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Review

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Antioxidant Activity/Capacity Measurement. 1. Classification, Physicochemical Principles, Mechanisms, and Electron Transfer (ET)-Based Assays

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ABSTRACT: Because there is no widely adopted "total antioxidant parameter" as a nutritional index for labeling food and biological fluids, it is desirable to establish and standardize methods that can measure the total antioxidant capacity (TAC) level directly from plant-based food extracts and biological fluids. In this review, we (i) present and classify the widely used analytical approaches (e.g., in vitro and in vivo, enzymatic and nonenzymatic, electron transfer (ET)- and hydrogen atom transfer (HAT)-based, direct and indirect assays) for evaluating antioxidant capacity/activity; (ii) discuss total antioxidant capacity/activity assays in terms of chemical kinetics and thermodynamics, reaction mechanisms, and analytical performance characteristics, together with advantages and drawbacks; and (iii) critically evaluate ET-based methods for analytical, food chemical, biomedical/clinical, and environmental scientific communities so that they can effectively use these assays in the correct places to meet their needs.

KEYWORDS: *antioxidant activity, total antioxidant capacity, electron transfer-based assays, antioxidant mechanisms, food analytical methods*

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1. INTRODUCTION

1.1. Scope. Oxidative stress is a pathological state in which reactive oxygen/nitrogen species (ROS/RNS) overwhelm antioxidative defenses of the organism, leading to oxidative modification of biological macromolecules (i.e., lipid, protein, DNA), tissue injury, and accelerated cellular death[1] as the foundation of many diseases. Measuring the antioxidant activity/capacity levels of biological fluids and foods is carried out for the diagnosis and treatment of oxidative stress-associated diseases in clinical biochemistry, for meaningful comparison of foods in regard to their antioxidant content, and for controlling variations within or between products. Antioxidant measurements have not yet been adapted to standard protocols in medical diagnosis and treatment despite evidence-based changes of serum antioxidant capacity in certain diseases [e.g., overall total antioxidant capacity (TAC) of human serum was increased in dialysis patients of chronic renal failure due to high urate values, but there was a marked reduction after hemodialysis; antioxidant activity (AOA) of serum was markedly lower in acute myocardial infarction and chronic lymphocytic leukemia patients compared to those of controls]. Therefore, ideally one should be able to detect/screen certain oxidative stress-originated diseases and monitor the course of medical treatments by considering the changes in TAC values of intracellular fluids and blood plasma/serum of a given individual measured by standardized methods. The complexities of food and physiological applications of antioxidants, separately and combined, require rigorous consideration and analysis of all aspects of the chemistry, reaction mechanisms, and reaction/radical/target specificity in various test systems, as well as careful and accurate quantitation of all reactants and products involved. The current literature clearly states that there is no widely adopted "total antioxidant parameter" as a nutritional index available for the labeling of food and biological fluids due to the lack of standardized quantitation methods. Therefore, it is desirable to establish and standardize methods that can measure the TAC level directly from plant-based food extracts and biological fluids. Ideally, agreement on standardized methods for antioxidant testing requires (i) general guidance for the application of assays, including exact description of test conditions, experimental apparatus, and stability of reagents; (ii) all new methods to be properly validated within their framework, associated with intra- and interlaboratory validation (including the production of repeatability, reproducibility, and recovery data), standardization, internal quality control, proficiency testing, and analytical quality assurance; (iii) the results to enable a reasonable comparison of the antioxidant content of foods, pharmaceuticals, and other commercial products; (iv) provision of a measure for meeting the need of quality standards for regulatory issues and health claims.[2,3]

To date, many in vitro tests are available from a chemical assay performed in a homogeneous solution to more complex methods using exogenic probes to detect oxidation. Although many literature methods do not distinguish between "antioxidant capacity" and "antioxidant activity" of nonenzymatic antioxidants, end-point assays measuring the efficiency of antioxidant action (i.e., reactive species inactivation) should be differentiated from kinetic-based assays measuring reaction rate. TAC assays may be broadly classified as electron transfer

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(ET)- and hydrogen atom transfer (HAT)-based assays, although in some cases, these two mechanisms may not be differentiated with distinct boundaries [such as those utilizing 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical reagents]. In fact, most nonenzymatic antioxidant activity (e.g., scavenging of free radicals, inhibition of lipid peroxidation, etc.) is mediated by redox reactions. In addition to these two basic classes focusing on mechanism, ROS/RNS scavenging assays will also be taken into account. Complementary to existing methods, novel approaches have recently been developed such as cupric reducing antioxidant capacity (CUPRAC) TAC assay (introduced by our research group to the world literature in 2004), its modified ROS scavenging assays, and other modifications (e.g., antioxidant sensor, postcolumn online HPLC technology). The current direction of CUPRAC methodology can be best described as a self-sufficient and integrated train of measurements providing a useful "antioxidant and antiradical assay package".

The complexity and diversity of research topics investigated have led to the development of a multitude of tests, but unfortunately none has gained universal acceptance. Thus, one of the major challenges in antioxidant testing is to know which method is best suited for a particular application. Because antioxidants may exert their effect through various mechanisms such as scavenging radicals, sequestering transition metal ions, decomposing hydrogen peroxide or hydroperoxides, quenching active prooxidants, and repairing biological damage, it should be clarified which function of antioxidants is being measured and, accordingly, the antioxidant assay method should be selected considering the function to be evaluated.[4] Because of the wide divergence of results for natural antioxidants in food systems, more valid and rigorous guidelines and assay protocols are required to bring some order and agreement to this important field. Naturally, it should be remembered that TAC and AOA are not like elemental analysis parameters for which the analyst has to obtain more or less the same result from different techniques (e.g., calcium in a milk sample measured by different techniques should yield identical results within tolerable limits), because it is possible to get quite different TAC or AOA results using the same probe under different experimental conditions. Our understanding of the effects of antioxidant compounds can only be improved with a deeper chemical insight if more specific methodology is used and is capable of defining what products are formed and inhibited by antioxidants depending on conditions, systems, and targets of protection.

1.2. Technical Issues with TAC Assays. The technical issues associated with current "state-of-the-art" TAC assays that require special consideration can be summarized as follows:

(i) There is an almost total lack of standardization in experimental procedures and expressing results.

(ii) Too many assays not having a demonstrated clear chemistry give rise to inconsistent results, inappropriate application and interpretation of assays, and improper specification of TAC. Naturally, statistical validation (e.g., using a certified reference material) of a given assay is not possible in view of the different reaction kinetics and chemistry of the assays producing different results.

(iii) Most assays developed for TAC screening are devoid of detailed investigations related to underlying initiators, targets, antioxidant interactions, kinetics, effects of solvent, concentration, pH, etc. In earlier work regarding TAC assays, there seems to be some lack of relevant classification according to chemical reaction mechanisms and kinetics. Molecular accessibility problems of relevant reagents (such as steric hindrance, inner- and outer-sphere electron transfer mechanisms of transition metal-based chromophores/fluorophores, etc.) have not been discussed.

(iv) Several studies on natural phytochemical compounds produced conflicting results because of the nonspecific "one-dimensional" character of methods used to evaluate antioxidant activity. Because most natural antioxidants and phytochemicals are multifunctional (i.e., due to variations in system composition, type of oxidizable substrate, media of initiation and acceleration of oxidation, methods to assess oxidation and to quantify antioxidant activity), a reliable antioxidant protocol requires the measurement of more than one property relevant to either foods or biological systems.[5]

(v) A noteworthy question is that, if the protective action of an antioxidant is being assayed in a selected test, what biomolecule (lipid, protein, DNA, etc.) is the antioxidant supposed to protect and how? Is the tested antioxidant at a significantly lower concentration than that of the protected substrate? Are relevant ROS/RNS utilized in assaying the antioxidant?[6] In AOA and TAC assays covering a simulated oxidant−antioxidant reaction, chromophore or luminescent probes capable of accepting H atoms or electrons from antioxidants do not necessarily protect relevant substrates (such as lipid, protein, DNA) from oxidation and, therefore, these TAC assay results may not consequently reflect the capacity to retard or suppress oxidation.[7]

(vi) Antioxidant activity (i.e., related to the kinetics of antioxidant action for quenching reactive species, usually expressed as reaction rates or scavenging percentages per unit time) and antioxidant capacity (i.e., thermodynamic conversion efficiency of reactive species by antioxidants, such as the number of moles of reactive species scavenged by 1 mol of antioxidant during a fixed time period) are both important in antioxidant research, and care must be exercised to distinguish between these two terms, which are often used interchangeably and therefore confused.[8] Because the term "total antioxidant capacity" (TAC) is indicative of the collaborative (additive and possibly synergistic/antagonistic) action of all antioxidants present in a complex sample, it is considered by most researchers as a more useful parameter to assess the antioxidative defenses in food or plasma than separately determining the concentrations of individual antioxidant constituents.

(vii) Other than reactivity toward ROS/RNS, several factors such as concentration, distribution, localization, fate of antioxidant-derived radical, interaction with other antioxidants, and metabolism should be evaluated.[4] The question of bioavailability and the fate of metabolites of the antioxidant components (e.g., whether they undergo further redox cycles) must be addressed in the case of in vivo assays.[9] Also, in vitro antioxidant assays carried out at unrealistic pH values (i.e., far from physiological pH, in either alkaline or acidic range) cannot have much meaning for in vivo estimations of antioxidant action. In tests carried out at weakly acidic pH, most oxidation reactions for phenolic antioxidants are incomplete within the protocol time of the assay.

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(viii) Due to the inadequacies in analytical methodology, the antioxidant activity of proteins (i.e., first-line defense elements against ROS attack in the human body) and specifically of protein thiols are often ignored in most antioxidant assays, because proteins are separated by precipitation from the main matrix and their contribution to serum TAC is left unmeasured.[10] Thus, the precise determination of serum TAC incorporating the "antioxidant gap" originating from protein components is believed to be potentially useful to biochemists investigating the diagnosis, treatment, prognosis, and follow-up of diseases utilizing TAC measurement.[11]

(ix) Possible interactions of antioxidants among themselves (i.e., synergistic or antagonistic effects) should be taken into account to better visualize the collaborative action of antioxidants in the organism.

(x) Prooxidant effects of antioxidants, especially dependent on the composition of medium in which antioxidant measurements are made, should also be considered; for example, transition metal (especially iron) complex-based TAC assay reagents have the possibility of redox cycling at their lower oxidation states that may falsify antioxidant test results.[12]

(xi) Antioxidant capacity assay results have not been effectively correlated to tests measuring oxidative damage, such as the thiobarbituric acid-reactive substances (TBARS) test in lipids or carbonyl test in proteins. Normally, the difference in oxidative status of a medium undergoing ROS/RNS attack, to which a food or biological extract was added, should correlate to TAC of the extract under investigation.[13] Additionally, antioxidant activity needs to be correlated to electrochemical behavior of antioxidants.

As required parameters, a standardized TAC method (i) utilizes a suitable oxidant that actually is or is a simulator of a biologically relevant reactive species; (ii) is simple, practical, and versatile; (iii) uses a method with a defined end-point and a clear chemical mechanism; (iv) has readily available and preferably low-cost instrumentation; (v) is reproducible with good within-run and between-run precision; (vi) can assay both hydrophilic and lipophilic antioxidants; (vii) does not generate new reactive species that may cause under- or overestimated TAC readings; and (viii) is suitable for "high-throughput" analysis for routine quality control of food and biological extracts.[2,14]

1.3. Purpose. The aims of this comprehensive review series are (i) to present a brief panorama of the most widely used methods and of new analytical approaches for evaluating antioxidant capacity/activity; (ii) to discuss TAC/AOA assays in terms of chemical kinetics and thermodynamics, reaction mechanisms, analytical performance characteristics (linear concentration range, recovery, repeatability, reproducibility, and recognition of interfering substances, etc.), and advantages and drawbacks; (iii) to bring in terms of definitions or definition-like characterization and classification of the chemical and biochemical methods of antioxidant assays as well as related antioxidant chemistry; and finally (iv) to provide a critical evaluation of this topic to analytical, food chemical, biomedical/clinical, and environmental scientific communities so that they can effectively use these assays in their correct places to meet their needs. The literature gap summarized in the above "technical issues with TAC assays" is endeavored to be filled. The basic criteria of classification of antioxidant assays, such as in vitro and in vivo, enzymatic and nonenzymatic, ET- and HAT-based, direct and indirect assays, have been addressed. The essential ET-based assays with possible advantages/drawbacks have been critically evaluated, and at the same time, various methodologies of the main and modified CUPRAC procedures regarding TAC and ROS/RNS-scavenging assays have been unified and summarized. However, in the first review of this series, the stress will be upon ET assays, and HAT and mixed-mode (ET/HAT) assays, together with lipid peroxidation assays, ROS/RNS scavenging assays, and oxidative stress biomarkers, are the subjects of the following reviews.

2. CLASSIFICATION OF AOA AND TAC ASSAYS

"Antioxidants" are natural or synthetic substances that may prevent or delay oxidative cell damage caused by physiological "oxidants" having distinctly positive reduction potentials, covering ROS/RNS and free radicals (i.e., unstable molecules or ions having unpaired electrons). The terms "oxidant" and "antioxidant" have complementary meanings in the sense that these compounds neutralize the effects of each other. On the other hand, a "prooxidant" may not have a large reduction potential by itself, but may induce oxidative damage to various biological targets such as DNA (e.g., nucleic base modification and single/double strand breaks), lipids (e.g., structural changes in fatty acid composition and lipid peroxidation), and proteins (e.g., protein carbonylation and oxidation of certain amino acid moieties).[15] For example, transition metal ions at their lower oxidation states are not oxidant species by themselves, but may induce the generation of ROS/RNS with hydrogen peroxide or molecular oxygen, thereby acting as prooxidants. Antioxidants may be broadly defined as "substances that, when present at *relatively* low concentrations compared with those of the oxidizable substrates, significantly delay or inhibit oxidation of those substrates".[16,17] Although the term "oxidizable substrate" includes every type of molecule found in vivo, it is generally understood as biomacromolecules such as lipid, protein, and DNA. This definition emphasizes the importance of the selected damage target and the source of ROS/RNS used when antioxidants are tested and their actions are examined.[18] However, Finley et al.[19] points to the ambiguity of this definition in that the term "antioxidant" may have different connotations to different audiences, such as the capability of quenching metabolically generated ROS (to biochemists and nutritionists), the functionality of retarding food oxidation (to food scientists), or the property of yielding high TAC values in ET- and HAT-based in vitro assays (presumably to a wider community of food science, commerce, and industry). For convenience, antioxidants have been traditionally divided into two classes: primary or chain-breaking antioxidants (mainly acting by ROS/RNS scavenging) and secondary or preventative antioxidants (usually acting by transition metal ion chelation).[20] Thus, an antioxidant may act directly by scavenging reactive species or inhibiting their generation. It may also act indirectly, for example, by up-regulating endogenous antioxidant defenses.[6,21] This work is concerned with the corresponding methods of antioxidant capacity/activity assay capable of measuring chain-breaking or preventive antioxidant ability. In lipid peroxidation experiments, antioxidants acting only by metal chelation are essentially maintained, whereas chain-breaking antioxidants are consumed. Most of the time, this difference is reflected in a lag (retardation) phase of the peroxidation process by chain-breaking antioxidants, compared

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to a constant inhibition by metal-binding antioxidants throughout the reaction.[6,21] However, the scope of this review is limited to the integrated activity of nonenzymatic antioxidants, meaning that endogenous antioxidative enzymes such as superoxide dismutase (scavenging superoxide anion radicals), catalase, and glutathione peroxidase (able to remove hydrogen peroxide) are not considered.

Chain-breaking mechanisms regarding the breaking of the oxidation chain of lipid radicals ($L^{\bullet}$, $LOO^{\bullet}$, or $LO^{\bullet}$) involve the sacrificial consumption of antioxidants (AH) to produce antioxidant radicals ($A^{\bullet}$) protecting lipid molecules (L):

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet} \quad (1)$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet} \quad (2)$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet} \quad (3)$$

Thus, radical initiation (by reacting with a lipid radical) or propagation (by reacting with lipid peroxyl or alkoxyl radicals) steps are inhibited. Because chain-breaking antioxidants exert their action through either hydrogen atom ($H^{\bullet}$) and electron (e^{-}) donation or both (i.e., proton-coupled electron transfer), such AOA measurement methods are commonly classified as HAT- and ET-based assays according to mechanism.

On the other hand, secondary (or preventive) antioxidants retard or prevent lipid oxidation. For example, transition metal ion [e.g., Fe(II) or Cu(I)] chelator antioxidants may inhibit Fenton-type reactions that produce hydroxyl radicals, which may cause oxidative degradation of biological macromolecules (lipids, proteins, DNA, etc.):

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + {}^{\bullet}OH + OH^{-} \quad (4)$$

$$Cu(I) + H_2O_2 \rightarrow Cu(II) + {}^{\bullet}OH + OH^{-} \quad (5)$$

Therefore, preventive antioxidant assay methods should measure transition metal ion chelating ability.

The transition metal chelation functionality of antioxidants covers a neutralization reaction between a Lewis base (antioxidant) and a Lewis acid (metal ion), without involving the donation of H atoms or electrons by the antioxidant. Therefore, the measurement of such preventive AOA should be covered under a different category. On the other hand, hindering the formation of ROS/RNS should be considered as a preventive action,[7] whereas scavenging of ROS/RNS is closely related to HAT and ET mechanisms for the measurement of chain-breaking AOA. Nevertheless, techniques for the measurement of scavenging activity of reactive species (e.g., ROS essentially cover hydroxyl radical, $^{\bullet}OH$; superoxide anion radical, $O_2^{\bullet-}$; singlet oxygen, $^1O_2$; hydrogen peroxide, H_2O_2; and hypochlorous acid, HOCl; RNS mainly cover peroxynitrite, $ONOO^{-}$; and nitric oxide, $^{\bullet}NO$) are investigated under a separate subtitle.

