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Novel spectroscopic sensor for the hydroxyl radical scavenging activity measurement of biological samples

Burcu Bekdeşer, Mustafa Özyürek, Kubilay Güçlü, Reşat Apak*

Department of Chemistry, Faculty of Engineering, Istanbul University, Avcilar 34320, Istanbul, Turkey

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ABSTRACT

A novel spectroscopic sensor was developed and validated for hydroxyl radical scavenging (HRS) activity estimation using terephthalate (TP) as probe. This sensor was designed by electrostatic immobilization of the chromogenic oxidizing agent of the *CUP*ric *R*educing *A*ntioxidant *C*apacity (CUPRAC) method, Cu(II)-Neocuproine (Cu(II)-Nc) complex, on a Nafion cation-exchange membrane, and the spectrophotometric assay developed in aqueous–alcoholic solutions was integrated to the CUPRAC sensor. Hydroxyl radicals ($^{\bullet}$OH) generated from an equivalent mixture of Fe(II)+EDTA with hydrogen peroxide attacked both the probe and the $^{\bullet}$OH scavengers in 37 °C-incubated solutions for 1/2 h. The HRS activity was measured using the decrease in CUPRAC absorbance at 450 nm – arising from the reduction of Cu(II)-Nc reagent to the Cu(I)-neocuproine chelate – of the hydroxylated probe (TP) undergoing radical attack in the presence of $^{\bullet}$OH scavengers. The HRS activity was evaluated as the second-order rate constants of biologically active compounds for $^{\bullet}$OH scavenging and also as the percentage scavenging of a measured compound or sample relative to a reference compound. Using this reaction, a kinetic approach was adopted to assess the HRS activity of amino acids, plasma- and thiol-antioxidants. This assay, applicable to small molecule antioxidants and tissue homogenates, proved to be efficient for serine and albumin for which the widely used TBARS (thiobarbituric acid-reactive substances) test is nonresponsive. Under optimal conditions, about half of the probe (TP) was converted into 2-hydroxyterephthalate (hTP), and this monohydroxylated derivative, being the only product of hydroxylation, was a more specific marker of $^{\bullet}$OH than the non-specific malondialdehyde end-product of the TBARS test. The sensor gave a linear response to scavenger concentration in the competition kinetic equation.

1. Introduction

Hydroxyl radical ($^{\bullet}$OH) is the most reactive oxygen species (ROS), which can react with most organic molecules by addition to unsaturated bond, hydrogen abstraction, or electron transfer (its reduction potential = 1.9 V) [1]. However, in case of excessive $^{\bullet}$OH generation or insufficient cellular antioxidant defense, this can stimulate free radical chain reactions by interacting with cellular macromolecules including carbohydrates, lipids, proteins, and nucleic acids that may eventually give rise to various health disorders like cancer, cardiovascular disorders, atherosclerosis, and Alzheimer's disease [2]. So it is important to eliminate excessive $^{\bullet}$OH *in vivo* to prevent $^{\bullet}$OH-originated disease using antioxidant defense systems consisting of low and high molecular weight constituents to defend against ROS attack. The altered levels of thiols (reduced forms) and their disulfides (oxidized forms) in physiological liquids have been linked to specific pathological conditions [3]. Since prooxidant activity tests carried out with the use of different oxidation agents give rise to a wide range of reactive species in biological samples, the antioxidant power of various protective (reactive species scavenging) compounds needs to be assessed in a broader sense so as to better understand the resistance or susceptibility of a tissue to oxidative stress under different conditions [4].

Some biochemical researchers have argued that the measurement of HRS activity in biological media may be irrelevant because almost all molecules of biological importance may scavenge $^{\bullet}$OH with appreciable rates. This is not valid based on many recent applications and requirements in biological chemistry. For example, cyclic ischemia and reperfusion in animal tests (e.g., mediated by partial urinary bladder outlet obstruction) resulting in the generation of ROS (mainly hydroxyl radicals) may be expected to increase the level of free radicals and to decrease the level of protective antioxidants [5], and the production of hydroxyl radicals as measured by selective and sensitive probes should better correlate with the reactivity of antioxidants

* Corresponding author. Tel.: +90 212 473 7028; fax: +90 212 473 7180.
E-mail address: rapak@istanbul.edu.tr (R. Apak).

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found within the ischemia/reperfusion-obstructed tissue as compared to the control tissue. Another requirement may be exemplified from cancer research. Since certain chemotherapeutic agents (like cis-diamminedichloroplatinum(II), known as cisplatin) may induce the generation of hydroxyl radicals causing tissue damage and toxic effects including nephrotoxicity, attempts for amelioration of $^{\bullet}$OH accumulation by pharmacological scavengers such as NAC and dimethylthiourea [6] may involve the use of rapid, efficient and inexpensive HRS activity assays.

Various detection probes, being critical components of free radical scavenging assays, have been developed for monitoring $^{\bullet}$OH concentration and $^{\bullet}$OH scavenging capacity. The most sophisticated ESR spin-trapping methods, though having excellent sensitivity and specificity, are often not carried out under biologically relevant conditions such as pH and require expensive equipment, whereas MS detections require expensive instruments and are difficult to implement. This leaves molecular spectroscopic (i.e., absorption and fluorescence spectrometric) probes as good potential detection methods [7] with low cost and high practical applicability to relatively less equipped conventional laboratories. Unfortunately, from most spectroscopic probes, a mixture of isomers are produced as a result of hydroxylation upon $^{\bullet}$OH attack, rendering mechanistic interpretation of the hydroxylation reaction difficult. The attack of $^{\bullet}$OH on a benzoate probe may occur at *ortho*-, *meta*-, or *para*-positions, whereas hydroxylation of a salicylate probe produces 2,3-, 2,4-, and 2,5-dihydroxybenzoates and catechol [8]. When a salicylate probe is used *in vivo* as hydroxyl radical detector, its radical metabolism produces two main hydroxylated derivatives (2,3- and 2,5-dihydroxybenzoic acids: DHBAs). Since the latter acid can be also produced by enzymatic pathways through the cytochrome P-450 system while the former acid is reported to be solely formed by direct $^{\bullet}$OH attack, measurement of 2,3-DHBA, following oral administration of the drug acetyl salicylate, was proposed for assessment of oxidative stress *in vivo* [9]. The hydroxylation reaction of phenylalanine has been proposed as an alternative to salicylate, but the disadvantage of multiple hydroxylation products remains, since reaction of phenylalanine with $^{\bullet}$OH yields a mixture of the 2-, 3- and 4-hydroxylated products, *o*-tyrosine, *m*-tyrosine, and *p*-tyrosine [10]. Therefore, development of a practical and specific assay (i.e., producing a single hydroxylation product) for the HRS activity measurement is both important and a challenging task owing to the high activity, short lifetime, and low concentration of $^{\bullet}$OH.

