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tert-Butylhydroquinone as a Spectroscopic Probe for the Superoxide Radical Scavenging Activity Assay of Biological Samples

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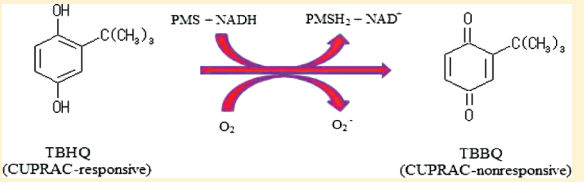
Supporting Information

ABSTRACT: As a more convenient and less costly alternative to electron spin resonance (ESR) and nonspecific nitroblue tetrazolium (NBT) and cytochrome *c* assays of superoxide radical (SR, $O_2^{\bullet-}$) detection, a novel probe, *tert*-butylhydroquinone (TBHQ), is introduced for SR nonenzymatically generated in the phenazine methosulfate−β-nicotinamide adenine dinucleotide (PMS−NADH) system. SR attacks both TBHQ and SR scavengers incubated in solution for 30 min where scavengers compete with TBHQ for the $O_2^{\bullet-}$ produced. TBHQ, but not its $O_2^{\bullet-}$ oxidation product, *tert*-butyl-1,4-benzoquinone (TBBQ), is responsive to the CUPRAC (cupric reducing antioxidant capacity) spectrophotometric assay. The CUPRAC absorbance of the ethyl acetate extract of the incubation solution arising from the reduction of Cu(II)−neocuproine reagent by the remaining TBHQ was higher in the presence of $O_2^{\bullet-}$ scavengers (due to less conversion to TBBQ), the difference being correlated to the SR scavenging activity (SRSA) of the analytes. With the use of this reaction, a kinetic approach was adopted to assess the SRSA of amino acids, vitamins, and plasma and thiol antioxidants. This assay, applicable to small-molecule antioxidants and tissue homogenates, proved to be efficient for cysteine, uric acid, and bilirubin, for which the widely used NBT test is nonresponsive. Thus, conventional problems of NBT assay arising from formazan insolubility and direct reduction of NBT by tested scavengers were overcome.



Reactive oxygen species (ROS), including superoxide anion radical ($O_2^{\bullet-}$), have gained great attention due to their role in the progression of a number of human diseases and carcinogenesis. So it is important to eliminate excessive $O_2^{\bullet-}$ in vivo to prevent $O_2^{\bullet-}$-originated disease. All cells possess elaborate antioxidant defense systems consisting of low and high molecular weight components to defend against ROS attack. These protective systems include enzymatic and nonenzymatic antioxidants that are both endogenous (produced in the body) and exogenous (supplied through diet). Biologically important antioxidative compounds within cells, cell membranes, and extracellular fluids can be up-regulated and mobilized to neutralize excessive and inappropriate ROS formation.[1]

The superoxide radical (SR) can act both as a reducing and oxidizing agent for a variety of compounds and is a key intermediate for more active oxygen species, such as hydroxyl radical and singlet oxygen. It has been documented that $O_2^{\bullet-}$ formation occurs continuously in the cells as a consequence of both enzymatic and nonenzymatic reactions. Xanthine oxidase (XO) is a complex enzyme in endothelial cells that leads to the generation of $O_2^{\bullet-}$. XO catalyzes the oxidation of xanthine to uric acid yielding $O_2^{\bullet-}$ and raises the oxidative level in an organism.[2] This enzymatic system is frequently used as a generator of $O_2^{\bullet-}$ (eq 1):

$$\text{xanthine} + 2O_2 + H_2O \rightarrow \text{uric acid} + 2O_2^{\bullet-} + 2H^+ \quad (1)$$

There are reports documenting that some kinds of thiols, including dithioerythritol (DTE), acted not only as $O_2^{\bullet-}$ scavengers but also as XO inhibitors.[3] To date, several nonenzymatic methods employing other $O_2^{\bullet-}$-generating systems have been identified, for example, a H_2O_2 degradation system in alkaline dimethyl sulfoxide, a riboflavin irradiation system, and a phenazine methosulfate (PMS)−β-nicotinamide adenine dinucleotide (NADH) oxidation system.[4] In the present study, $O_2^{\bullet-}$ is generated under aerobic conditions in a PMS−NADH system by oxidation of NADH[5,6] in a two-step reaction (eqs 2 and 3):

$$\text{NADH} + H^+ + \text{PMS} \rightarrow \text{NAD}^+ + \text{PMSH}_2 \quad (2)$$

$$\text{PMSH}_2 + 2O_2 \rightarrow 2O_2^{\bullet-} + 2H^+ + \text{PMS} \quad (3)$$

A large number of assays have been developed to detect $O_2^{\bullet-}$,[7] all employing a "probe", i.e., a molecule that reacts with $O_2^{\bullet-}$ producing a detectable product. The most commonly utilized spectrophotometric detection methods for $O_2^{\bullet-}$ use cytochrome *c*[8] or nitroblue tetrazolium (NBT)[9] as spectrophotometric probes. Since cytochrome *c* (Cyt *c*−Fe^{3+} → Cyt *c*−Fe^{2+}; λ_{max}: 550 nm) is easily reduced by reductases such as NAD(P)H reductase and other reducing agents, it is necessary to consider possible contaminants in the samples. The NBT method is based on the generation of water-insoluble blue formazan dye (λ_{max}: 560 nm) by a reaction with $O_2^{\bullet-}$.[10] In this case, $O_2^{\bullet-}$ acts as a reducing agent toward NBT, since the standard reduction

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potentials for the $O_2/O_2^{\bullet-}$ and $NBT^{2+}/NBTDF^{2-}$ (diformazan) redox couples are −0.330[11] and −0.080 V,[12] respectively. Consequently, a nonhomogeneous suspension of mixed formazans is created that causes problems in the reproducibility of data, and therefore, a modified NBT assay with staining on gels was developed due to the problems encountered when NBT is used for assaying superoxide dismutase (SOD) in solution.[13] Moreover, NBT is reduced by various reducing agents (i.e., cellular reductases, fructosamines)[14] and used for the detection of keto-amines as an index of diabetic control.[15] The chemiluminescence probes (i.e., lucigenin, luminol, luciferin derivative) used for $O_2^{\bullet-}$ detection can also be applied for SR scavenging activity (SRSA) assay. The lucigenin assay has been criticized, because lucigenin itself can react to produce $O_2^{\bullet-}$ and in some cases can stimulate $O_2^{\bullet-}$ production by intact cells. Luminol, unlike lucigenin, is oxidized by $O_2^{\bullet-}$. This leads to a complex series of reactions among luminol, luminol radicals, oxygen, and $O_2^{\bullet-}$, ultimately producing a luminol endoperoxide, which decomposes with the release of a photon. Since $O_2^{\bullet-}$ is involved as both initiator and intermediate in the reaction, objections have been raised to the use of luminol as a quantitative measure of $O_2^{\bullet-}$ production.[7] As electron spin resonance (ESR) techniques along with a spin-trapping agent (e.g., 5,5-dimethyl-1-pyrroline *N*-oxide)[4] require a specialized and expensive ESR spectrometer, novel $O_2^{\bullet-}$ detection methods are needed with more readily available equipment. No matter which assay is used, it is important to include proper controls in the experimental design in order to be certain that any signal detected is due to $O_2^{\bullet-}$. A positive control experiment with SOD may be useful; i.e., if the signal is not abolished by SOD, it does not represent $O_2^{\bullet-}$ production.

