and oxygen, and this light emission is sensitive to interference by radical scavenging antioxidants, but will be restored when all of the added antioxidants have been consumed in the reaction. The antioxidant capacity is measured as the time of depressed light emission (*t*), which is arbitrarily measured at 10% recovery of light output.

Chemiluminescence is characterized by very low emission intensity, tens to a few thousand counts per second in contrast to millions of counts for fluorescence. Thus, CL detection requires special equipment that both places samples close to the detector and detects light at single photon levels and, in addition, provides temperature control (*49*). Nevertheless, CL can be quite sensitive in detecting low-level reactions because it provides a detectable response below the detection limit of most chemical assays.

Advantages/Disadvantages of CL. Chemiluminescence reactions are adaptable to automation and can be run in microwell plates. The choice of emitter is a critical consideration. Lucigenin undergoes redox-cycling and actually produces superoxide anion, and so is not preferred for some antioxidant applications. Luminol has been extensively used to study radical reactions and is acceptable when single oxidants are being measured. However, because the intensity of emissions varies considerably with the oxidant, use of luminol in systems with mixed oxidants is not straightforward. In addition, the activated product of luminol itself is redox active.

Photochemiluminescence (PCL) Assay: General Chemistry. This assay was described by Popov and Lewin (*50*−*52*), was commercialized by Analytik Jena AG (Jena, Germany), and is sold as a complete system under the name PHOTOCHEM.

The assay involves the photochemical generation of superoxide $O_2^{\bullet-}$ free radicals combined with CL detection. The assay is initiated by optical excitation of a photosensitizer (S), resulting in the generation of the superoxide radical anion (*51*).

$$S + h\nu + O_2 \rightarrow [S^* O_2] \rightarrow S^{\bullet+} + O_2^{\bullet-}$$

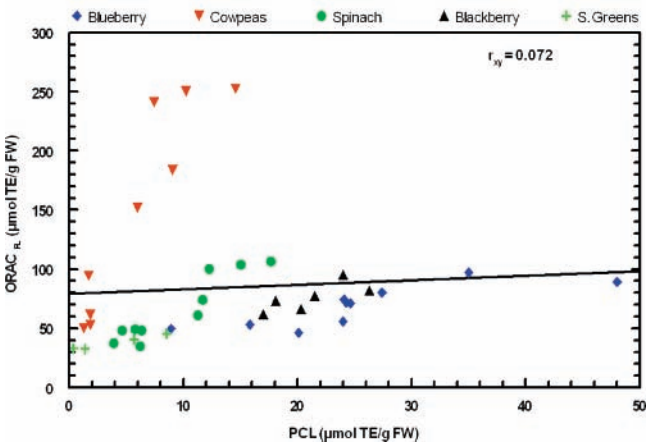

Figure 2. Relationship between ORAC$_{FL}$ and PCL antioxidant capacity in different foods (Luke Howard, unpublished data, personal communication).

The complete reaction mechanism is not known (*52*). There are two basic kinds of radicals present in the PCL measuring system: $O_2^{\bullet -}$ and luminal radicals; thus, in the strictest sense, the antioxidant capacity represents an antiradical capacity (*52*). The free radicals are detected with a CL reagent, luminol, which acts as a photosensitizer as well as an oxygen radical detection reagent. The ACW and ACL kits provided by the manufacturer are used to measure hydrophilic and lipophilic AOC, respectively, of biological samples. The hydrophilic AOC is assayed by means of the lag phase (L) in seconds

$$L = L_0 - L_1$$

where L_0 and L_1 are the respective parameters of the blank and sample. The lipophilic AOC is assayed by the degree of PCL inhibition (I), according to the calculation

$$I = 1 - S/S_0$$

where S_0 is the integral under the blank curve and S is the integral under the sample curve. Ascorbic acid and Trolox are typically used as calibration reagents for hydrophilic and lipophilic AOC, respectively, at measuring ranges of 0−2 nmol. In contrast to other commonly used AOC assays, the PHOTOCHEM method is not restricted to a specific pH value or temperature range.

Advantages/Disadvantages of the PHOTOCHEM System. This system is marketed as a time- and cost-effective system for the determination of the integral antioxidative capacity toward superoxide. Reagents for the lipophilic and hydrophilic assays are available only from the manufacturer. Because only one sample can be measured at a time, it is not, in its present configuration, adaptable to a high-throughput assay system. The assay has been used to measure antioxidant capacity in berries (*53*) and other foods. Data from Dr. Luke Howard (**Figure 2**; personal communication) clearly point out that there is little relationship between ORAC and the PHOTOCHEM data across a variety of foods. This is not unexpected in that two completely different radical sources are being evaluated. Additional work will be necessary in order to have a better understanding of the potential importance of having data using the superoxide radical and how it might help in relating to potential in vivo effects.

