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Effects of Molecular Structure on Kinetics and Dynamics of the Trolox Equivalent Antioxidant Capacity Assay with ABTS$^{+\bullet}$

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ABSTRACT: Reaction kinetics in the Trolox equivalent antioxidant capacity (TEAC) assay between ABTS$^{+\bullet}$ [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical] and compounds with different structure, molecular weight, number of OH groups, and redox potential were investigated by recording loss of ABTS$^{+\bullet}$ absorbance (734 nm) continuously over time. Curves showed six distinguishable kinetic patterns, including both immediate and extended reaction components. Radical quenching rates in the immediate component most relevant to reactions in foods and tissues depended on phenol structure and steric accessibility to the hindered radical, while reaction stoichiometry correlated with the number of phenol groups (>0.81) but not redox potential. Current assay procedures measure antioxidant capacity under conditions not relevant to actual applications and do not determine radical quenching rates. Results raise serious questions regarding the ability of reactions with the hindered ABTS$^{+\bullet}$ to rank actual radical quenching by compounds with different structures and invalidate reporting antioxidant activity as Trolox equivalents.

KEYWORDS: *antiradical activity, phenols, phenolic structure, Trolox Equivalent Antioxidant Capacity (TEAC), hydrogen atom transfer, electron transfer, ABTS$^{+\bullet}$, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) cation radical, radical quenching kinetics, radical quenching capacity*

INTRODUCTION

Natural compounds with ability to limit radical reactions by transferring hydrogen atoms or electrons have been shown to be important factors reducing or preventing oxidative stress-related diseases in vivo[1−3] as well as stabilizing foods during processing and storage.[4−6] Synthetic antioxidants such as BHA and BHT used traditionally in foods have demonstrated toxicity[7] so are being replaced by a variety of natural antioxidants. Thus, for both health and stability reasons, antioxidant capacity and constituents in foods we consume have become a topic of intense interest to the general public as well as to medical and nutritional experts and food science research.

Numerous assays have been developed to provide fast prediction of antioxidant or antiradical activity of natural compounds and extracts.[8] Currently, the most widely used methods include oxygen radical absorbance capacity (ORAC),[9] Trolox equivalent antioxidant capacity (TEAC),[10] 2,2-diphenyl-1-picrylhydrazyl (DPPH),[11] ferric reducing ability of plasma (FRAP),[12] and cupric iron reducing antioxidant capacity (CuPRAC)[13] assays. However, each of these assays has limitations, and they are difficult to compare directly because they differ from each other in terms of substrates, probes, and quantitation methods.[14] Even within the same assay, lack of standard procedures makes it difficult to compare data from lab to lab.[8] Most importantly, radical quenching is not a single reaction but embraces multiple mechanisms including hydrogen atom transfer, electron transfer, metal chelation, and others.[15] So far, no single assay accurately reflects them all.[16]

The complexity of chemistry of antioxidant assays and inconsistent procedures have created considerable disorder and controversy in antioxidant reporting. Without standardization of analytical methods, reliable measures of rates, extent, and conditions for radical quenching by natural compounds of different structures cannot be provided for the food and nutraceutical industries.[16] Too much emphasis on fast screening of antioxidant action with inadequate attention to the fundamental chemistry of compounds and reactions, including kinetics, has often led to erroneous interpretation of experimental results of assays and inappropriate application of antioxidants. Finding methods that accurately reflect antioxidant chemistry has been a particular problem.

As part of a larger project studying mechanisms and standardizing procedures in antioxidant activity assays, this research reinvestigated the chemistry and reaction conditions of the total equivalent antioxidant capacity (TEAC) assay (also known as the ABTS assay) to elucidate active reaction mechanisms, optimize methodology, provide a basis for standardization, and determine appropriate applications. To learn more about reaction kinetics, absorbance changes were monitored continuously as well as before and after reaction as in conventional procedures. This approach revealed significant differences in reaction kinetics for different phenols and identified marked dependence on molecular size and concentration. We report here kinetic data and reactivity patterns that raise serious questions about current methodology and, even more, about valid applications of this assay.

MATERIALS AND METHODS

Chemicals and Reagents. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was obtained from Sigma-

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Aldrich (St. Louis, MO). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%), 4-hydroxy-3-methoxybenzoic acid (ferulic acid) (97%), α-D-glucose (A.C.S. reagent), coniferyl alcohol (purified by preparative HPLC), 3-methylcatechol (99%), and pyrogallol (99%, A.C.S. reagent) were products of Aldrich Chemical Co. (Milwaukee, WI). Uric acid (~99%), protocatechuic acid, chlorogenic acid (minimum 95% by titration), catechin (minimum 98%, HPLC), catechin gallate (minimum 98%, HPLC), gallocatechin (>98% HPLC), gallocatechin gallate from green tea (minimum 98%, HPLC), benzoic acid (minimum 99.5%), *n*-propyl gallate, rutin (hydrate, minimum 95%), L-cysteine (97%), caffeic acid, *p*-coumaric acid, and catechol (~99%) were obtained from Sigma-Aldrich (St. Louis, MO). Hydroquinone was obtained from Allied Chemical (NY). Resorcinol and reduced glutathione were purchased from Nutritional Biochemicals Corporation (Cleveland, OH). Quercetin (99%, by HPLC) was purchased from Indofine Chemical Company, Inc. (Hillsborough, NJ). High purity water (18 MΩ resistivity) generated in a Milli-Q four-cylinder water purification system (Milli-Q Corp., Milford, CT) was used in all analyses.

Determination of Antioxidant Activity. The ability of antioxidants to quench the $ABTS^{+\bullet}$ cation radical [16] (Figure 1) was

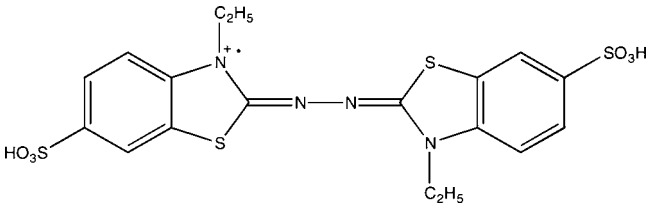

Figure 1. Structure of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) ($ABTS^{+\bullet}$).

determined by a modification of the improved ABTS method of Re et al.,[10] sometimes called TEAC II. Compounds tested for reactivity included simple phenols and catechins with different numbers of phenolic groups and ring adducts, as well as nonphenolic compounds known to have radical quenching ability; nonantioxidant compounds (urea, methyl indole, sugars) provided specificity controls. A 7 mM stock solution was prepared by dissolving ABTS in Milli-Q water. Potassium persulfate (2.45 mM final concentration) was added to this stock solution, and the mixture was allowed to stand for 12−16 h at room temperature until a dark blue-green color developed.[10] Fresh stock solutions of $ABTS^{+\bullet}$ were prepared weekly. Before each analysis session, aliquots of the $ABTS^{+\bullet}$ stock solution were diluted to an absorbance of ~1.0 (~6.67×10^{-5} mol) using Milli-Q water. Although Re et al.[10] recommended $ABTS^{+\bullet}$ with absorbance ~0.7, we found that using a higher concentration ($A \approx 1.0$, 67 μM) allows testing of a more extended range of antioxidant concentrations. Stock solutions (10 mM) of test antioxidant compounds were prepared in water sparged with argon. Final reaction concentrations of antioxidant were 0.5 mM, 0.25 mM, 0.1 mM, 0.05 mM, 0.005 mM, and 0.001 mM. These covered a range from well below [$ABTS^{+\bullet}$], to allow full reaction, to well above $ABTS^{+\bullet}$ saturation levels, to eliminate possible diffusion effects. Final pH of the reaction mixture was 4.7.

