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Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: The CUPRAC method

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Abstract

Background: Tests measuring the combined antioxidant effect of the nonenzymatic defenses in biological fluids may be useful in providing an index of the organism's capability to counteract reactive species known as prooxidants, resist oxidative damage and combat oxidative stress-related diseases. The selected chromogenic redox reagent for the assay of human serum should be easily accessible, stable, selective, respond to all types of biologically important antioxidants such as ascorbic acid, α-tocopherol, β-carotene, reduced glutathione (GSH), uric acid and bilirubin, regardless of chemical type or hydrophilicity. Currently, there is no rapid method for total antioxidant assay of human serum meeting the above criteria.

Methods: Our recently developed cupric reducing antioxidant capacity (CUPRAC) spectrophotometric method for a number of polyphenols and flavonoids using the copper(II)-neocuproine reagent in ammonium acetate buffer was now applied to a complete series of plasma antioxidants for the assay of total antioxidant capacity (TAC) of serum, and the resulting absorbance at 450 nm was recorded either directly (e.g. for ascorbic acid, α-tocopherol and glutathione) or after incubation at 50°C for 20 min (e.g. for uric acid, bilirubin and albumin), quantitation being made by means of a calibration curve. The lipophilic antioxidants, α-tocopherol and β-carotene, were assayed in dichloromethane (DCM). Lipophilic antioxidants of serum were extracted with n-hexane from an ethanolic solution of serum subjected to centrifugation. Hydrophilic antioxidants of serum were assayed after perchloric acid precipitation of proteins in the centrifugate.

Results: The molar absorptivities, linear ranges and trolox equivalent antioxidant capacity (TEAC) coefficients of the serum antioxidants were established with respect to the CUPRAC spectrophotometric method, and the results (TEAC, or TEAC coefficients) were evaluated in comparison to the findings of the ABTS/TEAC reference method using persulfate as oxidant. As for hydrophilic phase, a linear correlation existed between the CUPRAC and ABTS findings ($r = 0.58$), contrary to current literature reporting that either serum ORAC or serum ferric reducing antioxidant potency (FRAP) does not correlate at all with serum TEAC. The analytical responses of serum antioxidants were shown to be additive, enabling a TAC assay. The intra- and inter-assay CVs were 0.7 and 1.5%, respectively, for serum.

Conclusions: The CUPRAC assay proved to be efficient for glutathione and thiol-type antioxidants, for which the FRAP test was nonresponsive. The findings of CUPRAC completely agreed with those of ABTS-persulfate for lipophilic phase. The additivity of absorbances of all the tested antioxidants confirmed that antioxidants in the CUPRAC test did not chemically interact among each other so as to cause an intensification or quenching of the theoretically expected absorbance. As a distinct advantage over other electron-transfer based assays (e.g. Folin, FRAP, ABTS, DPPH), CUPRAC is superior in regard to its realistic pH close to the physiological pH, favourable redox potential, accessibility and stability of reagents and applicability to lipophilic antioxidants as well as hydrophilic ones.

Keywords: *CUPRAC antioxidant capacity, human serum, plasma antioxidants, ABTS assay, uric acid, bilirubin*

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Introduction

When natural defenses of the organism (of enzymatic, non-enzymatic or dietary origin) are overwhelmed by an excessive generation of reactive oxygen species, a situation of oxidative stress occurs, in which cellular and extracellular macromolecules (proteins, lipids and nucleic acids) can suffer oxidative damage, causing tissue injury [1,2]. Living organisms have developed complex antioxidant systems to counteract reactive species and to reduce their oxidative damage [3]. These antioxidant systems include enzymes such as superoxide dismutase, catalase and glutathione peroxidase [4]; macromolecules such as albumin, ceruloplasmin and ferritin; and an array of small molecules, including ascorbic acid, α-tocopherol, β-carotene, ubiquinol-10, glutathione (GSH), methionine, uric acid and bilirubin [5]. Several methods have been developed to measure the TAC of biological fluids such as human serum or plasma [6–13], and these have been discussed in relevant reviews [14,15]. Antioxidant activity assay methods existing in literature based on the measurement of radical scavenging activity of antioxidant compounds suffer from the difficulties encountered in the formation and stability of colored radicals [16] such as ABTS (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)) [9] and DPPH (2,2′-diphenyl-1-picrylhydrazyl) [17]. Re et al. developed an improved ABTS radical cation decolorization assay using persulfate as the oxidant, and thereby compensated for the weaknesses of the original ferryl myoglobulin/ABTS assay [18]. The total radical trapping parameter (TRAP) assay of Wayner et al. [6] was the most widely used method of measuring total antioxidant capacity (TAC) of plasma or serum during the last decade. However, it suffered from the major drawback of oxygen electrode end-point in that the electrode would not maintain its stability over the required time period [19]. Antioxidant assays based on spectrophotometric methods of thiobarbituric acid-reactive substances (TBARS) formation have poor reproducibility due to instability of substrates used for lipid peroxidation [20]. The inhibition of accumulation of colored radical reagents in the presence of antioxidants is expressed in the units of "lag time" (i.e. the time period required for the colored radical to emerge in the reaction medium), constituting a rather unobjective approach for antioxidant assay, because "lag time" is not always linearly correlated to antioxidant concentration. The major limitation of the $ORAC_{PE}$ (ORAC test based on B-phycoerythrin: B-PE) has been reported to be the use of B-PE as the fluorescent probe, in that B-PE produces inconsistency from lot to lot, resulting in variable reactivity to peroxyl radical, and additionally, B-PE is not photostable and can be bleached after extended exposure to excitation radiation. However, the alternative fluorescent probe, fluorescein, developed to overcome the drawbacks of B-PE, reports extremely high ORAC values (as trolox equivalents) for a number of antioxidant compounds that are quite inconsistent with those of conventional assays [21]. On the other hand, the ferric reducing antioxidant potency (FRAP) assay of antioxidants [13], which is based on ferric-to-ferrous reduction in the presence of a Fe(II)-stabilizing ligand such as tripyridyltriazine (TPTZ), is both unrealistic (i.e. the colored complex is formed at a definitely acidic pH such as pH = 3.6, much lower than the physiological pH) and insufficiently reactive to thiol-type (i.e. —SH containing) antioxidants like cysteine and glutathione [22].