Croton or β-Carotene Bleaching by LOO•: General Chemistry. Carotenoids bleach via autoxidation, oxidation induced by light or heat (*54*), or oxidation induced by peroxyl radicals (e.g., AAPH or oxidizing lipids) (*55*, *56*), and this decolorization can be diminished or prevented by classical antioxidants that donate hydrogen atoms to quench radicals. Although β-carotene is often used as the target (*54*), its decolorization at 470 nm can occur by multiple pathways, so interpretation of results can be complicated. In contrast, crocin, first championed by Bors and colleagues (*57*), has straightforward reactions and bleaches only by the radical oxidation pathway, so it has become the reagent of choice over β-carotene.

$$\text{crocin-H (orange)} + \text{ROO}^{\bullet} \rightarrow \text{crocin}^{\bullet}\ \text{(bleached)} + \text{ROOH}$$

$$\text{crocin-H (orange)} + \text{ROO}^{\bullet} + \text{AH} \rightarrow \text{crocin}^{\bullet} + \text{ROOH} + \text{A}^{\bullet}$$

Color loss is followed optically at 443 nm ($\epsilon_{443} = 89000\ M^{-1}\ cm^{-1}$ in phosphate buffer, pH 7.4) (*58*), so the reaction requires no special instrumentation.

Advantages/Disadvantages of Croton Bleaching. Carotenoid bleaching is readily adaptable to high-throughput methodology such as microplates. However, temperature control is critical, and increased variability in the external wells has been noted (*59*). Because of the need to calculate the IC$_{50}$, multiple dilutions of the same sample need to be run so that only three samples can be run in duplicate per plate. Additional limitations are that crocin is not available commercially and so must be extracted, and there are no standard formats for expressing results—every study has a different method for calculating inhibition kinetics.

Low-Density Lipoprotein (LDL) Oxidation: General Chemistry. Ex vivo oxidation of LDL was developed primarily as a measure of antioxidant status, but applications of LDL oxidation have also been adapted to assess antioxidant capacity in a more physiologically relevant system. LDL is isolated fresh from blood samples, oxidation is initiated by Cu(II) or AAPH, and peroxidation of the lipid components is followed at 234 nm for conjugated dienes or by peroxide values for lipid hydroperoxides (*60*, *61*).

Advantages/Disadvantages of the LDL Oxidation Assay. LDL oxidation utilizing AAPH as the radical source clearly has relevance to oxidative reactions that might occur in vivo. On a limited group of samples, a good relationship was observed between LDL oxidation using AAPH and the ORAC value (*60*); however, the relationship was not present when Cu(II) was used as the oxidant. The method has a major drawback in that LDL must be isolated on a regular basis, and because of the necessity to obtain blood samples from different individuals, it is not possible to get consistent preparations. Thus, this method is not conducive to the development of a consistent, reproducible high-throughput AOC assay.

AOC Methods Utilizing SET Reaction Mechanisms. *Ferric Reducing Antioxidant Power (FRAP): General Chemistry.* The FRAP assay was originally developed by Benzie and Strain (*62*, *63*) to measure reducing power in plasma, but the assay subsequently has also been adapted and used for the assay of antioxidants in botanicals (*64*−*68*). The reaction measures reduction of ferric 2,4,6-tripyridyl-*s*-triazine (TPTZ) to a colored product (**Figure 3**) (*62*, *63*).

The reaction detects compounds with redox potentials of <0.7 V (the redox potential of Fe^{3+}-TPTZ), so FRAP is a reasonable screen for the ability to maintain redox status in cells or tissues. Reducing power appears to be related to the degree of hydroxylation and extent of conjugation in polyphenols (*69*). However, FRAP cannot detect compounds that act by radical quenching (H transfer), particularly thiols and proteins (*65*). This causes a serious underestimation in serum.

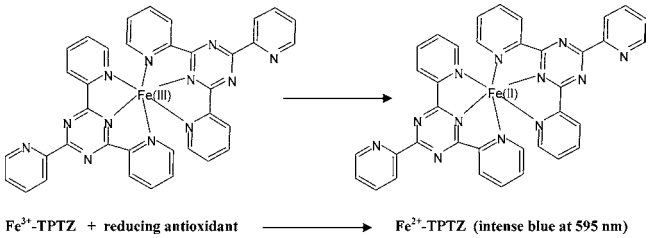

Figure 3. Reaction for FRAP assay.