In initial testing, we found that $ABTS^{+\bullet}$ reactions occurred faster than mixing and transfer of standard cuvettes. Therefore, to provide more rapid and consistent mixing, structure−reactivity relationship assays were performed in 96-well transparent plates using a Biotek Synergy 2 Multi-Detector Microplate Reader (Biotek, Winooski, VT) with dispensers. Ten microliter test antioxidant solutions were deposited manually into separate wells. A 190 μL $ABTS^{+\bullet}$ working solution was then dispensed automatically into each well sequentially, and absorbance data recording was initiated within 0.02 s. Preliminary experiments with long recording times showed that reactions of very active compounds were complete within seconds and slower reactions were largely complete within minutes. Furthermore, reactions of likely target hydroxyl ($HO^{\bullet}$), superoxide ($O_2^{-\bullet}$), and lipid oxyl ($LOO^{\bullet}$ and $LO^{\bullet}$) radicals in vivo or in foods are very fast (milliseconds to seconds)[17] and will not survive for "slow" reactions over extended periods. Thus, routinely monitoring absorbance changes for only 6 min was considered adequate for detecting critical radical-quenching chemistry.

TEAC values were expressed as Trolox equivalents[10] calculated from $(A_i - A_f)_{antioxidant}/(A_i - A_f)_{Trolox}$ at 0.05 mM concentrations, and from the ratio of the slopes of the concentration−response curves, AOX/Trolox.[18] The conventional TEAC expressed as the concentration of Trolox giving the same percentage reduction of absorbance at 734 nm as the 1 mM antioxidant solution was not used because 1 mM antioxidant is considerably in excess of the $ABTS^{+\bullet}$ in solution. Reaction kinetics were evaluated by plotting initial immediate drops in $ABTS^{+\bullet}$ versus antioxidant concentration for fast reactors, or final absorbance drops versus antioxidant concentration for slow reactors that showed no initial absorbance drop. First-order rate constants determined from slopes of the concentration plots provided objective measures of concentration dependence and, inversely, steric hindrance; shapes of the response curves provided a basis for assessing types of reaction and steric effects. All analyses were performed at least in triplicate. Pearson correlation coefficients between various antioxidant properties and their reactions responses were calculated using the statistical functions in Excel.

RESULTS AND DISCUSSION

Most studies using the $ABTS^{+\bullet}$ assay evaluate a single AOX concentration, record only the absorbance reduction at the end of 6 min or varying longer times (up to several hours), and calculate TEAC values as $[A_{final} - A_{initial}]_{test\ compd}/[A_{final} - A_{initial}]_{Trolox}$ or variants thereof. This method of recording only beginning and ending $ABTS^{+\bullet}$ absorbance values provides a fast screening tool and a value for comparison but provides only time-dependent stoichiometry of reaction (mols $ABTS^{+\bullet}$ quenched per OH group over a given time). Most importantly, it does not measure reaction rate, so it misses the critical initial fast reaction and eliminates distinction between fast and slower reacting antioxidants. In contrast, recording absorbance changes continuously over time and running the assay over a range of antioxidant concentrations provides kinetic information and reveals important structure−reactivity patterns of different classes of antioxidant compounds.

Continuous absorbance monitoring shows that antioxidants fall into several reactivity classes determined by rates of initial and subsequent reaction and patterns of concentration dependence. Table 1 lists characteristics of six reactivity patterns observed in this study.

Group 1 (Figure 2): Reaction complete within mixing time, instantaneous absorbance drop, and no further reaction thereafter. Examples: Trolox, hydroquinone, chlorogenic acid, and ascorbic acid.

Group 2 (Figure 3): Instantaneous drop but some slight further reaction at higher concentrations; all reaction responses registered within a few seconds. Examples: protocatechuic acid, 3-methylcatechol, and catechol.

Group 3 (Figure 4): Smaller initial absorbance drop and continued reaction afterward. Examples: pyrogallol, gallic acid, *n*-propyl gallate, caffeic acid, ferulic acid, coniferyl alcohol, catechin, gallocatechin, catechin gallate, gallocatechin gallate, rutin, and uric acid.

Group 4 (Figure 5): Threshold for reaction; no reaction at low concentrations, and slow continuous reaction at higher concentrations. Examples: resorcinol, *p*-coumaric, and glutathione.

Group 5 (Figure 6): No initial fast reaction, gradual drop from starting absorbance. Examples: quercetin, curcumin.

Table 1. Patterns of ABTS$^{+\bullet}$ Reaction Responses for Various Phenolic and Other Structures

	compound	initial reaction	concentration curve (final drop)
group 1	hydroquinone	instantaneous drop, no further reaction thereafter	linear over entire concentration range
	cholorogenic acid		
	Trolox		
	ascorbic acid		
group 2	catechol protocatechuic acid	lower instantaneous drop followed by short slower reaction at higher concentrations, no continued reaction afterward	slight departure from linear
	3-methylcatechol		
group 3	ferulic acid coniferyl alcohol	instantaneous drop, followed by continuing gradual absorbance drop	saturating curve, response decreases as [antioxidant] increases
	pyrogallol		
	gallic acid		
	n-propyl gallate		
	caffeic acid		
	catechin		
	gallocatechin		
	catechin gallate		
	gallocatechin gallate		
	rutin		
	rosmarinic acid		
	uric acid		
group 4	*p*-coumaric acid resorcinol	no initial fast reaction, gradual drop from the same initial absorbance	polynomial curve, response decreases as [antioxidant] increases
	glutathione		
group 5	curcumin quercetin	no reaction for low concentrations, slow continuing reaction for higher concentrations	threshold at low concentration
group 6	vanillin	little or no reaction	flat line
	phenol		
	1-methylindole		
	urea		
	glucose		
	benzoic acid		
	sucrose		

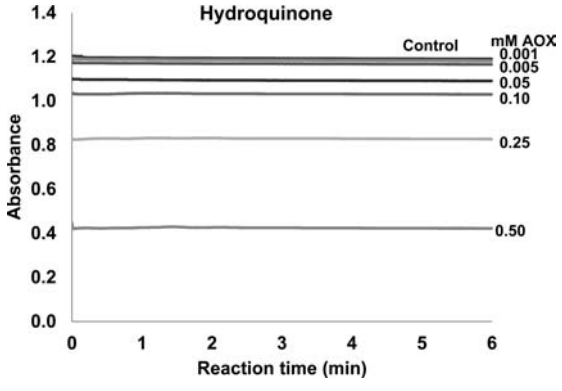

Figure 2. ABTS$^{+\bullet}$ reaction time curves for instantaneous reactors (Group I). Curves shown are for hydroquinone; similar curves were given by chlorogenic acid, Trolox, and ascorbic acid.

