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The main and modified CUPRAC methods of antioxidant measurement

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The antioxidant activity/capacity levels of biological fluids and foods are measured for the diagnosis and the treatment of oxidative stress-associated diseases in clinical biochemistry, and for meaningful comparison of the antioxidant content of foods. Currently, there is no "total antioxidant" as a nutritional index available for food labeling and biological fluids due to the lack of standardized quantitative methods.

The CUPRAC (CUPric Reducing Antioxidant Capacity) method of antioxidant measurement, introduced by our research group, is based on the absorbance measurement of Cu(I)-neocuproine (Nc) chelate formed as a result of the redox reaction of chain-breaking antioxidants with the CUPRAC reagent, Cu(II)-Nc, where absorbance is recorded at the maximal light-absorption wavelength of 450 nm.

We introduce the main CUPRAC method and describe modifications to it in the past six years.

Keywords: Ascorbic-acid determination; CUPRAC antioxidant capacity/activity assay; CUPRAC sensor; Food antioxidant; Hydrogen-peroxide scavenging; Hydroxyl-radical scavenging; Post-column detection; Serum antioxidant; Total antioxidant capacity; Xanthine-oxidase inhibition

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1. Introduction

Reactive oxygen and nitrogen species (ROS/RNS) that emerge as a result of the respirative cycle of oxidative phosphorylation may attack biological macromolecules (e.g., cellular DNA), giving rise to single-strand and double-strand breaks that may eventually cause cell ageing, cardiovascular diseases, mutagenic changes and cancerous tumor growth. Consumption of foods naturally bearing antioxidant activity is considered to be the most efficient way of combating such undesired transformations and health risks. It is important to measure the antioxidant potency of food material and human plasma to assess food quality and to diagnose and treat diseases.

Recent literature states that a single "total antioxidant capacity (TAC)" index for food labeling may not be adequate because of the lack of standardized quantitative methods [1], so several methods have to be employed to assess the antioxidant quality of food. TAC measurement methods existing in literature have been classified under four categories [2]:

(1) based on measurement of the time for the consumption of all antioxidants in a sample;
(2) based on radical consumption (quenching) measurement, when the antioxidant compound to be analyzed is added to a medium containing a free radical;
(3) based on observation of the rate of a given free-radical process, and evaluation of the decrease of this rate upon the addition of the antioxidant sample to be analyzed; and,
(4) correlating the total amount of antioxidants to the reducing capacity of the samples.

In general, antioxidant activity (AOA) measurements are associated with reaction kinetics (rate) whereas antioxidant capacity (AOC) measurements are associated with reaction thermodynamics (i.e. conversion efficiency in terms of the number of radicals quenched or electrons transferred per molecule of antioxidant). These methods may further be classified (e.g., '*in vitro*' and '*in vivo*'; enzymatic and non-enzymatic; or, direct and indirect). A widely accepted mechanistic classification

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involves two major categories: hydrogen-atom transfer (HAT)-based and electron-transfer (ET)-based assays that cannot be differentiated with distinct boundaries [3,4]. It has been hypothesized that no single AOC assay will truly represent the TAC of a particular sample, and that additional tests reflecting both hydrophilic and lipophilic TACs as well as the protective effects against both ROS/RNS are required to fully elucidate AOC [4]. Consequently, in spite of the presence of a wide variety of methods in antioxidant literature, there is no single, versatile AOC or AOA assay that is approved by a majority of analytical, biological and food chemists and that can be applied to different matrices.

The two widely used HAT-based assays {i.e. TRAP (Total Radical-trapping Antioxidant Parameter) [5] and ORAC (Oxygen Radical Absorbance Capacity) [6,7]} essentially measure the ability of an antioxidant to interfere with the reaction between peroxyl radicals and a target probe (undergoing $ROO^{\cdot}$ attack), the probe being less damaged in the presence of antioxidants capable of quenching peroxyl radicals. Both HAT methods are rather slow in signal recovery from complex samples, so they are incapable of accurately evaluating induction period (lag time).

The most important disadvantage of the TRAP method is the instability of the oxygen electrode acting as an end-point indicator. As the most widely-used HAT-based assay, ORAC measures the fluorescence decay of a target probe under $ROO^{\cdot}$ attack. Initially, the selected probe was β-phycoerythrin, which varied from one production lot to another, then it was fluorescein [8]. ORAC evaluates the area under curve (AUC) of fluorescence decay of probe *versus* time in the absence and the presence of antioxidants, and the difference between AUC of sample and blank is correlated to antioxidant concentration in the sample. ORAC has been shown to have certain limitations in terms of the requirements for the strict control of temperature, oxygen, reagent (fluorescein and azide) and sample concentrations, and fluorescein interactions with sample molecules (e.g., proteins and polyphenols) may give rise to erroneously high "apparent" ORAC values [9].