Terephthalate (TP) is a known probe for $^{\bullet}$OH that produces a highly fluorescent (excitation 315 nm, emission 425 nm) monohydroxylated product, 2-hydroxyterephthalate (hTP), upon hydroxylation. The hydroxylation of TP is often used a specific and sensitive marker for $^{\bullet}$OH formation when compared with other chemical probes [10]. A major advantage of this system is that, apart from some minor fragmentation products occurring in all aromatic hydroxylation processes, the symmetry of the molecule leads to a single hydroxylated product, hTP. This stable product is highly fluorescent and can be detected with a standard fluorometer, whereas TP is practically non-fluorescent. In contrast to other probes (phenylalanine, salicylate, 4-hydroxybenzoic acid), neither the ROS trap (TP) nor the hydroxylated product (hTP) is endergenously present or metabolized. Moreover, TP has been known to be non-toxic and not accumulative [11]. Although the oxidation potential of TP to hTP has not been reported in literature, the hydroxylation of TP is specific for highly reactive oxygen species as it requires the high redox potentials of $^{\bullet}$OH or ferryl species (>1.6 V), and TP has no significant reactivity toward other ROS such as superoxide anion radical, H_2O_2, ROO$^{\bullet}$, and singlet oxygen [11–14]. In addition to the selectivity of TP for $^{\bullet}$OH, TP was preferred as the $^{\bullet}$OH probe in the developed colorimetric assay because the CUPRAC absorbance of the parent probe was quite low whereas of its hydroxylation product (of definite identity) relatively high.

There is very limited study about the usage of optical sensors for molecular spectroscopic HRS activity assays. Naughton et al. developed a hydroxyl radical fibre-optic sensor by using an $^{\bullet}$OH radical-sensitive reagent phase (nitrophenol) which was immobilized onto XAD-7 methacrylate beads. Nitrocatechol, generated from the attack of $^{\bullet}$OH on nitrophenol, exhibits a strong absorption band in the visible region of the electromagnetic spectrum ($\lambda_{max}=510$ nm) [15]. An optical nano-based sensor was designed by attaching coumarin-3-carboxylic acid (CCA) onto the NP surface. The detection of this probe was based on the irreversible hydroxylation of a nonfluorescent form of CCA, resulting in a fluorescent product (7-hydroxycoumarin-3-carboxylic acid) [16]. The HRS activity measurement of thiols was also realized by using an electrochemiluminescence (ECL) sensor based on the quenching of the chemiluminescence produced from thioglycolic acid-capped CdSe quantum dots (QDs) film/peroxide aqueous system [17].

The CUPRAC method of antioxidant capacity determination [18] is currently evolving into an ''antioxidant measurement package'' comprising many assays. A number of $^{\bullet}$OH scavenging assays using different probes based on modified CUPRAC methods comprised the determination of $^{\bullet}$OH scavenging rate constants of certain water-soluble compounds [19] and of polyphenolics [20]. In this study, the developed HRS activity assay of biological samples using TP probe was incorporated into the mentioned package so as to improve selectivity and expand applicability. The CUPRAC sensor was designed by electrostatic immobilization of the chromogenic oxidizing agent of the CUPRAC method, Cu(II)-Nc complex, on a Nafion membrane, and the spectrophotometric assay developed in aqueous–alcoholic solutions was integrated to the CUPRAC sensor [21]. Nafion is a perfluorosulfonate polymer in which hydrophilic perfluorinated ether side chains terminate with cation-exchanger sulfonate groups, which are periodically attached to a hydrophobic perfluoroethylene backbone. Absorbance values of the final products (i.e., hTP) formed from the testing reactions on the cupric neocuproine-attached Nafion membrane sensor was measured, and correlated to HRS activities of the biological samples. Naturally, linearity of sensor response over a reasonable concentration range is a prerequisite for meaningful comparison of HRS activities of biological samples. Consequently, the proposed CUPRAC sensor for practical, versatile and low-cost HRS activity measurement is believed to broaden the applicability of the method to antioxidant researchers in all fields comprising bioanalytical, medicinal and food chemistry.

2. Experimental

2.1. Reagents and apparatus

The following chemical substances of analytical reagent grade were supplied from the corresponding sources: Neocuproine (2,9-dimethyl-1,10-phenanthroline), L-serine, L-valine, L-threonine, L-leucine: Sigma (Steinheim, Germany); uric acid, L-ascorbic acid, 2-thiobarbituric acid (TBA), Nafion 115 perfluorinated membran (thickness 0.005 in.): Aldrich (Steinheim, Germany); copper(II) chloride dihydrate, L-tryptophan, L-glutathione reduced, L-proline, hydrogen peroxide (35 wt%), iron(II) chloride tetrahydrate, sodium hydroxide, potassium dihydrogen phosphate, concentrated H_3PO_4, concentrated HCl, Na_2HPO_4, $NaH_2PO_4\cdot 2H_2O$, albumin from bovine serum, disodium terephthalate (Na_2-TP): Merck (Darmstadt, Germany); ammonium acetate, iron(III) chloride, ethyl acetate (EtAc): Riedel-de Haen (Steinheim, Germany); L-lysine, L-glutathione oxidized, L-cysteine, L-methionine, *N*-acetyl-L-cysteine, 2-deoxy-D-ribose, ethylenediaminetetraacetic acid

disodium salt (Na_2-EDTA): Fluka (Buchs, Switzerland). The biological samples were supplied from the Faculty of Veterinary Medicine of Istanbul University.

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 were Telstar Cryodos freeze dryer (Terrassa, Spain) and a BIOSAN Programmable rotator-mixer Bulti Bio RS-24 (Riga, Latvia). The chromatograph was a Waters Breeze™ 2 Model HPLC system (Milford, MA, USA) equipped with a 2998 photo-diode array detector (Chelmsford, MA, USA), and ACE C18 column (4.6 mm × 250 mm, 5 μm particle size) (Milford, MA, USA). Data acquisition was accomplished using Empower PRO (Waters Associates, Milford, MA, USA).