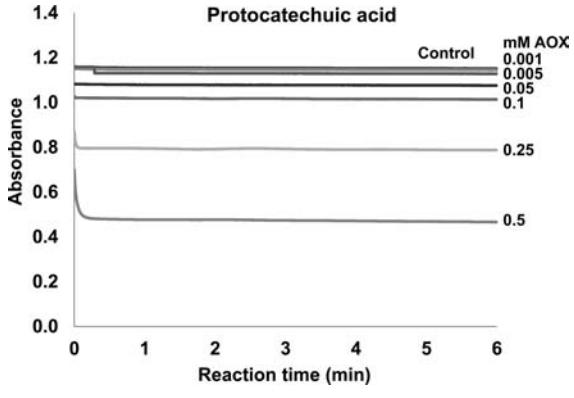

Figure 3. ABTS$^{+\bullet}$ reaction time curves for fast reactors (Group 2). Reactors in this Group gave reaction patterns similar to Group 1, but with slower initial reaction and some continued short-term reaction. Curves shown for protocatechuic acid; similar curves were given by 3-methylcatechol and catechol.

Group 6 (Figure 7): Little or no reaction. Examples: phenol, vanillin, benzoic acid, 1-methylindole, urea, glucose, and sucrose.

Differences in kinetic patterns corresponding to groups 1 and 2, and 4 and 5, have previously been reported[19−21] but do not appear to be widely recognized. While at first consideration it may seem surprising that these kinetic differences have never been incorporated into reactivity calculations, the oversight perhaps arises because the strong concentration dependence of antioxidant−ABTS$^{+\bullet}$ reactions, the very fast initial reaction and marked differences in kinetic patterns, and the time dependence of results obtained create distinct challenges in expressing antioxidant kinetics quantitatively in a way that accurately reflects the reaction chemistry in this assay.

Conversion of Reaction Curves into Numerical Values That Reflect Radical Scavenging Capabilities. Several methods of quantitating the ABTS$^{+\bullet}$ reaction were compared to determine which ones accurately reflect the radical quenching

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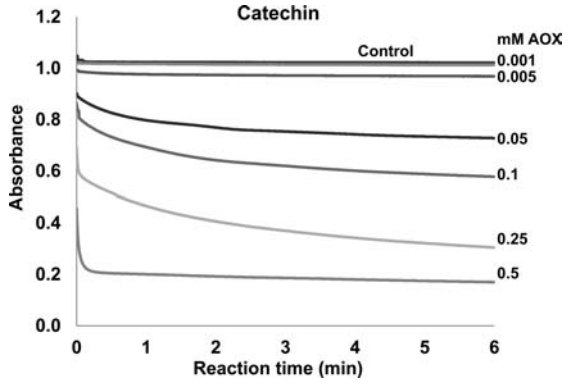

Figure 4. ABTS$^{+\bullet}$ reaction time curves for fast reactors (Group 3). Reactors in this class had continued reaction after initial absorbance drop. Curves shown are for catechin; similar curves were given by ferulic acid, coniferyl alcohol, caffeic acid, pyrogallol, gallic acid, 2-propyl gallate, gallocatechin, catechin gallate, gallocatechin gallate, rutin, and uric acid.

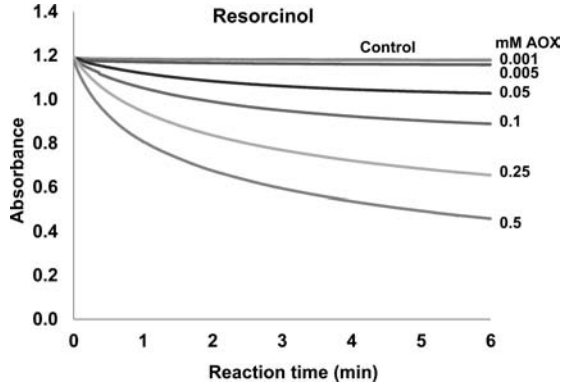

Figure 5. ABTS$^{+\bullet}$ time curves for slow reactors (Group 4). No initial fast reaction was observed; all reactions occurred gradually over time. Curves shown are for resorcinol; similar curves were given by *p*-coumaric acid and glutathione.

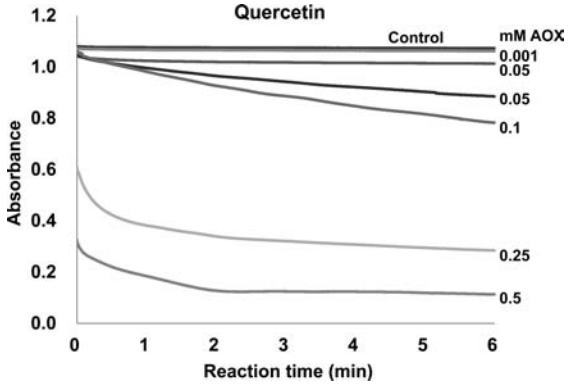

Figure 6. ABTS$^{+\bullet}$ time curves for slow reactors (Group 5). Compounds in this class had no reaction at low concentrations but showed slow continuing reaction at higher concentrations. Curves shown for quercetin; similar curves are given by curcumin.

kinetics displayed in the total reaction curves (Figures 2−7). In conventional TEAC calculations using final absorbance drops, all test compounds except curcumin had TEAC values greater than Trolox, with preference given to polyphenols and to simple phenols with three phenolic groups (Table 2). This is not surprising because the full reaction time allows for diffusion

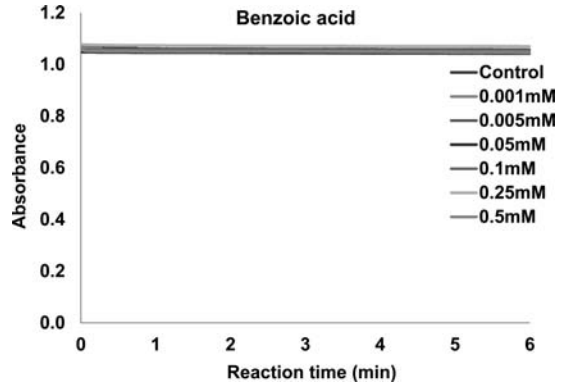

Figure 7. ABTS$^{+\bullet}$ time curves for nonreactors (Group 6). Curves shown for benzoic acid; similar curves are given by vanillic acid, 1-methylindole, phenol, urea, glucose, and sucrose.

of the antioxidants and more complete reaction of all the phenol groups. Thus, larger multiphenolic compounds that need time to reorient for docking with the ABTS radical are weighted more heavily than simpler molecules whose reactions are complete within minutes. However, the TEAC value order was inconsistent with initial reaction kinetics in that some antioxidants showing immediate fast reaction had the lowest TEAC values.