The CUPRAC (cupric reducing antioxidant capacity) method of antioxidant measurement, introduced by our research group to world literature,[16] 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, Cu(II)−neocuproine, where absorbance is recorded at the maximal light absorption wavelength of 450 nm. Properly located phenolic hydroxyls are oxidized to the corresponding quinones in the CUPRAC redox reaction. *tert*-Butylhydroquinone (TBHQ) is a $O_2^{\bullet-}$ scavenger with IC_{50} of 18.1 μM.[17] Quinones shuffle electrons enzymatically or nonenzymatically among their reduced form (hydroquinone), oxidized form, and/or their semiquinone radicals to construct redox cycles. TBHQ can be oxidatively converted to the corresponding semiquinone radical ($TBQ^{\bullet-}$, one-electron oxidation product) or TBBQ (fully oxidized by two electron loss),[18,19] the redox potential of the TBBQ/$TBQ^{\bullet-}$ pair being $E_{1/2}$ = 0.52 V.[20] Since the standard reduction potential, $E°$, of the $O_2^{\bullet-}/H_2O_2$ redox couple in acidic medium is 0.940 V, $O_2^{\bullet-}$ can oxidize TBHQ to TBBQ (the latter redox couple having $E°$ = 0.360 V).[21] As opposed to its role toward NBT, $O_2^{\bullet-}$ then acts as an oxidizing agent toward TBHQ. Thus, the idea in the present study is to use TBHQ probe with the PMS−NADH nonenzymatic $O_2^{\bullet-}$-generating system for SRSA assay of thiol-type antioxidants (e.g., glutathione, cysteine), amino acids (e.g., serine, threonine), plasma antioxidants (e.g., bilirubin, albumin), and other antioxidants (e.g., methionine) by measuring the CUPRAC absorbance of the remaining TBHQ in the reaction medium (TBHQ is CUPRAC-reactive and is isolated by ethyl acetate extraction from other CUPRAC-reactive interferents in the aqueous phase). As a result, the difference in the CUPRAC absorbance of the TBHQ probe in the absence and presence of $O_2^{\bullet-}$ scavengers is measured, because the incubation reaction medium would show a greater CUPRAC absorbance when scavengers are present (i.e., the CUPRAC-reactive TBHQ is converted to a lesser extent into the CUPRAC-nonresponsive TBBQ in the presence of SR scavengers, as shown in Figure 1). The tissue homogenates (kidney and liver) were evaluated for their SRSA using the proposed method in comparison with the high-performance liquid chromatography (HPLC) and NBT reference methods.

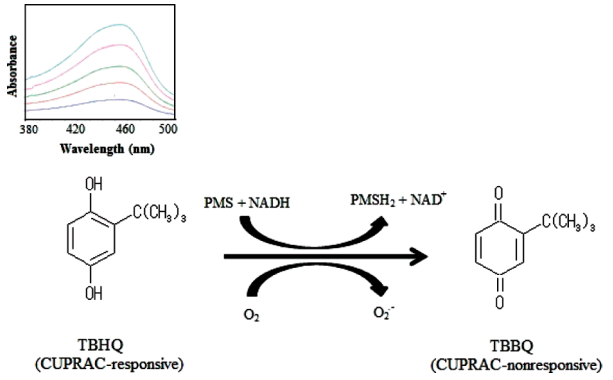

Figure 1. Oxidation of TBHQ to TBBQ by $O_2^{\bullet-}$ generated in the PMS−NADH system.

EXPERIMENTAL SECTION

Reagents and Apparatus. The following chemical substances of analytical reagent grade were supplied from the corresponding sources: β-nicotinamide adenine dinucleotide reduced dipotassium salt (β-NADH), L-threonine, L-leucin, L-phenylalanine, L-serine, 1,4-dithioerythritol (DTE), bilirubin, neocuproine (2,9-dimethyl-1,10-phenanthroline), superoxide dismutase from bovine liver (30 KU) (SOD), *tert*-butylhydroquinone (TBHQ), α-tocopherol, uric acid, 2-*tert*-butyl-1,4-benzoquinone (TBBQ), and nitroblue tetrazolium chloride (NBT) were from Sigma-Aldrich (Steinheim, Germany); copper(II) chloride dihydrate, L-tryptophan, L-glutathione reduced, L-ascorbic acid (AA), and albumin from bovine serum were from Merck (Darmstadt, Germany); ammonium acetate, dimethyl sulfoxide (DMSO), and ethyl acetate (EtAc) were from Riedel-de Haen (Steinheim, Germany); phenazine methosulfate (PMS), L-glutathione oxidized, L-cysteine, L-methionine, and *N*-acetyl-L-cysteine were from Fluka (Buchs, Switzerland).

The visible spectra and absorption measurements were recorded in matched quartz cuvettes using a Varian Cary Bio 100 UV−vis spectrophotometer (Mulgrave, Victoria, Australia). Other related apparatus and accessories were a J. P. Selecta water bath (Barcelona, Spain) and Telstar Cryodos freeze-dryer (Terrassa, Spain). The chromatograph was from a Waters Breeze 2 model HPLC system (Milford, MA, U.S.A.) equipped with a 2998 photodiode array detector (Chelmsford, MA, U.S.A.), and ACE C18 column (4.6 mm ×250 mm, 5 μm particle size) (Milford, MA, U.S.A.). Data acquisition was accomplished using Empower PRO (Waters Associates, Milford, MA).

Preparation of Solutions. The TBHQ solution at 1.0×10^{-3} M was prepared in EtOH/water (1:9, v/v). β-NADH (468 μM), PMS (60 μM), NBT (300 μM), $CuCl_2$ (10 mM), and ammonium acetate buffer solutions (1 M, pH 7) were all prepared in pure distilled water (Millipore Simpak1 Synergy 185, U.S.A.),

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and neocuproine solution (7.5 mM) was prepared in absolute ethanol. All thiol-type antioxidants and amino acids, ascorbic acid, and SOD were freshly prepared in distilled water at 1.0×10^{-2}–5.0×10^{-5} M concentration, bilirubin and uric acid were in 0.5 M NaOH, and α-tocopherol was in EtOH at 1.0×10^{-4} M concentration prior to measurement.

Preparation of Tissue Homogenates. CD-1 mice were obtained from the experimental animal facility of the Faculty of Veterinary Medicine of Istanbul University. The mice were housed in polycarbonate cages (450 cm^2 area per animal), acclimatized under laboratory conditions (23 ± 2 °C, humidity 50–60%, 12 light/dark cycles), and fed by standard mice food. Liver and kidney tissues were isolated after sacrifice by decapitation from mice. The tissue samples were washed with 0.9% NaCl, weighed (10%, w/v), and homogenized by adding cold 1.15% KCl in a glass homogenizer. Homogenates were immediately frozen in liquid nitrogen and kept at −80 °C until analysis.[22] Homogenates were separated from proteins by adding EtOH, centrifuging (4000 rpm, 5 min), and filtering through a 0.45 μm membrane filter before analysis.

Modified CUPRAC Assay (Proposed Method). Superoxide anion radicals are generated nonenzymatically by a PMS–NADH system.[5] To a test tube was added 0.5 mL of 1.0 mM TBHQ (probe material), 0.5 mL of scavenger solution at a suitable concentration or water (for reference), 2.0 mL of 468 μM NADH, and 1.0 mL of 60 μM PMS, rapidly in this order. The reaction was started by adding PMS solution. The mixture in a total volume of 4.0 mL was incubated for 30 min in a water bath kept at 25 °C. At the end of this period, the remaining TBHQ together with its oxidation product (TBBQ) was extracted by adding 3.0 mL of ethyl acetate (extraction of probe) and vortexed for 20 s. To 1.0 mL of the EtAc extract, the modified CUPRAC method[16] was applied in the following manner:

1.0 mL of Cu(II) + 1.0 mL of Nc + 1.0 mL of NH_4Ac buffer

+ 1.0 mL of EtAc extract + 0.1 mL of EtOH (V_{total} = 4.1 mL)

The absorbance at 450 nm of the final solution at 4.1 mL of total volume was recorded 30 min later against a reagent blank. The colors of the incubation solution in the presence and absence of SOD (scavenger) were compared with respect to their stabilities, the corresponding CUPRAC absorbances being recorded between 0 and 35 min.