2.2. Preparation of solutions

The Na_2-TP at 10 mM concentration was prepared by dissolving 0.105 g in distilled water (DH_2O) and diluting to 50 mL. Fe(II) at 20 mM concentration was prepared by dissolving 0.1988 g $FeCl_2 \cdot 4H_2O$ with 2 mL of 1 M HCl, and diluting to 50 mL with DH_2O. Na_2-EDTA at 20 mM concentration was prepared by dissolving 0.372 g of the salt in DH_2O and diluting to 50 mL. Hydrogen peroxide at 10 mM concentration was prepared from a 0.5 M intermediary stock solution, the latter being prepared from 35% H_2O_2 and standardized with permanganate. The NaH_2PO_4–Na_2HPO_4 buffer solution (pH 7.4) at 200 mM was prepared in DH_2O. Copper(II) at 1.0×10^{-2} M was prepared by dissolving 0.4262 g of $CuCl_2 \cdot 2H_2O$, and diluting to 250 mL. Ammonium acetate (NH_4Ac: 1.0 M) aqueous solution contained 19.27 g of the salt in 250 mL. Nc at 7.5×10^{-3} M was prepared by dissolving 0.078 g of the free base in EtOH, and diluting to 50 mL with EtOH (prepared fresh). Trichloroacetic acid at 2.8% (w/v) was prepared in DH_2O, and TBA at 1% (w/v) in 50 mM aqueous NaOH. Deoxyribose at 10 mM, and EDTA at 1 mM were prepared in DH_2O. $FeCl_3$ at 0.5 mM concentration was prepared by dissolving 0.0135 g $FeCl_3 \cdot 6\ H_2O$ with 2 mL of 1 M HCl, and diluting to 50 mL with DH_2O. Ascorbic acid, 1.0 mM, and KH_2PO_4–KOH buffer solution (pH 7.4) at 100 mM (total orthophosphate) were prepared in DH_2O. All scavengers were freshly prepared in DH_2O at various concentrations.

2.3. 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 cycle), and fed by standart 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, centrifuged (4000 rpm, 5 min), and filtered through a 0.45 μm membrane filter (Chromofil, Germany) before analysis.

2.4. General procedures

2.4.1. Solution-based CUPRAC method for HRS activity measurement

To a test tube were added 1.5 mL of phosphate buffer (pH 7.4), 0.5 mL of 10 mM TP (probe material), 0.25 mL of 20 mM Na_2-EDTA, 0.25 mL of 20 mM $FeCl_2$ solution, $(2.0-x)$ (mL) H_2O, x (mL) scavenger sample solution (x varying between 0.1 and 2.0 mL) at various concentrations, and 0.5 mL of 10 mM H_2O_2 rapidly in this order. The mixture in a total volume of 5 mL was incubated for 30 min in a water bath kept at 37 °C. At the end of this period, the reaction was stopped by adding 0.5 mL of 1.0 M HCl solution and 5 mL EtAc vortexed for 30 s. To 1.0 mL of the EtAc extract, the solution-based CUPRAC method [18] was applied in the following manner:

$$1.0\ \text{mL Cu(II)} + 1.0\ \text{mL Nc} + 1.0\ \text{mL } NH_4\text{Ac buffer} + 1.0\ \text{mL EtAc extract} + 0.5\ \text{mL EtOH} \xrightarrow{A_{450}}$$

The absorbance at 450 nm of the final solution at 4.5 mL total volume was recorded 30 min later against a reagent blank.

The second-order rate constants of the scavengers were determined with the use of competition kinetics by means of a linear plot of A_0/A as a function of $C_{scavenger}/C_{probe}$, where A_0 and A are the CUPRAC absorbances of the system in the absence and presence of scavenger, respectively, and C is the molar concentration of relevant species [19]. The second-order rate constants found with the modified CUPRAC procedure [19] were then compared with those found with optical sensor-based CUPRAC and TBARS methods [23].

The inhibition ratio of scavengers and biological samples (%) was calculated using the following formula:

$$\text{Inhibition ratio } (\%) = 100\left[(A_0 - A)/A_0\right] \quad (1)$$

2.4.2. Optical sensor-based CUPRAC method for HRS activity measurement

The commercial Nafion membrane was sliced into 4.5 × 0.5 cm pieces, immersed into a tube containing 2.0 mL of 1.0×10^{-2} M Cu(II) + 2.0 mL of 7.5×10^{-3} M Nc + 2.0 mL of 1 M NH_4Ac + 2.2 mL of H_2O, and agitated for 30 min in a rotator. The reagent-impregnated membrane was taken out and immersed in a tube containing 2.0 mL of EtAc extract + 6.2 mL of EtOH. The tube was placed in a rotator and agitated for 30 min so as to enable color development. The colored membrane was taken out and placed in a 1 mm optical cuvette containing H_2O (to prevent sticking of slices to the walls of the cuvette), and its absorbance at 450 nm was read against a blank membrane prepared under identical conditions excluding analyte.

2.4.3. Thiobarbituric acid reactive substances (TBARS) method

The colorimetric TBARS method – widely used method for assessment of oxidative damage and HRS activity measurement – was applied as the reference method of comparison for determining the HRS activity of scavengers and biological samples [23,24]. The reacting mixture for this assay contained in a final volume of 1.0 mL the following reagents: 200 μL KH_2PO_4–KOH (100 mM), 200 μL deoxyribose (15 mM), 200 μL $FeCl_3$ (500 μM), 100 μL EDTA (1 mM), 100 μL ascorbic acid (1 mM), 100 μL H_2O_2 (10 mM) and 100 μL sample. Reaction mixtures were incubated at 37 °C for 1 h. At the end of the incubation period, 1.0 mL of 1%(w/v) TBA was added to each mixture followed by the addition of 1.0 mL of 2.8% (w/v) TCA. The solutions were heated on a water bath at 80 °C for 10 min to develop the pink colored malondialdehyde-thiobarbituric acid: MDA-$(TBA)_2$ adduct, and the absorbance of the resulting solution (total volume = 3.0 mL) was measured at 532 nm [23]:

$$1.0\ \text{mL TBA} + 1.0\ \text{mL TCA} + 1.0\ \text{mL incubation solution} \xrightarrow{A_{532}}$$