The same paradox was presented when the initial and final absorbance drops were plotted as a function of antioxidant concentration (Figures 8−13), and the slopes were compared as absolute values or as TEAC calculations normalized to Trolox response (Table 2). Values of slopes in the limited linear ranges were lowest for fast reactors in groups 1 and 2 and increased with molecular weight and complexity (groups 3 and 4), a hierarchy opposite the reaction curve patterns. In explaining this paradox, it is important to recognize that higher slopes here do not indicate greater reactivity, only that the reaction efficiency increases with concentration. The efficiency issue is reflected also in the ratios of final to initial slopes. For the fast reactors, the initial and final slopes were essentially the same because the reaction was complete within seconds. However, for molecules that did not react instantaneously or had prolonged reaction components, the enhancement over time ranged to above 2. Such behaviors result when increasing concentrations force antioxidant molecules into closer juxtaposition with ABTS$^{+\bullet}$, facilitating reaction. At the same time, ring adducts or overall structures that introduced steric hindrance also reduced the concentration at which saturation occurred and the slope of the response curve decreased. Both TEAC values and slopes of concentration−response curves (each reflecting stoichiometry) had reasonable correlations with the numbers of phenol groups (0.81−0.86) but low correlation with redox potential (−0.21 to −0.28) (Table 3).

Because the ABTS$^{+\bullet}$ assay is known to be a capacity rather than reactivity assay,[10,16] we compared reaction stoichiometry by converting final reaction ΔA values to moles ABTS$^{+\bullet}$ consumed per mole antioxidant at 0.05 mM and 0.5 mM concentrations, corresponding to nonsaturating and saturating antioxidant, respectively. Theoretically, two electrons can be transferred from each phenol group. This expectation was met for only a few antioxidants under nonsaturating conditions (e.g., coniferyl alcohol, ferulic acid, pyrogallol, gallic acid, catechin, gallocatechin, catechin gallate) while under saturating conditions, radical quenching per OH group was limited for

Table 2. Molecular Properties and Reaction Characteristics of Antioxidant Compounds Reacting with $ABTS^{+\bullet}$

					TEAC		stoichiometry[a]		concn−response slope[b]			initial rxn, mol $ABTS^{+\bullet}$ s^{-1}	
group	compd	MW	phe-OH	E_7 (V)[c]	(ΔA)[d]	(slope)	0.05 mM	0.5 mM	ΔA_i	ΔA_f	ratio	/mol AOX	/phe-OH
1	Trolox	250.29	1	0.48[25]	1.00 ± 0.02[e]	1.00 ± 0.06[e]	1.0	1.0	1.15 ± 0.09[e]	1.09 ± 0.06[e]	0.95	0.67	0.67
	hydroquinone	110.11	2	0.46[22]	2.00 ± 0.02	1.73 ± 0.21	2.8	2.0	1.90 ± 0.25	1.90 ± 0.23	0.99	1.01	0.51
	chlorogenic acid	354.31	2	0.55[23]	1.82 ± 0.02	1.73 ± 0.03	2.7	1.8	1.96 ± 0.07	1.90 ± 0.03	0.99	0.88	0.44
	ascorbic acid	176.12	2	0.33[22]	2.18 ± 0.07	2.06 ± 0.10	3.0	2.0	2.12 ± 0.13	2.24 ± 0.11	1.06	0.93	0.47
2	protocatechuic acid	154.12	2		1.45 ± 0.01	1.55 ± 0.24	2.2	2.3	1.70 ± 0.22	1.60 ± 0.26	1.00	0.63	0.31
	3-methylcatechol	124.14	2	0.52[25]	1.64 ± 0.01	1.63 ± 0.06	2.0	2.0	1.72 ± 0.03	1.79 ± 0.07	1.03	0.85	0.43
	catechol	110.11	2	0.53[22]	2.36 ± 0.01	2.03 ± 0.17	3.4	2.5	1.38 ± 0.16	2.19 ± 0.18	1.60	0.65	0.33
3	uric acid	168.11	0[f]	0.59[24]	1.09 ± 0.03	0.95 ± 0.02	2.1	1.0	*f*	1.04 ± 0.02	–	0.28	–
	ferulic acid	194.18	1	0.60[23]	3.45 ± 0.02	3.30 ± 0.34	4.9	2.5	3.12 ± 0.32	3.55 ± 0.37	1.13	0.64	0.64
	coniferyl alcohol	180.20	1		2.91 ± 0.03	2.69 ± 0.05	3.6	2.4	1.57 ± 0.03	2.98 ± 0.05	1.89	0.92	0.92
	caffeic acid	180.16	2	0.54[23]	1.64 ± 0.00	1.46 ± 0.28	2.2	2.0	1.41 ± 0.17	1.54 ± 0.30	1.13	0.91	0.45
	pyrogallol	126.11	3	0.58[25]	6.73 ± 0.02	6.28 ± 0.21	9.5	2.7	2.94 ± 0.10	6.84 ± 0.23	2.33	1.29	0.43
	gallic acid	170.12	3	0.56[26]	4.73 ± 0.00	4.39 ± 0.15	6.5	2.7	2.48 ± 0.06	4.78 ± 0.16	1.93	1.17	0.39
	2-propyl gallate	212.20	3	0.41[26]	3.27 ± 0.02	3.27 ± 0.19	4.5	2.4	2.45 ± 0.18	3.56 ± 0.21	1.45	1.13	0.38
	catechin	290.27	4	0.57[25]	5.45 ± 0.01	5.43 ± 0.27	7.7	2.2	2.62 ± 0.31	5.92 ± 0.29	2.29	0.76	0.19
	gallocatechin	306.27	5	0.42[25]	7.27 ± 0.03	7.06 ± 0.50	10.6	2.7	3.55 ± 0.27	7.72 ± 0.54	2.16	1.32	0.36
	catechin gallate	442.37	7	0.51[27]	11.82 ± 0.07	11.37 ± 0.30	15.5	3.2	7.45 ± 0.25	12.39 ± 0.33	1.66	1.64	0.23
	gallocatechin gallate	458.37	8	0.43[27]	7.27 ± 0.02	6.36 ± 0.36	9.8	3.2	5.19 ± 0.21	7.57 ± 0.39	1.64	1.69	0.21
	rutin	610.52	4	0.60[25]	1.82 ± 0.02	1.46 ± 0.26	2.5	1.7	1.59 ± 0.06	3.30 ± 0.28	1.00	0.19	0.05
4	*p*-coumaric acid	164.16	1	0.59[23]	2.73 ± 0.02	2.70 ± 0.10	3.9	1.5	*f*	2.94 ± 0.11			
	resorcinol	110.11	2	0.81[22]	3.45 ± 0.02	3.03 ± 0.24	4.5	1.9	*f*	3.30 ± 0.26			
	glutathione	307.32	0	0.92[25]	4.00 ± 0.03	3.09 ± 0.44	5.6	1.4	*f*	3.37 ± 0.48			
5	curcumin	368.38	3	0.77[28]	0.55 ± 0.00	*f*	1.2	1	*f*	*f*			
	quercetin	302.24	4	0.33[25]	2.73 ± 0.04	*f*	2.4	2	*f*	*f*			
6	phenol	94.11	1	>0.80[22]	no rxn								
	benzoic acid	122.12	1		no rxn								
	vanillic acid	168.15	1		no rxn								
	glucose	180.16	5[g]		no rxn								
	sucrose	342.30	8[g]		no rxn								
	urea	60.06	0[h]		no rxn								
	1-methylindole	131.17	0		no rxn								