The range of tests used for antioxidant activity measurement is a testimony to the uncertainty surrounding the chemistry of antioxidant compounds. Thus for example, in tests where free radical oxidation is induced by a metal ion like Cu(II) or Fe(III), it is uncertain whether the test measures the ability of the antioxidant to interact with a free radical or its ability to bind the metal ion [23]. Current literature taking a philosophical look at antioxidant indexes clearly states that there is no "total antioxidant" as a nutritional index available for food labeling because of the lack of standard quantitation methods [24]. As a result, the antioxidant activities of common vegetables (total sample size: 927) collected from the US market, analyzed using the ORAC and FRAP procedures, did not correlate well [24]. Exactly, a similar situation exists for human plasma or serum where different tests yield different results that do not correlate well. For example, Cao and Prior observed a weak linear correlation between serum ORAC and serum FRAP, but no correlation either between serum ORAC and serum TEAC, or between serum FRAP and serum TEAC [3]. Total antioxidant capacity assays measure the capacity of biological samples only under defined conditions prescribed by the given method using different oxidants in each case. If the standard potential of the oxidant is too high (e.g. the potential of the ferric–ferrous couple is 0.77 V, that may significantly increase in the presence of ferrous-stabilizing ligands such as TPTZ or phenanthroline), then compounds other than the plasma antioxidants of interest, like glucose or citrate, the latter being used to preserve the plasma, may also be oxidized causing positive error. Some methods measure only the hydrophilic antioxidants, without caring for the lipophilic ones. Not all methods measure protein-thiols, or smaller molecule —SH compounds of different origin (such as GSH, with FRAP). To briefly summarize the current situation, there is no single, widely-acceptable assay method applicable to a reasonable variety of compounds in plasma and food matrices. Thus the aim of this work is to develop a simple, widely applicable antioxidant capacity index for human serum, as successively performed

previously for dietary polyphenols, vitamins C and E [25], utilizing the copper(II)–neocuproine (Cu(II)–Nc) reagent as the chromogenic oxidizing agent. Since copper(II) (or cupric) ion reducing ability is measured, the method is named by our research group as cupric reducing antioxidant capacity (CUPRAC) method. This method should be advantageous over FRAP since the redox chemistry of copper(II)—as opposed to that of chemically inert high-spin ferric ion having half-filled d-orbitals in its electronic configuration—should involve faster kinetics. Since the optimal pH of the method is close to the physiological one, there would be no risk of underestimation (under acidic conditions) or overestimation (under basic conditions) of TAC, due to either protonation of antioxidants or proton dissociation of phenolic compounds, respectively [15]. As in similar electron-transfer based assays, the antioxidant capacity is assumed to be equal to reducing capacity [15].

Materials and methods

Chemicals and instruments

Uric acid, ascorbic acid and neocuproine (2,9-dimethyl-1,10-phenanthroline) were purchased from Sigma Chemical Co.; trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Aldrich Chem. Co.; glutathione (reduced, GSH), α-tocopherol, ammonium acetate, copper(II) chloride, albumin fraction V (from bovine serum, BSA), potassium persulfate, dichloromethane (DCM) and 96% ethanol from E. Merck, β-carotene, bilirubin, ABTS (2,2′-azinobis(3- ethylbenzothiazoline-6-sulfonic acid) diammonium salt) and ascorbate oxidase from Fluka Chemicals and n-hexane from Riedel-deHaën. Serum samples from healthy adults were supplied freshly from Istanbul University, Cerrahpasa Faculty of Medicine, Central Laboratory, Istanbul, Turkey and citrated for a longer standing to access whenever necessary.

The absorbance measurements after reaction of serum antioxidant compounds and human serum samples with the CUPRAC reagent were made with the aid of a Varian CARY 1E UV–Vis spectrophotometer using a pair of matched quartz cuvettes. Serum centrifugation was made with a MSE Mistral 2000 centrifuge apparatus using 10 cm-tubes of 1.5 cm diameter. The pH measurements were made with a E512 Metrohm Herisau pH-meter equipped with a combined glass electrode.

Serum extraction

Plasma or serum samples that were freshly collected and kept at +4°C in a refrigerator just prior to analysis (or stored at −70°C as necessary, were thawed slowly), mixed well on a vortex, and centrifuged if needed. Serum extraction was based upon the procedure published by Aebischer et al. [26], and applied to plasma and serum samples by Prior et al. [27]. One ml of such a (serum) sample was transferred to a centrifuge tube, 2 ml of 96% ethanol and 1 ml of distilled water were added, and mixed well. Four milliliters of n-hexane were added to the mixture, again mixed and the final mixture was let to stand for a few minutes for the separation of phases. The solution was centrifuged at 5000 rpm (1500*g*) for 5 min. The upper organic phase was separated, and transfered to a dark tube. The procedure was repeated with extra 4 ml of hexane, i.e. 4 ml hexane was added to the remaining aqueous phase, mixed well, let to stand for a few minutes for the separation of phases and centrifuged again at 5000 rpm for 5 min. This second hexane extract was separated, and transferred to the original dark tube so as to combine with the first extract. The organic solution comprising combined hexane extracts was dried down under N_2 flow, and the residue was taken up with 1 ml of DCM for the assay of lipophilic antioxidants. The above procedure was repeated 8 times for the serum sample: The DCM phases were combined for the assay of lipophilic antioxidants.

The minute amounts of hexane remaining in the aqueous phase of each tube was removed by drying under nitrogen. Protein content of each tube was precipitated by adding 4 ml of 0.5 M $HClO_4$. The nature and relative quantity of this precipitant was as optimized by Prior et al. [27]. The aqueous solution was centrifuged at 5000 rpm (1500*g*) for 5 min. The upper clear phases of 8 tubes were combined for the assay of hydrophilic antioxidants. The combined acidic aqueous phase was neutralized with 10.1 ml of 1.0 M NaOH prior to analysis. Thus the serum was separated into two phases for the assay of lipophilic and hydrophilic antioxidants. For the application of standard addition technique (so as to observe whether the calibration curve of a given antioxidant compound in standard-added serum was parallel to the one obtained with the sole antioxidant), the lipophilic antioxidants, α-tocopherol and β-carotene were added one by one to the organic phase, and the hydrophilic antioxidants, bilirubin, uric acid, ascorbic acid, glutathione (GSH) and albumin were added one by one to the aqueous phase.

Preparation of standard solutions of CUPRAC reagents and plasma antioxidants

The CUPRAC reagent solutions were prepared as described in the original CUPRAC method developed for flavonoids [25]. Copper(II) chloride solution at a concentration of 10^{-2} M was prepared from $CuCl_2 \cdot 2H_2O$ weighing 0.4262 g, dissolving in H_2O and diluting to 250 ml with water. Ammonium acetate (NH_4Ac) buffer at pH = 7.0 was prepared by

dissolving 19.27 g of NH_4Ac in water and diluting to 250 ml. Neocuproine (Nc) solution at a concentration of 7.5×10^{-3} M was prepared by dissolving 0.039 g Nc in 96% EtOH, and diluting to 25 ml with ethanol. The standard solutions of plasma antioxidants were prepared at 1.0×10^{-3} M concentration. α-Tocopherol and β-carotene were dissolved in dichloromethane (DCM), and the β-carotene solution was further diluted with the same solvent at 1:50 volume ratio. Ascorbic acid and glutathione (GSH) solutions were prepared in distilled water. Uric acid (0.0168 g) was dissolved in 20 ml of 0.01 M NaOH, the excess base was neutralized with the addition of 0.01 M HCl, and finally diluted to 100 ml with H_2O. Bilirubin (0.0146 g) was dissolved using 1 ml of 0.1 M NaOH, excess base was neutralized with 0.1 M HCl, and finally diluted to 25 ml with water.