Because the redox potential of Fe(III)-TPTZ is comparable with that of $ABTS^{\bullet+}$ (0.68 V), similar compounds react in both the TEAC (see below) and FRAP assays. Reaction conditions differ, though: TEAC is carried out at neutral pH, and the FRAP assay is conducted at acidic pH 3.6 to maintain iron solubility. Reaction at low pH decreases the ionization potential that drives electron transfer and increases the redox potential, causing a shift in the dominant reaction mechanism (*70*, *71*). Thus, TEAC and TRAP may give comparable relative values, but TRAP values are usually lower than TEAC values for a given series of antioxidant compounds (*69*, *72*, *73*). Often, FRAP values have a poor relationship to other antioxidant measures.

It has been argued that the ability to reduce iron has little relationship to the radical quenching processes (H transfer) mediated by most antioxidants. However, oxidation or reduction of radicals to ions still stops radical chains, and reducing power reflects the ability of compounds to modulate redox tone in plasma and tissues. The FRAP mechanism is totally electron transfer rather than mixed SET and HAT, so in combination with other methods can be very useful in distinguishing dominant mechanisms with different antioxidants. In addition, because reduced metals are active propagators of radical chains via hydroperoxide reduction to $RO^{\bullet}$, it would be interesting to evaluate whether high FRAP values correlate with the tendency of polyphenols to become pro-oxidants under some conditions. This has been shown for some flavones and flavanones (*74*), which also have high FRAP values.

Advantages/Disadvantages of the FRAP Assay. Both the FRAP and TEAC assays evolve from assays that rely on the hypothesis that the redox reactions proceed so rapidly that all reactions are complete within 4 and 6 min, respectively, but in fact this is not always true. FRAP results can vary tremendously depending on the time scale of analysis. Fast-reacting phenols that bind the iron or break down to compounds with lower or different reactivity are best analyzed with short reaction times, for example, 4 min. However, some polyphenols react more slowly and require longer reaction times for detection, for example, 30 min. The order of reactivity of a series of antioxidants can vary tremendously and even invert, depending on the analysis time (*69*). Pulido and co-workers (*69*) recently examined the FRAP assay of dietary polyphenols in water and methanol. The absorption (A_{593}) slowly increased for polyphenols such as caffeic acid, tannic acid, ferulic acid, ascorbic acid, and quercetin, even after several hours of reaction time. Thus, a single-point absorption endpoint may not represent a completed reaction. The FRAP assay does not measure thiol antioxidants, such as glutathione. FRAP actually measures only the reducing capability based upon the ferric ion, which is not relevant to antioxidant activity mechanistically and physiologically. However, in contrast to other tests of total antioxidant power, the FRAP assay is simple, speedy, inexpensive, and robust and does not require specialized equipment. The FRAP assay can be performed using automated, semiautomatic, or manual methods.

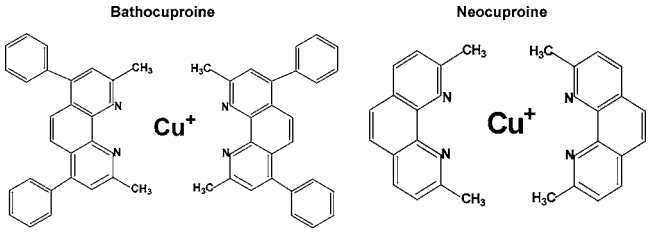

Figure 4. Structures of bathocuproine and neocuproine used in copper reduction assays.

Copper Reduction Assay (CUPRAC, AOP-90): General Chemistry. Variants of the FRAP assay using Cu instead of Fe have recently been introduced as Bioxytech AOP-490 (*75*) and CUPRAC (*76*). These assays are based on the reduction of Cu(II) to Cu(I) by the combined action of all antioxidants (reducing agents) in a sample. In the Bioxytech AOP-490 assay, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline) (**Figure 4**) forms a 2:1 complex with Cu(I), yielding a chromophore with maximum absorbance at 490 nm. Rate and reaction and concentration of products are followed by bathcuproine complexation of the Cu(I) produced. The CUPRAC assay uses a related compound, neocuproine (2,9-dimethyl-1,10-phenanthroline) (**Figure 4**), the Cu(I) complex of which absorbs at 450 nm. A dilution curve generated by uric acid standards is used to convert sample absorbance to uric acid equivalents.

Phenanthroline complexes have very limited water solubility and thus must be dissolved in organic solvents such as 95% ethanol and diluted. However, β-carotene will not react with the CUPRAC reagent in aqueous ethanol and requires dichloroethane, which limits miscibility (*76*). CUPRAC values are comparable to TEAC values for polyphenols, whereas FRAP values are usually considerably lower (*76*). Copper, free and in phenanthroline complexes, has a lower redox potential than iron, so its reactions are more selective; sugars and citric acid, common interferences with FRAP, are not oxidized in CUPRAC. At the same time, the low redox potential enhances redox cycling, so copper reduction may be an even more sensitive indicator of potential pro-oxidant activity of antioxidants.