[a]Moles ABTS reacted/mol antioxidant. [b]Slopes of [ABTS consumption vs antioxidant concn] curves, calculated in linear range 0.001−0.050 mM antioxidant; ratio expressed as $\Delta A_f / \Delta A_i$. [c]E^o reported vs normal hydrogen electrode and at pH 7; superscripts are reference numbers. [d]Determined at 0.05 mM. [e]Standard deviation. [f]Reaction nonlinear even at low concentrations. [g]Nonphenolic OH as potential H donor. [h]NH or NH_2 as potential H donor.

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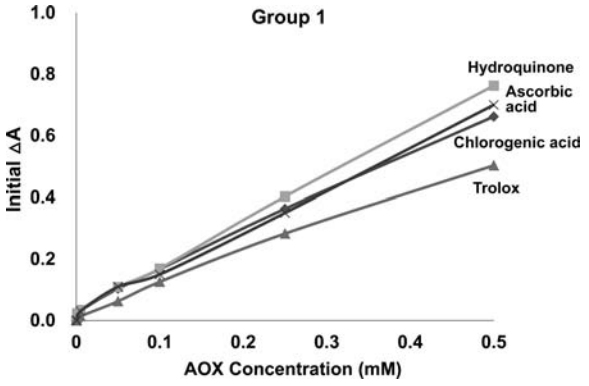

Figure 8. Effects of antioxidant concentration on reaction with $ABTS^{+\bullet}$: Group 1, simple phenols and ascorbic acid.

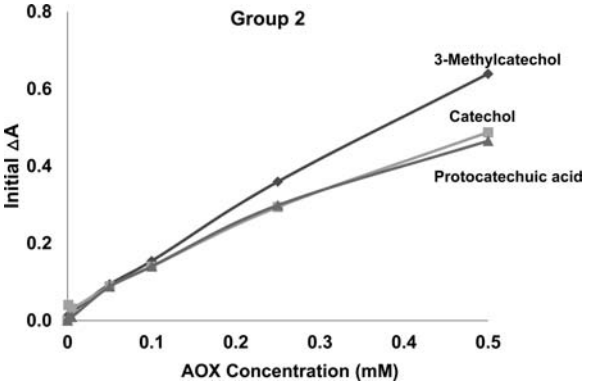

Figure 9. Effects of antioxidant concentration on reaction with $ABTS^{+\bullet}$: Group 2, simple phenols.

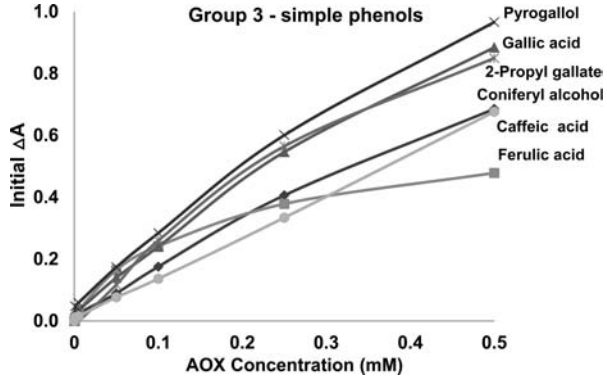

Figure 10. Effects of antioxidant concentration on reaction with $ABTS^{+\bullet}$: Group 3, phenols with ring adducts, cinnamic acids, and benzoic acids.

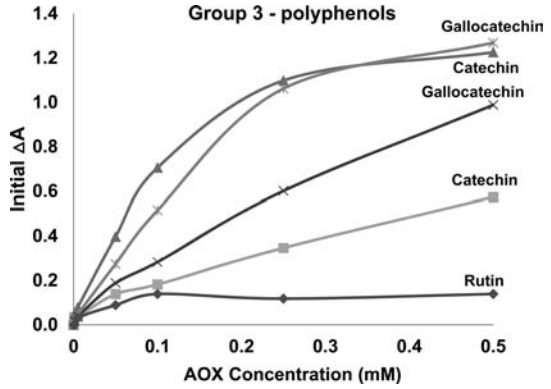

Figure 11. Effects of antioxidant concentration on reaction with $ABTS^{+\bullet}$: Group 3, polyphenols.

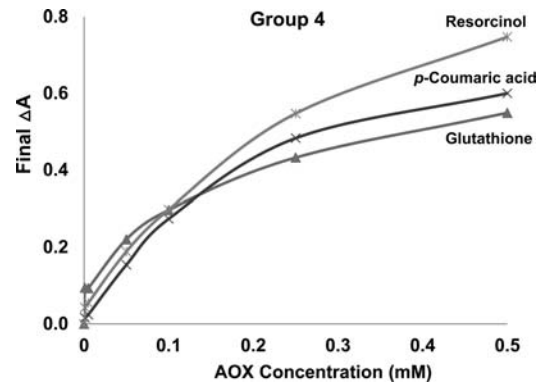

Figure 12. Effects of antioxidant concentration on reaction with $ABTS^{+\bullet}$: Group 4, *p*-coumaric acid, resorcinol, glutathione. Final absorbance drop was plotted because no initial reaction occurred.

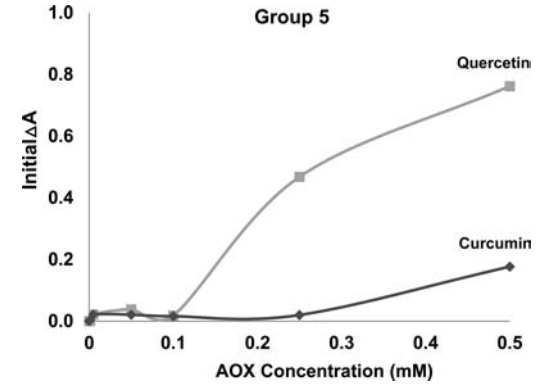

Figure 13. Effects of antioxidant concentration on reaction with $ABTS^{+\bullet}$: Group 5, curcumin and quercetin.

nearly all antioxidants. These results suggest three important points about this assay: (1) as indicated in the name of the assay, absorbance drops at the end of the reaction period reflect the extent of reaction (or antioxidant capacity) rather than the reaction rate (antioxidant reactivity), and the numbers of phenolic groups more than the total antioxidant structure; (2) the reaction is highly dependent on antioxidant concentration, so a range of concentrations should always be assayed to construct concentration curves from which second-order rate constants and response saturation can be determined; (3) contrary to some recommendations in the literature, the reaction should be run with $ABTS^{+\bullet}$ at least in slight excess of antioxidant concentrations for accurate determination of stoichiometry.