2.4.4. HPLC method

Samples containing TP were assayed in the absence and presence of $^{\bullet}$OH-scavenger with HLPC using UV detection. A

2.0 mL aliquot of EtAc extract was freeze-dried for 10 min. The remaining residues were dissolved with 2.0 mL of 1:1 (v/v) EtOH-H_2O mixture. The mobile phase consisted of two solvents, i.e., 100 mmol L^{-1} potassium phosphate solution including 2% KCl (pH 4.37) (A) and acetonitrile (B). The following parameters and gradient elution program were used for the analysis of EtAc extracts: (V_{sample}=20 μL; Flow rate=0.8 mL min^{-1}; λ_{TP}=254 nm): 1 min 100% *A* (slope 1.0); 8 min 90% *A*–10% B (slope 1.0); 13. min 80% *A*–20% B (slope 1.0). The capability of •OH scavenging was calculated using a modified version of Eq. (1):

$$\text{Inhibition ratio } (\%) = 100\left[(A_2 - A_1)/(A_0 - A_1)\right] \quad (2)$$

where A_1 and A_2 are the peak areas of the TP probe in the absence and presence of HR-scavenger, respectively, and A_0 is the peak area of the TP probe ($A_0 = 1.5 \times 10^7$) at initial concentration in the reaction mixture.

2.5. 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) [25].

3. Results and discussion

3.1. Optimization of the optical sensor-based CUPRAC method

An optical sensor-based CUPRAC method was applied to assess the HRS activities of amino acids (i.e., L-serine, L-threonine, L-leucine, L-proline, 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., uric acid, albumin). CUPRAC absorbance arises from the reduction of the Cu(II)-Nc reagent to the Cu(I)-chelate [18] by the hydroxylated product hTP (i.e., $hTP + 2 \cdot Cu(Nc)_2^{2+} \rightarrow$ oxidized-$hTP + 2 \cdot Cu(Nc)_2^{+} + 2H^{+}$), while the TP probe had a negligible original CUPRAC absorbance. A scheme of competitive reactions between the TP probe and cysteine as a representative •OH scavenger is shown in Fig. 1. TP is a known probe for •OH that produces a single hydroxylated product hTP. TP is a rare example of a molecule that, because of its symmetric configuration, will not result in differently hydroxylated stereoisomers. Hydroxyl radical attack at any of the four unsubstituted ring carbons in TP probe yields the same hydroxylation product, as opposed to the situation in benzoic acid probe where the attack of •OH may occur at *ortho*-, *meta*-, or *para*-positions [26].

The reproducibility and stability of the CUPRAC sensor were investigated. The inter-assay coefficient of variation (CV) for the HRS acitivity measurements of 1.0 mM GSH using five independently prepared CUPRAC sensors was 2.21%. The intra-assay CV for five repeated determinations of 1.0 mM GSH using CUPRAC sensors was 3.34%. The optical activity of the CUPRAC sensor remained 97% after 15 days of storage in distilled water. These results indicate that the CUPRAC sensor has good reproducibility and stability.

The effects of {Fe(II)-EDTA+H_2O_2} mixture on the CUPRAC absorbance of the TP probe (in the absence and presence of scavenger, GSH) were studied under the same experimental conditions, where neither the mixture constituents nor the scavenger compound in the EtAc phase gave a 450 nm absorbance. In other words, the only species giving rise to an absorbance in the system is the hydroxylation product of TP (Fig. 2). {Fe(II)-EDTA+H_2O_2} mixture can produce •OH, and these radicals are manifested in the increase of absorbance of the CUPRAC chromophore (A_{450}=0.876) as a result of TP oxidation with •OH to hTP, whereas the absorbance due to hTP decreases upon competition with GSH (A_{450}=0.672). The inhibition ratio (%) leading to HRS activity estimation is calculated from the relative decrease of CUPRAC absorbances of the EtAc extracts using Eq. (1). The competition with GSH of the TP probe for •OH can be followed simply by observing the changes in the concentration of the EtAc-extracted hTP without interference from water-soluble •OH-scavengers and other system constituents (i.e., Fe(II)-EDTA and H_2O_2).

Conversion of the TP probe to hTP and inhibition of this reaction with a scavenger (i.e., GSH) were followed by measuring the CUPRAC absorbance of the mixture as a function of time (Fig. 3). The optimal measurement time of 30 min was chosen. This optimal period is sufficient to achieve a stable absorbance difference between TP and its conversion product. Thus, due to the high conversion yield of the selected probe, HRS activity of the studied compounds could be rapidly and precisely determined by recording the relative absorbances within 30 min.

3.2. Comparison of the optical sensor-based CUPRAC and HPLC findings

Hydroxyl radical attack on TP produces the characteristic hydroxylation product, hTP, as CUPRAC reactive species (CUPRAC-rs). There is some change in product ratio when the 30 min assay is

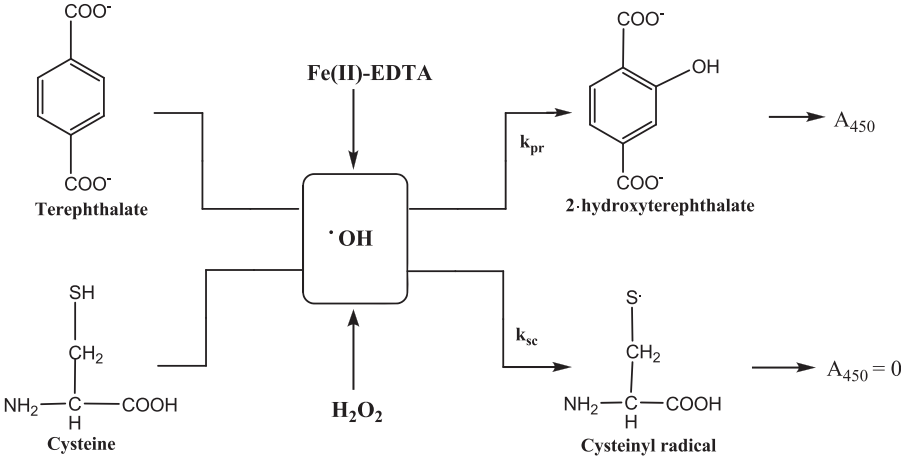

Fig. 1. Schematic representation of the competition between a scavenger compound (e.g., cysteine) and TP probe for •OH radicals.