Enzymatic antioxidants are either reductase enzymes [e.g., superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rx), and catalase] and their cofactors, which limit the cellular concentration of free radicals and prevent excessive oxidative damage or oxidase enzyme inhibitors. Therefore, the corresponding enzymatic AOA assays should either measure the enzymatic reduction ability of ROS or the inhibition of oxidases (e.g., xanthine oxidase, NADPH oxidase) capable of producing reactive species. On the other hand, nonenzymatic AOA/TAC assays utilize a relevant probe for simulating the antioxidative action toward oxidant species. Because antioxidant and oxidant-regenerating enzymes in blood cells and the blood vessel wall have a profound impact on the antioxidant properties of blood plasma (which is not reflected in the in vitro assays of isolated plasma), the term "TAC" has been claimed to measure only a part of antioxidant capacity, usually excluding enzymatic activities, and therefore "nonenzymatic antioxidant capacity" (NEAC) has been suggested as a more relevant term than TAC.[22] For example, nonenzymatic plasma antioxidants usually cover albumin and other related proteins containing thiols and other antioxidative amino acid residues, as well as small molecules such as α-tocopherol, bilirubin, ascorbic acid, uric acid, and reduced glutathione (GSH). In the literature, much more effort has been spent on developing nonenzymatic antioxidant assays covering a wide range of HAT- and ET-based assays and methods for measuring ROS/RNS scavenging activity. Recently, synthetic and natural phenolic antioxidants have been summarized, together with their mode of action, health effects, degradation products, and toxicology.[23] A general and updated overview of methods available for measuring antioxidant activity and the chemistry behind them has been provided.[24] Advantages and limitations of common testing methods have been listed, with a preference for method selection serving different needs.[25]

2.1. Measurement of Nonenzymatic Chain-Breaking Antioxidant Activity/Capacity. Nonenzymatic chain-breaking antioxidant ability can be measured by finding the rate (kinetic AOA methods) or thermodynamic conversion efficiency (TAC methods) for the reaction of a suitable oxidant probe with the antioxidant. AOA assays such as total peroxyl radical trapping antioxidant parameter (TRAP),[10,26] crocin bleaching,[27,28] oxygen radical absorbance capacity (ORAC),[29,30] total oxyradical scavenging capacity (TOSC),[31,32] etc., are usually competitive (Figure 1) and work on a HAT

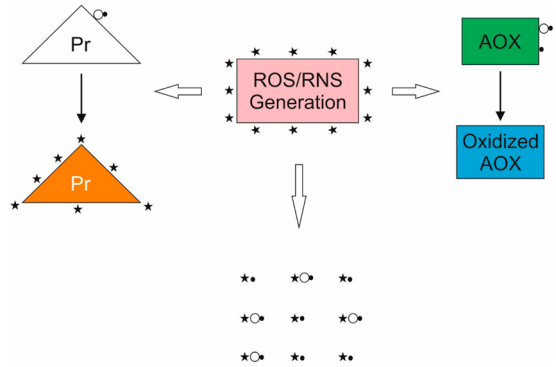


Figure 1. Direct (competitive) antioxidant assay, involving a fluorogenic or chromogenic probe and biologically relevant ROS/RNS.

mechanism, whereas TAC measurement methods are usually noncompetitive (Figure 2) ET and mixed-mode (ET/HAT) assays. In a competitive inhibition (or scavenging) assay, the oxidant reacts with a probe leading to changes in its absorbance, fluorescence, luminescence, or any other measurable property, where antioxidants compete with the probe for the oxidant and repair the oxidized probe.[33] Due to the competition between the probe and antioxidants for reactive species, the probe undergoes less oxidative conversion by ROS/RNS in the presence of antioxidants. A major criticism directed at HAT-based competitive assays using fluorescent probes is that the concentration of the target species (i.e., assay probe simulating a biological substrate) is usually smaller than that of tested antioxidants,[14] which contradicts the basic "definition of antioxidant", because, in real

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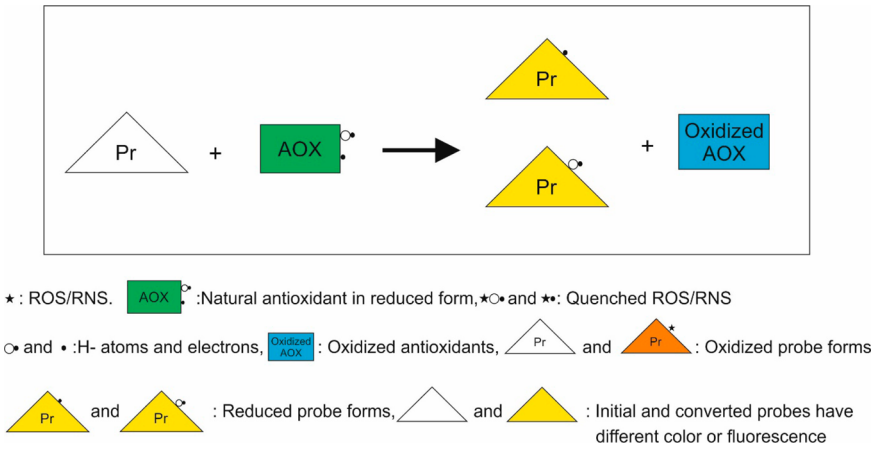


Figure 2. Indirect (noncompetitive) antioxidant assay, in which physiological redox reactions (i.e., oxidant-antioxidant interactions) are simulated on an artificial probe without biologically relevant ROS/RNS.

life, antioxidants exert their protective effects even when they are at much lower concentration than that of the biological (oxidizable) substrate.[15] On the other hand, in ET-based assays, either the probe undergoing reduction with the antioxidant is converted to a colored, fluorescent, chemiluminescent, etc., species or the initial absorbance/fluorescence is attenuated as a result of the antioxidation reaction. ET-based assays have been criticized mainly because the utilized probe acting as the oxidizing agent does not involve physiologically important oxidants such as ROS/RNS, causing a discrepancy between the simulated assay and the real-life antioxidant action.

The most widely used chromophores in ET-based spectrophotometric TAC assays of Folin−Ciocalteu,[34,35] ABTS/TEAC (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid/Trolox-equivalent antioxidant capacity),[36,37] DPPH 2,2-diphenyl-1-picrylhydrazyl,[38,39] CUPRAC (cupric reducing antioxidant capacity),[40,41] FRAP (ferric reducing antioxidant power),[42−45] ferricyanide,[46,47] ferric-phenanthroline,[48] and ferric-ferrozine[49] assays are phospho-tungsto-molybdate(V), $ABTS^{\bullet+}$ radical cation, $DPPH^{\bullet}$ radical, cuprous neocuproine $[Cu(Nc)_2]^{+}$ chelate, ferrous tripyridyltriazine $[Fe(TPTZ)_3]^{2+}$ chelate, Prussian blue $K[Fe(Fe(CN)_6]$ heteropoly acid salt, ferrous phenanthroline $[Fe(phen)_3]^{2+}$ chelate, and ferrous ferrozine: $[Fe(FZ)_3]^{2+}$ chelate, respectively. When transition metal ion-based probes such as ferric and cupric chelates oxidize the antioxidant compounds to be assayed, the metal ion placed at the coordination center of the complex is reduced to the lower oxidation number and its chelate has strong intermolecular charge-transfer interactions; that is, visible light absorbed by the chelate causes a partial transfer of electronic charge from the metal ion to the ligand (giving rise to very high molar extinction coefficients for these chromophores, thereby raising the sensitivity for antioxidant determinations). Some authors classify DPPH and ABTS tests as mixed-mode (i.e., having both ET and HAT mechanisms) assays.[2] TAC assays using Cr(VI) as chromate[50] and Ce(IV)[51−53] as oxidant are also ET-based methods, where antioxidants reduce these species to Cr(III) and Ce(III), respectively; naturally, certain measures have to be taken to decrease the oxidizing ability of these reagents so that antioxidants but not other organic compounds (e.g., citric acid and common sugars in foods and beverages) are oxidized in the assays. When gold nanoparticles (AuNPs) generated from $HAuCl_4$ upon reduction with phenolic acid antioxidants are used as colored probes exhibiting localized surface plasmon resonance (LSPR) absorption, the highest capacity of reducing gold(III) to elemental gold nanoparticles corresponds to the highest antioxidant activity, consistent with the tendency of phenolic antioxidants to donate electrons.[54] The same reasoning applies for the formation of silver nanoparticles (Ag-NPs) coatings generated from $AgNO_3$ with phenolic antioxidants onto citrate-stabilized silver seeds, where the enlargement of preformed Ag-NPs gave rise to enhanced LSPR absorption linearly dependent on polyphenol concentration.[55]

2.1.1. HAT-Based Methods. HAT-based assays measure the capability of an antioxidant to quench free radicals (generally peroxyl radicals) by H atom donation. Peroxyl radicals are generally chosen as the reactive species in these assays because of their higher biological relevance and longer half-life (compared to hydroxyl and superoxide radicals). The HAT mechanism of antioxidant action, in which the hydrogen atom ($H^{\bullet}$) of a phenol (Ar—OH) is transferred to an $ROO^{\bullet}$ radical, can be summarized by the reaction

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet} \quad (6)$$

where the aryloxy radical ($ArO^{\bullet}$) formed from the reaction of antioxidant phenol with peroxyl radical is usually stabilized by resonance. The AH and ArOH species denote the protected biomolecules and antioxidants, respectively. Effective phenolic antioxidants need to react more quickly than biomolecules with free radicals to protect the latter from oxidation.[56] Major criticisms directed at HAT-based assays having a "lag-phase" approach (i.e., measuring the retardation time for the initiation of oxidative probe conversion) to quantification of antioxidant capacity include (i) not every antioxidant possesses an apparent lag-phase (i.e., to have a distinct lag-time, an antioxidant should have a rate constant for the tested radical much higher than that of the probe such that the antioxidant should be consumed up by the time when the probe oxidation is observable);[22] (ii) ambiguity in end-point observation makes interlaboratory comparison of generated data difficult; and (iii) the antioxidant capacity profile of samples following the lag phase is disregarded.[15] Because in HAT-based antioxidant assays using a fluorescent probe both the probe and antioxidants react with $ROO^{\bullet}$, the antioxidant activity can be determined from competition kinetics (Figure 1) by measuring the fluorescence decay curve of the probe in the absence and presence of antioxidants and integrating the area under these curves (AUC approach). The AUC difference between a sample and a

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reagent blank is then related to antioxidant concentration in the sample.[2,14]

2.1.2. ET-Based Methods. The ET mechanism of antioxidant action with a biologically relevant radical is based on the reactions

$$ROO^{\bullet} + AH/ArOH \rightarrow ROO^{-} + AH^{\bullet+}/ArOH^{\bullet+} \quad (13)$$

$$AH^{\bullet+}/ArOH^{\bullet+} + H_2O \leftrightarrow A^{\bullet}/ArO^{\bullet} + H_3O^{+} \quad (14)$$

$$ROO^{-} + H_3O^{+} \leftrightarrow ROOH + H_2O \quad (15)$$

where the reactions are generally assumed to be relatively slower than those of HAT-based assays (although for metal−complex probes capable of outer-sphere electron transfer, this assumption was shown to be invalid as a function of solvent type) and are both solvent- and pH-dependent. The pH-dependency is apparent from the above reaction sequence of ET mechanism. For example, phenolic compounds (Ar—OH) having weakly acidic −OH groups dissociate to a greater extent at higher pH and become more susceptible to oxidation. Thus, most ET reactions occur at a higher rate at higher pH. The reactivity of the $ABTS^{\bullet+}$ cation radical toward ascorbic acid at neutral pH is expressed with a second-order rate constant of $8 \times 10^6\ M^{-1}\ s^{-1}$, whereas in acidic pH, this rate constant decreases by almost 2 orders of magnitude.[57] Generally, ionization potentials of phenolic antioxidants decrease with increasing pH, which causes an increase in electron-donating capacity concomitant with deprotonation.[2] Iron(III)-based antioxidant assays conforming to ET mechanism [excluding the sole hexacyanoferrate(III) complex not in combination with ferric ion] have to be carried out at an acidic pH to prevent the hydrolysis of trivalent iron (e.g., giving rise to $FeOH^{2+}$ and further hydrolysis complexes).[48] The aryloxy radical ($ArO^{\bullet}$) is subsequently oxidized to the corresponding quinone (Ar═O). The more stabilized the aryloxy radical is, the easier will be the oxidation from ArOH to Ar═O due to reduced redox potential[56] and the stronger is the antioxidant.

ET-based (or reduction-based) assays can be better understood considering the fact that antioxidants are also good reducing agents capable of reductive quenching of ROS/RNS. However, these assays do not necessarily use biologically relevant reactive species (such as peroxyl radicals); instead, they use artificial probes that change color or fluorescence when reduced by antioxidants (Figure 2).[14,56] For example, the cupric- or ferric-reducing ability measured for a biological sample may indirectly but efficiently reflect the total antioxidant power of the sample even though no radical species are involved in the assay. The change in absorbance or fluorescence of the probe (at a prespecified wavelength) upon reduction with antioxidants is a measure of the total concentration of antioxidants in a sample, or TAC. This TAC is usually expressed in terms of a reference compound, such as Trolox for hydrophilic antioxidants, α-tocopherol (vitamin E) for lipophilic antioxidants, and gallic acid for aqueous solutions of polyphenols, where trolox-equivalent and gallic acid-equivalent TAC values are denoted as TE and GAE, respectively. The TEAC coefficient is a unitless value, defined as the reducing potency—in Trolox millimolar equivalents—of 1 mM antioxidant solution under investigation. Usually in spectrophotometric TAC assays, this TEAC coefficient is found from the ratio of the slope of the calibration curve (drawn as absorbance versus concentration) of the tested compound to that of Trolox obtained under identical conditions. The TAC value, in mM-TE units, of a complex antioxidant mixture (composed of antioxidants: 1, 2, 3, ..., *i*, ..., *n*) is the sum of the products obtained by multiplying the mM concentration (C_i) of each antioxidant with its TEAC coefficient ($TEAC_i$): $TAC_{(mixture)} = \Sigma C_i(TEAC)_i$.

Naturally this equation is valid as long as the principle of additivity of absorbances is retained for a complex mixture conforming to Beer's law of spectrophotometry; that is, the TAC of a complex antioxidant mixture is the sum of the TAC values of individual antioxidants comprising the mixture.

In general, ET-based TAC assays have good precision, because the difference in absorbance or fluorescence intensities of the reduced and original probes can be directly measured. Because most of the time a single reduced species is produced from the probe upon chemical reduction with antioxidants, the absorbance changes of a single chromogenic product at a fixed pH and wavelength usually vary almost perfectly linearly with total antioxidant concentration (in TE units). Thus, within the linear concentration range obeying Beer's law, additivity of absorbances (and therefore additivity of TAC values of individual constituents forming an antioxidant mixture) is usually attained. Reduction-based assays have generally been criticized for not involving biologically relevant radicals. However, their simulated conditions can well imitate the media in food and biological fluids such as redox potential, pH, and lipophilic/hydrophilic balance of solvent mixtures and microemulsions. With the exception of the Folin−Ciocalteu reagent having an indefinite redox potential, most ET reagents have standard potentials in the useful range of 0.6−0.7 V relevant for food and biological fluids; that is, they can oxidize important antioxidants to measure their TAC values. The reaction conditions of reduction-based TAC assays should be well-defined so as to maintain reproducibility, because interassay results without detailed description of conditions cannot be compared. The time and temperature of measurements should always be indicated in regard to repeatability of reaction kinetics, because some oxidation reactions of ET probes with antioxidants may not reach saturation within the prespecified protocol time of the assays. For example, high-spin Fe(III) having half-filled d-orbitals may show a kinetic inertness to thiols, some phenolic acids, and flavonoids such that the envisaged oxidation cannot be completed within the protocol time period of the FRAP assay and similar Fe(III)-based assays.[48]

When ET-based reagents, especially metal complexes utilizing Fe(III) or Cu(II), are to be used in the TAC determination of plasma or serum, it should be remembered that these samples should be preserved cold with either heparin or citrate and not EDTA, because EDTA usually stabilizes the higher oxidation state [such as Fe(III) of Cu(II)] in preference to the lower one [e.g., Fe(II) or Cu(I)] by forming a more stable complex, and this decreases the Nernst potential of the concerned redox couple in the presence of a chromogenic ligand (e.g., tripyridyltriazine or neocuproine), thereby weakening the oxidizing power of the reagent against certain plasma antioxidants and causing negative errors in TAC measurement. Bartosz recommended the use of human plasma in TAC studies rather than serum, which should be analyzed immediately after blood collection; he also recommended the use of citrate or heparin rather than EDTA as anticoagulant for serum samples (EDTA may be permissible only at low concentrations) and pointed to cases in which serum yielded higher TAC values than plasma due to the possible release of antioxidants from blood platelets during the clotting process.[33]

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There is a lack of correlation between activities determined by the same antioxidant by different assays and between activities determined by the same assay in different laboratories.[58] Especially assays based on the inhibition of lipid peroxidation are known to correlate poorly with either HAT- or ET-based assays, meaning that an antioxidant with a high TEAC value in routine TAC assays may not perform well in preventing/retarding lipid peroxidation. The reason is that every assay has its own unique thermodynamic and kinetic characteristics, and the oxidizing power of each TAC reagent against a given antioxidant within a fixed time is naturally different from that of another. Even the same assay with slight differences in reagent preparation may give rise to serious differences in results: for example, the ORAC assay using two different probes, β-phycoerythrin and fluorescein, may give entirely different TEAC values for quercetin as 2.07 ± 0.05 and 7.28 ± 0.22, respectively; ABTS radical yields 0.55 and 0.94 TEAC values for glutathione using the ABTS/H_2O_2/HRP and $ABTS^{\bullet+}$ decolorization methods, respectively; the TEAC value for ascorbic acid may change 3-fold depending on the type of oxidant used for generating $ABTS^{\bullet+}$ radical, that is, MnO_2 versus persulfate.[33] Generally ET-based assays correlate well among themselves due to the similarity in mechanism of action, and therefore some antioxidant researchers consider the application of a series of ET-based assays redundant. On the other hand, it is well-known that HAT- and ET-based assays such as ORAC, ABTS/TEAC, and FRAP give none or weak correlations for plasma samples.[33] In this regard, the antioxidant activities of common vegetables (total sample size = 927) collected from the U.S. market, analyzed using the ORAC and FRAP procedures, did not correlate well.[59] Cao and Prior observed a weak linear correlation between serum ORAC and serum FRAP, but no correlation either between serum ORAC and serum ABTS/TEAC or between serum FRAP and serum ABTS/TEAC.[60] Prior et al. recommended the evaluation of overall antioxidant capacity by using multiple assays to generate an "antioxidant profile" encompassing reactivity toward both aqueous and lipid/organic radicals directly via radical quenching and radical reducing mechanisms and indirectly via metal complexing.[2] The use a variety of assays with different mechanisms (such as ET-, HAT-, and lipid peroxidation-based assays) may generally be recommended for complex samples to see the whole picture for antioxidant action.

2.1.2.1. Spectroscopic Methods. 2.1.2.1.1. Folin−Ciocalteu (FC) Assay. The Folin−Ciocalteu (FC) method was initially intended for the analysis of proteins, taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue, where tyrosine reacted with the heteropoly reagent to give a blue color proportionate to the protein content.[34] Much later, Singleton et al.[35] extended this assay to the analysis of total phenols in wine. Fundamentally, the FC assay is based on the oxidation of phenol compounds in alkaline (carbonate) solution with a molybdotungstophosphate heteropolyanion reagent ($3H_2O-P_2O_5-13WO_3-5MoO_3-10H_2O$), yielding a colored product with a broad band having an absorbance maximum (λ_{max}) lying between 750 and 765 nm. Although a similar phosphomolybdenum blue formation method, without tungstate in the reagent, was also reported for antioxidant capacity determination in acidic medium at elevated temperature,[61] it was not tested for a wide variety of antioxidants and its molar absorptivity for vitamin E was rather low, which possibly restricted further practice of the method.

The possible simultaneous occurrence of several reduced species in the FC heteropoly chromophore can account for the broad peaks. Although the color may be developed more quickly at warmer temperature, the loss of color with time is greater at higher temperatures. As tannic acid from different preparations of wines and spirits could vary, and other tannins covered a wide range of color yield per unit weight, Singleton et al.[35] replaced tannin (as a reference compound) with GAE in reporting FC results; the minimum detectable amount of phenols was at the order of 3 mg GAE/L, depending on optical cuvette thickness. The gallic acid added to wine was recovered quantitatively, and the absorbance produced from a mixture of natural phenols of different classes was equivalent to the sum of their individual contributions, meaning that chemical deviations from Beer's law was essentially absent in the FC system.