To determine reaction rates rather than stoichiometry, we tested most standard first- and second-order approaches, including plots of $\ln(\Delta ABTS^{+\bullet})$ over time and versus antioxidant concentration, plots with change as a function of $[\text{antioxidant}]^2$, etc., and found none that were able to describe a constant rate relationship for all compounds. Nearly all analyses showed narrow linear ranges about 0.0005 to 0.005 mM, with thresholds at lower concentrations, as well as slowing and saturation at higher concentrations. The fastest rates in all cases were within the first second. Thus, the important rates seem to

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Table 3. Pearson Correlations between Antioxidant Characteristics (numbers of phenolic groups and redox potentials) and Reaction Response Reported Directly or in Comparison to Trolox (TEAC)

	no. of phenolic groups	E^o
concentration slope (initial)	0.82	−0.21
concentration slope (final)	0.86	−0.28
TEAC (ΔA)	0.83	−0.28
TEAC (concn slope)	0.81	−0.28
initial rate		
ABTS/mol/s	0.75	−0.51
ABTS/mol/OH	−0.66	−0.07

be occurring on time scales not accessible to this method, and fast flow methods will be needed for accurate rate determinations. These analyses also showed that rates of antioxidant reactions with $ABTS^{+\bullet}$ were related to factor(s) other than numbers of phenol groups, redox potential, and inductive effects.

To obtain at least an estimate of initial reaction rates for correlation with antioxidant structure and kinetic patterns, we compared $ABTS^{+\bullet}$ reaction that occurred just during mixing, about 1 s in the plate reader. Because of the marked dependence on antioxidant concentration, we compared only 0.05 mM systems where antioxidant concentrations were close to but not exceeding $ABTS^{+\bullet}$ concentrations (0.067 mM), i.e., the system was not saturating in antioxidant. If it is assumed that the fast reactions were complete within 1 s, the initial absorbance drops converted to moles of $ABTS^{+\bullet}$ become estimates of reaction rates. $ABTS^{+\bullet}$ consumption in that 1 s normalized to moles of antioxidant and to number of phenolic OH in the antioxidant are given in the last two columns, respectively, of Table 2.

Estimated rates expressed per molecule of antioxidant once again seemed to favor polyphenols that reacted more slowly and to underrepresent compounds that reacted immediately and fully in reaction response curves (Figures 2−7). Normalization of $ABTS^{+\bullet}$ consumption to numbers of phenolic OH groups in each compound (i.e., rate per phenol group, Table 2, last column) revealed one explanation for this discrepancy. Single phenols reduced ≥0.67 mol of $ABTS^{+\bullet}$, but the reaction efficiency decreased with each additional OH, until in catechins, the seven to eight OH groups reduced only about 0.22 mol of $ABTS^{+\bullet}$ each. This suggests that the apparent higher reactivity of catechins resulted not from increased reactivity per se but from larger numbers of phenol groups each reacting at lower fractional efficiency.

The observed decrease in reaction rate and efficiency with molecular size and numbers of OH can reasonably be explained by decreased steric accessibility to the $ABTS^{+\bullet}$ radical site. Small molecules can approach the radical site in ABTS more readily, while more complex molecules (more OH groups, ring adducts, multiple rings) have their access impeded and their reaction efficiency decreases accordingly. Among the simple phenols, rate differences are somewhat consistent with ring adduct effects on electron distribution and redox potential. Unexpected high reactivity (more than two electrons per OH) in ferulic acid and coniferyl alcohol may result from generation of reactive secondary products such as quinones that also react with $ABTS^{+\bullet}$.[28,29] However, without molecular modeling it is difficult to distinguish these chemical influences from physical steric accessibility.

These observations raise a critical point to remember about antioxidant reactions: it is the stability, redox potential, and steric configuration of the radical rather than the antioxidant that creates the environment for quenching. Freely accessible and diffusible radicals with high redox potentials, such as hydroxyl radicals, can be quenched by many kinds of molecules, while sterically hindered radicals such as $ABTS^{+\bullet}$ do not adequately measure actual radical quenching rates of large molecules that have limitations in approach. In this regard, the $HO^{\bullet}$ radicals generated in the original $ABTS^{+\bullet}$ assay[30,31] was a better choice of radical targets. Unfortunately, results in this version of the assay were confounded by antioxidant reaction with both initiator and reporter molecules.

A second key point which cannot be overemphasized is that for radical quenching in real materials, quenching kinetics rather than total quenching capacity is more important because, unlike stable $ABTS^{+\bullet}$ radicals, the lifetimes of oxygen radicals normally being combated in vivo and in foods (Table 4) are far

Table 4. Lifetimes of Radicals Commonly Active in Living Tissues and in Foods[a]

radical (10^{-3} M, 37 °C)	lifetime
$HO^{\bullet}$	10^{-9} s
$L^{\bullet}$ (lipid alkyl)	10^{-8} s
$RO^{\bullet}$	10^{-6} s
$AnOO^{\bullet}$ (arachidonic acid)	10^{-5} s
$ROO^{\bullet}$	10 s

[a]Data excerpted from ref 17.

too short-lived to allow for molecular diffusion and reorientation for reaction.[17] Consequently, immediate reactions with $ABTS^{+\bullet}$ are most important and the relevance of following reactions over long times to detect slow reactors, "full reactivity", or activity of secondary products is highly questionable at best and is probably meaningless for radical quenching in foods and biological materials.

Single Electron Transfer (SET) vs Hydrogen Atom (HAT) Mechanisms of Radical Quenching. Quenching of $ABTS^{+\bullet}$ radicals has been attributed to both SET and HAT mechanisms as well as to mixtures of the two.[16] SET mechanisms are strongly solvent-dependent due to solvent stabilization of charged species, whereas HAT mechanisms are affected by hydrogen-bonding solvents.[32] Exploratory testing of fast and slow reactors over the pH range 5 to 9 to detect electron transfer and in water versus methanol to detect hydrogen atom transfer could not clearly distinguish active or dominant mechanisms in $ABTS^{+\bullet}$ quenching. Because the quenching sequence occurs within milliseconds, it is likely that the time scale of this assay is too long to detect differences in mechanisms and that fast flow-mix techniques will be required to make this distinction. It is also possible that neither mechanism is dominant, or that other unidentified mechanisms also contribute to the radical quenching.