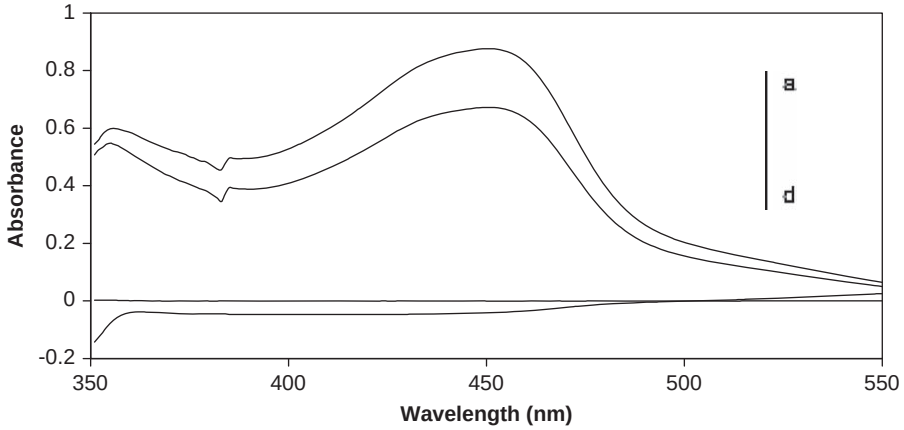

Fig. 2. Visible spectra of Cu(I)-Nc chelate in EtAc phase, produced as a result of CUPRAC reaction with (a) Fe(II)-EDTA+TP+H_2O_2 (b) Fe(II)-EDTA+TP+10^{-3} M GSH+H_2O_2 (c) Fe(II)-EDTA+10^{-3} M GSH+H_2O_2 (d) TP probe (all mixtures were incubated in phosphate-buffered medium).

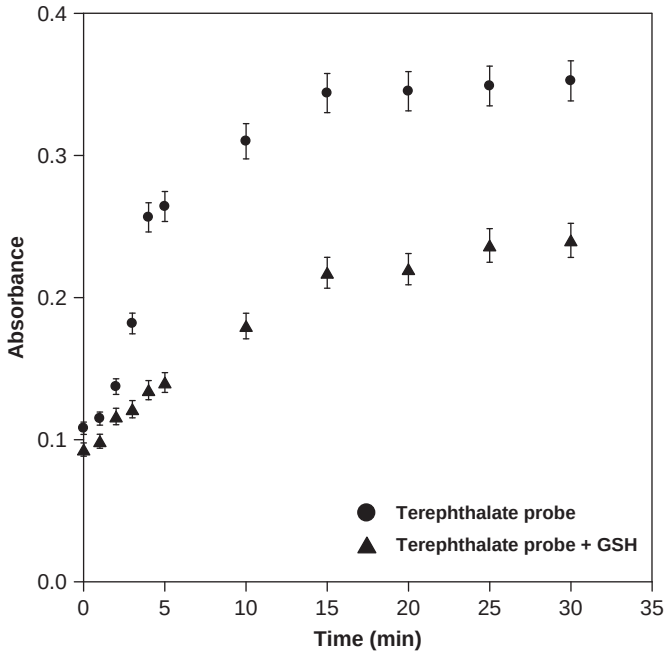

Fig. 3. CUPRAC absorbance *versus* incubation time curves in the presence (GSH) and absence (reference) of a •OH scavenger.

adopted. The required chromatograms for TP conversion upon •OH attack – in the absence and presence of the •OH scavenger – for the developed assay are provided in Fig. 4A and B. The product ratio largely depends upon the type of assay used for •OH generation, presence of other oxidants, and time [27,28]. The amounts of remaining TP in Fenton mixtures were found with the aid of calibration curves drawn as peak areas *versus* concentration in the absence and presence of scavengers. For example, approximately 47% of TP was converted into hTP in the absence of scavengers (Fig. 4A). In the presence of a potent scavenger such as GSH (Fig. 4B), the conversion ratio of TP was considerably smaller (11.6%), as is apparent from the significant lowering of the peak heights of the hydroxylation product hTP, e.g., the peak area for unchanged TP as 8.20×10^6 (reference) (Fig. 4A) increased to 1.34×10^7 with GSH (Fig. 4B). The percentage inhibition values of 2.0 mL GSH (1.0 mM) with respect to the optical sensor-based CUPRAC and HPLC methods were 48.02 ± 0.34, 49.57 ± 0.68, respectively.

The HRS activity of GSH (as inhibition %) was calculated with the proposed method using Eqs. (1) and (2), as well as with HPLC method. CUPRAC and HPLC assay results agreed among themselves with tolerable error; IC_{50} values of GSH with respect to the optical sensor-based CUPRAC and HPLC methods were 0.42 ± 0.01 and 0.41 ± 0.02 mM, respectively (Table 1).

3.3. Comparison of the optical sensor-based CUPRAC and TBARS methods for HRS activity assay

A rate constant for the reaction of the scavenger with •OH can be deduced from the inhibition of color formation on the CUPRAC sensor membrane due to decreased hydroxylation of the probe. In agreement with the findings of Halliwell et al. [24], the rate constant for the reaction of scavengers with •OH can be determined with the use of the following equation:

$$\frac{1}{A} = \frac{1}{A_0}\left(1 + \frac{k_{sc}[sc]}{k_{pr}[pr]}\right) \quad (3)$$

where A is the absorbance in the presence of •OH-scavengers (sc) at concentration [sc] and A_0 the absorbance in the absence of a scavenger; (k_{sc}) and (k_{pr}) are the rate constants of reactions in the presence or absence of scavenger, respectively, and [pr] is the concentration of terephthalate probe used in the experiment. The second-order rate constants were obtained with the aid of competition kinetic method using a TP probe of which the hydroxylation product (hTP) can be monitored at λ_{max}: 450 nm.

The requirement of linearity with respect to Eq. (3) does not exist for the relationship between percentage inhibition and scavenger concentration. In almost all curvi-linear calibrations of inhibition *versus* concentration, there is a saturation of the analytical response with respect to concentration at sufficiently high concentrations. In studying the •OH scavenging activity of either flavonoids [29] or plant extracts [30], there is a plateau region of scavenging percentage as a function of concentration. Likewise, mannitol was reported to inhibit tyrosine formation from phenylalanine in a Fenton reaction, where scavenging of •OH by mannitol reached saturation at high concentrations [31]. This probably arises from the fact that above a limiting concentration, scavenger antioxidants may not effectively compete with the probe for •OH absorption due to some self-quenching side-reactions [32].