Neither the exact chemical nature nor the redox potential of the FC phenol reagent is definitely known. It is a strong oxidizing agent and can nonspecifically oxidize many nonphenolic reducing compounds including weak reductants [e.g., aromatic amines, sulfite, ascorbic acid, Cu(I), Fe(II), etc.] along with phenolics.[15] Due to this oxidative ability, FC was proposed to be used as a TAC reagent in the reducing capacity assay for antioxidants, where the molybdenum center in the complex reagent is reduced from Mo(VI) to Mo(V) with an electron donated by an antioxidant to produce a blue color.[14] Unfortunately, the detailed molecular and electronic structures of the blue reduction products are unclear, but it is known that molybdates are more easily reduced than tungstates in heteropoly salts. The FC assay has certain advantages over some other TAC assays in that it is simple, fast, and robust, does not require specialized equipment, and the long-wavelength absorption of the chromophore minimizes interference from the sample matrix. However, a drawback of the FC assay is that reducing agents such as ascorbic acid, citric acid, simple sugars, or certain amino acids can interfere with the analysis and thus overestimate the content of phenolic compounds. Tryptophan, indoles, purines, guanine, xanthine, and uric acid were also reported to react with the FC reagent to yield molybdenum blue.[35] The conventional FC reagent is only applicable to water-soluble antioxidants and operates at an unrealistically high pH. To avoid the possible air oxidation of the tested phenols before the color reaction, the FC reagent should be added before alkali. Nevertheless, FC is routinely practiced in antioxidant research laboratories for testing food and plant extracts. Its fully automated−continuous flow (40 samples/hour) procedure was also adapted.[62]

Because most phenolic compounds are in dissociated form (as conjugate bases, mainly phenolate anions) at the working pH of the assay (pH ≈10), they can be more easily oxidized with the FC reagent, possibly giving rise to an overestimated TAC value.[14,56] The FC chromophore, the molybdotungstophosphate heteropolyanion ($PMoW_{11}O_{40}^{4-}$), does not have an affinity toward organic solvents owing to its quadruple negative charge[2] giving rise to strong ion−dipole interactions with solvent water molecules. Thus, the FC method was modified and standardized by Berker et al.[63] so as to enable simultaneous measurement of lipophilic and hydrophilic antioxidants in NaOH-added isobutanol−water medium. The modified procedure was successfully applied to the total antioxidant capacity assay of Trolox, quercetin, ascorbic acid, gallic acid, catechin, caffeic acid, ferulic acid, rosmarinic acid, glutathione, and cysteine, as well as of lipophilic antioxidants such as α-tocopherol

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(vitamin E), butylated hydroxyanisole, butylated hydroxytoluene, *tert*-butylhydroquinone, lauryl gallate, and β-carotene.[63]

2.1.2.1.2. FRAP Assay. The FRAP assay, first introduced by Benzie and Strain to determine the antioxidant capacity of plasma and later modified for application to other matrices such as tea and wine,[42−45] is based on the reduction of Fe(III) to Fe(II) by antioxidants in the presence of tripyridyltriazine tridentate ligand forming a colored complex with Fe(II):

$$\mathrm{Fe(TPTZ)_2}^{3+} + \mathrm{ArOH} \rightarrow \mathrm{Fe(TPTZ)_2}^{2+} + \mathrm{ArO}^{\bullet} + \mathrm{H}^{+} \quad (16)$$

TPTZ denotes the 2,4,6-tripyridyl-*s*-triazine ligand, and the absorption maximum lies at a wavelength of λ_{max} = 593 nm. Although Fe(III)−Fe(II) reduction may equally well produce colored chelates in the presence of *ortho*- and *batho*-phenanthrolines, yielding reduction potentials exceeding 1.0 V, TPTZ was carefully selected, because $E^0_{Fe(III)-Fe(II)}$ in the presence of TPTZ is only 0.70 V, which may selectively oxidize most antioxidants but not citric acid and simple sugars. The FRAP assay is simple, practical, and inexpensive and may offer a putative index of antioxidant capacity.[15] Because the FRAP reaction with antioxidants produces a single colored product, that is, Fe(II)−TPTZ, the FRAP absorbance versus concentration curves are well linear over a wide range, and TAC additivity is usually observed in mixtures—except for those containing small-molecular and protein thiols.[11,64]

Pulido et al.[45] compared the antioxidant efficiency of a number of antioxidant compounds with the use of equivalent concentrations (EC_1), defined as the concentration of antioxidant with a reducing effect equivalent to 1 mmol/L Fe(II), and found that polyphenols had lower EC_1 values, and therefore higher reducing power, than ascorbic acid and Trolox. Tannic acid and quercetin had the highest antioxidant capacity followed by gallic and caffeic acids, whereas resveratrol showed the lowest reducing effect and carotenoids had no ferric reducing ability. This inability of the FRAP method to assay thiols[64] and carotenoids,[45] possibly due to the kinetic inertness of high-spin Fe(III) and the problems associated with mutual solubility of reagent and analyte in the same solvent medium, respectively, has also been criticized in various research papers by other users of the method. Especially the inability of the FRAP reagent to effectively oxidize biothiols makes the method rather ineffective in evaluting the TAC of intracellular fluids and human plasma/serum.[11,64,65] Magalhaes et al.[15] attribute this specific inadequacy of FRAP (i.e., an ET reagent) to the mode of action of thiols and carotenoids (i.e., antioxidants mainly acting by H atom transfer). However, if the problem had merely arisen from H atom transfer, CUPRAC, as another well-known ET-based method, would not have responded to carotenoids in aqueous acetone solution.[66] High-spin iron(III) in the reagent has half-filled d-orbitals responsible for kinetic inertness, and thiol (RSH) oxidations usually proceed through thiyl ($RS^{\bullet}$) radical intermediates, which do not form appreciably at the acidic pH of the FRAP protocol.[67] Another fact worthy of comment is that polyphenols with slow kinetic behaviors such as caffeic acid, tannic acid, ferulic acid, *p*-coumaric acid, and quercetin cannot be fully oxidized within the protocol time (i.e. typically 4 min) of the FRAP assay.[14] FRAP reactions are carried out in acidic medium to suppress Fe(III) hydrolysis (i.e., at pH 3.6) where phenolic antioxidants are not dissociated, and therefore lower results than actual TAC are to be expected because phenolates are oxidized much more rapidly than the corresponding phenols. FRAP and similar Fe(III) reduction-based ET assays were also criticized for producing Fe(II) as the reduction product, which could give rise to the generation of reactive species (such as hydroxyl radicals) upon Fenton-like reactions with H_2O_2, thereby causing "redox cycling" of phenolics and yielding erroneous TAC results.[30] Antolovich et al.[68] are of the opinion that the measured ferric reducing capacity does not necessarily reflect antioxidant activity, but instead provides a very useful "total" antioxidant concentration, without measurement and summation of the concentration of all antioxidants involved. Pulido et al.[45] concluded that the antioxidant efficiency of polyphenols depended on the extent of hydroxylation and conjugation. Specifically for flavonoids, scientists investigating structure−activity relationships of antioxidants suggested that the free radical-scavenging ability increases when the following criteria are met: (i) the presence of a 3′,4′-dihydroxy structure in the B ring; (ii) the presence of a 2,3-double bond in conjunction with the 4-oxo group in the heterocycle, allowing for conjugation between the A and B rings; (iii) the presence of 3- and 5-hydroxyl groups in the A ring together with a 4-oxo function in the A and C rings (Figure 3).[69,70] The results of Pulido et al.[45]

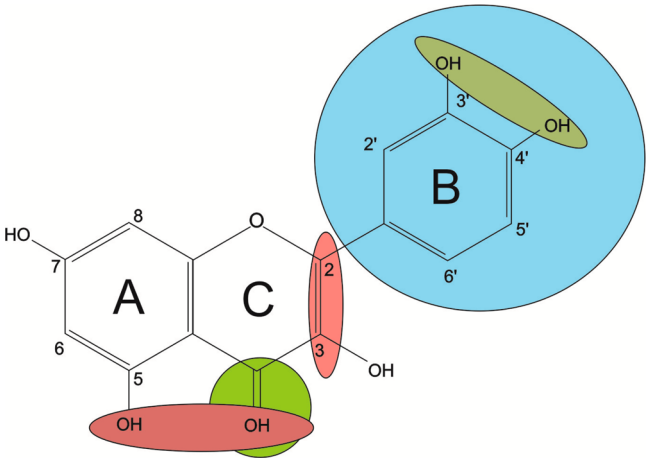


Figure 3. Characteristic functional groups having a key role in the high antioxidant capacity of a flavonoid (such as quercetin).

with flavonoids agreed with these criteria; for example, quercetin, meeting all of the listed three conditions, was more potent than rutin (a flavonoid glycoside) and catechin (lacking coplanarity due to the absence of 2,3-double bond and therefore having hindered conjugation/resonance stabilization over the whole molecule).

2.1.2.1.3. CUPRAC Assay. The CUPRAC assay of TAC determination is only 11 years old[40] but has branched into various modified methods of antioxidant capacity/activity measurement associated with Cu(II)−Cu(I) reduction in the presence of a selective Cu(I)-stabilizing ligand, neocuproine (2,9-dimethyl-1,10-phenanthroline). It has also been applied to various matrices containing both hydrophilic and lipophilic antioxidants.

The main method is based on the absorbance measurement of the CUPRAC chromophore, Cu(I)−neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine)copper(II) cation [Cu(II)-Nc], where absorbance is recorded at the maximal light absorption wavelength of 450 nm (Figure 4).

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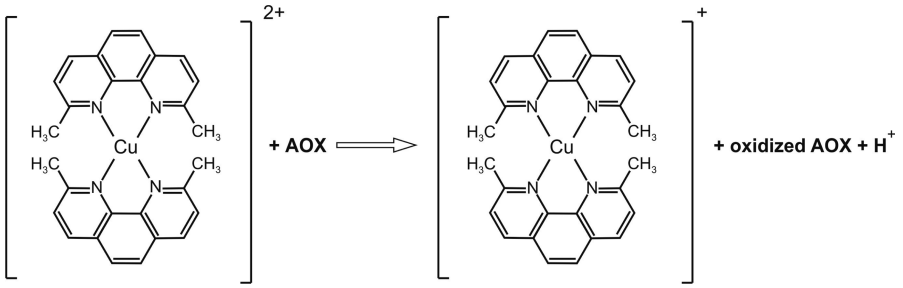


Figure 4. Reaction scheme for the CUPRAC antioxidant capacity assay (liberated protons are buffered by ammonium acetate).

The chromogenic oxidizing reagent of the developed CUPRAC method, Cu(II)-Nc, reacts with *n*-electron reductant antioxidants (AOX), according to eq 17:

$$n\mathrm{Cu(Nc)_2}^{2+} + n\text{-electron reductant (AOX)} \leftrightarrow n\mathrm{Cu(Nc)_2}^{+} + n\text{-electron oxidized product} + n\mathrm{H}^{+} \quad (17)$$

In this reaction, the oxidation of reactive Ar—OH groups of polyphenolic antioxidants to the corresponding quinones (Ar═O) of ascorbic acid to dehydroascorbic acid and of thiols to the corresponding disulfides occurs, whereas Cu(II)-Nc is reduced to the yellow-orange colored $Cu(Nc)_2^{+}$ chelate. Although the concentration of Cu^{2+} ions is in stoichiometric excess of that of Nc in the CUPRAC reagent for driving the redox equilibrium reaction to the right, the actual oxidant is the $Cu(Nc)_2^{2+}$ species and not the sole Cu^{2+}, because the standard redox potential of the Cu(II/I)-Nc is 0.6 V, much higher than that of the Cu^{2+}/Cu^{+} couple (0.17 V).[71] The reason is that Cu(I)-Nc is perfectly tetrahedral owing to the d^{10}-electronic configuration of Cu(I) having sp^3 hybridization, whereas two molecules of neocuproine give a distorted tetrahedral structure to Cu(II) having d^9-configuration [i.e., known as the Jahn−Teller effect in coordination chemistry, which is enhanced when solvent molecules are also attached to $Cu^{II}(Nc)_2$ in octahedral coordination], thereby selectively stabilizing Cu(I) over Cu(II) [i.e. the logarithmic stability constants of $Cu^{II}(Nc)_2$ and $Cu^{I}(Nc)_2$ are 12 and 19, respectively].[72] As a result, polyphenols are oxidized much more rapidly and efficiently with Cu(II)-Nc than with Cu^{2+}, and the amount of colored product [i.e., Cu(I)-Nc chelate] emerging at the end of the redox reaction is equivalent to that of reacted Cu(II)-Nc.[71] The liberated protons are buffered in ammonium acetate medium, which provides a pH of 7.0 basically conforming to physiological conditions. The highest antioxidant capacities in the CUPRAC method were observed for epicatechin gallate, rosmarinic acid, epigallocatechin gallate, quercetin, fisetin, epigallocatechin, catechin, caffeic acid, epicatechin, gallic acid, rutin, and chlorogenic acid in this order,[73,74] in accordance with theoretical expectations, because the number and position of the hydroxyl groups as well as the degree of conjugation of the whole molecule are important for easy electron transfer.[70]

Among the three substituted phenanthrolines, used before in antioxidant or protein assays, capable of selective stabilization of Cu(I), thereby increasing the Cu(II,I) reduction potential, namely, Nc (neocuproine), BCS (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline disulfonic acid),[75] and BCA [bicinchoninic acid, 2-(4-carboxyquinolin-2-yl)quinoline-4-carboxylic acid],[76] only neocuproine with the CUPRAC method has found wide use as an effective tool in antioxidant research.[40,41] Certainly, there are a number of reasons for this choice due to the more diverse areas of usage of the cupric-neocuproine reagent. For example, it is known that Cu(I)-BCS has a higher overall charge than Cu(I)-Nc due to the presence of negatively charged sulfonate groups on the phenanthroline ring, giving rise to stronger ion−dipole interaction of the former with water molecules and subsequent cell membrane impermeability. As a result, the Cu(I)-BCS method is expected to be less useful than CUPRAC for the TAC assay of tissue homogenates. ET-based antioxidant assays may show significant solvent dependencies and differences in proton-coupled electron transfer rate,[77] and it has been shown by Çelik et al.[78] that the cupric-BCS assay is not competent with conventional CUPRAC using cupric-neocuproine reagent in regard to reaction kinetics and response to lipophilic plasma antioxidants (e.g., β-carotene, α-tocopherol). The standard reduction potential of the Cu(II,I)-BCS couple was reported to be $E^0 = 0.844$ V,[79] a little higher than those of most widely used ET reagents, possibly affecting selectivity toward antioxidant compounds. On the other hand, although BCA provides a longer wavelength (558 nm) than Nc for cuprous chelate absorption, which may seem advantageous at first glance for preventing the possible interferences of plant pigments during measurement, Marques et al.[80] recently discovered that in the BCA assay, the concentration of free Cu^{2+} ions cannot be maintained in excess (i.e., required for the completion of certain oxidation reactions) because of the precipitation of the complex.[81] To guarantee a complete complexation without precipitation, the BCA ligand must be added in stoichiometric ratio or in excess. Therefore, the potential complexation of other metal ions that might be present in the sample (for example, Fe) cannot be ignored, and this situation limits the applicability of BCA.[80]

Owing to the favorable redox potential in neutral medium, the CUPRAC method has a better chance of simulating physiologically important redox reactions of antioxidant compounds, including serum antioxidants. In the normal CUPRAC method ($CUPRAC_N$), the oxidation reactions of most food/biological antioxidants are essentially complete within 30 min. Flavonoid glycosides require acid hydrolysis to their corresponding aglycons for fully exhibiting their antioxidant potency. Slow-reacting antioxidants may need elevated temperature incubation so as to complete their oxidation with the CUPRAC reagent.[40,41] Although the protocol time period of the CUPRAC assay is set at 30 min, a few antioxidants with high redox potentials, such as naringin, naringenin, and bilirubin, may not reach absorbance saturation so easily. On the other hand, because 80−90% of the peak absorbances for a great majority of antioxidants is reached within the first few minutes, online HPLC post-column detection and voltammetric modifications of CUPRAC may allow a reaction time of 1 min for antioxidants with the Cu(II)-neocuproine reagent.

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The CUPRAC method of TAC assay has been successfully applied to antioxidants in food plants and human serum and to hydroxyl and superoxide radical scavengers. In the assay of human serum antioxidants, hydrophilic antioxidants were measured after precipitation of proteins with perchloric acid (trichloroacetic acid, ammonium sulfate, and organic solvents are other known protein precipitation reagents), whereas lipophilic ones such as α-tocopherol and β-carotene were determined by *n*-hexane extraction and evaporation, followed by color development in dichloromethane (DCM) of the Cu(I)-Nc charge-transfer complex formed from their CUPRAC reaction.[41] Because the CUPRAC chromophore, that is, Cu(I)-neocuproine cation, has a large molecular size, it has a very weak hydration sphere and therefore is easily extracted into an organic solvent such as DCM due to its low hydration energy [for divalent chromophores such as Fe(II)-tripyridyltriazine cation or tetravalent Folin−Ciocalteu chromophore, phosphotungsto-molybdate anion, this extraction is not so easy because of enhanced ion−dipole interactions with solvent water molecules]. This feature of the Cu(I)-Nc chelate provides a great advantage for the CUPRAC method to be applicable to lipophilic antioxidants along with hydrophilic ones. In a miniaturized CUPRAC method without preliminary separation of lipophilic and hydrophilic serum antioxidants, serum samples were centrifuged after 10% TCA precipitation, and CUPRAC was directly applied to the supernate.[82]

Because essentially flavones and flavonols (and other flavonoids to a lesser extent) could be chelated with lanthanum(III) in the form of basically nonpolar complexes and ascorbic acid (AA) either did not complex or formed very weak hydrophilic complexes under the same conditions, AA assay with a high redox equilibrium constant of the CUPRAC reaction with preliminary extraction of flavonoids as their La(III) complexes was possible.[83] Lipophilic and hydrophilic antioxidants (e.g., β-carotene, α-tocopherol, AA, quercetin, etc.) could be simultaneously assayed with a modified CUPRAC method in the same solvent medium of acetone/water (9:1, v/v) with the aid of their inclusion complexes formed with 2% methyl-β-cyclodextrin (M-β-CD), because this oligosaccharide can form inclusion complexes (Figure 5) with lipophilic antioxidants in the interior part while the outer part binds hydrophilic antioxidants.[84]

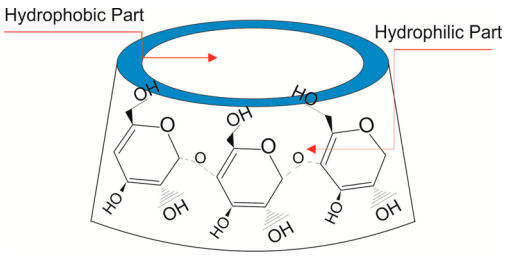


Figure 5. Methyl-β-cyclodextrin oligosaccharide, retaining lipophilic antioxidants in the hydrophobic inner core while holding hydrophilic antioxidants on the outer surface.

The CUPRAC assay could be further modified to fit the needs of scavenging activity determinations of ROS/RNS such as hydrogen peroxide, superoxide anion, and hydroxyl radicals, where either the original probe or the converted product had CUPRAC reactivity. In measuring the hydroxyl radical-scavenging activity of certain water-soluble compounds (metabisulfite, thiourea, glucose, lysine, etc.), the probes of *p*-aminobenzoate, 2,4-dimethoxybenzoate, and 3,5-dimethoxybenzoate were used to detect hydroxyl radicals, and the $^{\bullet}$OH scavenging rate constants of these compounds were determined by competition kinetics.[85] In the measurement of hydroxyl radical-scavenging activities of polyphenolics, special measures were taken to prevent the redox cycling of phenolic compounds that could otherwise produce unrealistic results. For this purpose, the Fenton reaction was stopped at the end of the 10th minute with the addition of catalase to annihilate hydrogen peroxide and cease $^{\bullet}$OH production, and the dihydroxybenzoates formed from the salicylate probe under hydroxyl radical attack (Figure 6) were measured with the CUPRAC method, rate constants being calculated with competition kinetics.[12]

In another modified CUPRAC method, the superoxide anion radical was generated with xanthine−xanthine oxidase (X-XO), and the inhibition of the enzyme was measured upon addition of polyphenolics to the system.[86]

The hydrogen peroxide scavenging (HPS) activity of the polyphenolics was measured in the presence of Cu(II) (as catalyst) with the HPS-CUPRAC method.[87] A low-cost optical antioxidant sensor (CUPRAC sensor) was developed by immobilizing the Cu(II)-Nc reagent onto a perfluorosulfonate cation-exchange polymer membrane matrix (Nafion),[73] and the colored Cu(I)-Nc cation was produced on the membrane without diffusing into solution. This membrane sensor provided great ease and convenience to TAC determinations, like a sensitive pH paper immersed in solution for hydrogen ion activity determination.