Until more definitive verification of mechanisms is obtained, on the basis of fundamental properties of SET vs HAT reactions and documented actions of some antioxidants, we propose that the instantaneous reactions result from dominant electron transfers from compounds or individual phenol groups with greatest steric access to the $ABTS^{+\bullet}$ radical site and that slow sustained reactions with no initial absorbance drop result from hydrogen atom transfers. Slow reactions following initial rapid absorbance drop result from either (a) large molecules

with slow diffusion and hindered steric access to the radical, or (b) molecules exhibiting both electron and hydrogen transfer.

Recommendations. After using the $ABTS^{+\bullet}$ assay to evaluate mixtures of antioxidants, Van den Berg et al.[33] concluded that "quantitative evaluation of antioxidant capacity using the TEAC assay can be troublesome or even impossible". Results of the current study support that conclusion. The TEAC assay using the stable ABTS cation radical has the advantage of simplicity and speed, but it suffers from many more detractions, including marked dependence on time and antioxidant concentration as well as steric limitations of the $ABTS^{+\bullet}$. Both reaction time and antioxidant concentration must be considered for results of this assay to make any chemical sense.

Time of Measurement and Antioxidant Action. Most active antioxidants have very rapid immediate reaction components that are ignored in current assays and do not lend themselves to easy or accurate rate calculations, even with direct dispensing plate readers. Test compounds exhibit different kinetic patterns that do not consistently match stoichiometry calculated from reaction over variable periods, in some cases many hours. Without continual absorbance monitoring to detect immediate reaction and determine kinetic patterns, especially in the initial second after mixing, inaccurate conclusions can be drawn about antioxidant reactivity.

Current assay procedures measure reaction stoichiometry or antioxidant capacity after extended reaction periods. Not surprisingly, quenching capacities over time parallel numbers of phenolic groups in the test compounds. However, total potential capacity is not the same as kinetic reactivity, which must be determined on very short time scales. Radical quenching rates, the critical values determining antioxidant effectiveness in real systems (e.g., tissues or foods), are difficult if not impossible to measure with current assay procedures. At a minimum, initial absorbance drops that occur during mixing should be measured, converted to $ABTS^{+\bullet}$ concentration, and normalized to the numbers of phenol groups as an approximation of antioxidant reaction rates. Should the $ABTS^{+\bullet}$ assay be continued in use, it must be converted to a rapid mixing, fast kinetic approach to determine reaction rates accurately.

Absorbance changes monitored from the mixing point suggest that molecular structure and steric accessibility to the hindered radical site in $ABTS^{+\bullet}$ are dominant factors controlling reaction rates with $ABTS^{+\bullet}$. Small single phenols react most completely within mixing time and may also generate secondary species that further quench $ABTS^{+\bullet}$. As numbers of phenolic groups increase, antioxidant access to $ABTS^{+\bullet}$ becomes correspondingly more limited and efficiency of radical quenching drops to a small fraction of the expected two electrons per phenol group. This pattern raises questions about radical quenching in situ, whether foods or biological tissues. It does not seem likely that similar steric hindrance occurs in antioxidant reactions with hydroxyl radicals, $HO^{\bullet}$, but lipid oxyl radicals may not be so accessible. Some computer molecular modeling to determine spatial and energetic aspects of monophenol versus polyphenol interactions with $ABTS^{+\bullet}$ and other radicals would be very useful to evaluate the potential impact of molecular size and configuration on lipid and other peroxyl radical quenching.

Antioxidant Concentration. Both rates and stoichiometry of antioxidant quenching of $ABTS^{+\bullet}$ are strongly dependent on antioxidant concentration. For most compounds, slopes of concentration−response curves increased and moles of $ABTS^{+\bullet}$ quenched per mole of antioxidant decreased with antioxidant concentration, and the effects were magnified in trihydroxy- and polyphenols. Steric accessibility to the $ABTS^{+\bullet}$ radical site plays a critical role in this behavior. Small monophenols react rapidly and completely within seconds, and their reactions increase linearly with concentration. Additional phenol groups (di- and triphenols) and rings (catechins) increase the total antioxidant capacity but also impede radical access and quenching efficiency, so reaction response drops off at higher concentrations. Slopes of concentration curves generally increase with the number of phenol groups and complexity of antioxidant structure, while radical quenching efficiency and antioxidant concentrations at which response saturation occurs decrease correspondingly. All of these effects result when reactions are diffusion-controlled or sterically limited so increased antioxidant concentrations initially enhance and then impede contact with the $ABTS^{+\bullet}$radical site.

Consequently, if this assay is to be used, single concentrations of antioxidant or extracts are inadequate for judging reactivity. Assays must be run with antioxidant concentrations that cover several orders of magnitude and bracket the $ABTS^{+\bullet}$ concentration. Plots of reaction response (initial and/or final absorbance drop) versus antioxidant concentration can then be used to determine reaction thresholds and saturation levels; slopes of these curves help assess steric effects on reactions.

Applicability of $ABTS^{+\bullet}$ Assay. Although we have presented recommendations for modification and improvement of this assay, overall we feel that the assay has many serious limitations and does not reflect true reactivity of antioxidants, which must necessarily include rate components as well as capacity. Reaction times that are much longer than normal radical lifetimes miss the rapid reactions that are critical for quenching radicals in real materials and overestimate actions of polyphenols. Use of a sterically hindered stable radical ensures that steric accessibility is as, or even more, important than chemical characteristics in observed radical-quenching action. Steric effects make it difficult to accurately compare reactivity of different classes of antioxidants, although the shapes of reaction curves can indicate dominance of small reducing molecules versus polyphenols. Thus, the $ABTS^{+\bullet}$ assay is not appropriate for ranking natural compounds with different structures or comparing extracts with very different compositions, and as a consequence, expression of antioxidant activity on a Trolox equivalence basis is invalid.

Nevertheless, the $ABTS^{+\bullet}$ assay may have some application in tracking changes in antioxidant activity in the same or similar materials over time or under different conditions. For example, we have used it to monitor changes in tocopherol activity after heat exposure in oils and in packaging film extrusion;[34] the assay has also been applied to determine loss of antioxidant activity in strawberries dried with different methods[35] and to monitor effects of coffee roasting on residual antioxidant activity.[36] In these cases, limitations of the assay remain, but antioxidant components are constant and variations in $ABTS^{+\bullet}$ accessibility are not the prime determinant of reactivity.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Uttara, B.; Singh, A. V.; Zamboni, P.; Mahajan, R. T. Oxidative stress and neurodegenerative diseases: A review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* **2009**, *7* (1), 65−74.

(2) Hamid, A. A.; Aiyelaagbe, O. O.; Usman, L. A.; Ameen, O. M.; Lawal, A. Antioxidants: Its medicinal and pharmacological applications. *African J. Pure Appl. Chem.* **2010**, *4* (8), 142−151.

(3) Bendich, A. Physiological role of antioxidants in the immune system. *J. Dairy Sci.* **1993**, *76* (9), 2789−2794.