The IC_{50} (50% inhibitive concentration) values of the scavengers were determined with the use of the NADH–PMS system by means of a linear plot of inhibition percent as a function of $C_{scavenger}$, where A_o is the initial CUPRAC absorbance of the original TBHQ probe solution, A_1 and A_2 are those of the TBHQ probe subjected to PMS–NADH action in the absence and presence of SR scavenger, respectively, and C is the molar concentration of relevant scavenger. The IC_{50} values were then compared with those found with the NBT method.[10] The CUPRAC absorbance of the ethyl acetate extract of the incubation solution arising from the reduction of Cu(II)–neocuproine reagent by the remaining TBHQ was higher in the presence of $O_2^{\bullet-}$ scavengers (due to less conversion to TBBQ); therefore, increased absorbance of the reaction mixture indicated increased SRSA.

The SRSA of various scavengers were calculated using the following equation:

$$\text{SRSA}\ (\%) = 100[(A_2 - A_1)/(A_o - A_1)] \qquad (4)$$

SRSA percentage (y) can be empirically correlated to concentration of scavenger (x) within an absorbance range over which Beer's law is valid:

$$y = mx + n \qquad (5)$$

where m and n are the slope and intercept of this linear correlation, respectively. IC_{50} can then be calculated for 50% inhibition (y = 50) such that

$$y = 50 = m(IC_{50}) + n \quad \text{or} \quad IC_{50} = (50 - n)/m \qquad (6)$$

Testing of TBHQ and Reaction Products in the Presence of AA with the UV Method. The tubes bearing the reaction mixtures (0.5 mL of TBHQ (1.0 mM) + 2 mL of NADH (468 μM) + 1 mL of PMS (60 μM) + 0.5 mL of AA (0.1–0.5 mM) or water) were incubated for 30 min in a 25 °C water bath. The mixture solution was then extracted with adding 3.0 mL of ethyl acetate and vortexed for 20 s. The UV spectrum of the extract containing TBHQ probe and its oxidation product (TBBQ) was recorded in the wavelength range of 260–320 nm against EtAc (reagent blank).

NBT Assay. The commonly used indirect reference method of determining $O_2^{\bullet-}$ is the reduction of NBT[10] to the insoluble blue formazan. SRSA was evaluated by spectrophotometric measurement of formazan formed from NBT reduction by $O_2^{\bullet-}$.

$$NBT^{2+} + 4O_2^{\bullet-} + 2H^+ \rightarrow \text{diformazan} + 4O_2 \qquad (7)$$

To a test tube was added (2.5 − x) mL of DMSO, x = 0–0.5 mL of scavenger solution at a suitable concentration, 2.0 mL of 468 μM NADH, 1.0 mL of 300 μM NBT, in this order. The reaction was started by adding 1.0 mL of 60 μM PMS solution to the incubation mixture. The mixture in a total volume of 6.5 mL was incubated for 5 min in a water bath kept at 25 °C, and the absorbance was read at 560 nm against DMSO. Decreased absorbance of the incubation reaction mixture indicated increased SRSA. The inhibition ratio of scavengers (%) was calculated using the following formula:

$$\text{inhibition ratio}\ (\%) = 100[(A_o - A)/A_o] \qquad (8)$$

where A_o and A are the absorbances of the incubation reaction mixture in the absence and presence of scavenger, respectively.

HPLC Assay. A 2 mL aliquot of EtAc extract was freeze-dried for 10 min. The remaining residues were dissolved with 2 mL of 1:1 (v/v) EtOH–H_2O mixture. The analyses were carried out using a reversed-phase Agilent C18 column (4.6 mm ×250 mm, 5 μm particle size) (Milford, MA, U.S.A.). The mobile phase consisted of two solvents, i.e., methanol (A) and bidistilled water (B). The following parameters and gradient were used for the analysis of EtAc extracts: (V_{sample} = 20 μL; flow rate = 1.0 mL min^{-1}; λ_{TBHQ} = 290 nm; λ_{TBBQ} = 252 nm): 1 min 60% A to 40% B (slope 1.0); 4 min 70% A to 30% B (slope 1.0); 10 min 80% A to 20% B (slope 1.0); 15 min 60% A to 40% B (slope 1.0). The capability of $O_2^{\bullet-}$ scavenging was calculated using a modified version of eq 4:

$$\text{inhibition ratio}\ (\%) = 100[(A_2 - A_1)/(A_o - A_1)] \qquad (9)$$

where A_1 and A_2 are the peak areas of the TBHQ probe in the absence and presence of SR scavenger, respectively; A_0 is the peak area of the TBHQ probe at initial concentration in the reaction mixture.

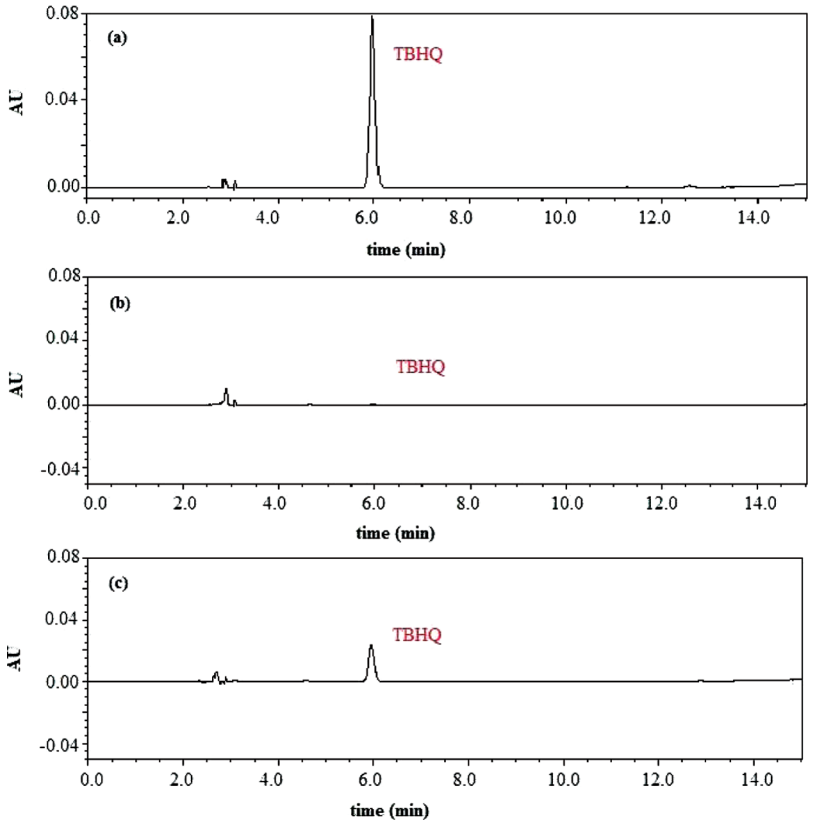

Figure 2. HPLC chromatograms for standard (original) and remaining TBHQ after PMS−NADH reaction in the presence and absence (reference) of AA (λ = 290 nm): (a) TBHQ standard; (b) reference (0.5 mL of TBHQ (1.0 mM) + 2 mL of NADH (468 μM) + 1 mL of PMS (60 μM) + 0.5 mL of water); (c) 0.5 mL of TBHQ (1.0 mM) + 2 mL of NADH (468 μM) + 1 mL of PMS (60 μM) + 0.5 mL of AA (0.3 mM).