Standard addition method applied to organic extract of serum

To a test tube were added 1 ml each of copper(II) chloride solution, neocuproine solution and NH_4Ac buffer solutions in this order. A suitable aliquot (0.8 ml) of the combined organic extract (of serum) was added to this tube (such that the initial absorbance of this extract with respect to the CUPRAC spectrophotometric method would be around 0.2). To this mixture, 3.2 ml of DCM were added, shaken and the organic phase was separated from the aqueous phase. Standard additions of α-tocopherol and β-carotene in varying concentrations were made to the serum (organic) extract so as to construct the calibration curves of these lipophilic antioxidants in organic serum extract of initial absorbance around 0.2. Absorbance reading was made against a reagent blank at 450 nm. Since the boiling temperature of DCM was low, the DCM used in the procedure was cooled to an initial temperature of +4°C to prevent evaporation losses. No elevated temperature incubation tests (as applied to hydrophilic antioxidants in the aqueous phase) were carried out with the organic extract.

Standard addition method applied to aqueous extract of serum

To a test tube were added 1 mL each of copper(II) chloride solution, neocuproine solution and NH_4Ac buffer solution in this order. A suitable aliquot (1.5 ml) of the combined aqueous extract (of serum) was added to this tube (such that the initial absorbance of this extract with respect to the CUPRAC spectrophotometric method would be around 0.2). Standard additions of bilirubin, uric acid, ascorbic acid and GSH in varying concentrations were made to this extract so as to construct the calibration curves of these hydrophilic antioxidants in aqueous serum extract of initial absorbance around 0.2. If (x) ml of the standard antioxidant solution was taken, then ($0.25 - x$) ml H_2O was added to make the final volume 4.75 ml. For dilution experiments of serum, 1.5 ml of the combined aqueous extract diluted with water at ratios varying between 1:1 and 1:10 was treated as the unknown sample, and 1.5 ml of this final diluted sample was subjected to CUPRAC analysis as stated above. Absorbance reading was made against a reagent blank at 450 nm. All hydrophilic antioxidants reacted instantly with the CUPRAC reagent except uric acid and bilirubin, which showed a slight absorbance increase upon standing at room temperature. Therefore, absorbance readings were recorded 30 min after the mixing of analyte solution with reagents. The results were evaluated by means of a calibration curve (line) for each antioxidant. Comparison with trolox as the reference compound was made using the room temperature molar absorptivity of trolox, i.e. $1.67 \times 10^4 \mathrm{l\,mol^{-1}\,cm^{-1}}$.

Standard addition method applied to aqueous extract of serum with incubation

The standard addition method applied to aqueous extract of serum was followed with the single difference of extract volume taken for analysis (i.e. 0.7 ml of combined aqueous extract of serum was suitable so that it would yield an initial absorbance of 0.2 with respect to the CUPRAC method). After the addition of (x) ml of standard hydrophilic antioxidant (bilirubin, uric acid, GSH and BSA) solutions, dilution was made with H_2O to 4.75 ml. The tubes were stoppered, and incubated at 50°C in a water bath for 20 min. (Tests were also performed to follow the color development kinetics of hydrophilic antioxidants for longer incubation periods at this temperature). The incubation period was selected with respect to the kinetic behavior of bilirubin, which required 20 min at 50°C for absorbance stabilization. Data for ascorbic acid was not collected at this stage, because ascorbic acid decomposed at elevated temperature incubation. The incubated tubes were let to cool to room temperature, and the 450 nm-absorbance was read as stated. The results were evaluated by means of a calibration curve (line) for each antioxidant. Comparison with trolox as the reference compound was made using the 50°C-incubated molar absorptivity of trolox, i.e. $1.86 \times 10^4 \mathrm{l\,mol^{-1}\,cm^{-1}}$.

Individual determination of ascorbic acid among hydrophilic serum antioxidants

The original ascorbate oxidase enzyme solution of initial activity 328 U/mg was diluted with water to a concentration of 4 U/ml. To a separate test tube was added 0.100 ml of 10^{-3} M ascorbic acid, and analyzed

conventionally with the CUPRAC method to yield an absorbance of 0.30. To another tube containing the same amount (0.100 ml) of ascorbic acid solution was added 0.200 ml of ascorbate oxidase solution, let to stand for 1 min, and analyzed with the CUPRAC method to observe at least 90% quenching of the absorbance due to ascorbic acid ($A_{450} = 0.03$).

A synthetic mixture of hydrophilic antioxidants was prepared to include 0.050 ml uric acid, 0.050 ml GSH and 0.100 ml ascorbic acid standard solutions. The CUPRAC absorbance of this mixture was 0.57. To another tube containing the same antioxidants mixture (with identical amounts) was added 0.200 ml ascorbate oxidase solution, and subsequently analyzed with the CUPRAC method to yield an absorbance of 0.26, the absorbance difference corresponding to ascorbic acid content of the mixture. Thus it was shown that ascorbic acid among hydrophilic antioxidants of serum could be individually quantified by the aid of two successive CUPRAC measurements of the antioxidant mixture with and without ascorbate oxidase, the ascorbic acid content being calculated from the difference.

ABTS assay of total antioxidant capacity of serum antioxidants

The ABTS-persulfate assay of Re et al. [18] was slightly modified for serum antioxidant assay. An ABTS chromogenic radical reagent solution at 7.0 mM concentration was prepared in water. To this solution was added $K_2S_2O_8$ (as an oxidant for conversion of ABTS into a radical cation) to yield a final persulfate concentration of 2.45 mM. The color of the resulting solution was blue–green. This radical solution was kept in a stoppered flask in the dark at room temperature for 12–16 h before use in actual measurements. The kept solution was diluted with 96% ethanol at a ratio of 1:10. The absorbance of the 1:10 diluted $ABTS^+$ radical cation solution was 1.28 ± 0.04 at 734 nm. To (x) ml of the sample solution (aqueous extract of serum) were added 1 ml of final $ABTS^+$ solution, and ($4 - x$) ml of 96% EtOH, and the change of absorbance during 6 min was recorded (usually the absorbance decrease at the 6th-min was used for calculations). As a convention, (x) was selected between 0.5 and 1.0 ml for the organic and aqueous extracts of serum, and the total volume was 5.0 ml.

Results and discussion

The copper(II)-neocuproine (2,9-dimethyl-1,10-phenanthroline) reagent, introduced for various reducing agents as a mild oxidant [28], was previously used by our research team to determine the biochemically important reductants such as cysteine [29] and vitamin E [30]. It has recently been used for ascorbic acid assay in foods and beverages [31], and for flavonoids as a total antioxidant capacity assay (CUPRAC assay) of food materials [25]. The antioxidant potency of flavonoids of similar conjugation level was roughly proportional to the total number of —OH groups in the CUPRAC assay, and was positively affected by the presence of o-dihydroxy moiety in the B-ring [25].