Advantages/Disadvantages of the Copper Reduction Assays. Copper has advantages over iron for antioxidant assays in that all classes of antioxidants, including thiols, are detected with little interference from reactive radicals and the copper reaction kinetics are faster than iron. The AOP-490 assay requires only 3 min; the CUPRAC assay is complete in minutes for ascorbic acid, uric acid, gallic acid, and quercetin, but requires 30−60 min for more complex molecules. Thus, the copper reduction assays have similar problems with a complex mixture of antioxidants in terms of selecting an appropriate reaction time.

AOC Methods Utilizing both HAT and SET Mechanisms. Although the TEAC and DPPH assays are usually classified as SET reactions, these two indicator radicals in fact may be neutralized either by direct reduction via electron transfers or by radical quenching via H atom transfer (*77*). Reactivity patterns and mechanisms are thus difficult to interpret without detailed information about the composition and structures of antioxidants being tested. Interpretation is particularly difficult when small molecule reducing agents such as ascorbic acid are present in extracts of phenols.

TEAC or Other ABTS Assays: General Chemistry. The TEAC assay was first reported by Miller and Rice-Evans (*78*), which is based on the scavenging ability of antioxidants to the long-life radical anion $ABTS^{\bullet+}$ (**Figure 5**). In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\bullet+}$, which is intensely colored, and AOC is

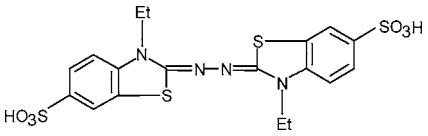

Figure 5. Structure of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{\bullet+}$).

measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\bullet+}$ radical. Results of test compounds are expressed relative to Trolox.

Originally, this assay used metmyoglobin and H_2O_2 to generate ferrylmyoglobin, which then reacted with ABTS to form $ABTS^{\bullet+}$ (*78*). The sample to be tested was added into the reaction medium before the radical was formed. This order of addition of reagents in the TEAC assay was then criticized as a major pitfall, because antioxidants can react with oxidizing agents themselves and, thus, lead to overestimation of antioxidant capacity (*79*). Thus, "post-addition" protocols were proposed to improve this assay (*80*, *81*). In these revised versions, the sample to be tested was added after generation and quantification of $ABTS^{\bullet+}$, which was expected to minimize the interference of compounds with oxidants during radical formation and prevent the possible overestimation. Besides this modification, other modifications in terms of the method used to generate $ABTS^{\bullet+}$, wavelengths that are used to monitor the reaction, and quantification methods were also made by different investigators, which have led to a number of diverse methods. Some modified methods have not used the name "TEAC", but they actually share the same reaction mechanism and use the same radical cation, $ABTS^{\bullet+}$.

According to Cano et al. (*82*), $ABTS^{\bullet+}$ can be generated by either chemical reaction [e.g., manganese dioxide (*83*), ABAP (*81*), potassium persulfate (*80*)] or enzyme reactions [e.g., metmyoglobin (*78*), hemoglobin, or horseradish peroxidase (*82*, *84*)]. Generally, chemical generation requires a long time (e.g., up to 16 h for potassium persulfate generation) or high temperatures (e.g., 60 °C for ABAP generation), whereas enzyme generation is faster and the reaction conditions are milder. Cano et al. (*85*) utilized horseradish peroxidase to generate $ABTS^{\bullet+}$ and have shown that the reaction can be studied over a wide range of pH values. However, the reaction mechanism may shift with pH; for example, electron transfer is facilitated at acid pH (*8*). This variation has been adapted also to measure selectively hydrophilic and lipophilic antioxidants by running the assay in buffered media and organic solvents, respectively (*82*, *86*, *87*), or by partitioning antioxidants in mixtures between hexane and aqueous solvents (*18*). However, water-soluble reactions appear to be favored (*88*).

The absorption maxima (λ_{max}) of $ABTS^{\bullet+}$ were shown to be at wavelengths of 415, 645, 734, and 815 nm. Among them, 415 and 734 nm were adopted by most investigators to spectrophotometrically monitor the reaction between the antioxidants and $ABTS^{\bullet+}$ (*87*). In terms of quantification methods, most recent revised methods measure the absorbance decrease of $ABTS^{\bullet+}$ in the presence of testing sample or Trolox at a fixed time point (4−6 min), and then antioxidant capacity was calculated as Trolox equivalents.