Statistical Analysis. Descriptive statistical analyses were performed using Excel software (Microsoft Office 2002) for calculating the means and the standard error of the mean. Results were expressed as the mean ± standard deviation (SD). Using SPSS software for Windows (version 13), the data were evaluated by two-way analysis of variance (ANOVA).[23]

RESULTS AND DISCUSSION

Optimization of the Method. A modified CUPRAC method was applied to assess the SRSA of amino acids (i.e., L-serine, L-threonine, L-leucin, L-phenylalanine, L-tryptophan), thiol-type antioxidants (i.e., L-cysteine, *N*-acetyl-L-cysteine, L-glutathione reduced, and L-glutathione oxidized), and plasma antioxidants (i.e., bilirubin, uric acid, albumin, α-tocopherol, ascorbic acid, and SOD). CUPRAC absorbance arises from the reduction of the Cu(II)−Nc reagent to the Cu(I) chelate[16] by the TBHQ probe (i.e., TBHQ + $2Cu(Nc)_2^{2+} \rightarrow$ TBBQ + $2Cu(Nc)_2^{+}$ + $2H^+$), whereas the quinonic product of the (TBHQ + NADH−PMS) incubation system, TBBQ, had a negligible original CUPRAC absorbance. A scheme of conversion of TBHQ to TBBQ by $O_2^{\bullet-}$ is shown in Figure 1. The chromatograms for TBHQ conversion upon $O_2^{\bullet-}$ attack—in the absence and presence of the SR scavenger (AA)—are provided in Figures 2 and 3.

TBHQ, by virtue of its two-electron oxidation to TBBQ, acts as a reductant toward the CUPRAC reagent, Cu(II)−Nc. The molar absorptivity for TBHQ in the CUPRAC method was $\varepsilon = 1.57 \times 10^4\ M^{-1}\ cm^{-1}$, and the linear concentration range was: 5.09×10^{-7}−7.60×10^{-5} M (correlation coefficient: $r = 0.998$), within which the relative standard deviation (RSD) was 3.4%. The limit of detection (LOD) and limit of quantification (LOQ) were calculated using the equations LOD = $3s_{bl}/m$ and LOQ = $10\ s_{bl}/m$, respectively, where s_{bl} is the standard deviation of a blank and m is the slope of the calibration line. The LOD and LOQ for TBHQ were found as 0.2 and 0.67 μM, respectively.

Conversion of the TBHQ probe to TBBQ and inhibition of this reaction with a SR scavenger (i.e., SOD) were followed by measuring the CUPRAC absorbance of the mixture as a function of time (Figure 4). Inhibition of this reaction proceeded slowly in the presence of SOD, and an optimal measurement time of 30 min was chosen (Figure 4). This optimal period is sufficient to achieve an absorbance difference between TBHQ and TBBQ. Thus, due to the high conversion yield of the selected probe, SRSA of the studied compounds could be rapidly and precisely determined by recording the relative absorbances within 30 min. For comparison, time-dependent absorbance changes were recorded using TBHQ standard solutions incubated under identical conditions. There was no noteworthy change in absorbance for TBHQ within the 0−30 min time interval (Figure 4), and autoxidation reaction (i.e., without PMS−NADH) did not occur in this system that would otherwise cause interference. Thus, the short-lived intermediate of TBHQ oxidation (i.e., semiquinone radical) was not detected. Li et al.[24] have demonstrated that no semiquinone radical was detectable by ESR spin trapping when 1.0 mM TBHQ was incubated in phosphate buffer saline (PBS) in the absence of added Cu(II), whereas the incubation system of this work, {PMS−NADH + TBHQ}, did not contain a Cu(II) catalyst, and TBHQ may possibly be

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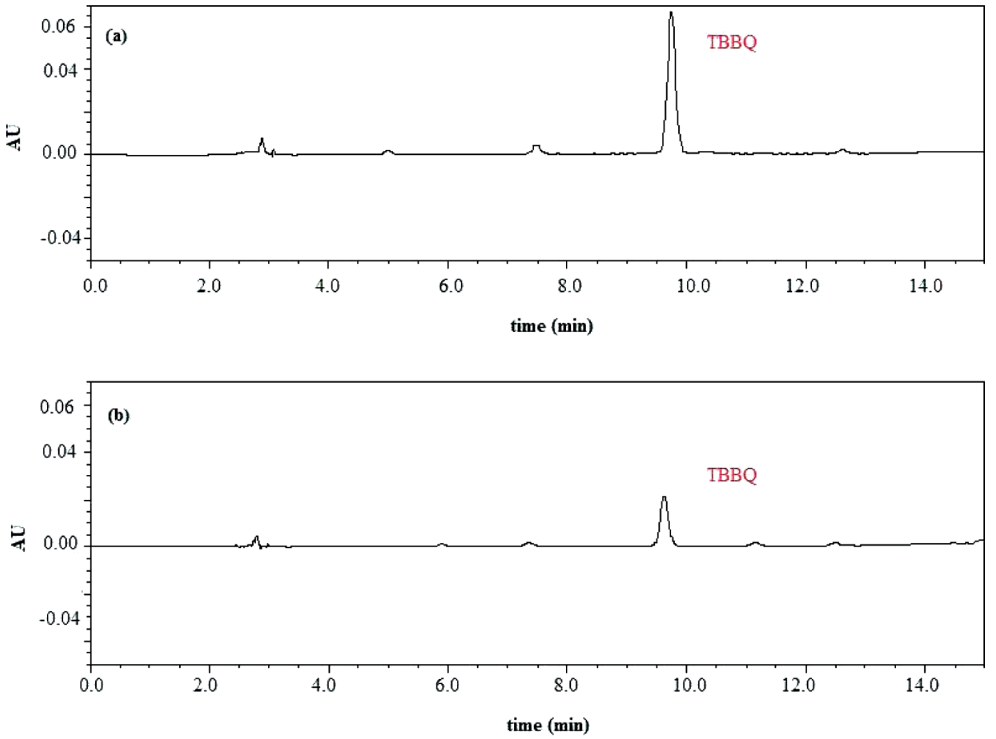

Figure 3. HPLC chromatograms for TBBQ (λ = 252 nm) formed from TBHQ in the PMS−NADH system in the presence and absence (reference) of AA: (a) reference (0.5 mL of TBHQ (1.0 mM) + 2 mL of NADH (468 μM) + 1 mL of PMS (60 μM) + 0.5 mL of water); (b) 0.5 mL of TBHQ (1.0 mM) + 2 mL of NADH (468 μM) + 1 mL of PMS (60 μM) + 0.5 mL of AA (0.3 mM).

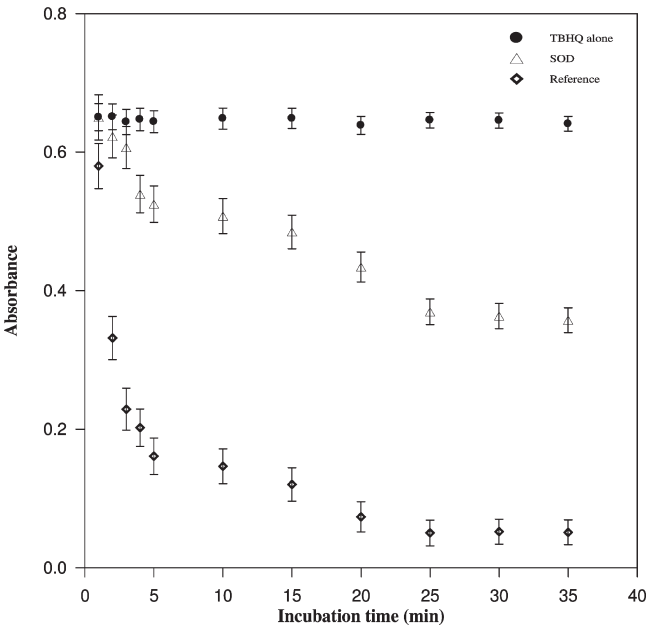

Figure 4. CUPRAC absorbance vs incubation time curves of TBHQ alone and TBHQ subjected to the PMS−NADH reaction in the presence (6.4 U mL^{-1} SOD) and absence (reference) of a $O_2^{\bullet-}$ scavenger.

oxidized by $O_2^{\bullet-}$ directly to TBBQ with the concomitant formation of H_2O_2.