In both the developed and reference assays, competition kinetics involving a probe and a scavenger for •OH reaction using various concentrations of the reactants should yield a straight line when A_0/A is plotted as a function of $C_{scavenger}/C_{probe}$ (Fig. 5), where the slope would yield the ratio of the associated rate constants (the means for $N=4$ or 5 data), and the intercept would be roughly equal to one. The calculated rate constants of scavengers were proportional to the slopes of these lines, and the

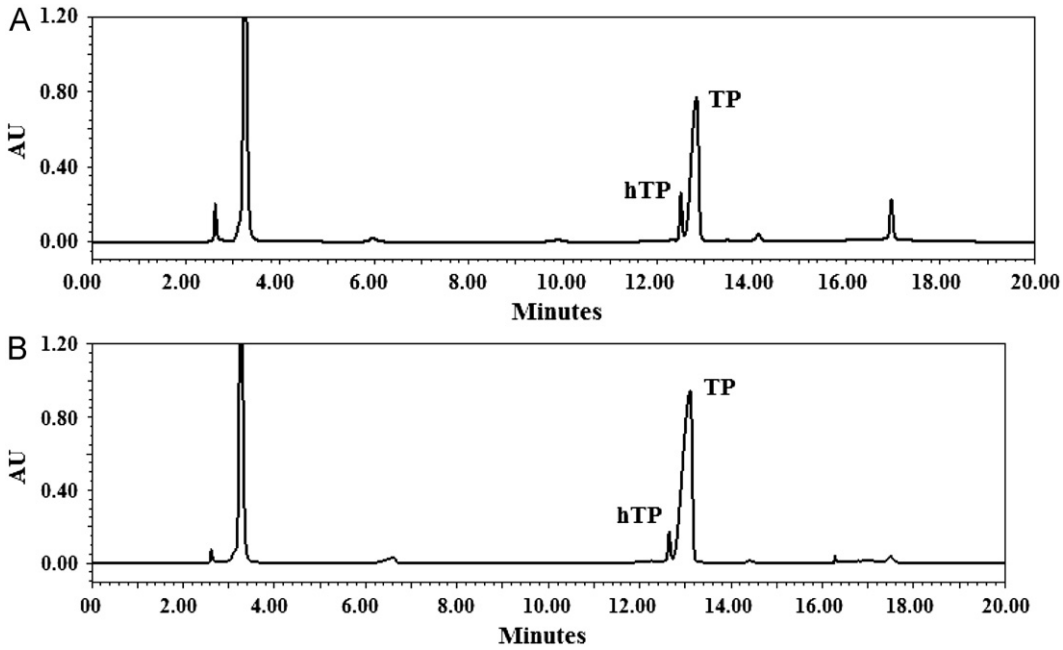

Fig. 4. The HPLC chromatogram for TP and its hydroxylation product (hTP) (A) in the absence of hydroxyl radical scavenger (reference); (B) in the presence of GSH sample (2.0 mL of 1.0 mM solution was taken).

Table 1
The variation of HRS activity of scavenger (GSH) with concentration, as measured with the proposed method in comparison with HPLC ($N=4$ or 5 data points).

Scavenger (GSH) concentration (mM)	Inhibition % with respect to optical sensor-based CUPRAC method	Inhibition % with respect to HPLC method
0.1	19.37	18.40
0.2	27.26	25.89
0.4	48.06	49.57
0.5	57.38	59.54

($P=0.05$, $F_{exp}=0.14$, $F_{crit\ (table)}=10.13$, $F_{exp}<F_{crit\ (table)}$) (Optical sensor-based CUPRAC *versus* HPLC method).

precision of data was reflected in the linear correlation coefficients. The HRS rate constant of TP is known from the literature that is $k=3.3\times10^9\ M^{-1}\ s^{-1}$ [26], generally in agreement with these values. Therefore, competition of the detector molecule with biomolecules existing under physiological conditions is expected to be efficient [26]. The HRS rate constant for 2-deoxy-D-ribose using the TBARS method was reported in the literature as $3.1\times10^9\ M^{-1}\ s^{-1}$ [33].

The calculated second-order rate constants for $^{\bullet}OH$ scavenger compounds in accordance with Eq. (3) are given in Table 2 for the solution- and optical sensor-based CUPRAC methods using a TP probe in comparison to those found with the TBARS method using a deoxyribose probe [24]. The proposed sensor-based method gave comparable rate constants (i.e., of the same order of magnitude) with those found by the TBARS method. In accordance with the results of the proposed method but not of TBARS (Table 2), the classic antioxidant uric acid was reported to exhibit a limited scavenging capacity toward hydroxyl radicals, suggesting relatively lower protection (than estimated by TBARS) afforded in the cellular environment against the potential toxicity of this reactive species [4].

Thiol compounds could be efficiently oxidized by $^{\bullet}OH$ with high rate constants between $10^9-3\times10^{10}\ M^{-1}\ s^{-1}$ under biologically relevant conditions. The sulfhydryl group was responsible for this quenching effect according to the following reaction [34]:

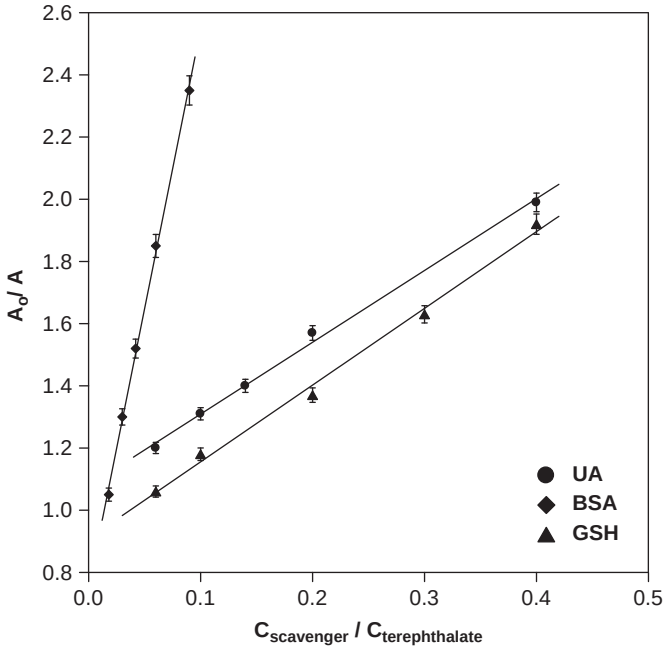

Fig. 5. Kinetic plots of $^{\bullet}OH$ radical scavenging action of certain compounds with respect to competitive kinetic equation using the optical sensor-based CUPRAC method together with the TP probe.