The trolox equivalent antioxidant capacity (TEAC) is defined as the millimolar concentration of a trolox solution having the antioxidant capacity equivalent to a 1.0 mM solution of the substance under investigation. The TEAC values of various antioxidants found according to the original ABTS method ($TEAC_{orig}$) [9,32], FRAP method ($TEAC_{FRAP}$) [13,33] and calculated with respect to the developed CUPRAC method ($TEAC_{CUPRAC}$) were very close to each other, except for hydroxycinnamic acids [25] for which the results of the CUPRAC method were more consistent with structure–activity relationships than those of the ABTS assay. The $TEAC_{CUPRAC}$ coefficients are simply calculated by dividing the molar absorptivity (ε) of the species under investigation by that of trolox under corresponding conditions (e.g. the ε values of normal and incubated solutions of trolox are 1.67×10^4 and $1.86 \times 10^4\,l\,mol^{-1}\,cm^{-1}$, respectively).

The molar absorptivities and linear working ranges obtained from normal and incubated solutions of plasma antioxidants are listed in Table I. Here it is apparent that the highest molar absorptivities were obtained for bilirubin (5.3×10^4, in the aqueous phase)

Table I. The CUPRAC molar absorptivities and linear working ranges of plasma antioxidants.

Antioxidant compound	ε ($l\,mol^{-1}\,cm^{-1}$)	Incubated ε ($l\,mol^{-1}\,cm^{-1}$)	Linear range (M)
Ascorbic acid	$(1.59 \pm 0.03) \times 10^4$	(Decomposes)	5.6×10^{-6}–8.5×10^{-5}
Bilirubin	$(5.3 \pm 0.1) \times 10^4$	$(8.0 \pm 0.15) \times 10^4$	3.23×10^{-7}–2.61×10^{-5}
Glutathione (GSH)	$(9.5 \pm 0.2) \times 10^3$	$(9.5 \pm 0.2) \times 10^3$	3.12×10^{-6}–1.48×10^{-4}
Uric acid	$(1.60 \pm 0.03) \times 10^4$	$(2.8 \pm 0.05) \times 10^4$	7.07×10^{-7}–8.64×10^{-5}
α-Tocopherol	$(1.83 \pm 0.03) \times 10^4$	–*	1.05×10^{-6}–7.67×10^{-5}
β-Carotene	$(5.6 \pm 0.1) \times 10^4$	–*	3.37×10^{-7}–2.49×10^{-5}

* Incubated absorptivity could not be measured in organic solution. Bovine serum albumin (BSA) only reacted in incubated solution with an absorptivity of $9.24\,ml\,mg^{-1}\,cm^{-1}$, reported as such since its molecular weight is too high (approximately given as $6.8 \times 10^4\,g\,mol^{-1}$); the linear equation of its abs./concn plot was: $A_{450} = 9.24 \times 10^{-3}$CBSA $- 4.78 \times 10^{-3}$ ($r = 0.9996$) where C_{BSA} was in $mg\,ml^{-1}$.

and β-carotene (5.6×10^4, in the organic phase). The TEAC coefficients of plasma antioxidants with respect to the CUPRAC method (i.e. the ratio of the molar absorptivity of antioxidant to that of trolox, measured under identical conditions) are listed and compared in Table II with those found by other widely-used methods currently employed, i.e. ORAC-peroxyl radicals [34], FRAP [13] and ABTS-persulfate [18] assays of TAC. The TEAC coefficients pertaining to the ABTS-persulfate method were simultaneously reported from the literature and experimentally found by us (see Table II). Inspection of data in Table II reveals that the FRAP method cannot measure glutathione, as criticized for not being capable of measuring thiol-type antioxidants [3]. In relation to cellular GSH and thiols metabolism, 2 molecules of GSH react with H_2O_2 or hydroperoxides through an enzymatic oxidation with glutathione peroxidase to form 1 molecule of glutathione disulfide (GSSG), where GSH acts as a 1 e-reductant [35]. Likewise, for a structurally similar compound, cysteine, two cysteine residues in proteins may undergo a reversible oxidation to form a disulfide bond, which often plays an important structural role (2 RSH═RSSR + $2H^+$ + $2e^-$). In accord with these roles, the TEAC coefficients of GSH found by ORAC and Randox-TEAC assays were 0.59 and 0.66, respectively [3]. Our (CUPRAC) TEAC coefficient of GSH was 0.57 (see Table II), again consistent with its physiological role as a (1 e-reductant) antioxidant. However, metal-catalyzed reactions of H_2O_2 or peroxynitrite with a thiol may produce sulfinic (—SO_2H) or sulfonic (—SO_3H) acids through sulfenic acid (—SOH) intermediates, which is less likely *in vivo* [35]. It is clear that the ABTS/persulfate assay treats GSH as a reductant capable of giving 2 or more electrons (The TEAC literature and experimental values of the latter assay for GSH were 1.28 and 1.51, respectively, as indicated in Table II). We think that our TEAC result of 0.57 is more reflective of the physiological role of GSH as an antioxidant. The exceptionally low TEAC values of the ORAC-peroxyl radical method for bilirubin and β-carotene in Table II is reminiscent of the fact that the fluorescent protein probe of the ORAC method, B-PE, as developed by Cao et al. [12] has interacted in a nonspecific manner—basically as hydrophobic interactions and H-bonding—with polyphenols, causing falsely low ORAC values for these polyphenols [21].

Possible ternary mixtures of the hydrophilic plasma antioxidants were synthetically prepared (for the lipophilic ones, a binary solution of α-tocopherol and β-carotene was prepared), and the suitably diluted solutions were analyzed for antioxidant capacity using the CUPRAC method. The experimentally measured capacities were generally within ±6% interval of the theoretically computed values using the formula:

$$\text{Capacity}_{\text{total}} = \text{TEAC}_1\ \text{concn.}_1 + \text{TEAC}_2\ \text{concn.}_2 + \text{TEAC}_3\ \text{concn.}_3 + \ldots \quad (1)$$

where 1,2,..., *i* denote the corresponding constituents of the synthetic mixture. The comparison of expected (using equation 1) and experimentally found antioxidant capacities of synthetic mixture solutions (as mM trolox-equivalents) were made, and depicted in Table III. The accordance of theoretical and experimental findings, combined with the parallelism of the linear calibration curves (absorbance/concentration plots) of each antioxidant compound (ascorbic acid, GSH, uric acid, bilirubin, α-tocopherol and β-carotene) tested in the presence and absence of the respective serum fraction, i.e. aqueous or organic extract containing hydrophilic or lipophilic antioxidants, respectively (Table IV), effectively demonstrated that there were no chemical interactions of interferent nature among the synthetic solution constituents, and that the antioxidant capacities of the tested antioxidants were additive. These abs. vs. concn. plots with or without serum extract were repeated for those hydrophilic antioxidants exhibiting an absorbance increase upon elevated temperature incubation (i.e. for bilirubin, glutathione and uric acid)

Table II. Trolox equivalent antioxidant capacity (TEAC) coefficients of plasma antioxidants.