TBHQ and TBBQ exhibit UV-absorbance spectra with peak absorbances at 290 and 252 nm, respectively (Figure 5). These results are confirmed in a study by Li et al.,[24] examining the Cu(II)-mediated redox-dependent activation of TBHQ. Upon mixing 1 mM TBHQ with $O_2^{\bullet-}$ generated by the PMS−NADH system in the presence of AA at concentrations 0.1−0.5 mM, the absorbance of TBHQ at 290 nm decreased with a corresponding increase in absorbance at 252 nm (Figure 5), indicating that the reaction of TBHQ with $O_2^{\bullet-}$ results in its oxidation to TBBQ. Thus, there is a proportional increase of 290 nm peak heights (Figure 5) with increasing concentration of scavenger (AA).

The effects of NADH and PMS, individually and simultaneously, on the CUPRAC absorbance of the TBHQ probe (in the absence and presence of scavenger, AA) were studied under the same experimental conditions, where neither compound in the EtAc phase gave a 450 nm absorbance (Table S-1, Supporting Information). In other words, the only constituent giving rise to an absorbance in the system is TBHQ, i.e., the CUPRAC-reactive substance (CUPRAC-rs) mentioned in Figure 1. PMS−NADH can produce $O_2^{\bullet-}$, and these radicals are manifested by the decrease in absorbance of the CUPRAC chromophore (A_{450} = 0.065) as a result of TBHQ oxidation with $O_2^{\bullet-}$ to TBBQ, whereas the absorbance due to the remaining TBHQ probe increases upon competition with AA (A_{450} = 0.490) (Table S-1, Supporting Information). The inhibition ratio (%) leading to SRSA estimation is calculated from the relative decrease of CUPRAC absorbances of the EtAc extracts using eq 4. The competition with AA of the TBHQ probe for $O_2^{\bullet-}$ can be followed simply by observing the changes in the concentration of the EtAc-extracted TBHQ probe without interference from water-soluble SR scavengers and other system constituents (i.e., PMS and NADH).

Superoxide and H_2O_2 can serve as substrates for the formation of hydroxyl radicals (·OH) having a potential to interfere with

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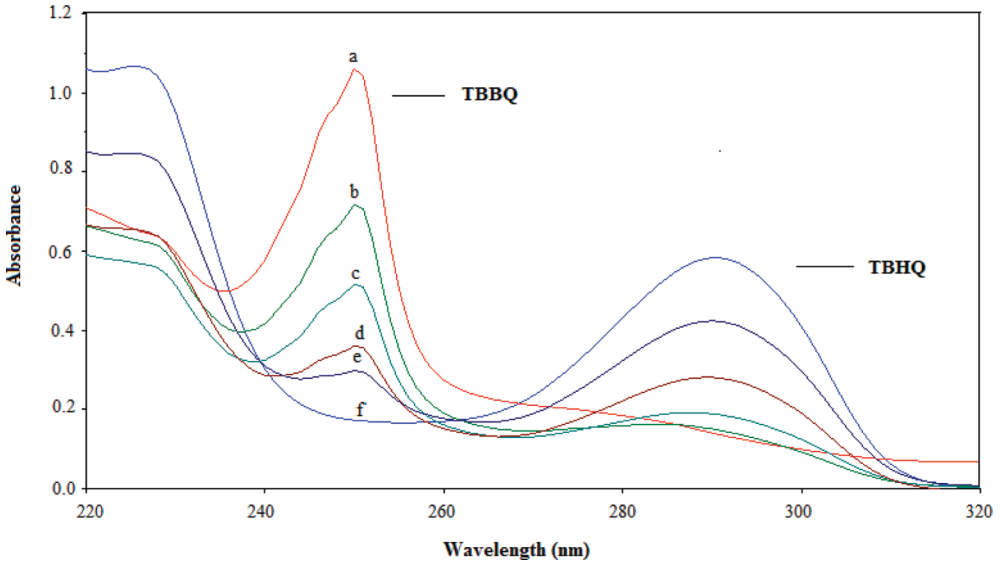

Figure 5. UV spectra of the remaining TBHQ and its oxidation product, TBBQ, as a result of PMS−NADH incubation in the presence of ascorbic acid (AA) at various concentrations: (a) 0 mM (reference, without scavenger), (b) 0.1 mM, (c) 0.3 mM, (d) 0.4 mM, (e) 0.5 mM AA, and (f) 0 mM (TBHQ standard).

the proposed assay only in a peroxidase-[25] or transition metal ion-catalyzed reaction,[26] whereas in this study, such catalyzers were absent. H_2O_2 is normally present at minute concentrations in leaf homogenates (at 40−120 nmol/g fresh weight)[27] and rat liver homogenate (at a concentration level of 0.1 μM).[28] When the possible effect of H_2O_2 at 0.1 and 1.0 μM levels was investigated in the nonenzymatic PMS−NADH incubation system with and without 721 U mL^{-1} catalase used as H_2O_2 scavenger (table not shown), it was seen that the added H_2O_2 did not interfere with the modified CUPRAC assay, producing A_{450} values in the EtAc phase between 0.058 and 0.072 absorbance units, i.e., not distinguishable from reference values. Besides added H_2O_2, any new formed H_2O_2 as a result of $O_2^{\bullet-}$ oxidation of the TBHQ probe was naturally eliminated by ethyl acetate extraction (i.e., H_2O_2 remained in the aqueous phase) followed by CUPRAC measurement in the organic phase.

Comparison of CUPRAC and HPLC Findings. In the chromatograms of this work (Figures 2 and 3), the retention times for TBHQ (detected at 290 nm) and its lone oxidation product TBBQ (detected at 252 nm) were 6.00−6.05 and 9.75 min, respectively. The amounts of TBHQ was found with the aid of the calibration curve drawn as peak area versus concentration, and the HPLC-found concentration of TBHQ multiplied by its absorptivity gave the experimentally measured CUPRAC absorbance. Concentrations of TBHQ (in μg mL^{-1}) remaining after PMS−NADH reaction in the presence of scavenger (AA) using the modified CUPRAC and HPLC methods were 2.18 ± 0.03 and 2.26 ± 0.05, respectively (initial concn of TBHQ was 27.76 ± 0.14, Figure 2a), and both methods correctly reflected the relative decrease in probe (TBHQ) conversion into TBBQ in the presence of AA (Figure 2, parts b and c).

SRSA activities of SOD (as IC_{50}) were calculated with spectrophotometric methods (i.e., modified CUPRAC, UV, and NBT methods) using eqs 4 and 8, as well as with HPLC. CUPRAC and HPLC assay results agreed among themselves with tolerable error (Table S-2, Supporting Information) (IC_{50} values of SOD with respect to the CUPRAC and HPLC methods were 8.66 ± 0.34 and 8.58 ± 0.68 U mL^{-1}, respectively). For the HPLC value, the peak areas were used to calculate the inhibition % of samples with respect to eq 9. The considerably larger IC_{50} value of SOD found with the use of the NBT method (Table S-2, Supporting Information) compared to those found by other methods may arise from numerous pitfalls met in assaying SOD by NBT conversion to formazan such as the SOD-noninhibitable portion of NBT reduction.[13] Likewise, it has earlier been reported that in many tissues where assay interferences were noted, no Mn-SOD activity could be detected using the NBT assay.[29] In the presence of potent scavengers such as AA, the conversion ratio of the probe was considerably smaller, as is apparent from the significant lowering of the peak heights of TBBQ, e.g., the peak area for TBBQ as 6.48 × 10^5 (reference) (Figure 3a) decreased to 3.61 × 10^5 with AA (Figure 3b). It has been reported that AA protects against oxygen poisoning with its role as a potential scavenger of $O_2^{\bullet-}$.[30] Nishikimi has found the second-order rate constant for the reaction of AA with $O_2^{\bullet-}$ to be 2.7 × 10^5 M^{-1} s^{-1},[30] in accordance with the value found in the present study (3.81 × 10^5 M^{-1} s^{-1}). The calculations for the second-order rate constant of $O_2^{\bullet-}$ scavenging by AA are illustrated in Table S-3 and Figure S-1 (Supporting Information).