Advantages/Disadvantages of TEAC. Because the TEAC assay is operationally simple, it has been used in many research laboratories for studying AOC. TEAC values of many compounds and food samples have been reported (*66*−*68*, *89*−*91*). $ABTS^{\bullet+}$ reacts rapidly with antioxidants, typically within 30 min. It can be used over a wide pH range and can be used to study effects of pH on antioxidant mechanisms (*8*). Also,

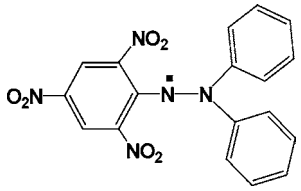

Figure 6. Structure of 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$).

$ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents and is not affected by ionic strength, so can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts and body fluids (*92*). TEAC reactions can be automated and adapted to microplates (*55*, *73*, *93*), to flow injection (*10*, *94*), and to stopped flow (*95*).

The ABTS radical used in TEAC assays is not found in mammalian biology and thus represents a "nonphysiological" radical source. Thermodynamically, a compound can reduce $ABTS^{\bullet+}$ if it has a redox potential lower than that of ABTS (0.68 V). Many phenolic compounds have low redox potentials and can thus react with $ABTS^{\bullet+}$. Also, the TEAC reaction may not be the same for slow reactions, and it may take a long time to reach an endpoint. Thus, by using an endpoint of short duration (4 or 6 min), one may be reading before the reaction is finished and result in lowered TEAC values. Van den Berg et al. (*81*) concluded that "quantitative evaluation of antioxidant capacity using the TEAC can be troublesome or even impossible, but it can be used to provide a ranking order of antioxidants".

2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay: General Chemistry. The $DPPH^{\bullet}$ (**Figure 6**) radical is one of the few stable organic nitrogen radicals, which bears a deep purple color. It is commercially available and does not have to be generated before assay like $ABTS^{\bullet+}$. This assay is based on the measurement of the reducing ability of antioxidants toward $DPPH^{\bullet}$. The ability can be evaluated by electron spin resonance (EPR) or by measuring the decrease of its absorbance. The widely used decoloration assay was first reported by Brand-Williams and co-workers (*96*). Antioxidant assays are based on measurement of the loss of DPPH color at 515 nm after reaction with test compounds (*97*), and the reaction is monitored by a spectrometer. The percentage of the DPPH remaining is calculated as

$$\%\ DPPH^{\bullet}_{REM} = 100 \times [DPPH^{\bullet}]_{REM}/[DPPH^{\bullet}]_{T=0}$$

The percentage of remaining $DPPH^{\bullet}$ ($DPPH^{\bullet}_{REM}$) is proportional to the antioxidant concentration, and the concentration that causes a decrease in the initial $DPPH^{\bullet}$ concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} is defined as T_{EC50}. Sánchez-Moreno and co-workers (*99*) further introduced another parameter to express antioxidant capacity, called "antiradical efficiency (AE)". It was defined as

$$AE = 1/EC_{50}T_{EC50}$$

The DPPH assay is considered to be mainly based on an ET reaction, and hydrogen-atom abstraction is a marginal reaction pathway (*2*).

Advantages/Disadvantages of the DPPH Assay. The test is simple and rapid and needs only a UV−vis spectrophotometer to perform, which probably explains its widespread use in antioxidant screening. However, interpretation is complicated when the test compounds have spectra that overlap DPPH at 515 nm. Carotenoids, in particular, interfere (*98*). Use of DPPH to measure AOC is plagued by many drawbacks. The assay is

not a competitive reaction because DPPH is both radical probe and oxidant. DPPH color can be lost via either radical reaction (HAT) or reduction (SET) as well as unrelated reactions, and steric accessibility is a major determinant of the reaction. Thus, small molecules that have better access to the radical site have higher apparent AOC with this test. DPPH has a relatively small linear reaction range of only 2−3-fold.

DPPH is a stable nitrogen radical that 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 due to steric inaccessibility. DPPH also is decolorized by reducing agents as well as H transfer, which also contributes to inaccurate interpretations of AOC. Thus, AOC is not fairly rated by the ability of antioxidants to react with DPPH.

Folin−Ciocalteu (F−C) AOC or Total Phenolics Assay. There is always the controversy over what is being detected in total antioxidant capacity assays—only phenols, or phenols plus reducing agents plus possibly metal chelators. The F−C assay has for many years been used as a measure of total phenolics in natural products, but the basic mechanism is an oxidation/reduction reaction and, as such, can be considered another AOX method.