$$RSH + {}^{\bullet}OH \rightarrow RS^{\bullet} + H_2O$$

$$RS^{\bullet} + RS^{\bullet} \rightarrow RS - RS$$

Reduced glutathione represents the most abundant intracellular thiol ($\approx$ 1–10 mM), where it functions as an important non-enzymatic antioxidant toward reactive oxygen species [35]. Antioxidant action of glutathione is based primarily on its reaction with ROS at the SH and –S–S groups, stepwise producing $GS^{\bullet}$ and GSSG [36].

Table 2
The •OH scavenging activity of various scavengers using the optical sensor-based and solution-based modified CUPRAC methods in comparison with the TBARS assay (k_{sc} with respect to Eq. (3), $N=4$ or 5 data points).

•OH scavengers	k_{sc}—optical sensor-based CUPRAC ($M^{-1}s^{-1}$)	k_{sc}—solution-based CUPRAC ($M^{-1}s^{-1}$)	k_{sc}—TBARS ($M^{-1}s^{-1}$)
Thiol-type antioxidants			
Glutathione	7.85×10^{9}	8.61×10^{9}	6.14×10^{9}
Glutathione oxidized	4.19×10^{9}	4.29×10^{9}	8.37×10^{9}
Cysteine	1.02×10^{9}	9.90×10^{8}	7.75×10^{8}
Cystine	3.74×10^{9}	3.86×10^{9}	6.48×10^{9}
N-acetyl cysteine	1.60×10^{10}	1.09×10^{10}	4.19×10^{9}
Homocysteine	1.06×10^{9}	1.08×10^{9}	2.85×10^{9}
Homocystine	4.65×10^{9}	4.81×10^{9}	8.62×10^{9}
Methionine	2.21×10^{9}	2.15×10^{9}	8.68×10^{8}
Amino acids			
Threonine	1.68×10^{8}	1.77×10^{8}	1.05×10^{9}
Proline	6.66×10^{8}	6.73×10^{8}	1.36×10^{9}
Arginine	1.15×10^{9}	1.27×10^{9}	1.52×10^{9}
Lysine	1.95×10^{9}	1.72×10^{9}	2.54×10^{9}
Leucine	1.19×10^{9}	1.29×10^{9}	6.23×10^{9}
Tryptophan	4.32×10^{9}	3.80×10^{9}	5.21×10^{9}
Valine	1.29×10^{9}	1.42×10^{9}	3.60×10^{9}
Serine	1.65×10^{8}	1.65×10^{8}	N.D.[a]
Plasma antioxidants			
Uric acid	7.62×10^{9}	7.33×10^{9}	1.01×10^{10}
Albumin	6.03×10^{10}	5.99×10^{10}	N.D.[a]

($P=0.05$, $F_{exp}=1.109$, $F_{crit\ (table)}=4.451$, $F_{exp}<F_{\text{crit }(table)}$) (Optical sensor-based CUPRAC *versus* solution-based CUPRAC).
($P=0.05$, $F_{exp}=4.303$, $F_{crit\ (table)}=4.543$, $F_{exp}<F_{\text{crit }(table)}$) (Optical sensor-based CUPRAC *versus* TBARS).

[a] N.D.: HRS activity at the studied concentration level could not be detected.

Although the glutathione radical (GS•) formed from the oxidation of GSH is a pro-oxidant radical, GS• can react with another GS• to yield oxidized-GSH (GSSG), which is then reduced to GSH by the NADPH-dependent GSH reductase [37]. GSSG scavenging of •OH is also possible according to the reaction (GSSG + •OH → GSOH + GS•) [38]. The second order rate constant of GSH was reported [38] as $8.0\times10^{9}\ M^{-1}\ s^{-1}$ which is compatible with the values found with the solution- and sensor-based CUPRAC methods (Table 2). The competition with GSH of the TP probe for •OH can also be followed by observing the changes in the concentration of the EtAc-extracted hTP product without interference from water-soluble scavengers and other system constituents (i.e., iron-EDTA, H_2O_2). If IC_{50} is defined as '50% inhibitive concentration' of the tested antioxidant with respect to hTP production (according to Eq. (1)), the HRS activities of GSH (as IC_{50}) calculated with fluorometric, optical sensor-based CUPRAC and solution-based CUPRAC methods were 3.48×10^{-4}, 4.20×10^{-4}, and 3.93×10^{-4} M, respectively.

NAC, a widely known antioxidant *in vivo* and *in vitro*, reacts with •OH with a rate constant of $1.36\times10^{10}\ M^{-1}\ s^{-1}$, as determined by pulse radiolysis [39]. The second-order rate constants of NAC for •OH scavenging were calculated by sensor- and solution-based CUPRAC methods as 1.6×10^{10} and $1.09\times10^{10}\ M^{-1}\ s^{-1}$, respectively. These values are compatible with those of the pulse hydrolysis study whereas the TBARS value ($4.19\times10^{9}\ M^{-1}\ s^{-1}$) is of lower order of magnitude.