In order to allow comparison among assays, it is useful to compare the obtained inhibition of the scavenger analyte with that obtained by the SOD enzyme, or by standard antioxidants, such as AA. Since the SRSA measurements were made under nonequilibrium conditions where $O_2^{\bullet-}$ was generated continuously, the results should be interpreted with caution.[31] SOD-equivalent SRSA activities of AA, L-serine, and NAC (at 0.5 mM scavenger concentration) calculated with CUPRAC, HPLC, UV, and NBT methods are presented in Table 1. The findings of modified CUPRAC and HPLC methods agreed among themselves with tolerable error (Table 1) (F_{exp} = 0.059, F_{crit} = 18.51, $F_{exp} < F_{crit}$ at P = 0.05). The problems encountered in observing very high SOD-equivalent SRSA values for *N*-acetyl cysteine (NAC) in the NBT method (Table 1) is thought to arise from the pitfalls met in assaying SOD with this method,[13] and not from the NAC assay itself, as the IC_{50} values of NAC found with the modified CUPRAC and NBT methods were alike (Table 2). By

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Table 1. SOD-Equivalent SRSA of Certain Scavengers Calculated with Respect to the Modified CUPRAC, HPLC, UV, and NBT Methods (N = 4 or 5 Data Points)[a]

SR scavengers	$SRSA_{CUPRAC}$ (U mL^{-1})	$SRSA_{HPLC}$ (U mL^{-1})	$SRSA_{UV}$ (U mL^{-1})	$SRSA_{NBT}$ (U mL^{-1})
ascorbic acid	10.56 ± 0.42	10.57 ± 0.31	12.65 ± 0.63	13.58 ± 0.95
N-acetyl cysteine	11.51 ± 0.69	9.94 ± 0.39	13.25 ± 0.66	44.21 ± 3.85
serine	1.70 ± 0.03	2.71 ± 0.13	5.65 ± 0.05	25.82 ± 1.54

[a] SOD equivalent was calculated for a 0.5 mM scavenger concentration. (The calculation of SOD-equivalent SRSA is given in the Supporting Information.)

Table 2. SRSA of Various Scavengers Using the Modified CUPRAC Method in Comparison with the NBT Method (IC_{50} with Respect to eq 5, N = 4 or 5 Data Points)[a]

SR scavengers	IC_{50} value with respect to CUPRAC method (mM)	IC_{50} value with respect to NBT method (mM)
	Thiol-Type Antioxidants	
glutathione (GSH)	0.51 ± 0.02	0.41 ± 0.03
glutathione oxidized (GSSG)	0.48 ± 0.01	0.38 ± 0.02
N-acetyl cysteine (NAC)	0.36 ± 0.03	0.37 ± 0.02
cysteine	0.28 ± 0.01	N.D.[b]
1,4-dithioerythritol	0.29 ± 0.02	0.63 ± 0.04
	S-Containing Antioxidants	
methionine	3.55 ± 0.21	4.37 ± 0.30
	Amino Acids	
serine	4.74 ± 0.19	51.00 ± 3.00
threonine	4.48 ± 0.27	52.00 ± 2.08
leucin	0.16 ± 0.01	4.39 ± 0.28
tryptophan	0.13 ± 0.01	2.45 ± 0.17
phenylalanine	0.35 ± 0.02	0.47 ± 0.02
	Plasma Antioxidants	
albumin	6.09 ± 0.31[c]	21.82 ± 1.74[c]
α-tocopherol	0.24 ± 0.01	N.D.[d]
uric acid	24.8 ± 0.96	N.D.[b]
ascorbic acid	0.41 ± 0.02	0.61 ± 0.04
bilirubin	0.11 ± 0.01	N.D.[d]
SOD	8.66 ± 0.34[e]	26.36 ± 2.37[e]

[a] P = 0.05, F_{exp} = 2.5499, $F_{crit(table)}$ = 4.965, $F_{exp} < F_{crit\ (table)}$). Data presented as (mean ± SD), N = 3. [b] Interference (reduction of NBT). [c] Unit of IC_{50} value is mg mL^{-1}. [d] N.D.: SRSA at the studied concentration level could not be detected. [e] Unit of IC_{50} value is U mL^{-1}.

similar reasoning, it can be understood that the IC_{50} values in Table 2 for SOD calculated by using the CUPRAC and NBT methods were quite different. The agreement between modified CUPRAC and NBT in IC_{50} calculation of NAC (Table 2) is in conformation with the findings of Aruoma et al.[32] who noted that there was no observable direct reduction of NBT with NAC, but at concentrations of 3 mM, NAC decreased the rate of NBT reduction by 13−15%.

Comparison of the Modified CUPRAC and NBT Methods for SRSA Assay. Under in vitro conditions, $O_2^{\bullet-}$ has been widely measured spectrophotometrically by means of the NBT method[33] due to its high sensitivity. NBT is known to be four-electron-reduced by $O_2^{\bullet-}$ to the water-insoluble blue-colored formazan (λ_{max}: 560 nm) with a high molar absorptivity (i.e., $\varepsilon > 10^4\ M^{-1}\ cm^{-1}$). Any added SR scavenger capable of reacting with $O_2^{\bullet-}$ inhibits the production of formazan. SRSA can therefore be expressed as the "percentage inhibition of NBT reduction". Since formazan is easily detected spectrophotometrically over a wide pH range, NBT has been used in clinical determination of SOD[23] and NADH or NADH-dependent enzyme (dehydrogenase) activities.[34] However, the mechanism of reduction of NBT is rather complicated,[35] and its half-reduced (two-electron-reduced) insoluble species (mono- and diformazans) may often lead to erratic absorbance readings in aqueous solution. In this study, DMSO was used as solvent for solubilizing the formazan compounds formed in the reference NBT assay.[36]

Although the reduction of 1 mol of NBT to 1 mol of formazan is assumed to theoretically require 4 mol of $O_2^{\bullet-}$,[9] the redox cycling of the intermediary reduction product of NBT (i.e., $NBT^{\bullet+}$ radical cation) with molecular oxygen,[37] combined with the generation of two different formazan species from NBT at the same redox potential[35] and with the existence of superoxide-independent pathways for the reduction of NBT,[38] are limiting factors to quantitative conversion of NBT to formazan as a probe for $O_2^{\bullet-}$ estimation. The NBT test producing the insoluble formazan product was reported not to permit a reliable quantitative photometric determination of $O_2^{\bullet-}$ in solution.[39] On the other hand, the conversion efficiency of TBHQ probe to TBBQ without any scavenger (reference) was >99%. As a result of the PMS−NADH reaction, product conversion varied with respect to the nature of scavenger, e.g., the conversion ratio of the original probe (TBHQ) to its only oxidation product (TBBQ) in the presence of 0.1 mM AA was approximately 90−92%, whereas with 0.3 mM AA, this ratio decreased to 64−66% (Figures 2 and 3).