A novel online HPLC-CUPRAC method was developed for the selective determination of polyphenols in complex plant matrices. This method combines chromatographic separation, constituent analysis, and postcolumn identification of antioxidants in plant extracts. Antioxidant polyphenols in complex samples can be separated on a C18 HPLC column (diode array detected at 280 nm) and further react with the Cu(II)-Nc reagent in a postcolumn reactor to yield the Cu(I)-Nc chromophore detected at 450 nm. Thus, twice as much information can be extracted from the same sample using these two chromatograms, that is, their separability through a C18 column and their CUPRAC reactivity in the postcolumn. This robust online chromatographic method enables individual detection/quantitation of antioxidant constituents as well as total measurement of TAC; moreover, non-antioxidants are not detected in the postcolumn chromatogram, increasing the selectivity of the method.[88]

In protein TAC determination, the classical buffer of the CUPRAC reaction, that is, ammonium acetate, should be replaced with urea at the same pH to prevent reprecipitation of dissolved proteins. CUPRAC in urea buffer also responded to thiol-containing proteins in foods and serum.[11,64] Another modified CUPRAC method is composed of a *tert*-butylhydroquinone (TBHQ) probe with the phenazine methosulfate/β-nicotinamide adenine dinucleotide (PMS/NADH) nonenzymic $O_2^{\bullet-}$-generating system for superoxide radical scavenging activity (SRSA) assay of thiol-type antioxidants (e.g., GSH, cysteine), amino acids (e.g., serine, threonine), plasma antioxidants (e.g., bilirubin, albumin), and other antioxidants (e.g., methionine); the SRSA method is based on the measurement of the CUPRAC absorbance of the remaining TBHQ in the reaction medium (TBHQ is CUPRAC-reactive, whereas its oxidation product is not (Figure 7), and this probe is isolated by ethyl acetate extraction from other CUPRAC-reactive interferents remaining in the aqueous phase).[89]

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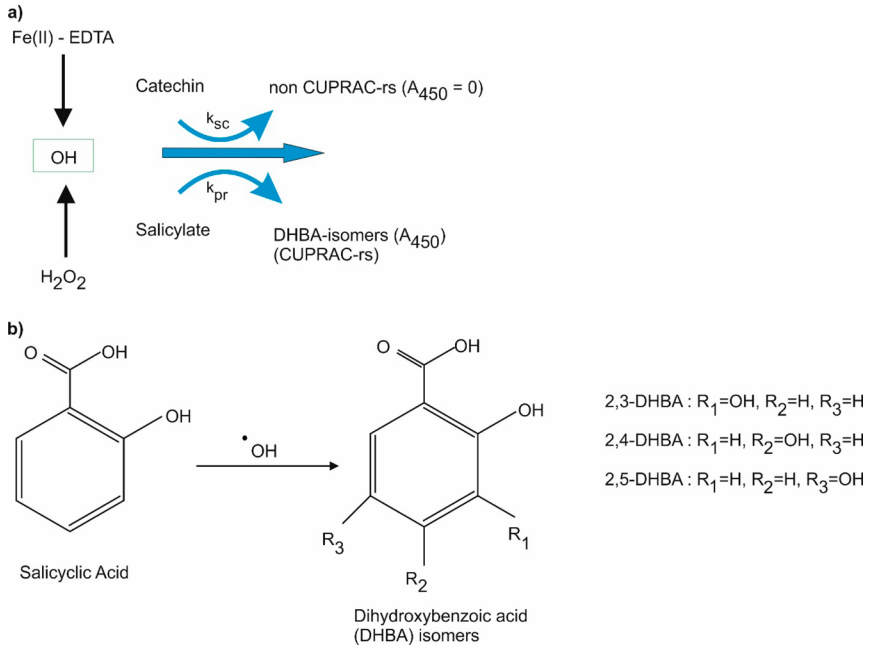


Figure 6. (a) Salicylate exposed to hydroxyl radicals generated in a Fenton system is converted to highly CUPRAC-reactive species (CUPRAC-rs), that is, dihydroxybenzoate (DHBA) isomers, enabling a turn-on colorimetric assay for hydroxyl radicals. (b) Primary hydroxylation products of salicylate.

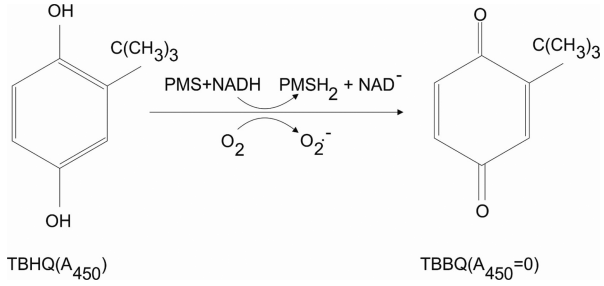


Figure 7. Superoxide anion radicals generated by a nonenzymatic PMS-NADH system attack the CUPRAC-reactive TBHQ probe, converting it to nonreactive TBB-quinone (TBBQ). This conversion is less in the presence of antioxidants, enabling a modified CUPRAC assay for superoxide scavenging antioxidants.

The CUPRAC assay and its modifications for ROS scavenging measurements have been summarized, and the methodology has been demonstrated to have certain advantages over other similar ET-based TAC methods, mentioned in a comprehensive review by Özyürek et al.:[8]

(i) The CUPRAC reagent, being an outer-sphere electron-transfer agent, is capable of rapidly oxidizing thiol-type antioxidants, whereas iron(III)-based ET-methods such as FRAP may only measure limited thiols such as GSH with serious negative error, possibly due to the kinetic inertness of high-spin Fe(III) and the inadequate formation of thiyl radicals (i.e., intermediary species of thiol oxidation) in acidic medium. Because the redox potential of oxidized and reduced forms of glutathione ($E^0_{GSSG/2GSH}$) is the basic indicator of the biological conditions of a cell and GSH acts as reconstituent of intercellular ascorbic acid from the dehydroascorbic acid, an ET assay should be capable of accurately measuring glutathione among plasma antioxidants.

(ii) The redox potential of Cu(II,I) chelated to neocuproine is only 0.6 V, close to that of $ABTS^{\bullet+}$/ABTS (E^0 = 0.68 V) and FRAP (E^0 = 0.70 V), all versus NHE. Simple sugars and citric acid, which are not classified as antioxidants, are not oxidized with the CUPRAC reagent. On the other hand, the ferric/ferrous potential in the presence of *ortho*-phenanthroline or *batho*-phenanthroline-type ligands is much higher, adversely affecting selectivity.

(iii) The reagent is much more stable and readily accessible than the chromogenic radical reagents [e.g., ABTS, DPPH, galvinoxyl and *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD)].

(iv) The CUPRAC method shows versatility to the determination of both hydrophilic and lipophilic antioxidants, because the CUPRAC chromophore, bis(neocuproine)-copper(I) chelate, has unipositive charge having less ion−dipole interaction with water, and the chelate rings are essentially hydrophobic. Thus, it is compatible with aqueous and organic solvents (alcohols, acetone, dichloromethane, etc.) and alcohol−water mixtures. The robustness of the CUPRAC assay to different solvents has been recently confirmed by Christodouleas et al.[90] in the TAC assay development study for edible oils.

(v) The redox reaction giving rise to the Cu(I)-Nc chromophore is relatively insensitive to a number of parameters (e.g., air, sunlight, humidity, and, to a certain extent, pH) adversely affecting radical reagents such as DPPH.

(vi) The CUPRAC reagent can be adsorbed on a perfluorosulfonate cation-exchanger membrane enabling the manufacture of a low-cost, linear response antioxidant sensor. CUPRAC is also adaptable to online HPLC applications with the use of a postcolumn reactor, enabling sensitive determination of antioxidants individually.

(vii) CUPRAC usually gives perfectly linear absorbance/concentration curves ($r \approx 0.999$) over a wide concentration

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range, as opposed to certain other methods yielding polynomial curves. The molar absorptivity (extinction coefficient) for *n*-electron reductants, that is, $(7.5-9.5) \times 10^3\ n)\ M^{-1}\ cm^{-1}$, is sufficiently high to enable sensitive determination of most phenolic antioxidants.

(viii) The CUPRAC spectrophotometric method obeys Beer's law in regard to the additivity of absorbances due to individual antioxidant constituents, because a single chromophore, cuprous-neocuproine, is formed upon reduction of the CUPRAC reagent with antioxidants. Consequently, the CUPRAC-TAC values of antioxidants in complex mixtures are perfectly additive (e.g., the TAC of a phenolic mixture is equal to the sum of individual antioxidant capacities of its constituent polyphenols).

(ix) CUPRAC operates at nearly physiological pH (pH 7 of ammonium acetate buffer) as opposed to the unrealistic acidic conditions (pH 3.6) of FRAP or alkaline conditions (pH 10) necessary for phenols to dissociate protons in the Folin–Ciocalteu assay. At more acidic conditions than the physiological pH, the reducing capacity may be suppressed due to protonation on antioxidant compounds, whereas in more basic conditions, deprotonation of phenolics enhances a sample's reducing capacity, thereby causing unrealistic TAC measurements.

(x) Because the Cu(I) ion emerging as a product of the CUPRAC redox reaction is in a coordinatively saturated state (i.e. two molecules of neocuproine tetrahedrally coordinate the cuprous ion), it cannot act as a prooxidant that may cause oxidative damage to biological macromolecules in body fluids. Fe(III)-based assays were criticized for producing Fe^{2+}, which may act as a prooxidant to produce $^{\bullet}OH$ radicals as a result of its reaction with H_2O_2 and subsequently cause a "redox cycling" of antioxidants during the assay, yielding unreliable results. Ferric or ferrous iron, even in full octahedrally coordinated (e.g., EDTA-chelated) state, can catalyze the decomposition of hydrogen peroxide to reactive species.[91] On the other hand, it was experimentally shown that the stable Cu(I)-Nc chelate did not react with H_2O_2, but the reverse reaction [i.e., oxidation of H_2O_2 with Cu(II)-Nc] is possible, excluding the possibility of redox cycling of antioxidants with the Cu(I)-Nc product.

(xi) The CUPRAC method has recently been implemented in microplate and flow modes, allowing the in vitro assessment of antioxidant capacity of endogenous and dietary molecules as well as the TAC determination of human biological samples.[92]

(xii) As noteworthy CUPRAC users, Gorinstein research groups stated that, as an advantage over other ET-based assays, the CUPRAC test gave reproducible values that were acceptable in regard to its realistic pH close to physiological pH in various food extracts (garlic,[93] onion, kiwi, etc.). Bean et al.[65] made a comparative evaluation of antioxidant reactivity within obstructed (i.e., resulting from the attack of ROS/RNS in cyclic ischemia and reperfusion) and control rabbit urinary bladder tissue using FRAP and CUPRAC assays and found that CUPRAC, but not FRAP, could detect a significant decrease in the reactivity of antioxidants found within the obstructed bladder tissue as compared to the control bladder tissue in both the muscle and mucosa. Bean et al.[65] concluded that, as the CUPRAC assay was responsive to hydrophilic, lipophilic, and thiol-containing antioxidants at physiological pH, it was a much better tool to analyze the reactivity found within tissues.

2.1.2.1.4. Ferricyanide (Hexacyanoferrate(III))–Prussian Blue Assay. The ferricyanide–Prussian blue assay is based on the following chemical reactions:

$$Fe(CN)_6^{3-} + ArOH \rightarrow Fe(CN)_6^{4-} + ArO^{\bullet} + H^+ \quad (18)$$

$$Fe(CN)_6^{4-} + Fe^{3+} + K^+ \rightarrow KFe[Fe(CN)_6] \quad (19)$$

with $\lambda_{max} = 700$ nm

In the conventional method,[46] the hexacyanoferrate(III) (common name, ferricyanide) reagent is first incubated in ($H_2PO_4^-/HPO_4^{2-}$) buffer at pH 6.6 with antioxidants (at 50 °C for 20 min), and the reduction product, hexacyanoferrate-(II) (common name, ferrocyanide), combines with the later added Fe(III) to produce Prussian blue, suspended in the medium. The method is also referred to as the "reducing power" assay.[94]

Although the Fe(III,II) standard reduction potential is 0.77 V, causing nonspecific oxidation of any species having a redox potential smaller than this value,[59] suitable selection of ligands may bring this value close to the range of common food and biological antioxidants (i.e., having standard potentials in the range of 0.2–0.6 V). In the case of cyanide complexes of iron in hexacyanoferrate complex ions, the logarithmic stability constant of Fe(III) complex is greater than that for Fe(II) [i.e., log β_6 values for $Fe(CN)_6^{3-}$ and $Fe(CN)_6^{4-}$ are 31 and 24, respectively]. Therefore, according to the Nernst equation, the reduction potential for hexanocyanoferrate (III,II) couple is 0.36 V versus NHE, less than that of Fe(III,II). This potential may not be sufficient for oxidizing certain antioxidants having a reduction potential (of the $ArO^{\bullet}$/ArOH couple) $E^0 > 0.4$ V, and therefore modification of the conventional ferricyanide assay[46] was a requirement for comprising diverse antioxidants. The wavelength of maximum absorption shifts to longer wavelengths in the order of ferric-complexing ligands: *ortho*-phen < *batho*-phen < tripyridyltriazine (FRAP) < ferricyanide. The bathochromic shift in λ_{max} and band broadening was strongest for Prussian blue in the ferricyanide method, because the bonding and antibonding energy levels were closest in $Fe[Fe(CN)_6]^-$ due to the interchanging oxidation states of iron centers in this complex salt. Longer wavelengths almost always constitute an important advantage in spectrophotometric method selection, because most plant pigments as well as some antioxidants show significant absorption at shorter wavelengths of the visible region, close to the UV range of the visible spectrum.[48]

The conventional ferricyanide assay was first modified by incorporating Fe(III) to the ferricyanide reagent in acidic medium and incubating at elevated temperature (30 min of incubation at 50 °C), but gave rise to overoxidation of certain antioxidants (especially of hydroxycinnamic acids such as caffeic and ferulic acids) causing distinct deviations from linearity of calibration curves.[48] Then the authors introduced a second modification of the method, by adding Fe(III) at the start and optimizing the acidity of the incubation medium as pH 1.6 [to prevent Fe(III) hydrolysis] and by adding the anionic surfactant SDS to stabilize the negatively charged Prussian blue complex ion: $Fe[Fe(CN)_6]^-$.[47] Compared to the original method,[46] stabilization with SDS enabled a color development time of 30 min at room temperature (instead of the 2 min reaction time of Oyaizu to avoid Prussian blue precipitation)

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and a wavelength (λ_{max}) shift from 700 to 750 nm. The modified procedure was named the "ferric−ferricyanide assay" because it was not clear whether Fe(III) or ferricyanide was the actual oxidant. Consequently, the oxidation equilibria for antioxidants by the combined [Fe(III) + $Fe(CN)_6^{3-}$] reagent significantly shifted to the right, possibly increasing the potential above that of the Fe(III,II) couple, owing to the formation of insoluble Prussian blue; that is, if the actual oxidant was Fe(III), then its reduced form, Fe(II), would again produce the same Prussian blue product with the ferricyanide constituent of the reagent:

$$Fe^{3+} + ArOH \rightarrow Fe^{2+} + ArO^{\bullet} + H^{+} \quad (20)$$

$$Fe^{2+} + Fe(CN)_6^{3-} + K^{+} \rightarrow KFe[Fe(CN)_6] \quad (21)$$

with $\lambda_{max} = 750$ nm

Finally, with the third modification of the method, Berker et al.[95] were able to measure lipophilic (e.g., α-tocopherol, BHT, and β-carotene) and hydrophilic antioxidants in the same solution comprising 1:9 (v/v) H_2O/acetone with or without 2% methyl-β-cyclodextrin; this was necessary, as the original ferricyanide method could only assay hydrophilic antioxidants. This modified assay was not adversely affected from citric acid and simple sugars and proved to be additive for TAC values of complex mixtures.[95]

2.1.2.1.5. ET-Based Spectrophotometric Assays Involving Strongly Oxidizing Reagents [Ce(IV), Cr(VI), and Mn(VII) Assays]. Strong oxidizing agents such as Ce(IV), Cr(VI), and Mn(VII) may be used as chromogenic TAC reagents only if their oxidizing powers are decreased to the level of specifically oxidizing common antioxidants but not other organic substances (i.e., their redox potentials should be brought to roughly 0.6−0.7 V of widely used TAC reagents). This can usually be accomplished by increasing the working pH [e.g., the extent of chromate(VI) and permanganate(VII) oxidations is a function of H^+ ion concentration] and/or by selectively stabilizing the higher oxidation state of the redox couple [e.g., by preferential complexation of Ce(IV) over Ce(III) with sulfate] to decrease the Nernst potential [$E = E^0 + (RT/nF)$ ln $[c_{ox}/c_{red}]$, where E and E^0 are the instantaneous and standard values of the reduction potential, respectively; R is the universal gas constant; T is the absolute temperature; n is the number of electrons involved in half-cell reaction; F is Faraday's constant; and c_{ox} and c_{red} are the concentrations of the oxidized and reduced forms, respectively, of the redox-active constituent of the TAC reagent]. Another disadvantage of the use of strong oxidizing agents in TAC assays is their hydrophilicity; that is, they cannot be applied to lipophilic antioxidant testing.

Özyurt et al.[51] developed a simple, sensitive, and low-cost indirect spectrophotometric method for the determination of Ce(IV) reducing antioxidant capacity (CERAC) of plant extracts, based on the oxidation of antioxidants with cerium(IV) sulfate in dilute sulfuric acid at room temperature. The spectrophotometric determination of the remaining Ce(IV) at 320 nm was performed after all antioxidants in solution were oxidized. Blank correction of significantly absorbing plant extracts at 320 nm could be made with the aid of spectrophotometric titration. Because the TEAC coefficients (found by CERAC) of naringin−naringenin and rutin−catechin pairs were close to each other, this assay was advantageous to accomplish the simultaneous hydrolysis of flavonoid glycosides to the corresponding aglycones and their subsequent oxidation such that the hydrolysis products exhibited antioxidant capacities roughly proportional the number of −OH groups in a phenolic molecule. Özyurt et al.[52] further developed the CERAC method by employing a medium of 0.3 M H_2SO_4 + 0.7 M Na_2SO_4 to finely tune the Nernst potential of the Ce(IV)/Ce(III) couple by maintaining sufficient acidity while selectively complexing Ce(IV) with sulfate in preference to Ce(III) to oxidize true antioxidants but not citric acid or simple sugars. The TEAC coefficients of this modified CERAC procedure for antioxidants were in the order quercetin > rutin > gallic acid > catechin > caffeic acid ≥ ferulic acid > naringenin ≥ naringin > Trolox ≥ ascorbic acid, in accordance with those found by other antioxidant assays. It was also possible for Özyurt et al.[53] to measure the fluorescence of the reduction product, Ce(III), with excitation at 256 nm and emission at 360 nm, and then correlate this fluorescence intensity to the TAC value of a sample. By measuring the produced Ce(III) fluorometrically instead of the remaining Ce(IV) spectrophotometrically, the linear range was widened (e.g., 5.0×10^{-7}−1.0×10^{-5} M for quercetin), and the possible interferences of plant pigments for absorbance measurement at 320 nm were eliminated [however, the linear correlation coefficient in fluorescence was lower than in spectrophotometry due to the fluorescence-quenching effect of Ce(IV)].