Antioxidant compound	$\text{TEAC}_{\text{CUPRAC}}$	Inc. $\text{TEAC}_{\text{CUPRAC}}$	Measd $\text{TEAC}_{\text{ABTS}}$	Lit. $\text{TEAC}_{\text{ABTS}}$	$\text{TEAC}_{\text{ORAC}}$	$\text{TEAC}_{\text{FRAP}}$
Ascorbic acid	0.96	–	1.03	1.05	0.52–1.12	0.95–1.05
Bilirubin	3.18	4.34	2.36	–	0.84	2.1 – 2.3
Glutathione (GSH)	0.57	0.57	1.51	1.28	0.68	Unmeasurable
Uric acid	0.96	1.54	1.11	1.01	0.92	1.0–1.2
α-Tocopherol	1.11	–	1.02	0.97	1.0	0.85–1.05
β-Carotene	3.35	–	2.80	2.57	0.64	Unmeasurable
Bovine serum albumin	–	0.033	–	–	–	0.05

Inc. $\text{TEAC}_{\text{CUPRAC}}$: TEAC measured in incubated solution (inc. at 50°C for 20 min); Measd. and Lit. $\text{TEAC}_{\text{ABTS}}$ values are experimentally measured and literature reported ABTS-persulfate values of TEAC coefficients, respectively; $\text{TEAC}_{\text{ORAC}}$ were extracted from the literature (ORAC-peroxyl radicals); $\text{TEAC}_{\text{FRAP}}$ values were calculated by dividing the literature FRAP values by 2, since original FRAP was reported as Fe(II) equivalents which is a 1-e reductant whereas conversion to trolox (2-e reductant) is required. The incubated $\text{TEAC}_{\text{CUPRAC}}$ values of ascorbic acid, α-tocopherol and β-carotene were not reported in Table II due to the reasons given in Table I.

Table III. The comparison of expected and found CUPRAC antioxidant capacities of synthetic mixture solutions (as mM trolox equivalents).

Composition of mixture	Capacity expected (as mM TR-equivalent)	Capacity found experimentally (as mM TR-equivalent)
50 μl of 1 mM AA 50 μl of 1 mM UA 50 μl of 1 mM GSH	2.62×10^{-2}	$(2.79 \pm 0.10) \times 10^{-2}$
50 μl of 1 mM AA 50 μl of 1 mM GSH 20 μl of 1 mM BIL	2.95×10^{-2}	$(2.9 \pm 0.11) \times 10^{-2}$
50 μl of 1 mM AA 50 μl of 1 mM UA 20 μl of 1 mM BIL	3.36×10^{-2}	$(3.23 \pm 0.12) \times 10^{-2}$
50 μl of 1 mM UA 50 μl of 1 mM GSH 20 μl of 1 mM BIL	2.95×10^{-2}	$(3.0 \pm 0.11) \times 10^{-2}$
50 μl of 1 mM UA (inc) 50 μl of 1 mM GSH (inc) 20 μl of 1 mM BIL (inc)	4.2×10^{-2}	$(4.0 \pm 0.15) \times 10^{-2}$
50 μl of 1 mM TP 0.5 ml of 1 mM CAR	1.9×10^{-2}	$(1.9 \pm 0.07) \times 10^{-2}$

AA—Ascorbic acid, GSH—Glutathione, UA—Uric acid, BIL—Bilirubin, CAR—β-Carotene, TP—α-Tocopherol; inc: incubated at 50°C.

(see Table IV, incubated measurements), and again a good parallelism of linear curves was observed in pure aqueous solution and in a real complex mixture of serum extract having an initial nonzero absorbance with the CUPRAC reagent. This confirmed that the constituents of a real matrix solution such as serum did not chemically interact with selected pure antioxidants, and that the antioxidant capacities were additive. Thus the proposed CUPRAC method may be effectively used for the antioxidant capacity assay of synthetic mixtures and real biological fluids. It should be mentioned here that the "competition kinetics"-based capacity assays [36] may not fully ensure ideal "additivity" (of antioxidant capacities), because the capacity of a complex mixture is defined as the sum of the products of the concentration of each antioxidant with its rate constant, and these rate constants may result from different kinetic models (i.e. reaction orders, such as first or second order reactions), and therefore may have different units.

The dilution sensitivity of serum extracts was evaluated using the CUPRAC method, and the found capacities (as micromolar trolox equivalents) were recorded against expected capacities at varying dilutions of the aqueous and organic extracts (see Figures 1 and 2). The excellent linear curves passing through the origin in each case was an advantage over the Randox-TEAC (i.e. the commercialized version of ABTS-TEAC) assay in which dilution of serum might produce up to a 15% increase in the TEAC values [3]. Another advantage of the current method over the Randox assay is that, due to the fixed-time inhibition of the ABTS radical utilized by Randox-TEAC, quercetin was reported to yield a nonlinear dose-response curve [3], whereas in the CUPRAC method, quercetin and other flavonoids were shown by us to yield excellently linear calibration curves over a wide concentration range [25].

The protein fraction may contribute significantly to the antioxidant capacity, which may mask responses, particularly if the interest lies in small molecular-weight antioxidants. Therefore protein removal was important, and effectively applied using a volume ratio of ethanol/plasma/H_2O/0.5 N $HClO_4$ solution as 2:1:1:4, as optimized by Prior et al. [27].

Figures 3 and 4 show the CUPRAC reaction kinetics with individual antioxidants measured at room temperature and incubated at 50°C, respectively. It is apparent from Figure 3 that among hydrophilic antioxidants, only uric acid and bilirubin showed an absorbance increase with time, which determined the time period of measurement (i.e. 30 min after the mixing of reagents with the analyte). As the reduction potential of the antioxidant approaches that of the reagent, the thermodynamic efficiency, and possibly the rate of the oxidation reaction decreases, which is the case for bilirubin and uric acid (E^{o}_{red} for the latter is 0.59 V). Albumin (BSA) was not shown in Figure 3, because it did not react with the CUPRAC reagent at room temperature, and required elevated temperature incubation (Figure 4) for the oxidation to proceed. Figure 4 shows that the initial absorbance of uric acid markedly increased with temperature (compared to that of room temperature) but rapidly stabilized, whereas the absorbances of bilirubin ans albumin continued to increase with time at elevated temperature, and stabilized within 20 min at 50°C. Figure 5 shows the ruggedness of the

Table IV. The slope, intercept and correlation coefficients of calibration equations of linear absorbance vs. molar concentration data of the tested antioxidants alone and in serum⋆.

Compound	Slope† $\times 10^{-4}$	Intercept†	Correlation coefficient† (r)	Slope‡ $\times 10^{-4}$	Intercept‡	Correlation coefficient‡ (r)
Ascorbic acid (AA)	1.56 (1.56)	− 0.024 (0.17)	0.998 (0.999)	–	–	–
α-Tocopherol (TP)	1.82 (1.84)	0.009 (0.20)	0.999 (0.999)	–	–	–
β-Carotene (CAR)	5.91 (6.15)	− 0.002 (0.18)	0.999 (0.999)	–	–	–
Bilirubin (BIL)	5.31 (5.18)	0.013 (0.15)	0.999 (0.999)	8.08 (8.18)	0.031 (0.25)	0.999 (0.999)
Uric acid (UA)	1.59 (1.58)	− 0.030 (0.16)	1.000 (0.999)	2.84 (2.83)	− 0.013 (0.23)	0.999 (0.999)
Glutathione (GSH)	0.97 (0.91)	− 0.003 (0.17)	0.999 (0.999)	0.947 (0.946)	0.0012 (0.24)	0.999 (0.999)
Albumin (BSA)-	–	–	–	0.059 (0.052)	0.011 (0.24)	0.999 (0.998)

⋆The first value in the column pertains to the calibration equation of antioxidant alone, the second (in paranthesis) to the calibration equation in serum. †Measurement conditions: room temperature for 30 min. ‡Measurement conditions: incubation at 50°C for 20 min.