Thiols like glutathione, *N*-acetyl cysteine, and cysteine were effective SR scavengers (Table 2) in accordance with literature reports.[40−42] Winterbourn and Metodiewa[41] examined the reaction of thiol compounds with $O_2^{\bullet-}$ as demonstrated by the loss of thiol groups and enhanced oxygen uptake in the xanthine oxidase incubation system. Among the biologically important thiol compounds tested, cysteine showed a high reactivity (i.e., exhibiting the highest superoxide-dependent thiol loss upon incubation)[41] in compliance with our finding (IC_{50} (mM): 0.28 ± 0.04) (Table 2). Okada and Okada[43] also reported a SOD-equivalent SRSA activity value of (2.73 ± 0.12 units mL^{-1}) for 100 μM cysteine ($SRSA_{CUPRAC}$: 4.73 ± 0.12 units mL^{-1}), whereas NBT assay was nonresponsive to cysteine, probably due to the direct reduction of NBT with cysteine. The overall equation for the reaction of cysteine with $O_2^{\bullet-}$ is given by eq 10:

$$2\text{cysteine(RSH)} + 2O_2^{\bullet-} + 2H^+ \rightarrow \text{cystine(RSSR)} + 2H_2O_2 \quad (10)$$

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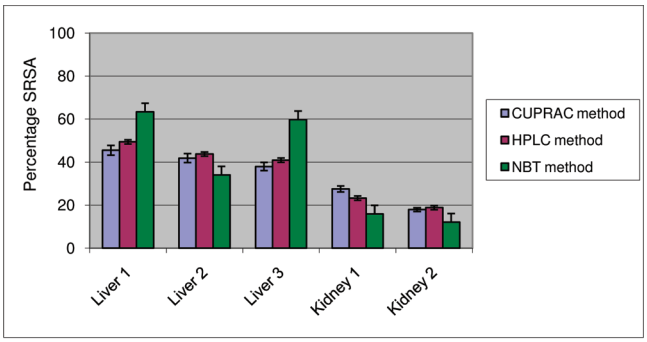
Nishikimi et al.[44] demonstrated that α-tocopherol (α-TOC) was oxidized to α-tocopheryl quinone by $O_2^{\bullet-}$ generated in the xanthine−xanthine oxidase system, and IC_{50} of α-TOC was found at the SOD-equivalent concentration of 0.19 μg mL^{-1} (the approximate IC_{50} value of α-TOC was 0.2 mM) in compliance with our finding (IC_{50} (mM): 0.24 ± 0.02) (Table 2). SRSA of α-TOC using the NBT method at the studied concentration level could not be detected.

The second-order rate constant for NBT reduction by $O_2^{\bullet-}$ ($k = 6.0 \times 10^4\ M^{-1}\ s^{-1}$)[45] is ~4 orders of magnitude lower than that of SOD ($k = 2.0 \times 10^9\ M^{-1}\ s^{-1}$),[46] and this may lead to underestimation of $O_2^{\bullet-}$ as a result of scavenging in some biological samples containing SOD. Compared to NBT reaction, the rate constant calculated for TBHQ oxidation by $O_2^{\bullet-}$ ($k_{TBHQ} = 4.7 \times 10^5\ M^{-1}\ s^{-1}$) (Supporting Information) was an order of magnitude higher, and this is believed to cause less interference to the SRSA measurement of biological samples. Moreover, Spitz and Oberley reported extremely prevalent tissue specific interferences, where spontaneous tissue-mediated NBT reduction occurred in assay mixtures without the initiation of $O_2^{\bullet-}$ generation.[29]

Following the general reasoning of competition kinetics, when inhibition percentage (y) was correlated to micromolar concentration of inhibitor antioxidant (x), the resulting linear equations conforming to eq 5 enabled the calculation of IC_{50} values of inhibitors using eq 6 (RSD: 6−7%). The precision of IC_{50} measurements was reflected in the r values of the competition plots which varied between 0.935 and 0.998 for both modified CUPRAC and NBT methods in the optimal concentration range of the tested SR scavenger (Figure S-2, Supporting Information).

The ANOVA comparison by the aid of F-test of the mean-squares of "between treatments" (i.e., IC_{50} values of different samples with respect to the modified CUPRAC and NBT methods depicted in Table 2) and of residuals[23] for a number of real samples (consisting of 11 SR scavengers) enabled us to conclude that there was no significant difference between treatments. In other words, the experimentally found CUPRAC results and NBT results were statistically alike at the 95% confidence level ($F_{exp} = 2.549$, $F_{crit} = 4.965$, $F_{exp} < F_{crit}$ at $P = 0.05$) (by exclusion of the values for compounds with highest IC_{50} variability, i.e., SOD, albumin, threonine, serine, leucin, and tryptophan; $F_{exp} = 1.252$, $F_{crit} = 5.987$, $F_{exp} < F_{crit}$ at $P = 0.05$). Thus, the proposed methodology was validated against the classical NBT method.

Application of the Method to Certain Tissue Homogenates and Possibility of Extension to Further Studies. The SRSA of tissue homogenates was determined by the modified CUPRAC, HPLC, and NBT methods as IC_{50} values in the PMS−NADH reaction mixture. The liver homogenates were generally shown to exhibit higher scavenging activity than kidney homogenates. Similarly, Nandi and Chatterjee[47] found that rat liver tissue homogenates (total SOD activity: 1700 ± 100 units per g tissue) are stronger SR scavengers than kidney homogenates (total SOD activity: 750 ± 80 units per g tissue). The SRSA of kidney homogenates as inhibition % versus homogenate volume is shown as a curve in Figure S-3 (Supporting Information). Generally, in the study of $O_2^{\bullet-}$ scavenging activity of complex plant extracts and biological fluids and homogenates, a plateau region of inhibition (or scavenging) percentage as a function of concentration was reported.[47] This plateau probably arises from the fact that, above a limiting concentration, scavengers may not effectively compete with the probe for $O_2^{\bullet-}$ quenching. Likewise, Logan et al.[48] indicated that SRSA showed a linear correlation with the volume of red wine up to a limited volume, and exceeding this critical volume resulted in some saturation resulting in a curved correlation. The SRSA values measured with the proposed CUPRAC and reference methods are comparatively depicted in a bar diagram (Figure 6); the percentage inhibitions of identical tissue homogenates found with CUPRAC were almost equal to those measured with HPLC.



$SRSA_{HPLC} = 1.16 \cdot SRSA_{CUPRAC} - 4.49$ $r = 0.981$
$SRSA_{HPLC} = 0.50 \cdot SRSA_{NBT} + 16.78$ $r = 0.888$

Figure 6. SRSA (%) of some tissue homogenates calculated with the modified CUPRAC method in comparison to NBT and HPLC methods.

In future work, the proposed assay may be extended to the SRSA measurement of phenolic superoxide scavengers. Cos et al.[49] have reported that flavonoids exhibit SRSA at micromolar concentration levels (e.g., the IC_{50} values of quercetin, apigenin, and myricetin were 1.63, 1.33, and 0.33 μM, respectively). Thus, it is required that the phenolics in a test sample would not show an initial CUPRAC absorbance, and yet, they would react with $O_2^{\bullet-}$ generated by a PMS−NADH reaction. After the necessary incubation, dilution, and extraction, the phenolics themselves would not give an appreciable absorbance (i.e., $A_{450} \leq 0.02$ absorbance units using a 1 cm optical cell). In that case, the measured CUPRAC absorbance at the end of the proposed assay utilizing the TBHQ probe at millimolar levels would definitely come from TBHQ remaining after PMS−NADH reaction, and not from the consumed micromolar levels of phenolic $O_2^{\bullet-}$ scavengers.