Işık et al.[50] developed the Cr(VI) reducing antioxidant capacity (CHROMAC) assay, involving the reduction of chromate(VI) with antioxidants to Cr(III) in acidic solution at pH 2.8, and after 50 min of reaction time, the remaining Cr(VI) was spectrophotometrically measured with 1,5-diphenylcarbazide (DPC) at 540 nm. The in situ-formed Cr(III) complex with the oxidation product of DPC (i.e., diphenylcarbazone) indicated the remaining chromate, and Cr(VI) consumption was correlated to antioxidant concentration in the sample. However, the TAC order of common antioxidants in CHROMAC did not well agree with those of other ET-based assays (e.g., rosmarinic acid and quercetin were found to be less potent than ascorbic acid, based on the comparison of TEAC values). If no measures were taken for regulating the Nernst potential of the Cr(VI)/Cr(III) redox couple, as in chemical oxygen demand (COD) tests performed in water treatment, the acidic dichromate reagent would oxidize all of the organic load of solution without discriminating antioxidant compounds.

Potassium permanganate is a strong oxidant widely used in analytical chemistry for redox titrations without an external indicator because of the intrinsic violet color of the reagent. A critical and comprehensive review of acidic potassium permanganate chemiluminescence was presented, together with a discussion on reaction conditions, the relationship between analyte structure and chemiluminescence intensity, and its application to determine a variety of compounds including antioxidants.[96] Francis et al.[97] reported the use of acidic potassium permanganate as a chemiluminescence reagent to rapidly assess the antioxidant status of fruit juices, teas, and other beverages. In the acidic $KMnO_4$ spectrophotometric assay of reducing capacity for antioxidants,[98] the sample was oxidized with acidic permanganate, leading to sample discoloration until no color was observed; subsequent decrease of potassium permanganate concentration was determined with the use of a calibration curve of absorbance at 535 nm versus concentration.[99] In the original assay, Cacig et al.[98] also observed MnO_2 particles in suspension, showing the nonstoichiometric character of oxidation [instead of a neat Mn(VII)−Mn(II)

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reduction]. Although the results of this method were claimed to correlate with those of other reducing assays and acidic permanganate was assumed to oxidize phenol by forming phenoxyl radical and manganic acid (H_2MnO_4) in the slowest step of a series of oxidation reactions, it is obvious that permanganate in sulfuric acid medium would nonspecifically oxidize any organic substance and the measured parameter would be "total organic status" of a sample rather than its TAC.

2.1.2.2. Electrochemical Methods. Direct electrochemical sensing methods for in vitro antioxidant capacity assessment have been reviewed by Blasco et al.[100] Cyclic voltammetry (CV) is an electrochemical technique in which a sample is introduced to a reaction chamber with three electrodes: working electrode [such as glassy carbon (GC)], reference electrode (e.g., Ag/AgCl), and auxiliary electrode (e.g., Pt wire), where an increasing potential is applied to the working electrode and current intensity versus potential is recorded.[33] Although not all antioxidants can give their electrons to the GC electrode at an appreciable rate, most antioxidants are CV-active reducing agents and can therefore be assayed by CV on the basis of their redox potentials. For example, in CV utilizing a GC electrode, chlorogenic and caffeic acids showed well-defined reversible waves in weakly acidic solution, whereas their electrooxidation assumed a quasi-reversible character at higher pH due to possible polymerization reactions at the electrode surface.[101] Chevion et al.[102] made some pioneering studies on the electrochemical determination of antioxidant capacity and defined cyclic voltammetry as a simple, sensitive, and reliable method for determining the TAC of human plasma originating from low molecular weight antioxidants (LMWA). The half-wave potential ($E_{1/2}$) indicated the specific constituent of the LMWA and its ability to donate electron(s), whereas current intensity at half-wave potential (I_a) indicated the concentration of this constituent. The $E_{1/2}$ and I_a values reflected the antioxidant capacity of the plasma, whereas the change of I_a upon exposure to copper ions, ionizing radiation, and peroxyl radicals represented the severity of the oxidative stress induced. In their later work,[103] the authors suggested another parameter better correlating with antioxidant concentration, namely, the AUC around the anodic wave potential, $E_{1/2}$. Using the AUC approach, LMWA constituents of human plasma and animal tissues were identified and further validated by reconstruction of the CV tracing and by HPLC with electrochemical detection. As the changes for individual constituents within a single anodic wave could be different, the changes in AUC would better represent the residual antioxidant capacity and allow better quantitation of the loss in a specific component upon oxidative stress. Chevion et al.[102] identified two critical $E_{1/2}$ values for human plasma, namely, 420 ± 25 mV (mainly derived from ascorbate and urate constituents of LMWA) and 920 ± 25 mV (derived from unidentified constituents, excluding simple and protein thiols). Kohen et al. later showed that this group of mostly unidentified LMWA giving rise to the high-potential anodic wave could be related to the oxidized form of lipoic acid.[104] Spiking brain tissue samples with relatively high concentrations (0.5 mM) of other antioxidants (carnosine, tryptophan, and melatonin) yielded an increase in the amplitude of the second anodic wave.[103] The authors also found that when the wave comprised several constituents characterized by close but different $E_{1/2}$ values, a change in the relative concentrations of the constituents would cause a small shift in the $E_{1/2}$ of their combined anodic wave.[102] Plasma samples should be preserved with heparin when necessary, but not with EDTA, which gave a specific anodic wave at $E_{1/2}$ > 900 mV. The major disadvantages of CV are low sensitivity for antioxidants (usually in several micromolar range) and the necessity for frequent electrode cleansing before each measurement due to adsorption of proteins and other macromolecules from biological fluids onto the glassy carbon working electrode.[33]

Piljac-Žegarac et al.[105] used CV to study the electrochemical properties of antioxidants in fruit tea infusions as well as to estimate the antioxidant capacity. The most easily oxidized compound (at approximately 130 mV) was ascribed to the oxidation of the ene-diol of ascorbic acid. A pronounced anodic current peak (corresponding to a quasi-reversible redox process, reflecting oxidation to a stable quinone) observed at 440 mV in all analyzed fruit teas indicated that *ortho*-dihydroxyphenol and gallate groups were the major contributors to the antioxidant capacity of investigated teas. The less oxidizable compounds presented an irreversible oxidation process between 670 and 700 mV, which was ascribed to the oxidation of the monophenol group or the *meta*-diphenols on the A ring of flavonoids that led to a phenoxy radical or a phenoxonium ion undergoing successive secondary reactions. The antioxidant capacity of these fruit teas was determined by estimating the integrated area (AUC) under the peak up to 600 mV.

Electrochemical techniques of antioxidant characterization consist of CV, differential pulse (DPV), and square-wave voltammetry (SWV), proposed as useful tools to investigate the electrochemical behavior of phenolic compounds in different food samples in conjunction with carbon, diamond, and graphite electrodes.[106,107] DPV is one of the most sensitive techniques and has a received a great deal of attention in recent years.

CV methods have also been described to detect ascorbic acid, citric acid, and sugars in both food products and pharmaceuticals.[108] Qualitative assessment of wine phenolics based on reducing strength was also realized by using CV at a GC electrode; although in this approach, the AUC, namely, the charge passed to 0.5 V potential during CV recording, is understood as a better estimate of the concentration of polyphenols with low oxidation potentials, quantitation efforts of all reducing compounds may provide only a qualitative picture in a complex mixture such as wine mainly because the magnitude of the response is not identical on a molar basis for dissimilar compounds.[109] The antioxidant capacities of several drugs containing acetylsalicylic acid were evaluated using a SOD-based biosensor method in comparison to ORAC−fluorometric and DPV methods; however, although the DPV-based method generally seemed to be sufficiently in line with the results of the other two methods, its precision was rather poor (RSD > 10%).[110] Novak et al. used square-wave voltammetry for investigating the electrochemical behavior of major green tea compounds such as epigallocatechin gallate, epigallocatechin, and gallic acid.[106] Magarelli et al. developed and validated a sensitive DPV method using a GC electrode for the determination of total phenolic acids in cotton cultivars,[107] but the authors studied only four polyphenolics (i.e., caffeic, chlorogenic, gallic, and gentisic acids) and presented one anodic peak at approximately 0.4 V that was assigned to the oxidation of phenolic hydroxyls leading to the formation of *o*-quinone via semiquinone form and, therefore, it may be argued whether these four easily oxidized phenolic acids are a true representative of "total phenolic acids" having diverse oxidation potentials.

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There is very limited study about the usage of GC electrodes as electrochemical TAC sensors. Milardović et al.[111] developed an amperometric method, based on the reduction of $DPPH^{\bullet}$ at the GC electrode, for measuring the antioxidant activity of pure compounds and their real mixtures such as tea, wine, and other beverages; because the cyclic voltammograms of a number of water- and ethanol-soluble common antioxidants gave either irreversible or undefined oxidation peaks, electrochemical reduction of $DPPH^{\bullet}$ was conducted in the presence and absence of antioxidants, but potential selection was critical due to the electrochemical interferences of caffeic acid and Trolox. The electrochemical $ABTS^{\bullet+}$ assay is based on measuring catalytic voltammetric currents caused by antioxidants acting as reductant toward the electrochemically generated $ABTS^{2+}$ (thereby enabling reoxidation of $ABTS^{\bullet+}$ on the electrode) in edible oils; this oxidative voltammetric current intensity increased with an increase in antioxidant concentration, enabling TAC determination of edible oils. This method produced rather high blank values in the Trolox calibration curve, and Trolox addition to sunflower oil matrices did not yield perfectly linear responses.[112] In an attempt to adapt the Ce(IV)-reducing TAC assay[51] to electroanalytical chemistry, Ferreira and Avaca[113] measured the ability of eight different compounds in reducing Ce^{4+} by chronoamperometric measurement of the remaining Ce^{3+} species and found the TAC order as tannic acid ≫ quercetin > rutin > gallic acid ≈ catechin > ascorbic acid > BHA > Trolox, agreeing well with FRAP. The electrochemical behavior of the $Cu(Nc)_2^{2+}$ complex was recently studied by cyclic voltammetry at a GC electrode.[114] The electroanalytical method was based on the reduction of $Cu(Nc)_2^{2+}$ to $Cu(Nc)_2^{+}$ by antioxidants and electrochemical detection of the remaining Cu(II)−Nc (unreacted complex), the difference being correlated to antioxidant capacity of the analytes. The calibration curves of individual compounds comprising polyphenolics and vitamin C were constructed, and their response sensitivities and linear concentration ranges were determined. The reagent on the GC electrode retained its reactivity toward antioxidants, and the measured TEAC values of various antioxidants suggested that the reactivity of the Cu(II)-Nc reagent is comparable to that of the solution-based spectrophotometric CUPRAC assay. This electroanalytical method better tolerated sample turbidity and provided higher sensitivity (i.e., lower detection limits) in antioxidant determination than the spectrophotometric assay.[114]

In a review work, Prieto-Simon et al.[115] made a generalization that electroanalytical biosensor-originated antioxidant activity/capacity measurements based on the reduction of hazard caused by $O_2^{\bullet-}$ essentially involve the use of (i) Cyt *c* heme protein, (ii) SOD enzyme, and (iii) DNA. Superoxide anion determination using a Cyt *c*-based sensor was reported not to be so selective mainly because this heme protein is not specific for $O_2^{\bullet-}$ and can simultaneously reduce endogeneous H_2O_2 in biological systems, whereas SOD-based biosensors were claimed to be more selective and sensitive.[115] When using DNA-based bioelectrosensors, the oxidizibility of DNA bases (i.e., maintaining DNA integrity) in the absence and presence of antioxidants was taken as a measure of antioxidant activity.

(i) $O_2^{\bullet-}$ was produced by the xanthine−XOD enzyme system, using a Cyt *c*-modified electrode, where Cyt *c* was reported to communicate with the nano-Au electrode through self-assembled monolayers of short-chain alkanethiols. The immobilized Cyt *c* was reduced by $O_2^{\bullet-}$ and rapidly regenerated at the surface of the electrode polarized at the oxidation potential, where the current generated by electron transfer from the radical to the electrode through Cyt *c* was proportional to the radical concentration.[116] In this process, antioxidants, when present, quenched the radicals and decreased their concentration reflected in a decrease of oxidation current. Using this bioelectrosensor, an antioxidant activity sequence was established for flavonoids in decreasing order: flavanols > flavonols > flavones > flavonones > isoflavonones.[117]

(ii) SOD enzyme was immobilized onto cysteine-functionalized (via self-assembled monolayers) Au nanoparticles-coated carbon fiber microelectrodes, enabling direct electron transfer with $O_2^{\bullet-}$,[118] catalyzing their dismutation to O_2 and H_2O_2:

$$SOD(Cu^{2+}) + O_2^{\bullet-} \rightarrow SOD(Cu^{+}) + O_2 \quad (22)$$

$$E^0 = +0.3\ V\ (vs\ Ag/AgCl)$$

$$SOD(Cu^{+}) + O_2^{\bullet-} + 2H^{+} \rightarrow SOD(Cu^{2+}) + H_2O_2 \quad (23)$$

$$E^0 = -0.2\ V\ (vs\ Ag/AgCl)$$

Naturally antioxidants, when present, would cause a decrease in $O_2^{\bullet-}$ concentration. Both oxidation and reduction of $O_2^{\bullet-}$ could be measured with high sensitivity and selectivity. Both dismutation products (i.e., H_2O_2 and O_2) could be easily detected simultaneously using amperometric transducers,[115] for example, the former with an electrodeposited polypyrrole covered glassy carbon microelectrode modified with horseradish peroxidase and the latter with the same electrode modified with superoxide dismutase enzyme embedded in polymer layers.[119]

(iii) DNA-based bioelectrosensors worked on the principle of immobilizing (usually calf thymus-originated) double-stranded (ds) DNA on screen-printed carbon electrodes (SPE), followed by the detection of the guanine oxidation peak between +0.8 and +1.0 V (vs Ag/AgCl) by square wave voltammetry. Because the peak current intensity was proportional to the concentration of DNA base, guanine, the immersion of the DNA-modified electrode into a Fenton solution [such as $Fe(II)+H_2O_2$] produced a signal decrease in the peak current intensity, whereas the presence of antioxidants restored the signal (demonstrating DNA integrity) due to hydroxyl radical quenching.[115] To measure this signal alteration more effectively, screen-printed carbon was doped with TiO_2 nanoparticles, creating a porous surface structure on which ds-DNA adsorbed better because of specific DNA phosphate−TiO_2 interactions.[120] A redox mediator, namely, tris-2,2′-bipyridine (bipy) ruthenium(II), was electro-oxidized on the electrode surface to subsequently oxidize both the adsorbed ds-DNA and the antioxidants in solution. Divalent and trivalent ruthenium-bipy species, that is, $Ru_{DNA}^{(II)}$ and $Ru_{DNA}^{(III)}$, represented those redox mediators that were generated in the vicinity of TiO_2 nanoclusters. Here, the oxidative damage was produced by $[Ru(bipy)_3]^{3+}$, an efficient oxidant

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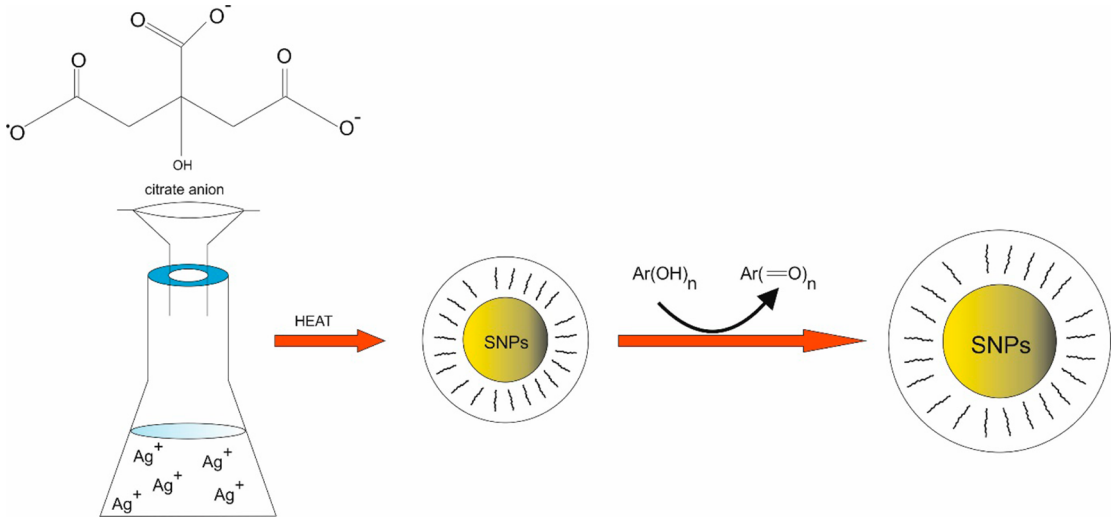


Figure 8. Enlargement of silver nanoparticles by antioxidant addition to a silver nitrate colloidal solution of citrate-stabilized Ag nanoparticles (via seed-mediated particle growth to generate core−shell AgNPs).

for the DNA bases, guanine and adenine, that are most sensitive to oxidation. The oxidative damage on adsorbed DNA was determined by square wave voltammetry via measuring the current intensity for the oxidation of $[Ru(bipy)_3]^{2+}$ catalyzed by the remaining ds-DNA on the electrode; antioxidants, when present, had a protective role reducing oxidative damage on DNA and were subsequently assayed through competition kinetics by comparing the rate constants for $Ru_{DNA}^{(III)}$ oxidation of antioxidant (in bulk solution) and of adsorbed DNA.[120]

The recently developed direct current (DC) polarographic assay for AOA estimation is based on the measurement of anodic current obtained by dropping mercury electrode (DME) in hydrogen peroxide solution upon the addition of antioxidant compounds. In this DC polarographic antioxidant assay, the decrease in anodic limiting current of the $[Hg(O_2H)(OH)]$ [hydroxoperhydroxo-mercury(II) complex], formed in alkaline solution of H_2O_2, at the potential of mercury oxidation, varied with the concentration of added antioxidant capable of H_2O_2 scavenging, and the method was validated against DPPH and Folin spectrophotometric assays for wines[121] and for teas and herbal infusions.[122] Although the signal decrease originated from the interaction of antioxidant with hydroperoxo radicals, the antioxidant did not directly scavenge H_2O_2 in the absence of Hg, and the decrease of Hg^{2+} cathodic current agreed with that of anodic current, leading to the assumption that mercury(II) reduction caused a decrease in concentration of Hg^{2+} available for hydroxoperhydroxo-mercury(II) complex formation, bringing about the decrease in its anodic current.[123]

2.1.2.3. Nanotechnological Methods. Nanotechnological methods of colorimetric TAC assay usually make use of either the formation or enlargement of noble metal (Au, Ag, etc.) nanoparticles, abbreviated as AuNPs or AgNPs, upon reaction of Au(III) or Ag(I) salts with antioxidant compounds. The standard reduction potentials for Au(III)−Au(0) and Ag(I)−Ag(0) redox couples are 1.5 and 0.8 V, respectively, and therefore many phenolic antioxidants can be favorably oxidized by simultaneous reduction of Au(III) and Ag(I) to the corresponding noble metal nanoparticles (i.e., AuNPs and AgNPs). However, the thermodynamic favorability of these redox reactions does not guarantee perfect heterogeneous phase kinetics, and therefore enlargement of previously formed NP seeds (e.g., citrate-stabilized AgNP seeds) via reaction with antioxidants is usually preferred, because coating of these NP seeds gives rise to better linearity of absorbance/concentration responses. The reason that nanomaterial-based methods have found little use in food science (specifically antioxidant research) is probably the substoichiometric character of the concerned reduction reactions by antioxidants leading to NP formation.[55] There are also other food constituents (in addition to antioxidants) causing AuNP or AgNP formation that may give rise to interferences in the assays.