General Chemistry of the F−C Method. The original F−C method developed in 1927 originated from chemical reagents used for tyrosine analysis (*100*) in which oxidation of phenols by a molybdotungstate reagent yields a colored product with λ_{max} at 745−750 nm:

$$Na_2WO_4/Na_2MoO_4 \rightarrow (\text{phenol}-MoW_{11}O_{40})^{-4}$$

$$Mo(VI)\ (\text{yellow}) + e^- \rightarrow Mo(V)\ (\text{blue})$$

The method is simple, sensitive, and precise. However, the reaction is slow at acid pH, and it lacks specificity. Singleton and Rossi (*101*) improved the method with a molybdotungstophosphoric heteropolyanion reagent

$$3H_2O - P_2O_5 - 13WO_3 - 5MoO_3 - 10H_2O$$

and

$$3H_2O - P_2O_5 - 14WO_3 - 4MoO_3 - 10H_2O$$

that reduced phenols more specifically; the λ_{max} for the product is 765 nm. They also imposed mandatory steps and conditions to obtain reliable and predictable data: (1) proper volume ratio of alkali and F−C reagent; (2) optimal reaction time and temperature for color development; (3) monitoring of optical density at 765 nm; and (4) use of gallic acid as the reference-standard phenol. The improved method outlined by Singleton and Rossi (S−R; *100*; *101*) specified the conditions to minimize variability and eliminate erratic results. The explicit conditions of the S−R method are as follows: mix 1 mL of sample (properly diluted) with at least 60 mL of water and 5 mL of F−C reagent; after 30 s and before 8 min, add 15 mL of Na_2CO_3; mix and bring to 100 mL total volume with water; incubate for 2 h at 75 °F and measure absorbance. Singleton and Rossi (*101*) concluded that "compared to permanganate oxidation or ultraviolet absorbance methods, the S−R method produces *predictable* results on a wide range of phenolics". Nevertheless, very few papers published in 2003 followed the exact steps of the improved F−C method. Different reagent concentrations and timing of additions and incubation are frequently used. In addition, a number of papers have replaced the recommended gallic acid reference standard with catechin equivalents (*102*, *103*), tannic acid equivalents (*104*), chlorogenic acid equivalents (*105*), caffeic acid equivalents (*106*), protocatechuic acid equivalents (*107*), vanillic acid equivalents (*108*), and ferrulic acid equivalents (*109*). Lack of standardization of methods can lead to several orders of magnitude difference in detected phenols. Total phenolics in blueberries, for example, ranged from 22 to 4180 mg/100 g of fresh weight depending mostly on assay conditions (*110*). Hence, continued efforts to standardize the assay are clearly warranted. Efforts are currently underway in the wine industry to standardize this method for the measurement of wine phenolics.

Advantages/Disadvantages of the F−C Assay. The F−C method is simple and can be useful in characterizing and standardizing botanical samples provided that some of the limitations and variations mentioned previously are properly controlled. The F−C method suffers from a number of interfering substances [particularly sugars, aromatic amines, sulfur dioxide, ascorbic acid and other enediols and reductones, organic acids, and Fe(II)], and correction for interfering substances should be made. Additional nonphenolic organic substances that react with the F−C reagent includd adenine, adenosine, alanine, aniline, aminobenzoic acid, ascorbic acid, benzaldehyde, creatinine, cysteine, cytidine, cytosine, dimethyaniline, diphenylamine, EDTA, fructose, guanine, guanosine, glycine, histamine, histidine, indole, methylamine, nitriloacetic acid, oleic acid, phenylthiourea, proteins, pyridoxine, sucrose, sulfanilic acid, thiourea, thymine, thymidine, trimethylamine, tryptophan, uracil, uric acid, and xanthine. Also, some inorganic substances such as hydrazine, hydroxyammonium chloride, iron ammonium sulfate, iron sulfate, manganese sulfate, potassium nitrite, sodium cyanide, sodium metabisulfite, sodium phosphate, sodium sulfite, and tin chloride may also react with the F−C reagent to give elevated apparent phenolic concentrations (*111*, *112*). The kind of phenolics that are included in the F−C method needs to be considered, the steps in analysis should be followed according to the original S−R modified method, proper corrections in the F−C analysis should be made as appropriate, and gallic acid should be used as a reference standard. If these factors are followed, a uniformly acceptable method of total phenolics analysis could be established, so that results can be compared rationally.

The relationship between the F−C method and AOC measurements by $ORAC_{FL}$ is usually good; however, differences in the way the antioxidant components in different foods react in this method differ (see **Figure 7**; compare cowpeas to blueberry and blackberry) from that of the HAT mechanism of $ORAC_{FL}$.

RECOMMENDATIONS FOR STANDARDIZED AOC MEASUREMENT

The advantages and disadvantages of some of the different AOC methods relative to simplicity, instrumentation required, biological relevance, mechanisms, endpoint, quantitation method, and potential for both lipophilic and hydrophilic AOC measurement are summarized in **Table 1**.