CONCLUSIONS

The proposed method renders the relatively specific detection of $O_2^{\bullet-}$ and is applied to SRSA estimation of a rich variety of biologically active compounds (i.e., amino acids, thiols, plasma antioxidants, and vitamins) and tissue homogenates. Thus, conventional problems of the widely used NBT method arising from the insolubility of formazan and from direct reduction of NBT by tested scavengers without involvement of $O_2^{\bullet-}$ were overcome. The modified CUPRAC results were close or comparable to those found by the conventional NBT and HPLC methods. This alternative cost-effective method emerges as a promising tool to better understand the role of $O_2^{\bullet-}$ in biology.

ASSOCIATED CONTENT

Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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REFERENCES

(1) Nijveldt, R. J.; van Nood, E.; van Hoorn, D. E. C.; Boelens, P. G.; van Norren, K.; van Leeuwen, P. A. M. *Am. J. Clin. Nutr.* **2001**, *74*, 418–425.
(2) Terada, L. S.; Leff, J. A.; Repine, J. E. *Methods Enzymol.* **1990**, *186*, 651–656.
(3) Corte, E. D.; Stirpe, F. *Biochem. J.* **1972**, *126*, 739–745.
(4) Unno, T.; Yayabe, F.; Hayakawa, T.; Tsuge, H. *Food Chem.* **2002**, *76*, 259–265.
(5) Nishikimi, M.; Rao, N. A.; Yagi, K. *Biochem. Biophys. Res. Commun.* **1972**, *46*, 849–854.
(6) Robak, J.; Grylewski, R. J. *Biochem. Pharmacol.* **1988**, *37*, 837–841.
(7) Bartosz, G. *Clin. Chim. Acta* **2006**, *368*, 53–76.
(8) Cohen, G.; Heikkila, R. E. *J. Biol. Chem.* **1974**, *249*, 2447–2452.
(9) Bielski, B. H. J.; Shiue, G. G.; Bajuk, S. *J. Phys. Chem.* **1980**, *84*, 830–833.
(10) Yu, H.; Liu, X.; Xing, R.; Liu, S.; Li, C.; Li, P. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 2659–2664.
(11) Lee, J.; Koo, N.; Min, D. B. *Compr. Rev. Food Sci. Food Saf.* **2006**, *3*, 21–33.
(12) Machado, R. A.; Vargas, F.; Arce, A. J.; Rivillo, D.; De Sanctis, Y.; Jorge, G.; Martinez, J. D.; D'Ornelas, L. *Ciencia* **2008**, *16*, 234–240.
(13) Beyer, W. F.; Fridovich, I. *Anal. Biochem.* **1987**, *161*, 559–566.
(14) Sutherland, M. W.; Learmonth, B. A. *Free Radical Res.* **1997**, *27*, 283–289.
(15) Johnson, R. N.; Metcalf, P. A.; Baker, J. R. *Clin. Chim. Acta* **1983**, *127*, 87–95.
(16) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. *J. Agric. Food Chem.* **2004**, *52*, 7970–7981.
(17) Badary, O. A.; Taha, R. A.; Gamal El-Din, A. M.; Abdel-Wahab, M. H. *Drug Chem. Toxicol.* **2003**, *26*, 87–98.
(18) Kim, M. C.; Pratt, D. E. In *Phenolic Compounds in Food and Their Effects of Health (II)*; Ho, C. T., Lee, C. Y., Huang, M. T., Eds.; American Chemical Society: Washington, DC, 1992; pp 200−218.
(19) Bergmann, B.; Dohrmann, J. K.; Kahl, R. *Toxicology* **1992**, *74*, 127–133.
(20) Pinkus, R.; Weiner, L. M.; Daniel, V. *J. Biol. Chem.* **1996**, *271*, 13422–13429.
(21) Van Ommen, B.; Koster, A.; Verhagen, H.; Van Bladeren, P. J. *Biochem. Biophys. Res. Commun.* **1992**, *189*, 309–314.
(22) Alía, M.; Horcajo, C.; Bravo, L.; Goya, L. *Nutr. Res. (N. Y.)* **2003**, *23*, 1251–1267.
(23) Miller, J. C.; Miller, J. N. *Statistics for Analytical Chemists*, 3rd ed.; Ellis Horwood and Prentice Hall: New York and London, 1993.
(24) Li, Y.; Seacat, A.; Kuppusamy, P.; Zweier, J. L.; Yager, J. D.; Trush, M. A. *Mutat. Res.* **2002**, *518*, 123–133.
(25) Chen, S.-X.; Schopfer, P. *Eur. J. Biochem.* **1999**, *260*, 726–735.
(26) Pasternack, R. F.; Halliwell, B. *J. Am. Chem. Soc.* **1979**, *101*, 1026–1031.
(27) Veljovic-Jovanovic, S.; Noctor, G.; Foyer, C. H. *Plant Physiol. Biochem.* **2002**, *40*, 501–507.
(28) Gonzalez-Flecha, B.; Cutrin, J. C.; Boveris, A. *J. Clin. Invest.* **1993**, *91*, 456–454.
(29) Spitz, D. R.; Oberley, L. W. *Anal. Biochem.* **1989**, *179*, 8–18.
(30) Nishikimi, M. *Biochem. Biophys. Res. Commun.* **1975**, *63*, 463–468.
(31) Frankel, E. N.; Meyer, A. S. *J. Sci. Food Agric.* **2000**, *80*, 1925–1941.
(32) Aruoma, O. I.; Halliwell, B.; Hoey, B. M.; Butler, J. *Free Radical Biol. Med.* **1989**, *6*, 593–597.
(33) Beauchamp, C.; Fridovich, I. *Anal. Biochem.* **1971**, *44*, 276–287.
(34) Ishiyama, M.; Miyazono, Y.; Shiga, M.; Sasamoto, K.; Ohkura, Y.; Ueno, K. *Anal. Sci.* **1996**, *12*, 515–519.
(35) Umemoto, K. *Bull. Chem. Soc. Jpn.* **1989**, *62*, 3783–3789.
(36) Scudiero, D. A.; Shoemaker, R. H.; Paull, K. D.; Monks, A.; Tierney, S.; Nofziger, T. H.; Currens, M. J.; Seniff, D.; Boyd, M. R. *Cancer Res.* **1988**, *48*, 4827–4833.
(37) Tarpey, M. M.; Fridovich, I. *Circ. Res.* **2001**, *89*, 224–236.
(38) Thayer, W. S. *Arch. Biochem. Biophys.* **1990**, *276*, 139–145.
(39) Frahry, G.; Schopfer, P. *Planta* **2001**, *212*, 175–183.
(40) Winterbourn, C. C.; Metodiewa, D. *Free Radical Biol. Med.* **1999**, *27*, 322–328.
(41) Winterbourn, C. C.; Metodiewa, D. *Arch. Biochem. Biophys.* **1994**, *314*, 284–290.
(42) Benrahmoune, M.; Therond, P.; Abedinzadeh, Z. *Free Radical Biol. Med.* **2000**, *29*, 775–782.
(43) Okada, Y.; Okada, M. *J. Agric. Food Chem.* **1998**, *46*, 401–406.
(44) Nishikimi, M.; Yamada, H.; Yagi, K. *Biochem. Biophys. Res. Commun.* **1980**, *627*, 101–108.
(45) Bielski, B. H. J.; Richter, H. W. *J. Am. Chem. Soc.* **1977**, *99*, 3019–3023.
(46) Fridovich, I. *Annu. Rev. Pharmacol. Toxicol.* **1983**, *23*, 239–257.
(47) Nandi, A.; Chatterjee, I. B. *J. Biosci.* **1988**, *13*, 305–315.
(48) Logan, B. A.; Hammond, M. P.; Storno, B. M. *Biochem. Mol. Biol. Educ.* **2008**, *36*, 39–42.
(49) Cos, P.; Ying, L.; Calomme, M.; Hu, J. P.; Cimanga, K.; Poel, B. V.; Pieters, L.; Vlietinck, A. J.; Berghe, D. V. *J. Nat. Prod.* **1998**, *61*, 71–76.