When AgNPs are dispersed in liquid media, they exhibit a strong UV−vis absorption band not present in the spectrum of the bulk metal. This surface plasmon resonance (SPR) absorption is attributed to the collective oscillation of electrons in the conduction band of these particles in resonance with the wavelength of incoming light, with a periodic change in electron density at the surface. The SPR absorption of nanosized particles having near-zero dielectric constant gives rise to a LSPR band. Although AgNPs have very high molar absorptivities ($\varepsilon \approx 3 \times 10^{11}$ M^{-1} cm^{-1})[124] and are expected to allow higher sensitivity in optical detection, this ε is a corrected value calculated on the basis of the molar amount of nanoparticles per unit volume of bulk metal. Furthermore, ideal sensitivity in NP-based TAC assays cannot be achieved because of various factors affecting LSPR absorption such as reaction stoichiometry, particle size and shape, and dielectric constants of both the metal and the surrounding medium. Because the properties of surface plasmons can be tailored as a result of altering the NP surface and, specifically, the shell thickness, the seed-mediated particle growth technique was adopted (Figures 8 and 9) for developing a AgNPs (SNPs)-based TAC assay.[55]

Because the redox potential of the oxidized/reduced forms of glutathione (GSSG/2GSH) is a basic indicator of the redox environment within a cell, selective quantification of biothiols is an important topic in bioanalytical chemistry.[125] An optical sensor for biologically important thiol quantification was designed with the use of Ellman's reagent [5,5′-dithiobis(2-nitrobenzoic acid), DTNB]-adsorbed gold nanoparticles (AuNPs) (DTNB-AuNP) in a colloidal solution.[126] DTNB,

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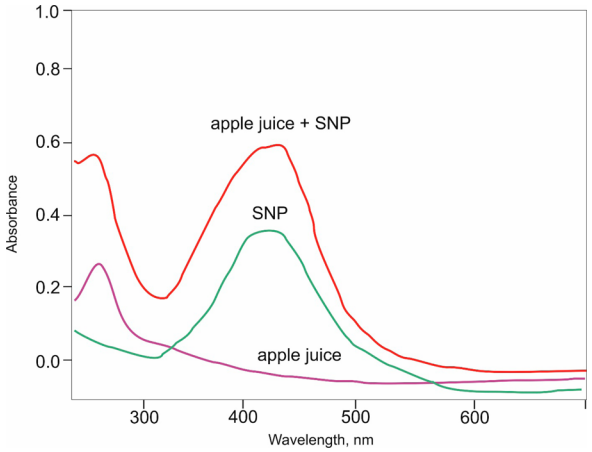


Figure 9. Surface plasmon resonance absorption of citrate-stabilized AgNPs is intensified by the addition of apple juices, corresponding to seed-mediated particle growth.

a well-documented thiol-selective compound, was adsorbed through noncovalent interaction onto AuNPs, and the absorbance changes associated with the formation of the yellow-colored 5-thio-2-nitrobenzoate (TNB^{2-}) anion as a result of reaction with biothiols were measured at 410 nm (Figure 10). The DTNB reagent could be selectively desorbed from the derivatized AuNPs surface to give Ellman's reaction with thiols due to preferential adsorbabilities of thiols over disulfides[127] and to thermodynamic/kinetic favorability of the thiol-exchange reaction. The linear response of the sensor for cysteine, glutathione, homocysteine, cysteamine, dihydrolipoic acid, and 1,4-dithioerythritol was better than that of Nile Red dye-derivatized AuNPs sensor for thiol determination.[128] Common biological sample ingredients such as amino acids, flavonoids, vitamins, and plasma antioxidants did not interfere with thiol sensing (Figure 10).[126]

As H_2O_2 is a cell membrane-permeable biological oxidant, noble metal nanoparticle-based methods were also developed to measure H_2O_2 scavenging antioxidant activity. H_2O_2 was found to enlarge the AuNPs on the surface of gold nanoshells (GNS)s precursor nanocomposites (SiO_2/AuNPs), and the preadsorbed AuNPs served as nucleation sites for Au deposition. AuNPs on the SiO_2 cores were enlarged by increasing concentrations of H_2O_2, concomitant with spectral changes corresponding to AuNP plasmon absorption bands, and antioxidants restricted H_2O_2-mediated formation of GNSs, enabling the determination of their inhibitive activity.[129] In another example, H_2O_2-induced growth of GNSs was inhibited by the addition of phenolic acids, which affected the peak wavelength of surface plasmon absorption. Among the tested antioxidants, caffeic acid was found to be the most efficient H_2O_2 scavenger, whereas *trans*-cinnamic acid exhibited the weakest activity.[130]

2.1.3. Mixed-Mode (ET- and HAT-Based) Methods. Mixed-mode assays are generally based on the scavenging of a stable radical chromophore (such as $ABTS^{\bullet+}$ and $DPPH^{\bullet}$) or fluorophore by antioxidants, in which HAT, ET, and proton-coupled electron transfer (PCET) mechanisms may play different roles to varying extents, depending on pH, solvent, and other reaction conditions. Schaich and co-workers have recently directed extensive criticisms at ABTS and DPPH assays[131−133] on the grounds that these assays use sterically hindered, N-centered free radicals as targets to antioxidants rather than biologically active short-lived radicals (e.g., hydroxyl, superoxide, and lipid oxyl radicals having lifetimes ranging from nanoseconds to 10 s) and that their action as radical scavenger should be irrelevant in vivo.[134]

2.1.3.1. ABTS/TEAC Assay. ABTS/TEAC assays use intensely colored cation radicals of $ABTS^{\bullet+}$ as useful colorimetric probes accepting hydrogen atoms or electrons supplied by antioxidant compounds. Although lag-phase assays were preferred at the initial stage of method development, the requirements for reproducibility and minimizing errors later evolved the assay to a decolorization strategy, where the initially formed (by H_2O_2/peroxidase, MnO_2 or persulfate) and stabilized $ABTS^{\bullet+}$ radical was allowed to react with the added antioxidant, causing an absorbance decrease at the characteristic wavelengths. Antioxidant capacity is measured as the ability of the test compound (e.g., Ph−OH) to decrease $ABTS^{\bullet+}$ color by intercepting initial oxidation and preventing $ABTS^{\bullet+}$ production or reacting directly with the preformed radical cation. Even when a fixed-time ABTS assay is preferred, the results may greatly vary for the same compound (e.g., GSH) depending on the oxidizing agent used to generate the stable colored radical.[33]

$$\text{ABTS} + \text{oxidizing agent (such as } K_2S_2O_8) \rightarrow ABTS^{\bullet+} \quad (24)$$

$$(\lambda_{max} = 734\ \text{nm})$$

$$ABTS^{\bullet+} + \text{PhOH} \rightarrow \text{ABTS} + \text{PhO}^{\bullet} + H^{+} \quad (25)$$

2.1.3.2. $DPPH^{\bullet}$ Radical Scavenging Assay. The stable chromogen radical $DPPH^{\bullet}$ was first proposed for quantitating antioxidant content nearly half a century ago, when Blois used the thiol-containing amino acid cysteine as his model antioxidant.[38] Later it was used as a phenol reagent.[135] The more recently introduced method of Brand-Williams and colleagues[136] has been used as a reference point by several groups of workers.[137,138] Reaction with DPPH was adapted for

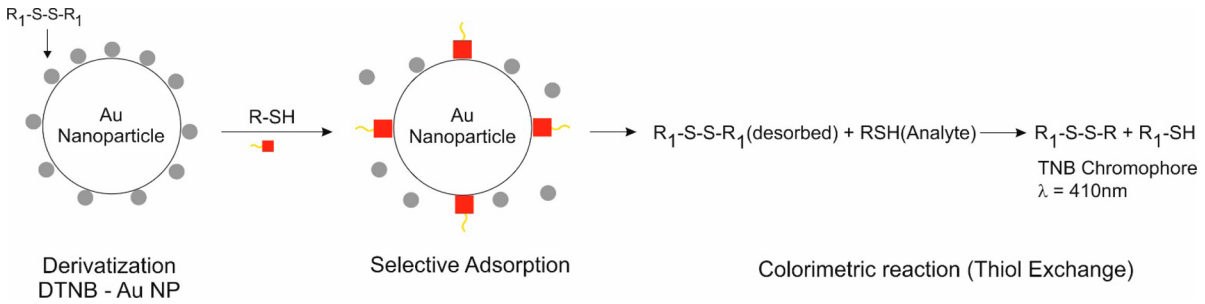


Figure 10. AuNPs-adsorbed Ellman's reagent (DTNB-disulfide) is exchanged for thiols (−SH) in solution, enabling a colorimetric thiol assay.[126]

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measuring radical quenching kinetics,[139,140] and since then numerous variations in methods and time for following the reaction as well as for calculating relative antioxidant action by reaction stoichiometry have evolved.[131,136] The reaction equation can be formulated with respect to the HAT mechanism, although the proton-coupled ET-mechanism cannot be excluded, especially in phenol-ionizing solvents:

$$DPPH^{\bullet} + PhOH \rightarrow DPPH_2 + PhO^{\bullet} \quad (27)$$

where $DPPH^{\bullet}$ is a stable chromogen radical with λ_{max} = 515 nm.

2.1.3.3. DMPD Radical Scavenging Assay. In the presence of ferric iron or reactive species such as hydroxyl radicals, *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) is converted to the colored $DMPD^{\bullet+}$ radical cation, which is scavenged by antioxidant molecules present in test samples, forming the principle of the $DMPD^{\bullet+}$ assay. Antioxidant compounds that are able to transfer a hydrogen atom (or an electron) to $DMPD^{\bullet+}$ cause rapid decolorization of the solution (manifested by an absorbance descrease at λ_{max} = 505 nm) with a stable end-point. The use of $DMPD^{\bullet+}$ has been widely extended to evaluate the antioxidant capacity of different food products such as fruits, vegetables, and wines.[141−143] However, less reproducibility was obtained in the presence of hydrophobic antioxidants such as tocopherol or BHT.[144] Mehdi and Rizvi modified the DMPD-based method for the measurement of plasma oxidative capacity during human aging.[145] Recently, in a population-based cohort study from Germany, Schöttker et al. measured the derivatives of reactive oxygen metabolites (d-ROM) in human sera as a proxy for ROS concentration with the use of a DMPD-based d-ROM colorimetric kit.[146] Çekiç et al. simultaneously measured the oxidative status (OS) and antioxidant activity with the aid of a sensor technique by retaining the pink-colored, positively charged chromophore of DMPD-quinone (resulting from the reaction between DMPD and ROS) on a Nafion membrane, where the 514 nm absorbance of the sensor membrane was a measure of OS (e.g., derived from hydroperoxides and labile iron compounds of sera), whereas antioxidants caused an absorbance decrease on the membrane due to their ROS scavenging action.[147]

Other radical probes used for free radical-scavenging activity measurement are Fremy's salt (galvinoxyl radical, potassium nitrosodisulfonate)[148] and the more recently developed aroxyl radical [2,6-di-*tert*-butyl-4-(4′-methoxyphenyl)phenoxyl radical] methods,[149] but these techniques have been much less frequently preferred than the widely used ABTS and DPPH assays.

2.1.4. ROS/RNS Scavenging Methods. ROS is a collective term often used to include oxygen radicals [superoxide ($O_2^{\bullet-}$), hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), and alkoxyl ($RO^{\bullet}$)] and certain nonradicals that are either oxidizing agents or easily converted into radicals, such as HOCl, ozone (O_3), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), and H_2O_2. RNS is a similar collective term that includes nitric oxide radical ($^{\bullet}NO$), $ONOO^-$, nitrogen dioxide radical ($^{\bullet}NO_2$), other oxides of nitrogen, and products arising when NO reacts with $O_2^{\bullet-}$, $RO^{\bullet}$ and $ROO^{\bullet}$.[150] Although ROS and RNS are essential to human health, an unbalanced excess of these reactive species may cause oxidative stress-related diseases.[7,151]

In ROS/RNS scavenging activity assays, the reactive species generated enzymatically or by redox-active chemical reagents are allowed to attack a probe, and the subsequent conversion of this probe is measured spectroscopically or electrochemically, where the extent of conversion on the probe is a measure of ROS/RNS concentration and its attenuation indicates the scavenging activity of the tested putative antioxidants. Most conventional fluorescence (FL) probes for ROS detection react by a free radical mechanism and are rather nonselectively converted into FL-enhanced or FL-quenched end-products, whereas the more recently developed boronate probes may act more selectively as they undergo nucleophilic attack by oxidant species to release the hidden fluorescence of a fluorophore containing a blocking group. Biological macromolecules such as lipids, proteins, sugars, and DNA have been subjected to the attack of biologically relevant reactive species and used as oxidative stress biomarkers, where various oxidative transformations on these macromolecules have been measured. For example, ethane and pentane, conjugated dienes, hydroperoxides, aldehydes, and ketones from lipids; and nitro-, chloro-, and bromo-amino acid residues; as well as carbonyls, −SS−, SOH, and −SOOH compounds, dimers, cross-linked, modified, and cleavage products from proteins; nitrated, chlorinated, brominated, and 8-hydroxylated nucleic bases from DNA can be formed upon oxidation.[7,58] In the case of biomarkers, the activity of an antioxidant can be indirectly measured by its attenuation of the oxidative hazard formed on the marker macromolecule. For example, antioxidants may decrease the color intensity of ferric thiocyanate or ferric-xylenol orange complexes used to measure lipid hydroperoxides or of the TBARS chromophore used to measure lipid-derived aldehydes and ketones (e.g., malondialdehyde) with questionable specificity.

The selectivity of ROS/RNS assays has been questioned, because frequently there is more than one reactive species capable of probe conversion, and absorptimetric measurements in the UV range (such as the hydrogen peroxide scavenging assay carried out at the intrinsic maximal absorption wavelength of H_2O_2 at λ = 230 nm)[152] suffer from the interference of many organic compounds absorbing at the same wavelength, thereby yielding inconsistent blanks. The redundancy of hydroxyl radical scavenging assays have occasionally been discussed in the literature because almost any biological molecule (not necessarily an antioxidant) can react with this radical at extremely high rates. The possible drawbacks of ROS/RNS scavenging assays may be listed as follows: (i) If the tested reactive species are produced enzymatically (e.g., superoxide anion radical by xanthine/xanthine oxidase, reactive species from H_2O_2 by peroxidase, HOCl from H_2O_2 and chloride by myeloperoxidase, reduction of residual nitrate to nitrite in the course of nitric oxide radical scavenging by NADH-dependent nitrate reductase), then it is not clear how to differentiate between the ROS/RNS scavenging and enzyme inhibition actions of antioxidants, and therefore ESR/spin trap methods should accompany conventional spectroscopic methods in such ambiguous cases; (ii) The tested reactive species should not react too rapidly with the selected probe, because in that case, a wide range of antioxidants reacting with quite different rates cannot be measured. For example, superoxide radical reacts with the cytochrome *c* probe much more rapidly than it does with NBT, rendering the competition of certain antioxidants with the former probe much less efficient;[153] (iii) The tested antioxidant or its oxidation product can enter a direct redox reaction with the probe via incompletely reacting with the subject ROS/RNS. For example, ascorbic acid can easily reduce ferricytochrome *c* in superoxide radical scavenging assay.[21] Another example is that in the HOCl scavenging assay in which

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TNB is oxidized by HOCl to DTNB, thiol-type antioxidants react with DTNB to produce excessive TNB chromophore.[154] (iv) The probe or its conversion product may be unstable or may itself generate reactive species during the course of measurement. As a result, such assays should be cautiously interpreted because they are prone to errors from the above-mentioned and other sources,[155] and quantification of ROS/RNS scavenging is additionally complicated by the esssentially nonlinear character of either ROS production or consumption with respect to concentration. Another challenge for the synthesis of future ROS/RNS probe molecules is tailoring them at a suitable redox potential to specifically quench a given reactive species but not others.

2.1.5. Cellular Antioxidant Activity (CAA) Assays. Because CAA assays are performed within the cell medium, they are assumed to better consider the uptake, distribution, and metabolism of antioxidants within cells.[156] López-Alarcón and Denicola[157] have recently reviewed cellular antioxidant activity assays in comparison to classical chemical assays and, to compensate for the possible complexities of antioxidant action, have recommended the use of CAA assays to assess the genuine antioxidant activity of a compound or extract. To measure CAA, Wolfe and Liu[156] used the peroxyl radical oxidation reaction of the nonfluorescent probe 2′,7′-dichlorofluorescin (DCFH) entrapped in human hepatocarcinoma HepG2 cells, where the presence of antioxidants attenuated the cellular fluorescence of the oxidation product, dichlorofluorescein (DCF). This probe may exhibit several disadvantages such as photochemical instability, incomplete trapping by cells, or decreased oxidation in the presence of endogenous antioxidants.[156] Also, the classical order of antioxidant effectiveness was not followed in these assays, and the results did not correlate with those of ORAC.[156,158] Halliwell and Whiteman[18] directed certain criticisms to cellular assays, such as interference of enzymic/nonenzymic endogenous antioxidants to the measurement procedure, intrinsic oxidative stress generation by the cell culture process, difficulty in distinguishing between intracellular and extracellular fluorescence from chemical reactions in the culture medium, and inability of DCF fluorescence measurement to specifically differentiate several reactive species. Effective use of cellular probes also necessitates a full understanding of the involved mechanistic pathways, environmental factors (e.g., O_2 and pH), distribution, and possible intermediary products of the probe, combined with instrumental artifacts and actual competition of the measured antioxidants with the probe for reactive species.[159]

3. PHYSICOCHEMICAL ASPECTS OF ANTIOXIDANT ACTION

3.1. Kinetic Solvent Effects and Structure−Activity Relationships. Antioxidant activity/capacity assays should be tested with several reactive species and oxidizing probes, because the reactivities of known antioxidants toward different ROS/RNS are quite different. For example, *N*-acetylcysteine is a powerful scavenger of HOCl and also reacts with hydroxyl radical at a rate constant of 1.36×10^{10} M^{-1} s^{-1}, but reacts only slowly with H_2O_2 and does not react at all with superoxide anion radical.[160] Antioxidant activity also strongly depends on the solubility/localization and distribution of antioxidants between different phases; for example, restricted diffusion of α-tocopherol may reduce its antioxidant activity in membranes, and a synergistic effect between ascorbic acid and α-tocopherol was observed under conditions of inhibited peroxidation of linoleate in SDS micelles. A water-soluble form of α-tocopherol complexed with bovine serum albumin (α-toc:BSA) proved to be an effective antioxidant for hindering the autoxidation of linoleate in SDS micelles, whereas α-toc:BSA required a long equilibration time with liposomes before α-toc was transferred to the liposomes to provide effective antioxidant action.[161] Antioxidants shown to be very effective in in vitro TAC assays may exhibit a conflicting order of antioxidant potency in different systems of varying lipophilic/hydrophilic balance, depending on their differential abilities to penetrate and interact with the lipid bilayers. A hierarchic order (i.e., pecking order) of antioxidants in relation to their free radical-scavenging ability can be predicted, on the basis of the comparison of one-electron reduction potentials of the scavenged reactive species with the corresponding reduction potentials of aryloxy/phenol redox couples. For example, the formal reduction potentials (at pH 7) of reactive species such as $^{\bullet}OH$ (2.31 V), alkoxyl radical (1.60 V), alkyl peroxyl radical (1.00 V), superoxide anion radical (0.94 V), singlet oxygen (0.65 V), unsaturated fatty acid radical (0.60 V), and H_2O_2 (0.32 V) are listed in a comprehensive review.[162] Hydrogen peroxide and superoxide anion radical can act as both an oxidant and reductant, depending on the substrate and medium. Moreover, the average lifetimes of radicals commonly active in living tissues and in foods vary over 10 orders of magnitude, that is, $^{\bullet}OH$ (10^{-9} s), $^{\bullet}OR$ (10^{-6} s), and $ROO^{\bullet}$ (10 s).[132] Thus, disregarding the distribution between phases, it is natural that different antioxidants would show different ROS/RNS scavenging abilities based on their redox potentials. Despite their different distributions between lipid membrane and aqueous phases, the fact that vitamin C (ascorbic acid) can physiologically regenerate vitamin E (α-tocopherol) can be understood by considering the reduction potentials of α-tocopheroxyl (0.50 V) and ascorbate (0.282 V) radicals, because α-tocopheroxyl radical can oxidize ascorbate and is itself converted back to α-tocopherol. Likewise, among flavonoids, quercetin and tea catechins, having reduction potentials of the corresponding aryloxy radical/phenol couple <0.4 V, should be able to regenerate α-tocopherol from α-tocopheroxyl radical.[163] However, it should be borne in mind that, although a relatively large difference between the standard reduction potentials of the oxidant and reductant ensures a high equilibrium constant for a nearly complete redox reaction between the concerned antioxidant and the reactive species it scavenges, it does not guarantee a high kinetic rate in a given medium, because equilibrium constant is the ratio of the rate constant of the forward reaction to that of the backward reaction.