(4) Bañón, S.; Díaz, P.; Rodríguez, M.; Garrido, M. D.; Price, A. Ascorbate, green tea and grape seed extracts increase the shelf life of low sulphite beef patties. *Meat Sci.* **2007**, *77* (4), 626−633.

(5) Bera, D.; Lahiri, D.; Nag, A. Studies on a natural antioxidant for stabilization of edible oil and comparison with synthetic antioxidants. *J. Food Eng.* **2006**, *74* (4), 542−545.

(6) Paradiso, V. M.; Summo, C.; Trani, A.; Caponio, F. An effort to improve the shelf life of breakfast cereals using natural mixed tocopherols. *J. Cereal Sci.* **2008**, *47* (2), 322−330.

(7) Shahidi, F.; Zhong, Y. Antioxidants: Regulatory status. In *Bailey's Industrial Oil and Fat Products*, 6th ed.; Shahidi, F., Ed.; John Wiley & Sons, Inc.: Hoboken, 2005; Vol. *1*, pp 491−512.

(8) Huang, D.; Qu, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.

(9) Prior, R. L.; Cao, G. H. Analysis of botanicals and dietary supplements for antioxidant capacity: A review. *J. AOAC Int.* **2000**, *83* (4), 950−956.

(10) Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biol. Med.* **1999**, *26*, 1231−1237.

(11) Sánchez-Moreno, C. Review: Methods used to evaluate the free radical scavenging activity in foods and biological systems. *Food Sci. Technol. Int.* **2002**, *8* (3), 121−137.

(12) Ghiselli, A.; Serafini, M.; Natella, F.; Scaccini, C. Total antioxidant capacity as a tool to assess redox status: Critical review and experimental data. *Free Radical Biol. Med.* **2000**, *29*, 1106−1114.

(13) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CuPRAC method. *J. Agric. Food Chem.* **2004**, *52*, 7970−7981.

(14) Frankel, E. N.; Meyer, A. S. The problems of using one dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925−1941.

(15) Ishige, K.; Schubert, D.; Sagara, Y. Flavonoids protect neuronal cells from oxidant stress by three distinct mechanisms. *Free Radical Biol. Med.* **2001**, *30*, 433−446.

(16) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(17) Pryor, W. A. Oxy-radicals and related species: Their formation, lifetimes and reactions. *Annu. Rev. Physiol.* **1986**, *48*, 657−667.

(18) Othman, A.; Jalil, A. M. M.; Weng, K. K.; Ismail, A.; Ghani, N. A.; Adenan, I. Epicatechin content and antioxidant capacity of cocoa beans from four different countries. *African J. Biotechnol.* **2010**, *9* (7), 1052−1059.

(19) Barton, H. J. A "zero sample concentration approach": Standardization of methods for the estimation of total antioxidant activity by the use of extrapolation to zero sample concentration. A novel standard. 1. ABTS cation radical scavenging. *J. Agric. Food Chem.* **2010**, *58*, 8918−8926.

(20) Kim, D.-O.; Lee, K. W.; Lee, H. J.; Lee, C. Y. Vitamin C equivalent antioxidant capacity (VCEAC) of phenolic phytochemicals. *J. Agric. Food Chem.* **2002**, *50*, 3713−3717.

(21) Walker, R. B.; Everette, J. D. Comparative reaction rates of various antioxidants with ABTS radical cation. *J. Agric. Food Chem.* **2009**, *57* (4), 1156−1161.

(22) Steenken, S.; Neta, P. One-electron redox potentials of phenols. Hydroxy- and aminophenols and related compounds of biological interest. *J. Phys. Chem.* **1982**, *86* (18), 3661−3667.

(23) Foley, S.; Navaratnam, S.; McGarvey, D. J.; Land, E. J.; Truscott, T. G.; Rice-Evans, C. A. Singlet oxygen quenching and the redox properties of hydroxycinnamic acids. *Free Radical Biol. Med.* **1999**, *26* (9−10), 1202−1208.

(24) Simic, M. G.; Jovanovic, S. V. Antioxidant mechanisms of uric acid. *J. Am. Chem. Soc.* **1989**, *111* (15), 5778−5782.

(25) Jovanovic, S. V.; Steenken, S.; Hara, Y.; Simic, M. G. Reduction potentials of flavonoid and model phenoxyl radicals. Which ring in flavonoids is responsible for antioxidant activity? *J. Chem. Soc., Perkin Trans. 2* **1996**, 2497−2504.

(26) Nemeikaite-Ceniene, A.; Imbrasaite, A.; Sergediene, E.; Cenas, N. Quantitative structure-activity relationships in prooxidant cytotoxicity of polyphenols: Role of potential of phenoxyl radical/phenol redox couple. *Arch. Biochem. Biophys.* **2005**, *441* (2), 182−190.

(27) Jovanovic, S. V.; Hara, Y.; Steenken, S.; Simic, M. G. Antioxidant potential of gallocatechins. A pulse radiolysis and laser photolysis study. *J. Am. Chem. Soc.* **1995**, *117* (39), 9881−9888.

(28) Hotta, H.; Nagano, S.; Ueda, M.; Tsujino, Y.; Koyama, J.; Osakai, T. Higher radical scavenging activities of polyphenolic antioxidants can be ascribed to chemical reactions following their oxidation. *Biochim. Biophys. Acta, Gen. Subj.* **2002**, *1572* (1), 123−132.

(29) Hotta, H.; Sakamoto, H.; Nagano, S.; Osakai, T.; Tsujino, Y. Unusually large numbers of electrons for the oxidation of polyphenolic antioxidants. *Biochim. Biophys. Acta, Gen. Subj.* **2001**, *1526* (2), 159−167.

(30) Miller, N. J.; Rice-Evans, C. A.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84* (4), 407−412.

(31) Miller, N. J.; Sampson, J.; Candeias, L. P.; Bramley, P. M.; Rice-Evans, C. A. Antioxidant activities of carotenes and xanthophylls. *Fed. Eur. Biochem. Soc. Lett.* **1996**, *384*, 240−242.

(32) Foti, M.; Ruberto, G. Kinetic solvent effects on phenolic antioxidants determined by spectrophotometric measurements. *J. Agric. Food Chem.* **2001**, *49* (1), 342−348.

(33) Van den Berg, R.; Haenen, G. R. M. M.; Van den Berg, H.; Bast, A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chem.* **1999**, *66*, 511−517.

(34) Lang, J. C. Tocopherol stability in controlled release packaging films. M.S. Thesis, Dept. of Food Science, Rutgers University, New Brunswick, NJ, 2009.

(35) Wodjdylo, A.; Figiel, A.; Oszmiański, J. Effect of drying methods with the application of vacuum microwaves on the bioactive compounds, color, and antioxidant activity of strawberry fruits. *J. Agric. Food Chem.* **2009**, *57* (4), 1337−1343.

(36) Del Castillo, M. D.; Ames, J. M.; Gordon, M. H. Effect of roasting on the antioxidant activity of coffee brews. *J. Agric. Food Chem.* **2002**, *50* (13), 3698−3703.