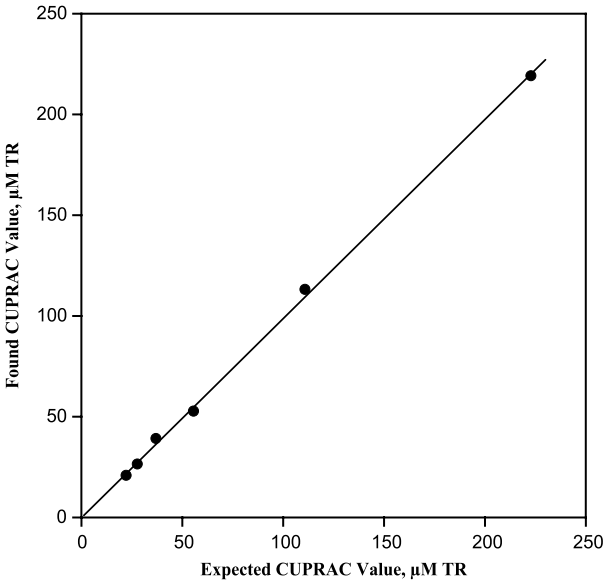

Figure 1. CUPRAC values of serum extract (hydrophilic phase) at different dilutions ($r = 0.999$).

CUPRAC procedure for aqueous extracts of serum samples, where the intra- and inter-assay CVs were around 0.7% and 1.5%, better than those of most methods.

The total antioxidant capacities of the serum samples (samples as described in materials and methods section) using the CUPRAC, incubated CUPRAC, and ABTS-persulfate methods applied on the aqueous and organic fractions of serum are listed in Table V. The results of hydrophilic and lipophilic antioxidants assays in Table V could be added to yield a sum as a measure of TAC of a sample [27]. Both CUPRAC and ABTS methods yielded close results

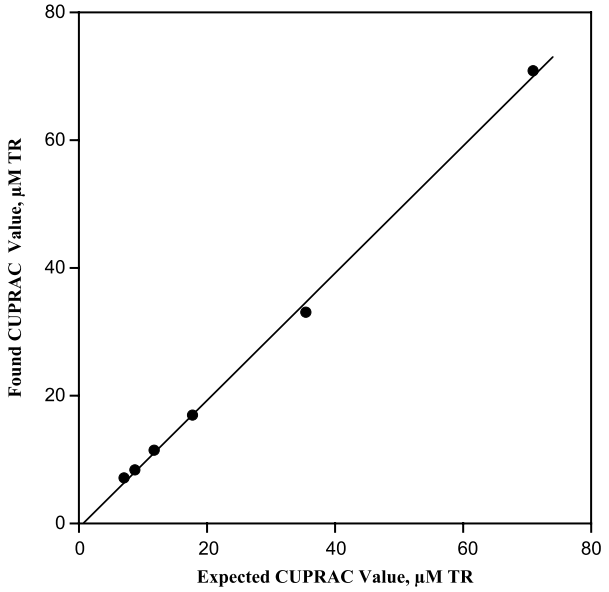

Figure 2. CUPRAC values of serum extract (lipophilic phase) at different dilutions ($r = 0.998$).

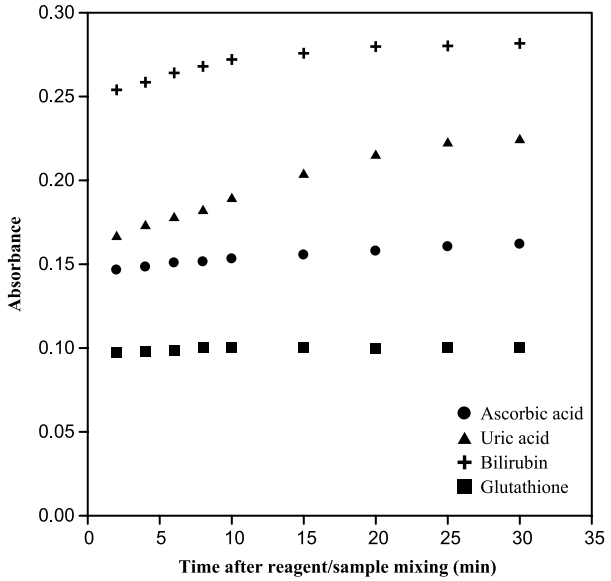

Figure 3. CUPRAC reaction kinetics with individual antioxidants; rate of increase in absorbance at 450 nm for 1 mM solutions of antioxidants (at room temperature).

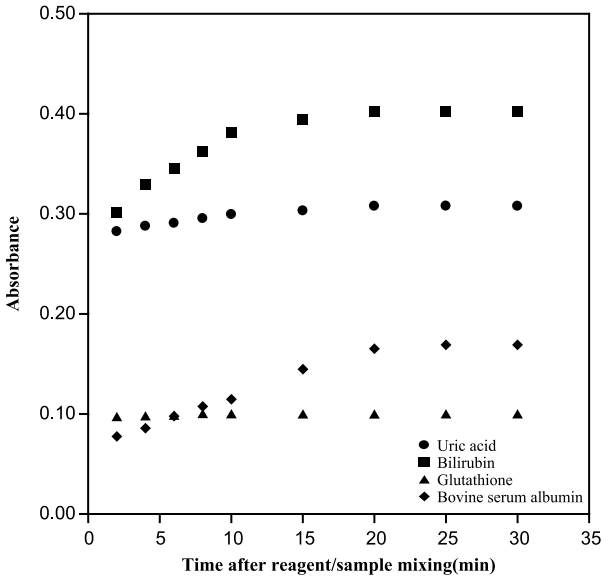

Figure 4. CUPRAC reaction kinetics with individual antioxidants; rate of increase in absorbance at 450 nm for 1 mM solutions of bilirubin, glutathione, uric acid and 300 mg l^{-1} solution of BSA (incubated measurement).

for the lipophilic antioxidants (organic fraction). The CUPRAC assay results of the aqueous extracts (for the hydrophilic antioxidants) as the outcome of room temperature measurements significantly increased upon incubation. Since uric acid, bilirubin and albumin, constituting a great majority of TAC of serum [13,36], all showed an absorbance increase upon elevated temperature incubation, the almost double-fold increase of CUPRAC capacities of aqueous extract of serum as a result of incubation is expectable. The significantly higher results of the ABTS-persulfate assay compared to those of CUPRAC for the aqueous extract was probably due to the higher TEAC coefficient ascribed by ABTS to thiols of various origin (1.5 compared to 0.5, as seen in Table II), and to the interaction of ABTS radical with the unidentified antioxidants possibly contributing at 1/3 ratio to the observed capacity [37]. A linear correlation existed between the CUPRAC and ABTS findings for hydrophilic antioxidants measurements carried out both at room temperature ($r = 0.58$) and in 50°C-incubated solution ($r = 0.53$). This is also an advantage of the developed method, as relevant literature reports that either serum ORAC or serum FRAP does not correlate at all with serum TEAC [3].