The rate and extent of lipid peroxidation can be measured from oxygen uptake, substrate consumption, or product formation.[58] Some secondary oxidation products (e.g., hexanal and 2,4-decadienal) or transition metal ions can act as prooxidants and catalyze lipid oxidation at initial stages, causing early consumption of antioxidants.[164] In Cu(II)-induced lipid peroxidation reactions, the kinetic profile of peroxidation is characterized by three major parameters: the "lag time" preceding rapid oxidation, the maximal rate of oxidation (V_{max}), and the maximal accumulation of oxidation products (OD_{max}). A distinction between various antioxidants with respect to their mechanism of action can be made based on observing the different impacts upon these three parameters; for example, antioxidative effects due to copper-chelating or blockade of copper binding sites can be distinguished from the effects of free radical quenching.[165] The effects of three

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different flavonoids of similar structure, that is, quercetin, morin, and catechin, as potential antioxidant protectors were studied in a linoleic acid emulsion to yield an inhibitive order of morin > catechin ≥ quercetin, in agreement with that of formal reduction potentials versus NHE at pH 7, that is, 0.60, 0.57, and 0.33 V for morin, catechin, and quercetin, respectively. Morin showed antioxidant effect at all concentrations, whereas catechin and quercetin showed both antioxidant and prooxidant effects depending on their concentrations. The structural requirements for antioxidant activity in flavonoids interestingly coincided with those for Cu(II)-induced prooxidant activity, because as the reducing power of a flavonoid increases, Cu(II)−Cu(I) reduction is facilitated that may end up with the production of reactive species.[166]

The thermodynamics and kinetics of antioxidant action cannot be properly understood without understanding solvent effects on HAT- and ET-based reactions, especially the latter.[167−169] Although HAT-based reactions have been claimed to be relatively rapid at least at their initial stages, hydrogen bonding in polar solvents may induce dramatic changes in the H atom donor activities of phenolic antioxidants and consequently affect the measured reducing antioxidant capacity.[170,171] The rates of oxidation reactions of phenolic compounds (by either HAT or PCET mechanism) by ROS/RNS are deeply influenced by H-bond accepting (HBA) and anion solvation abilities of solvents, as well as by the nature and position of phenol ring substituents [for instance, the rate constants (k, M^{-1} s^{-1}) for oxidation of phenoxide anions by ClO_2 radical, having a standard potential of 0.94 V versus NHE, were in the descending order of 10^9, 10^8, 10^7, 10^5, and 10^3 for resorcinol, *p*-methoxyphenol, simple phenol, *p*-nitrophenol, and *p*-cyanophenol, respectively, progressively decreasing with the removal of electron-donating substituents and/or with the increase in electron-withdrawing substituents on the phenolic ring];[172] there is a delicate balance between the three different, nonexclusive mechanisms of antioxidant action, namely, HAT, proton-coupled ET, and sequential proton loss ET, depending on both the environment and the reactants.[77,173] Choe and Min also discussed the effects of substituents and steric crowding around the phenolic hydroxyl groups as important parameters determining antioxidant activity.[174] As an example of the HBA ability of solvent on TAC, in the study of the effects of polar (e.g., acetonitrile and *tert*-butyl alcohol) and nonpolar (e.g., cyclohexane) solvents on the peroxyl radical-trapping antioxidant activity of some flavonoids, catechol derivatives, hydroquinone, and monophenols, phenols with two *ortho*-hydroxyls were the most active antioxidants, with inhibition rate constants (k_{inh}) in the range of (3−15) × 10^5 M^{-1} s^{-1} (in cyclohexane), whereas in the strong H-bond acceptor solvent *tert*-butyl alcohol, these rate constants dramatically declined; however, in the weaker H-bond acceptor solvent acetonitrile, k_{inh} values were restored close to the values in cyclohexane.[175] 3,5-Di-*tert*-butylcatechol, a very active H atom donor to DPPH in hexane, showed a dramatic loss of activity in HBA solvents, especially acetone.[176] In general, the aryloxy radicals formed from the oxidation of catechol (*o*-dihydroxy phenol) moieties of phenolic compounds are stabilized in non- or weak-hydrogen-bonding solvents by intramolecular H-bonding, through the interaction of two adjacent substituents on catechol, that is, −C(O$^\bullet$)···(HO)C−, bringing about the delocalization of the odd electron over the whole molecule. An extended delocalization and conjugation of the π-electrons, enhanced by resonance effects and planarity, favor the lowering of both ionization potentials (IP) and PhO−H bond dissociation energies (BDE) and affect strongly the capacity of antioxidants to donate a single electron.[177] It should be emphasized that the relative magnitudes of BDE and IP determine whether the HAT or ET mechanism is predominant for a given PhOH (and these values may show significant variations in polar or nonpolar solution and gas phases), as a low BDE is required for a strong phenolic antioxidant essentially acting by H atom donation, whereas a low IP is necessary for a strong antioxidant functioning essentially via electron donation.[177] The intramolecular H-bonding stabilization of the one-electron oxidized catechols will also lower the standard redox potential of the aryloxy radical/catechol couple, making the phenolic compound a stronger antioxidant in ET reactions.[173] The strong antioxidant properties of catechol or pyrogallol moieties of polyphenols may be predominantly attributed to the intramolecular H-bonding stabilization of aryloxy radicals produced from one-electron oxidation of these moieties,[178] whereas the intermolecular H-bonding abilities of phenolic hydroxyl groups with HBA solvent molecules lower antioxidant activity. The antioxidant activities of *o*-methoxyphenols were decreased in hydrogen bond accepting media.[179] Thus, in evaluating H atom transfer kinetics of polyphenols, the ability to form a linear H-bond [with a solvent (S) molecule, in the form of ArOH···S] should be distinguished from a nonlinear H-bond (intramolecular H-bond), because only the former may restrict H atom abstraction from a phenolic compound by a free radical.[180] Most of the PhO−H bond energy differences in H-bonding and nonbonding solvents (calculated from the enthalpy of the reaction between di-*tert*-butyl peroxide and phenol) could be accounted for from the known hydrogen-bonding equilibrium between the solvents and the phenol.[181] For strong antioxidant activity, the presence of an *o*-dihydroxyphenol (catechol) moiety in an antioxidant compound has an additional advantage of chelating transition metal ions such as iron and copper, thereby hindering transition metal-initiated Fenton-type reactions generating reactive species. On the other hand, the abnormally high rate constants in alcohols for H atom abstraction from 13 hindered and nonhindered phenols by DPPH$^\bullet$ were attributed to the partial ionization of the phenols (Ph−OH) in alcohols and a very fast electron transfer from phenoxide anion (Ph−O$^-$) to DPPH$^\bullet$; this also applies for low pK_a phenols in nonhydroxylic polar solvents such as di-*n*-butyl ether, acetonitrile, tetrahydrofuran, and dimethyl sulfoxide.[182] The rate constants for DPPH oxidation of phenols in alcohols were increased by the addition of sodium methoxide and were decreased by added acetic acid. The initial fast chemistry of the oxidation of curcumin with DPPH$^\bullet$ was attributed to the presence of curcumin anions present at low equilibrium concentrations in alcohols, whereas upon rapid depletion of these "preformed" anions, ionization of curcumin became partially rate-limiting.[77] In this regard, the TEAC coefficients (with respect to the CUPRAC method) of the antioxidant compounds quercetin, catechin, and BHT were higher in pure MeOH than in pure EtOH, probably due to facilitated electron transfer in ionizing solvents capable of anion (phenolate) solvation, because MeOH is the alcohol that best supports ionization.[173] The structure of the B ring in flavonoids seemed to be the primary determinant of antioxidant activity when studied through fast reaction kinetics with an oxidizing reagent such as ABTS$^{\bullet+}$.[183] When the H atom donating abilities of 15 flavonoids were studied by ESR, the second-order reaction rates, primarily governed by O−H bond dissociation energies,

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were myricetin > morin > quercetin > fisetin catechin > kaempferol ≈ luteolin > rutin > D-α-tocopherol > taxifolin > tamarixetin > myricetin 3′,4′,5′-trimethyl ether > datiscetin > galangin > hesperitin ≈ apigenin.[184] Antioxidant effectiveness and reactivity were highly dependent on the configuration of −OH groups on the flavonoid B and C rings, with a very minor contribution from the A ring. The rate constants for the free radical scavenging action of flavone, chrysin, and flavonol were low, indicating that the reactivities of 5- and 7-OH groups at the A ring and the 3-OH group at the C ring were very weak and almost negligible; rutin and quercetin with 3′- and 4′-OH groups at the B ring showed high reactivity, indicating that the *o*-dihydroxyl (catechol) structure in the B ring was the obvious radical target site for flavonoids.[185] Highest reaction rates and stoichiometries were observed with flavonols capable of being oxidized to *ortho*-quinones or extended *para*-quinones.[184] The stabilizing effect of electron-donating groups near phenolic hydroxyls may be exemplified in the much lower reactivity of 4-methoxy-2,3,5,6-tetramethylphenol (TMMP) than that of α-tocopherol against peroxyl radicals, because in TMMP, the methoxy group is twisted out of the plane of the aromatic ring by steric forces and, consequently, the p-type lone pair on the methoxyl oxygen cannot help stabilize the phenoxy1 formed upon abstraction of the phenolic hydrogen, whereas in tocopherols, the chroman ring system holds the ethereal oxygen's p-type lone pair nearly perpendicular to the aromatic ring, thereby providing additional stabilization for the resultant phenoxyls.[186] The structural requirements for strong antioxidant action with respect to both thermodynamic efficiency and kinetic rate have been excellently reviewed by Rice-Evans et al.[70] The three criteria for effective radical scavenging of flavonoids were established as the *o*-dihydroxy structure in the B ring, the 2,3-double bond in conjugation with a 4-oxo function in the C ring, and the 3- and 5-OH groups with 4-oxo function in the A and C rings, as characteristically demonstrated in the strong antioxidant flavonoid quercetin.[69,187] Flavanonols and flavanones, due to the lack of conjugation enhancing resonance stabilization over the entire molecule, are weak antioxidants.[163] The half-peak oxidation potentials ($E_p/2$) of flavonoids <0.2 V are defined as readily oxidizable and therefore good scavengers of reactive species.[163] The reduction potentials (at pH 7) of the aryloxy radical/phenol couple for quercetin (0.33 V) and myricetin (0.36 V) are lower than those for catechin (0.57 V), luteolin (0.60 V), and kaempferol (0.75 V), making the former two flavonoids stronger antioxidants in most in vitro tests. The high antioxidant activities of phenolic and hydroxycinnamic acids may be attributed to the number and position of phenolic hydroxyls, the presence of electron-donating substituents (e.g., methoxy groups) in *ortho*- and *para*-positions relative to the phenolic −OH, thereby stabilizing the produced aryloxy radicals via oxidation and the double bonds in side chains. For example, the order of antioxidant effectiveness of hydroxycinnamates on the induction period of autoxidizing fats was found as caffeic > ferulic > *p*-coumaric acid,[188] in accordance with the ET-based CUPRAC results (but not with the ABTS/TEAC results, in which caffeic acid proved to be less effective than ferulic and *p*-coumaric acids).

Recent theoretical analyses of antioxidant action revealed that the kinetics for the free radical scavenging of polyphenols (formerly assumed to consist of merely HAT and ET modes) can be further subdivided into three basic mechanisms involving H atom, proton (H^+), and electron (e^-) transfer.[189]

(i) In pure HAT, the phenolic proton and electron of the donated H atom (PhO−H) are transferred to the same atomic orbital of the free radical, whereas PCET involves several molecular orbitals; however, in both HAT and PCET, the proton and electron are transferred in one kinetic step, shown by the equation

$$PhOH + R^{\bullet} \rightarrow PhO^{\bullet} + RH$$

In fact, HAT may be visualized as a special case of concerted PCET involving electronically adiabatic proton transfer. The thermochemistry of PCET and its implications have been excellently reviewed by Warren et al.[190]

(ii) Electron transfer−proton transfer (ET-PT) is a two-step mechanism started by an e^- transfer and followed by a H^+ transfer:

$$PhOH + R^{\bullet} \rightarrow PhOH^{\bullet +} + R^-$$

$$PhOH^{\bullet +} + R^- \rightarrow PhO^{\bullet} + RH$$

In the case when PT is very fast, ET-PT is reduced to a HAT process.

(iii) Sequential proton loss−electron transfer (SPL-ET) is quite different from HAT and occurs (in three consecutive steps) by following the reverse order of ET-PT: it starts with a proton release (acidic dissociation) forming a phenolate anion, which subsequently transfers an electron. SPL-ET is favored when the phenolate anion (PhO^-) remains stable during the ET step before reprotonation.

$$PhOH \leftrightarrow PhO^- + H^+$$

$$PhO^- + R^{\bullet} \rightarrow PhO^{\bullet} + R^-$$

$$R^- + H^+ \rightarrow RH$$

Although these three mechanisms are thermodynamically equivalent (in terms of Gibbs free energy change), the competition between the different mechanisms is governed by the kinetics of the limiting step of each mechanism (atom transfer for PCET and electron transfer for both ET-PT and SPL-ET). By performing quantum chemical calculations on the primary roles of the *o*-dihydroxycatechol moiety and the 3-OH group of quercetin as scavengers of different types of free radicals, Di Meo et al. reached the conclusion that in nonpolar environments (e.g., lipid bilayer membranes) and at low pH (e.g., as in the stomach), PCET is the only possible mechanism, whereas in polar solvents and at pH where quercetin is partly deprotonated (e.g., in blood plasma), the faster and therefore more predominant process (SPLET) effectively competes with PCET.[189] Thus, the contribution of SPLET to the overall antioxidant activity of quercetin (also extrapolable to those of other flavonoids) was reported to increase with a rise in pH where phenols are dissociated to phenolates.[189] Amić et al. theoretically investigated the double-PCET (i.e. $1H^+/1e^-$ and $2H^+/2e^-$) processes of free radical scavenging by flavonoids and stressed that the significant contribution of the second PCET mechanism, resulting in the oxidative formation of a quinone/quinone methide, can effectively distinguish the active flavonoids from inactive ones.[191] In other words, the double-PCET descriptors such as the second O−H bond dissociation enthalpy related to HAT mechanism in the gas phase and lipid media and the second electron transfer enthalpy related to SPLET mechanism in an aqueous (polar) medium were found to better describe the quantitative structure−activity

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relationships of the studied set of 21 flavonoids.[191] Recently, Bakhouche et al. theoretically investigated the antioxidant activity of four forms of tocopherol using the HAT, ET-PT, and SPLET mechanisms and calculated the O−H bond dissociation free energy (BDFE), ionization potential (IP), proton dissociation free energy (PDFE), proton affinity (PA), and electron transfer free energy (ETFE) parameters in the gas phase and solvent media.[192] α-Tocopherol was shown to be the most reactive form in gas phase with the lowest BDFE, IP, and PA values. In the gas phase and nonpolar media, the HAT mechanism was predominant due to the fact that BDFE was lower than PA and IP, whereas in aqueous and polar solvents (e.g., DMSO and MeOH), SPLET was more effective because the PA of all forms of tocopherol was considerably lower than BDFE and IP.[192]

In ET-based TAC assays, kinetic solvent effects work in a different way. If the redox couple of the TAC assay reagent is a coordinatively saturated metal complex [involving different oxidation states of a given metal ion in the same ligand environment such as bis(neocuproine)copper(II,I), tris(1,10-phenanthroline)iron(III,II), hexacyanoferrate(III,II)] capable of outer-sphere electron-transfer with the polyphenol,[193] then a minor reorientation (but not substitution) of the already existing ligands around the central metal ion may be expected in the formation of a transient intermediate during the ET process and, consequently, the reaction rate may only be affected to a limited extent by solvent polarity. In this case, the magnitude of solvent effects may be determined by the extent of geometric rearrangement around the coordination center during ET. However, inner-sphere ET reactions of the assay reagent (e.g., hexaaqua-solvated Fe^{3+}) with phenolics will naturally be affected by the hydrogen-bonding behavior of the solvent due to stabilization or inhibition of the intermediary complex formed during electron transfer. When other factors are disregarded or assumed to remain constant, HAT-based TAC assays (e.g., ORAC, TRAP, and mixed-mode ABTS) are generally affected to a greater extent by the solvent behavior (polarity, HBA, etc.) than ET-based methods relying on outer-sphere electron transfer (e.g., CUPRAC, ferricyanide, and FRAP), provided that the ET reagent chromophore is soluble at effective concentrations in the solvent of concern.[173]

3.2. Partitioning of Antioxidants and the Polar Paradox Hypothesis. The capacity for inhibition of lipid peroxidation was measured in many different systems such as homogeneous solutions, aqueous miscelle dispersions, liposomal membranes, isolated low-density lipoproteins (LDLs), red blood cells, and plasma. Other than the major parameters of antioxidant activity/capacity, localization of antioxidant, fate of antioxidant-derived radical, interaction with other antioxidants, and mobility of antioxidant at the microenvironment are also important.[58]

The polar paradox (PP) hypothesis describing the varying behavior of an antioxidant at different locations can provide limited information about the actually complex behavior of antioxidants in oil, lipid, and fatty acid emulsions (in water) due to a number of constraints:

(i) The PP hypothesis simply states that due to different locations of polar and nonpolar antioxidants in bulk oil and water, nonpolar antioxidants should be more effective than their polar homologues in oil-in-water emulsions because of their better enrichment at the oil−water interface where oxidation primarily takes place. Even though this was basically confirmed in earlier cases, recent experiments carried out by varying the polarity of antioxidants by esterification with various alkyl chain lengths showed that esterified phenolic antioxidants (such as those of chlorogenic, rosmarinic, and dehydrocaffeic acids, tyrosol and hydroxytyrosol fatty acids, and rutin) essentially obeyed the PP hypothesis up to C_8−C_{12} carbon atom chain length, beyond which antioxidant activity sharply declined (called the "cutoff effect"), not conforming to expectations.[194−196] Bulky sized phenolic antioxidants (such as those with long side chains), despite their hydrophobic character, may also show lower mobility because of steric hindrance and thus decreased diffusibility toward reactive centers at the interface.[197] It is also probable that the longer chain antioxidants may form mixed micelles with emulsifiers used to prepare the emulsions resulting in their migration away from the emulsion droplets.[194−196] Emulsifiers may alter the physical location of antioxidants and the activity of prooxidants; they can also compete with antioxidants for localization at the interface.[197]

(ii) The PP hypothesis disregards the possible prooxidant effects of antioxidants under pH- and concentration-dependent conditions. The concentration dependency is so important that the hypothesis may be applicable only when the antioxidant reaches a critical concentration so that interfacial phenomena are predominant over solubility effects.[197] Some polyphenolics may act as prooxidants within certain concentration ranges by reducing transition metal ions to their lower oxidation states, thereby enhancing Fenton-type oxidation reactions,[4] which may give rise to "false antioxidant activity" measurement with respect to polarity.[197]

(iii) Excluding surfactant effects, antioxidants can exhibit unexpected interactions with oxidation promoters and prooxidants (redox reactions) and with trace metal ions (complexation or chelation) or even among themselves (e.g., through H-bonding and association), resulting in synergistic or antagonistic effects to change the kinetics of lipid peroxidation[198] and thus deviate from PP hypothetical expectations.