A primary factor to consider in selecting a method relates to the mechanism of reaction and its relationship to what might occur in the target application. For classical antioxidant action, an assay based on a HAT mechanism is preferred over a SET reaction mechanism because the peroxyl radical is the predominant free radical found in lipid oxidation in foods and biological systems. However, it may also be important to develop assays using other radical sources such as the hydroxyl, superoxide,

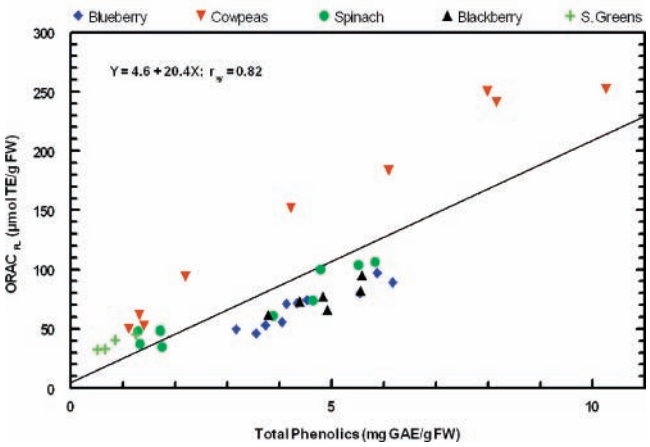

Figure 7. Relationship between $ORAC_{FL}$ and antioxidant capacity measurement by the F–C method in different foods (Luke Howard, unpublished data, personal communication).

and peroxynitrite, because these are active in cells and tissues of plants and animals alike, and it is clear that not all antioxidants behave the same toward different radical sources. No single assay can be considered a "total antioxidant capacity assay" even though it can be performed both in an aqueous solution and in a lipophilic environment. However, to fully elucidate a full profile of antioxidant capacity against various ROS/RNS, such as $O_2^{\bullet -}$, $HO^{\bullet}$, and $NO^{\bullet}$, the development of different methods specific for each ROS/RNS may be needed.

Among other factors that are important and influence the selection of a good method are biological relevance and endpoint as well as method of quantitation. ORAC, TRAP, and LDL oxidation are considered to be the most biologically relevant assays (**Table 1**). The antioxidant capacity from these in vitro methods may more closely reflect in vivo action. For this reason they have advantages over the methods adopting less relevant or irrelevant free radicals in a biological system. Choices of endpoint and quantitation methods are related to whether a given method can accurately assess different samples or not. A good method should be suitable to assess different antioxidants or antioxidant mixture and give an accurate value. Furthermore, a good method should be able to distinguish the antioxidant(s) with different reaction kinetics. Numerous studies have shown that different antioxidant(s), especially for food samples with complicated antioxidant composition, have different reaction curves. For instance, in decoloration assays, there are three typical curves that could be observed in following the decrease of free radical or probe (**Figure 8**) whether by UV−vis spectrometry, fluorescence, or luminescence. For methods using

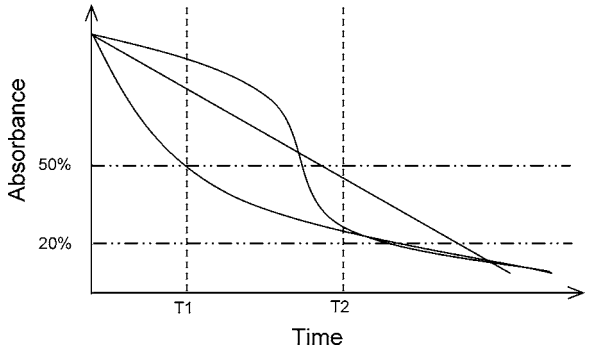

Figure 8. Example comparison of utilizing fixed-time endpoints or fixed-percent inhibition on AOC data.

a fixed time or inhibition degree as endpoint, the time or inhibition degree selection is critical to the assay. From **Figure 8**, it is clear that the different time points T1, T2, and T3 or different inhibition degrees (50 or 20%, respectively) will give quite different AOC values and may even change the ranking. Any activity of the reaction after the fixed point is totally ignored in the computed AOC value. However, for methods utilizing AUC such as ORAC, it has a clear starting point (baseline) and a clear endpoint (back to baseline). The calculation of AUC utilizes both inhibition time and degree, thus reflecting the different reaction kinetics. From this standpoint, we believe that assays using AUC provide better data than methods using a fixed time point or inhibition degree.