The aliphatic C–H bonds of amino acids react with •OH by H atom abstraction [40]. As might be expected by analogy with other aromatic and heterocyclic compounds (e.g., tryptophan), •OH reacts predominantly by addition to the ring of such amino acids [41]. Tryptophan bearing an indole ring showed the highest HRS activity among the tested amino acids with the exception of sulfur-containing compounds, yielding rate constants of 4.32×10^{9} and $3.80\times10^{9}\ M^{-1}\ s^{-1}$ calculated by sensor- and solution-based CUPRAC methods, respectively. The absolute reactivities of •OH with amino acids vary considerably with the structure of the compound and with pH. The simple aliphatic amino acids appear to be the least reactive although when –OH groups or branched chains are present in the molecules, inductive effects tend to increase the reactivity. Serine has the lowest reaction rate constant ($3.2\times10^{8}\ M^{-1}\ s^{-1}$) in literature, in accordance with the findings of the optical sensor-based CUPRAC method (Table 2), because of its simple molecular structure. The known rate constants from the literature for proline, threonine, lysine, valine, leucine, and arginine are 2.9×10^{8}, 3.6×10^{8}, 6.0×10^{8}, 6.6×10^{8}, 1.6×10^{8}, and $3.5\times10^{9}\ M^{-1}\ s^{-1}$, respectively [41]. The calculated rate constants with CUPRAC and TBARS methods for these aliphatic amino acids are compatible with the above values. The HRS rate constant of albumin according to optical sensor- and solution-based CUPRAC methods calculated from Eq. (3) gave the values of 6.03×10^{10} and $5.99\times10^{10}\ M^{-1}\ s^{-1}$, respectively, which are very close to the range of values tabulated for albumins, i.e., 1.0×10^{10}–$5.0\times10^{11}\ M^{-1}\ s^{-1}$ [42].

The two-way ANalysis Of VAriance (ANOVA) comparison with the aid of *F*-test of the mean-squares of 'between-treatments' (i.e., IC_{50} values of different samples with respect to the optical sensor-based CUPRAC and TBARS methods depicted in Table 2) and of residuals [25] for a number of real samples (consisting of sixteen •OH-scavengers) enabled to conclude that there was no significant difference between treatments. In other words, the experimentally found optical sensor-based CUPRAC results and TBARS results were statistically alike at 95% confidence level ($F_{exp}=4.303$, $F_{crit}=4.543$, $F_{exp}<F_{crit}$ at $P=0.05$). Seemingly, the findings of optical sensor-based CUPRAC and solution based-CUPRAC methods (for eighteen •OH-scavengers) agreed among themselves with tolerable error (Table 2) ($F_{exp}=1.109$, $F_{crit}=4.451$, $F_{exp}<F_{crit}$ at $P=0.05$). Thus, the proposed methodology was validated against the classical TBARS method of wide use.

3.4. Application of the CUPRAC sensor to certain tissue homogenates

The CUPRAC sensor was applied to tissue homogenates, and the results were compared with those obtained by TBARS method as % inhibition values in the Fenton reaction mixture. The liver homogenates were generally shown to exhibit the highest HRS activity. The inhibition percentages measured with the proposed CUPRAC and reference methods are comparatively depicted in a bar diagram (Fig. 6); the percentage inhibitions of identical tissue homogenates found with optical sensor-based CUPRAC method were very close to those measured with the TBARS method. The HRS activities in rat liver, kidney and heart tissues were reported as 63.8%, 68.4%, and 57.8% inhibitions, respectively [43]. Since catalase catalyzes the decomposition of H_2O_2 to water and oxygen [44], catalase in tissue homogenate is expected to indirectly prevent •OH generation in the Fenton reaction system incorporating H_2O_2. Therefore, it may be anticipated that higher catalase activity should result in stronger HRS activity. The relative HRS activities of liver and tissue homogenates shown in Fig. 6 support the findings of Casalino et al. [45] who reported that catalase activities in rat liver tissue and kidney homogenates were 383 ± 39 and 158.2 ± 16.1 units per mg protein, respectively.

4. Conclusions

Currently, there is no colorimetric hydroxyl radical sensor in either the market or literature sources of analytical sciences capable of selectively working in a reasonable concentration range with good precision and accuracy, and this work aims to

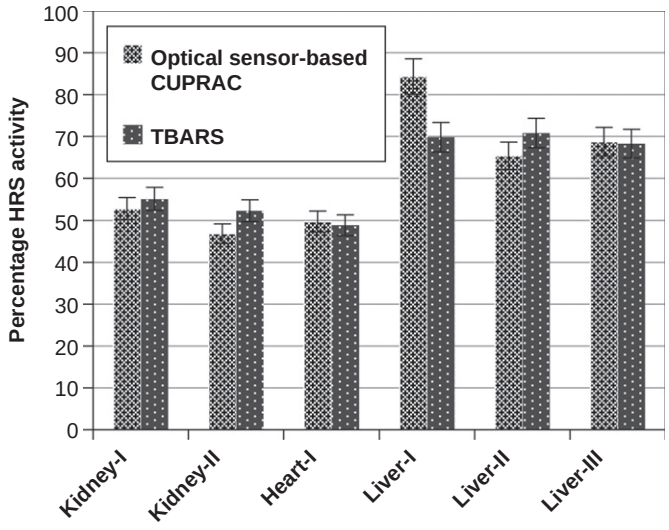

Fig. 6. The HRS activity (%) of some tissue homogenates calculated with the optical sensor-based CUPRAC method in comparison to TBARS assay. Data are presented as (mean ± SD) (error bars), $N=3$. ($P=0.05$, $F_{exp}=0.021$, $F_{crit\ (table)}=6.608$, $F_{exp} < F_{crit\ (table)}$).

fill such a literature gap. Since a single and stable hydroxylated product emerges from $^{\bullet}$OH oxidation of terephthalate due to the symmetry of the probe molecule, possible measurement errors of selected species among a mixture of isomeric products are avoided, and mechanistic interpretation of the hydroxylation reaction is facilitated. The probe is selective to $^{\bullet}$OH, because it has no significant reactivity toward $O_2^{\bullet-}$, ROO$^{\bullet}$, H_2O_2, and singlet oxygen. This work combines the idea of sensitive and selective detection of $^{\bullet}$OH with novel colorimetric sensor design based on CUPRAC measurement widely used in antioxidant research, and demonstrates that the CUPRAC sensor can be used for the determination of $^{\bullet}$OH levels, through which one can evaluate the HRS activities of various antioxidant reagents. The CUPRAC sensor results were close or comparable to those found by the conventional TBARS and HPLC methods. This study extended the use of the CUPRAC sensor into a new field for the detection of biologically important short-lived species such as $^{\bullet}$OH without a necessity for the more sophisticated and high-cost ESR and MS techniques. This alternative cost-effective method emerges as a promising tool to better understand the role of $^{\bullet}$OH in biological chemistry. The proposed methodology is expected to be of further use in measuring hydroxyl radical production in test animals after ischemia-reperfusion sequence, and in research attempting to detect and measure $^{\bullet}$OH generation in oxidative diseases such as diabetes, myocardial infarction, rheumatoid arthritis, cancer, and ageing [8].

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