Assigning serum concentrations (as micromol/l) of (605 ± 34) for albumin, (257 ± 71) for uric acid, (24.4 ± 4.9) for α-tocopherol, (42.3 ± 15.5) for ascorbic acid, (9.05 ± 2.84) for bilirubin, and 0.35 for GSH, as reported by Cao and Prior [3], and multiplying with $TEAC_{CUPRAC}$ coefficients for converting into trolox-equivalent concentrations, the reported CUPRAC capacities in Table II could be largely accounted for: α-tocopherol represented about 1/3 of the lipophilic capacity; uric acid, ascorbic acid, and bilirubin explained almost all the hydrophilic capacity obtained with the aid of room temperature measurements; and uric acid, bilirubin, and albumin could explain a significant percentage of the hydrophilic CUPRAC capacity upon incubation. The CUPRAC results were generally consistent with those of FRAP and ORAC (the latter using acetone-treated serum), both reference methods reporting around 0.4 mmol trolox equivalent per liter [3]. The Randox-TEAC assay also gave the same result for serum [38]. Differences in reported antioxidant

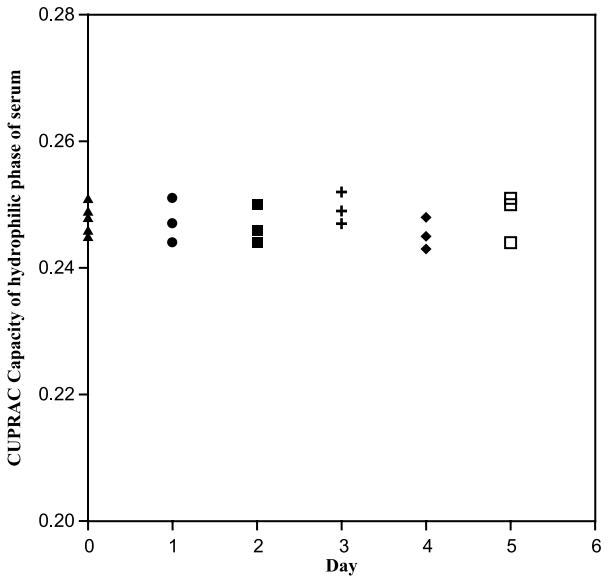

Figure 5. Ruggedness of the CUPRAC procedure for serum samples, showing intra- and inter-assay variations.

Table V. Lipophilic and hydrophilic total antioxidant capacities of serum samples using the CUPRAC (normal and incubated) and ABTS-persulfate assays ($N = 5$).

Sample solutions	Method	Organic extract (mM TR)*	Aqueous extract (mM TR)*	Aqueous extract (inc) (mM TR)*
Serum 1	CUPRAC	0.08	0.27	0.54
	ABTS	0.08	0.84	–
Serum 2	CUPRAC	0.07	0.23	0.46
	ABTS	0.06	0.90	–
Serum 3	CUPRAC	0.08	0.19	0.39
	ABTS	0.08	0.73	–
Serum 4	CUPRAC	0.05	0.21	0.43
	ABTS	0.06	0.72	–
Serum 5	CUPRAC	0.06	0.25	0.51
	ABTS	0.06	0.78	–

Hydrophilic phase (comparison) $A_{ABTS} = 0.472 + 1.4A_{CUPRAC}$ ($r = 0.58$) at room temperature vs. $A_{ABTS} = 0.483 + 0.67A_{CUPRAC}$($r = 0.53$) in incubated solution. inc: incubated at 50°C.

* All measurements showed deviations approximately ± 1.7% of the mean $\left(x = \bar{x} \pm \frac{t_{95S}}{\sqrt{N}}\right)$.

capacity of biological fluids determined with different techniques are considered acceptable, because this capacity is assumed to depend on which technology and free radical generator or oxidant is used in the measurement [39]. Taking a closer look at the FRAP and CUPRAC methods (reporting similar capacities for serum) reveals that both methods use chromogenic oxidizing agents, i.e. Fe(III)-TPTZ and Cu(II)-Nc, not involving radical species. However the redox potential of the Fe(III)-Fe(II) couple in the presence of TPTZ as a Fe(II)-stabilizing ligand increases the 0.77 V standard potential of iron, which may give rise to the oxidation of substances which are not true antioxidants (such as the anticoagulant citrate used for preserving biological fluids). On the other hand, the 0.17 V standard potential of the Cu(II)–Cu(I) redox couple is shifted to a higher potential around 0.6 V in the presence of neocuproine as a Cu(I)-stabilizing ligand, in close proximity to the potential of reactive oxygen species (e.g. the E^o for HO_2^-/OH^- couple is 0.87 V) against which one tries to measure antioxidant defenses of the organism. The Cu(II)–Nc reagent is a mild oxidant which may easily oxidize the biologically important antioxidants having standard potentials less than 0.2 V, and thereby produce the highly colored Cu(I)–Nc chelate useful for absorbance measurement. As a fortunate coincidence, the standard potential of the $ABTS^{+\cdot}/ABTS$ couple is 0.68 V, close to that of Cu(II,I)–Nc, which may produce the same effect [40]. Compared to FRAP in terms of reaction kinetics, CUPRAC also shows a rather slowed reaction for albumin, bilirubin and uric acid, somewhat resembling the behaviour of FRAP [13], but its distinct advantage over FRAP is its capability to oxidize thiols that are very important as a first line of antioxidant defense in plasma, whereas the FRAP assay criticized for not responding to thiol-type antioxidants [3] may be assumed to involve the kinetically inert high-spin iron(III) having half-filled d-orbitals as the redox centre.

Although the exact physical meaning of TAC of plasma is dubious, an increase of the antioxidant capacity of plasma indicates absorption of antioxidants and an improved *in vivo* antioxidant status, or an adaptation mechanism to an increased oxidative stress in cases of disease such as renal failure (uric acid), icteric status (bilirubin), or hepatic damage (hypoalbuminemia) [41]. The TAC of plasma was remarkably lower in cancer patients [42] or in total body irradiated patients [43]. The cooperation among different antioxidants provides a greater protection against attack by reactive oxygen or nitrogen radicals than any single compound alone. For example, the simultaneous inactivation of ascorbate and thiol groups produces a loss in antioxidant activity of plasma greater (26%) than the sum of the decreases produced by separate inactivation of each of the two compounds [8]. Concentrations of specific antioxidants cannot predict the antioxidant capacity of samples that depends on a variety of antioxidant compounds some of which might escape detection [44]. Thus the overall antioxidant capacity may give more relevant biological information compared to that obtained by measuring individual parameters, as it considers the cumulative effect of all antioxidants present in serum or other body fluids [45]. The *in vitro* nature of TAC assays should not compromise their value in guiding clinical research [15]. The total capacity is believed to be a useful measure of how much the antioxidants present can protect against oxidative damage to membranes and other cellular components [46]. An increased antioxidant capacity in plasma or serum is not necessarily a desirable condition if it is due to an adaptive response to increased oxidative stress, whereas a decrease in capacity may not be an undesirable condition when the production of reactive species decreases [47]. A "battery" of measurements may be necessary to adequately assess oxidative stress in biological systems rather than single measurements [47].