Future comparison of results from different AOC methods is not likely to produce much new information because it is not possible to observe good agreement between methods across a diverse group of botanicals, particularly if the reaction mechanisms differ. High correlations have been observed between FRAP and $ORAC_{FL}$ in some foods, but little or no relationship in other foods (*65*). In assaying AOC of extracts from natural materials, it is important to recognize that antioxidants encompass a wide range of polyphenols, reducing agents, and nucleophiles that vary in (a) solubility and phase of localization, (b) redox potential, and (c) specificity and mechanism of action. In addition, when the physiological effects of antioxidants are considered, it is important to also consider reducing activity that may be involved in maintaining redox tone, in signal transduction, and in metal cycling and possible pro-oxidant effects. At the present time, no single assay available provides all of the information desired, so evaluation of overall antioxidant capacity may require multiple assays to generate an "antioxidant profile" encompassing reactivity toward both aqueous and lipid/organic radicals directly via radical quenching

Table 1. Comparison of Methods for Assessing Antioxidant Capacity Based upon Simplicity of Assay, Instrumentation Required, Biological Relevance, Mechanism, Endpoint, Quantitation Method, and Whether the Assay Is Adaptable To Measure Lipophilic and Hydrophilic Antioxidants

antioxidant assay	simplicity	instrumentation required	biological relevance	mechanism	endpoint	quantitation	lipophilic and hydrophilic AOC
ORAC	++[a]	+	+++	HAT	fixed time	AUC	+++
TRAP	−−−[b]	−− specialized	+++	HAT	lag phase	IC_{50} lag time	−−
FRAP	+++	+++	−−	SET	time, varies	ΔOD fixed time	−−−
CUPRAC	+++	+++		SET	time	ΔOD fixed time	−−−
TEAC	+	+	−	SET	time	ΔOD fixed time	+++
DPPH	+	+	−	SET	IC_{50}	ΔOD fixed time	−
TOSC	−	−	++	HAT	IC_{50}	AUC	−−−
LDL oxidation	−	+++	+++	HAT	lag phase	lag time	−−−
PHOTOCHEM	+	−− specialized	++	?	fixed time	lag time or AUC[c]	+++

[a] +, ++, +++ = desirable to highly desired characteristic. [b] −, −−, −−− = less desirable to highly undesirable based upon this characteristic. [c] The lipophilic assay is quantitated by AUC measured over a defined measuring time, and the hydrophilic assay is quantitated based upon the lag phase.

and radical reducing mechanisms and indirectly via metal complexing.

Validation Issues. The principal reasons for the failure of many validation studies of analytical methods have often resulted from (1) failure to optimize the ruggedness of the test, (2) failure to clearly describe the method, (3) too many analytes, (4) too wide a range of concentrations of analytes in the test material, (5) lack of analyst training, (6) qualifications of the laboratory, and (7) failure to recognize or control the presence of interfering substances. Problems often overlooked in collaborative studies have included sample homogeneity, failure to maximize extraction efficiency, failure to identify critical control points, and failure to adhere to good quality control procedures. Included in the standardization protocols should be extraction and sampling procedures, critical handling considerations including identification of interferences and procedures for eliminating them, storage procedures, detailed procedures for the analysis, and statistical analysis. Consistent extraction methods will be critical. Because of the diversity of antioxidant phytochemicals in botanicals, no single solvent system is likely to be optimal for all. Thus, some compromises may need to be made. These specific issues have not been dealt with in detail in this overview, but will be important as actual validation of standardized methods is begun.

Recommendations for AOC Methods Standardization. From this evaluation, it is clear that no one AOC assay will truly reflect the "total antioxidant capacity" of a particular sample. The total antioxidant capacity needs to reflect both lipophilic and hydrophilic capacity, and at least for physiological activity it needs to reflect and differentiate both hydrogen atom transfer (radical quenching) and electron transfer (radical reduction). In addition, to fully elucidate a full profile of antioxidant capacity, tests evaluating effectiveness against various reactive oxygen species/reactive nitrogen species such as $O_2^{\bullet -}$, $HO^{\bullet}$, and $ONOO^-$ are needed, and this may require the future development of additional methods specific for each radical source.

With these factors in mind, it is proposed that three of the methods discussed in this review (ORAC, F−C phenolics assay, and TEAC) should be standardized for use in the routine quality control and measurement of AOC of dietary supplements and other botanicals. However, in suggesting multiple methods, it is not clear whether we have really helped the nutraceuticals industry. There is not going to be any "standard" relationship between the methods, and thus one must decide upon a single method or use multiple assays to compare foods or dietary supplements. Standardization of additional methods may be needed in the future as methods utilizing other radical sources are developed. This choice of methods is based upon two methods with differing reaction mechanisms, with one utilizing the peroxyl radical because of its predominance in biological systems and the other the SET mechanism utilizing the $ABTS^{\bullet +}$ radical. The F−C phenolics assay provides a third option for a simple, speedy, inexpensive, and robust assay that does not require specialized equipment, but can be automated for high-throughput assay. The $ORAC_{FL}$ assay represents a biologically relevant mechanism, one that can measure both lipophilic and hydrophilic AOC and is adapted for high-throughput assay.

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