(iv) If the changes in polarity of the phenolic antioxidant are introduced by adding or removing ring substituents, then such an addition/removal may actually change the O−H BDE and hence the antioxidant potency of the molecule.[197]

3.3. Possible Interactions among Antioxidant Constituents. The coexistence of multiple antioxidants usually results in additive effects, whereas synergistic or antagonistic interactions may appear in extreme cases. Synergism or antagonism occurs when the antioxidative protection/scavenging of two antioxidants is greater or smaller than the sum of their individual effects, respectively. Choe and Min discussed the possible reasons of synergism in antioxidant activity.[174]

Synergy and antagonism are two important issues in food and nutrition science because of the requirement to choose food combinations exhibiting maximal synergism and minimal antagonism in antioxidant activity. Antioxidative synergy may arise when two antioxidants (extendable to a greater number of antioxidants) in admixture show the following behaviors: (i) They display regenerative action, where the primary

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(stronger) antioxidant is regenerated by the weaker (secondary) one; for instance, carotenoids may be regenerated by α-tocopherol, which in turn may be regenerated by ascorbate, because the order of reduction potentials for their corresponding oxidized forms (radicals) is $E_{carotenoid} > E_{tocopherol} > E_{ascorbate}$.[162,199] It should be added that this protective action may also suppress the possible prooxidative action of tocopherol, because the tocopheroxyl radical at relatively high concentrations with respect to lipids may abstract an H atom from lipids and produce lipid radicals, whereas ascorbate may obstruct this pathway by regenerating tocopherol from tocopheroxyl radical.[200] Likewise, inhibited oxygen uptakes of a combination of α-tocopherol and flavonoids measured in *tert*-butyl alcohol confirmed the existence of an antioxidant interaction, which produced a more effective inhibition of lipid peroxidation, and the extended lag times provided evidence for the regeneration of α-tocopherol by quercetin.[201] (ii) They exhibit cooperative mechanisms of action; for instance, quercetin may act as a metal chelator, whereas α-tocopherol functions as a radical scavenger.[202] On the other hand, although there is no systematic theory of antagonistic interactions among antioxidant compounds, it is thought to arise from the regeneration of the minor (less effective) antioxidant from the major (more effective) one, competition between the formation of antioxidant−radical adducts, and changing of the microenvironment suitable for one antioxidant in the presence of the other,[174] as observed in binary mixtures of α-tocopherol with either caffeic acid or rosmarinic acid or in binary mixtures of caffeic acid with either catechin or quercetin.[203]

From our own laboratory experience with spectrophotometric ET-based TAC assays, we observed that if antioxidant concentrations in mixtures are appropriately selected to obey Beer's law, then the principle of additivity of absorbances (due to the color change of ET reagent) would lead to the additivity of total antioxidant capacities of mixture constituents:

$$TAC_{(x1,\ x2\ \ mixture)} = TAC_{(x1)} + TAC_{(x2)}$$

in mmol (or micromol) trolox-equiv./L

Only in this case, real synergistic or antagonistic interactions (if any) would manifest themselves. If the results of spectrophotometric ET-based (e.g., CUPRAC, FRAP, ferricyanide) or mixed-mode (e.g., ABTS and DPPH radical scavenging) assays relying on a fixed-time end-point are evaluated with the above approach, one would see that most data assumed to originate from synergistic or antagonistic interactions (via calculations involving quasi-linear combinations of lag time or inhibition percentage) are in fact additive,[11,64] because in accordance with Beer's law, absorbance varies linearly with concentration within a reasonable range, which is not valid for the essentially nonlinear parameters of lag time or inhibition percentage.[204,205] The synergy observed with the CUPRAC method in (BHT + BHA) or (BHT + TBHQ) mixtures[173] has not yet been fully interpreted, but is thought to arise from enhanced solubilization and stabilization of the semiquinone radical adducts formed in the course of oxidation via intermolecular H-bonding and dimerization. Thiol mixtures seemingly exhibiting synergy in FRAP and ABTS assays may involve further oxidations in the presence of multiple thiols (e.g., the partially reversible oxidation reaction expected to generate disulfides may go beyond that stage, giving rise to sulfenic and sulfinic acids).

Synergy and antagonism remain to be hardly quantifiable behaviors of diverse antioxidant mixtures. Recently, extensive efforts are being spent quantifying synergy or antagonism in antioxidant activity using different reagents and calculation methods. For instance, Skroza et al. observed synergy in the binary mixtures of resveratrol with catechin and gallic acid.[206] Prieto et al. used a dose-dependent mathematical model based on probability functions, where the interactive effects between antioxidants were introduced into the model with simple auxiliary functions that describe the parametric variations induced by each antioxidant in the presence of the other and a collective index of parametric responses was generated, and experimentally demonstrated this effect using DPPH and ABTS assays, extendable to other ET assays.[207]

3.4. Enhanced Solubilization of Antioxidants via Coupled Oxidation Using TAC Reagents. Because dietary fiber phenolic compounds (DF-PC) are made up of bound hydroxycinnamic acids, TAC of cereal fiber has been disputed, and therefore the actual TAC of DF-PC has been largely underestimated because of their low water and organic solvent solubilities. In fact, a proper assessment of DF-PC antioxidant capacity requires a multiple-step extraction and an appropriate chemical hydrolysis to release PC and to permit them to exert antioxidant activity in the in vitro assays. For many years, it was not clear whether the water-insoluble DF-PC fraction could exert any antioxidant action itself, that is, without any chemical hydrolysis.[208] Serpen et al.[209] were the first researchers to demonstrate the concept of antioxidant activity of insoluble material measured in a different way. In reality, the slow and continuous release of DF-PC from the insoluble material (known to survive for a considerable time) in the human gastrointestinal tract has been established to occur and, in particular, DF-PC may favorably act in vivo, quenching the soluble radicals that are continuously formed in the intestinal tract.[208] Tufan et al.[210] determined the TAC of cereals (i.e., barley, wheat, rye, oat) by the "QUENCHER procedure" (involving forced solubilization of bound phenolics by oxidizing TAC reagents) with the direct use of copper(II)-neocuproine [Cu(II)-Nc] reagent of the CUPRAC assay; the assay operated without a need for completely solubilizing or extracting antioxidant constituents of insoluble matrices before the CUPRAC reaction, because the driving force for the CUPRAC oxidation of bound phenolics was greater than that for their solubilization, making the whole coupled-oxidation process thermodynamically favorable. The authors effectively measured the TAC values of cereals by their proposed QUENCHER-CUPRAC assay, and their results linearly correlated with those of reference methods (ABTS and DPPH). They found additive responses of polyphenolic mixtures in a cellulose matrix with their proposed method.[210] Solvent effects in the QUENCHER approach were recently discussed.[211] Enhanced solubilization of antioxidant compounds via coupled oxidation is an active area of research, with many potential contributions in food analytical chemistry that may allow the building of unique databases for determining the antioxidant capacity of foods having insoluble moieties, for example, the polysaccharide matrix.[212]

4. A PRACTICAL GUIDE TO ANTIOXIDANT ASSAY SELECTION

In conclusion, we also propose a rather subjective guide (primarily stemming from our laboratory's experiences) for assay selection, briefly summarizing which assay can be chosen (or not to be preferred) to serve a specific purpose. Ideally, the chosen test should be sensitive, selective, robust, and reproducible, use conventionally available reagents and instruments,

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and measure a wide variety of antioxidant types including both lipophilic and hydrophilic antioxidants, but unfortunately these criteria are not met by a single specific assay. It is obvious that one has to decide at the start whether antioxidant activity (basically reaction rate) or antioxidant capacity (stoichiometric conversion efficiency) is to be measured. Antioxidant activity tests having an AUC approach toward fluorescence decay of a probe attacked by reactive species in the presence of antioxidants (such as ORAC) can simultaneously measure the lag phase, initial rate, and total extent of inhibition of antioxidants. On the other hand, such tests may not differentiate between reaction rate and radical scavenging efficiency and may give positively biased results for slowly reacting antioxidants. It is also recommended that test probe selection should be made in accordance with the classical definition of an "antioxidant"; that is, the concentration of the tested antioxidant should be much smaller than that of the probe, a requirement that is not strictly obeyed in certain HAT-based assays using fluorescent probes.

(i) The working pH is an important parameter of antioxidant assay selection. FRAP, CUPRAC, and Folin methods can be used for acidic (pH 3.6), neutral (pH 7.0), and alkaline (pH 10) media measurements, respectively. It should be borne in mind that phenolic antioxidants are undissociated, partly dissociated, and predominantly dissociated at the working pH values of FRAP, CUPRAC, and Folin methods, respectively, possibly reflecting an underestimation (in the case of FRAP) or overestimation (in the case of Folin) of the in vitro antioxidant action simulating physiological conditions. Classical "ferric reducing assay" utilizing ferricyanide incubation with the antioxidant followed by ferric ion addition at the end of the reaction (prior to color development) works at pH 6.6, whereas modified ferricyanide assay initially incorporating Fe(III) in the incubation solution has a pH of 1.7. A mixed-mode (HAT/ET) free radical scavenging assay such as the DPPH assay may also be very sensitive to pH, as the rate-determining step of proton-coupled electron transfer may be the acidic dissociation of a phenolic proton, followed by a fast electron transfer from the phenolate anion to DPPH. In general, a rise in pH significantly enhances both the rate and efficiency of phenolics oxidation in ET and mixed-mode assays, owing to deprotonation.

(ii) Use of natural materials in the selected assay may adversely affect the reproducibility of results. For example, the natural pigment crocin or the natural protein β-phycoerythrin may contain impurities and may vary from lot to lot in production. The same applies for lipids (e.g., purified LDL) in testing antioxidative action against lipid peroxidation.

(iii) Multicharged chromophores of TAC redox probes strongly interact with solvent water molecules by ion−dipole interaction, and therefore respond less to hydrophobic antioxidants. CUPRAC and ABTS methods, having monopositive charged chromophores, can simultaneously measure hydrophilic and lipophilic antioxidants, whereas ORAC, FRAP, ferricyanide, and Folin methods having either hydrophilic or multicharged chromophores require the use of cyclodextrins (CDs) or micellar solutions to enhance the solubilization of lipophilics. Unfortunately, cyclodextrins form inclusion complexes with antioxidants, which may adversely affect subsequent reaction with a redox probe if the antioxidant−CD complex is too stable at relatively high concentrations of CDs necessarily used for solubilization.

(iv) Colorimetric reagents having chromophores absorbing distinctly in the visible range of the electromagnetic spectrum (i.e., as far as possible to the UV range) are the appropriate methods of choice to deal with the background color of plant pigments and other UV-absorbing substances. The background color arising from the matrix shows maximal adverse effects on the precision of color-fading reactions (such as DPPH and ABTS) rather than on that of color-forming reactions (such as FRAP, CUPRAC, and ferricyanide). Antioxidant activity assays based on diene formation or hydrogen peroxide scavenging, all involving UV measurements, may be adversely affected from UV-absorbing interferents. This applies well to most plant extracts and pharmaceutical preparates, where many constituents strongly absorb in the UV range.

(v) Redox potential of the selected probe is important in antioxidant assay selectivity. CUPRAC, ABTS, and FRAP methods use oxidant probes having 0.60, 0.68, and 0.70 V reduction potentials (vs NHE), which are at the right potential to oxidize many common antioxidants lying in the 0.2−0.6 V redox potential range, whereas the conventional ferricyanide test has an insufficient potential (0.36 V vs NHE) to oxidize certain antioxidants and the Folin test has an indefinitely high potential (enabling the oxidation of citric acid, reducing sugars, and some amino acids, which are not "true" antioxidants). The newly developed colorimetric redox reagents containing Ce(IV), Cr(VI), and Mn(VII) centers have to be tested for longer periods of time to see the exact interference results, but it may be speculated that their high redox potentials, even when restricted by certain measures, would not enable highly selective TAC assays as other organics would be co-oxidized.

(vi) When metal ion-containing probes (such as those of FRAP and CUPRAC) are used, strong chelating or complexing agents may interfere with the assay. For example, we have observed that EDTA at sufficiently low concentrations to preserve serum samples does not interfere with the CUPRAC assay, but at higher concentrations, it may partly displace the neocuproine ligand from the coordination sphere of Cu(II) and decrease the redox potential of the Cu(II,I) couple, thereby preventing the oxidation of certain antioxidants. The same applies for FRAP when strongly complexing ligands displace the TPTZ ligand from the coordination sphere of Fe(III) ion.

(vii) Metal ion-containing probes may also suffer from undesired redox cycling of antioxidants, causing serious errors in TAC calculation. For example, the relative concentration of free Fe^{3+} ion in the FRAP reagent is higher than stoichiometrically required for the 1:2 Fe(III)−TPTZ complex. As the close standard potentials of $Fe^{3+,2+}$ and $Fe^{III,II}(TPTZ)_2$ redox couples are 0.77 and 0.70 V, respectively, significant amounts of Fe^{2+} may be formed along with $Fe^{II}(TPTZ)_2$ upon reduction reaction with antioxidants and may subsequently give rise to Fenton-type reactions with H_2O_2 or dissolved O_2.

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Production of reactive species may give rise to redox cycling of antioxidants causing erroneous TAC results. On the other hand, the standard potentials of $Cu^{2+,1+}$ and $Cu^{II,I}(Nc)_2$ redox couples are quite different in magnitude, being 0.17 and 0.60 V, respectively, and therefore no redox cycling is observed because the reduction product of the CUPRAC reagent with antioxidants is definitely $Cu^{I}(Nc)_2$, which may not be reoxidized with H_2O_2.

(viii) The active constituent of FRAP reagent is the ferric−TPTZ complex bearing high-spin iron, which exhibits slow kinetics, and therefore FRAP does not sufficiently respond to thiols mainly due to kinetic rather than thermodynamic reasons. As a result, due to its inefficiency in oxidizing biothiols (including both small-molecule and protein thiols), FRAP method may not be recommended for biological samples. We have observed in our laboratory that CUPRAC and ABTS methods give satisfactory results for measuring the TAC values of serum samples, due to their capability of simultaneously measuring lipophilic and hydrophilic serum antioxidants together with thiols. The results of ABTS test for biothiols should be carefully interpreted, because the TEAC coefficients of ABTS test for biothiols vary roughly between 1.2 and 1.6 depending on the method of $ABTS^{\bullet+}$ radical preparation, hinting at the fact that thiol (RSH) oxidation in ABTS assay proceeds further beyond disulfides (i.e., RSSR, encountered in CUPRAC test with a TEAC coefficient of roughly 0.5 for RSH) to higher oxidation products.

(ix) Structural limitations may be responsible for the slow kinetics of phenolic antioxidants having bulky substituents. For example, mixed-mode assays using ABTS and DPPH hindered radicals may suffer from steric accessibility problems caused by polymeric phenols having multiple hydroxyl groups and ring adducts (e.g., tannins). Use of assays using outer-sphere electron-transfer agents (such as CUPRAC, FRAP, and ferricyanide) may be more appropriate in such samples. It should always be remembered that reaction kinetics is a function of antioxidant structure, pH, temperature, and solvent type, and some ET (e.g., FRAP) or mixed-mode (ABTS and DPPH) assays cannot go to completion within the protocol time of the assay.

(x) In competitive assays, one has to avoid "short-circuit" reactions. The tested antioxidant may react directly with the probe (e.g., thiol−NBT reaction in superoxide scavenging test) instead of competing with the radical. Similar reactions may occur in enzyme-mediated generation of reactive species (e.g., peroxidase may directly oxidize ABTS in hydrogen peroxide scavenging test without adding H_2O_2).

(xi) Several assays, preferably working in different modes (i.e., HAT, ET, mixed-mode), may be used together to reveal the whole picture of antioxidative action. It should be borne in mind that a fast-reacting antioxidant may stay behind a slow-reacting one in antioxidant ranking when an end-point assay is used because of the fact that the latter quenches a greater number of free radicals per molecule. Thus, it may be advisible to use both AOA and TAC assays in biologically relevant antioxidant evaluation.

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ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-methylpropionamidine) dihydrochloride
ABAP, 2,2′-azobis(2-amidinopropane) dihydrochloride
ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)
AOA, antioxidant activity
AOX, antioxidants
AgNPs, silver nanoparticles
AUC, area under curve
AuNPs, gold nanoparticles
β-PE, *β*-phycoerythrin
CERAC, Ce(IV) reducing antioxidant capacity
CHROMAC, Cr(VI) reducing antioxidant capacity
CL, chemiluminescence
CUPRAC, cupric reducing antioxidant capacity
Cu(II)-Nc, bis(neocuproine) copper(II) cation
$[Cu(Nc)_2]^+$, cuprous neocuproine
CV, cyclic voltammetry
DCM, dichloromethane
DHBA, dihydroxybenzoic acid
DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride
DPPH, 2,2-diphenyl-1-picrylhydrazyl
DPV, differential pulse voltammetry
DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)
TNB, 5-thio-2-nitrobenzoate
ECL, electrochemiluminescence
EPR, electron paramagnetic resonance
ESR, electron spin resonance
ET, electron transfer
ETPT, electron transfer proton transfer
FRAP, ferric reducing antioxidant power
GAE, gallic acid equivalent
GC, glassy carbon
GSH-Px, glutathione peroxidase
GSH-Rx, glutathione reductase
HAT, hydrogen atom transfer
H_2O_2, hydrogen peroxide
HOCl, hypochlorous acid
HPS, hydrogen peroxide scavenging
LMWA, low molecular weight antioxidants
LSPR, localized surface plasmon resonance
KMBA, *α*-keto-*γ*-methiolbutyric acid
M-*β*-CD, methyl-*β*-cyclodextrin
NBT, nitroblue tetrazolium
Nc, neocuproine

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•NO, nitric oxide radical
•NO_2, nitrogen dioxide radical
$^1O_2$, singlet oxygen
$O_2^{\bullet-}$, superoxide anion radical
•OH, hydroxyl radical
$ONOO^-$, peroxynitrite anion
ORAC, oxygen radical absorbance capacity
PCA, principal component analysis
PCET, proton coupled electron transfer
PLS, partial least-squares
PP, polar paradox
$RO^{\bullet}$, alkoxyl radical
$ROO^{\bullet}$, peroxyl radical
ROS, reactive oxygen species
RNS, reactive nitrogen species
SNPs, silver nanoparticles (as in Figure 8)
SOD, superoxide dismutase
SPE, screen-printed carbon electrode
SPLET, sequential proton loss electron transfer
SPR, surface plasmon resonance
SRSA, superoxide radical scavenging activity
SWV, square-wave voltammetry
TAC, total antioxidant capacity
TBARS, thiobarbituric acid-reactive substances
TBHQ, *tert*-butylhydroquinone
TE, Trolox equivalent
TEAC, trolox-equivalent antioxidant capacity
TOSC, total oxyradical scavenging capacity
TPTZ, 2,4,6-tripyridyl-*s*-triazine
TRAP, total peroxyl radical trapping antioxidant parameter
XOD, xanthine oxidase

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