The advantages of the CUPRAC method may be summarized as follows:

– The CUPRAC reagent is fast enough to oxidize thiol-type antioxidants, whereas according to the protocol developed by Benzie et al. [13], the FRAP method does not measure thiol-type antioxidants like glutathione [21]. The reason for this may be the half-filled d-orbitals of high spin Fe(III) attributing it a chemical inertness, while the electronic structure of Cu(II) enables fast kinetics. A redox reaction of cysteine with iron(III) has been reported to proceed slowly in the presence of 1,10-phenanthroline, but the reaction could be accelerated in the presence of copper(II) as catalyst [48]. On the other hand, glutathione and cysteine are fast 1-electron reductants toward the Cu(II)-Nc reagent [28,29], and their oxidation is completed within 2 min at room temperature. Another reason for this difference between the kinetic behaviours of Fe(III) and Cu(II) toward thiols may be the softer character (with respect to the "Hard and Soft Acids and Bases", HSAB Theory) of Cu(II) enabling the coordination of the latter to the soft -SH groups as the electron donor.
– Reagent is selective, because it has a lower redox potential than that of the ferric–ferrous couple in the presence of phenanthroline- or TPTZ-type ligands. The standard potential of the Cu(II,I)-Nc redox couple is 0.6 V, close to that of $ABTS^{+\cdot}$ /ABTS, i.e. 0.68 V. Simple sugars and citric acid are not oxidized with the CUPRAC reagent.
– The reagent is much more stable and easily accessible than the chromogenic radical reagents (e.g. ABTS, DPPH, etc.). The cupric reducing ability measured for a biological sample may indirectly but efficiently reflect the total antioxidant power of the sample even though no radicalic species are involved in the assay.
– The method is easily and diversely applicable in conventional laboratories using standard colorimeters rather than necessitating sophisticated equipment and highly qualified operators.
– The redox reaction giving rise to a colored chelate of Cu(I)–Nc is relatively insensitive to a number of parameters adversely affecting radicalic reagents such as DPPH, e.g. air, sunlight, humidity and pH, to a certain extent.
– The redox reactions concerned may be easily forced to reach completion by incubation at 50°C (i.e. the oxidation reactions of uric acid and bilirubin may be completed).
– The analytical response (i.e. absorbance) vs. concentration curves are perfectly linear in the CUPRAC method over a wide range, unlike those of other methods yielding polynomial curves. The molar absorptivity of the method, i.e. $(7.5–9.5 \times 10^3\,n)$ $l\,mol^{-1}\,cm^{-1}$ for n–e reductants, is sufficiently high to determine biologically important antioxidants.
– As opposed to certain procedures of antioxidant activity assay (such as the TBARS spectrophotometric method) which cannot measure ascorbate as a radical-trapping agent [20], the CUPRAC method can measure ascorbate efficiently over a wide linear range both as a contributor to TAC, and simultaneously as an individual antioxidant present in a synthetic mixture. The $TEAC_{CUPRAC}$ coefficient for ascorbate is approximately 1, that is consistent with its electron transfer behaviour, since both trolox and ascorbic acid are 2-e reductants.
– The redox reaction producing colored species is carried out at nearly physiological pH (pH 7 of ammonium acetate buffer) as opposed to the unrealistic acidic conditions (pH 3.6) of FRAP or basic conditions (pH 10, necessary for phenols to dissociate protons) of Folin-Ciocalteau (FC) assay. At more acidic conditions than the physiological pH, the reducing capacity may be suppressed due to protonation on antioxidant compounds, whereas in more basic conditions, proton dissociation of phenolics would enhance a sample's reducing capacity [15].
– The method can simultaneously measure hydrophilic as well as lipophilic antioxidants (e.g. β-carotene and α-tocopherol). The lipophilic antioxidants of serum may be assayed separately from the hydrophilic ones by hexane extraction of serum, followed by colour development in DCM. The hydrophilic (after $HClO_4$ precipitation) and lipophilic capacities found for serum samples can be added together to yield a total capacity. The room temperature measurements of the CUPRAC method yielded total antioxidant capacities for serum generally in accord with those of FRAP and Randox-TEAC assays, while the sum of lipophilic and 50°C-incubated hydrophilic capacities yielded an overall capacity consistent with the findings of the ORAC assay. As an advantage to the widely used FC assay, it is known that the latter could not be adapted to measure lipophilic antioxidants [15].
– The intra- and inter-assay CVs of the CUPRAC method for human serum (0.7 and 1.5%) are much lower than those of most methods that find wide use in total antioxidant assays. The CV (RSD) data of CUPRAC were definitely better than kinetic-based assays where even the intra-assay CV may reach up to 8% [36].
– Since the Cu(I) ion emerging as a product of the CUPRAC redox reaction is in chelated state (i.e. Cu(I)–Nc), it cannot act as a prooxidant that may cause oxidative damage to biological macromolecules in body fluids. The ferric ion-based assays were criticized for producing Fe^{2+}, which may act as a prooxidant to produce $\cdot OH$ radicals as a result of its reaction with H_2O_2 [47]. The stable Cu(I)-chelate was shown by us not to react with hydrogen peroxide, but the reverse reaction, i.e. oxidation of H_2O_2 with Cu(II)–Nc, is possible.

– The CUPRAC antioxidant assay of biological fluids may be expected to facilitate experimental and clinical studies investigating the relationship among antioxidant status, dietary habits, and risk and progression of diseases.

Conclusion

It has been shown in this work that copper(II)–neocuproine (Nc) as the CUPRAC reagent effectively oxidizes small molecular-weight plasma antioxidants; ascorbic acid, α-tocopherol, β- carotene, reduced glutathione (GSH), uric acid, and bilirubin, with some oxidizing effect on albumin, regardless of chemical type and hydrophilicity of the antioxidants concerned, and is itself reduced in this redox reaction to the highly colored Cu(I)–Nc chelate useful for absorbance measurement at 450 nm. The CUPRAC assay of TAC may be successfully applied to individual antioxidants as well as to their mixtures and human serum. Since the color development is relatively fast, and the required reagents are relatively stable and cheap, the developed method is much simpler and expected to be more widely applicable in the near future than the existing methods. As a distinct advantage over other electron-transfer based assays (e.g. Folin, FRAP, ABTS, DPPH), CUPRAC is superior in regard to its realistic pH (close to that of physiological pH), favourable redox potential, accessibility and stability of reagents, and applicability to lipophilic antioxidants as well as hydrophilic ones.

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