The major ET-based AOC assays are ABTS/TEAC [10], DPPH [11,12], FRAP [13], and CUPRAC [14], though the first two assays using radical reagents, $ABTS^{\cdot+}$ (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) and $DPPH^{\cdot}$ (2,2′-diphenyl-1-picrylhydrazyl), are considered by some researchers to have a mixed mechanism (i.e. both ET-based and HAT-based). The stability of these chromogenic radicals depends upon many factors, and, in these systems, it cannot be exactly determined whether the reduction phenomena made feasible by a given compound arises from its radical scavenging capability or its lowering of the initial rate of radical formation [2]. $ABTS^{\cdot+}$ radical is soluble in both aqueous and organic solvent media, so it enables the simultaneous determination of hydrophilic and lipophilic antioxidants. In this assay, TAC is not expressed with an absolute unit, but with a relative unit obtained by proportioning the observed capacity to that of a reference trolox antioxidant standard measured under identical conditions (trolox equivalent antioxidant capacity, TEAC).

The ABTS assay has been criticized for being too dependent on the chromogenic radical-generation method, and, even in TAC measurement *via* decolorization of the $ABTS^{\cdot+}$ cation formed by persulfate oxidation, different results are obtained as a result of different modifications [15]. An end-point achieved within a protocol time of 6 min for ABTS/persulfate may not be suitable for slow reacting polyphenols [4]. $ABTS^{\cdot+}$ is an N-centered radical with sterically limited access to polymeric phenols giving rise to slow reactions [9]. The DPPH method, though simpler and of lower cost, has been reported to be much influenced by light, air oxygen, pH and type of solvent [16]. Since $DPPH^{\cdot}$ is essentially soluble in organic solvent media, it brings an important limitation to the determination of hydrophilic antioxidants. Flavonoids and other complex phenols generally exhibit moderate-to-slow reaction with $DPPH^{\cdot}$ [9]. The ferric-reducing antioxidant power (FRAP) method [13] is based on the reduction of the Fe(III)-complex of 2,4,6-tripyridyl-*s*-triazine (TPTZ) by antioxidants to the intensely colored Fe(II)-TPTZ chelate showing maximum light absorption at 593 nm. The FRAP assay employed at pH 3.6 does not work at physiological pH, so the adaptability of TAC results to redox reactions occurring in the human body should not be expected. Although the assay is simple, speedy, inexpensive and robust, it is not responsive to glutathione (GSH), the major thiol-type antioxidant peptide of cells, and oxidation of certain hydroxycinnamic acids and flavonoids with the FRAP reagent is not complete within the protocol time period of the assay [17]. High-spin Fe(III) bound to TPTZ has an inherently slow kinetics due to half-filled d-orbitals of ferric ion, and the trivalently-charged Fe(III)-TPTZ complex has more affinity toward the aqueous phase (due to ion-dipole interaction with solvent water molecules) than organic solvents [18], limiting the determination of lipophilic antioxidants especially in human plasma.

The Folin-Ciocalteau assay originally developed for tyrosine and tryptophan [19] and later modified for total phenolics analysis [20] has been suggested as an ET-based TAC assay, but, unfortunately, it is not specific for antioxidants, because it responds equally well to simple phenol, citric acid, many amines, amino acids, and sugars [4], due to the indefinitely high redox potential of the Folin reagent. Moreover, the molybdo-phosphotungstate heteropoly anion as the Folin chromophore is (4-) charged, exhibiting high ion-dipole interactions with water molecules, so this assay is unsuitable for lipophilic antioxidants [17].

Within the framework of suggesting solutions to the problems introduced by the above methods, we need to

develop a relatively objective antioxidant assay to enable precise, reproducible measurement of TAC of a variety of foodstuffs and biological fluids, thereby permitting the classification of food materials with respect to their antioxidant values, and showing good prospects of being useful to a wide range of scientists dealing with oxidative stress. This requirement was the essential aim of developing the CUPric Reducing Antioxidant Capacity (CUPRAC) method. In this method, developed in the analytical chemistry laboratories of Istanbul University, the copper(II)-neocuproine (2,9-dimethyl-1,10-phenanthroline) reagent can oxidize antioxidant compounds that are soluble in water or oil, itself being reduced to the colored copper(I)-neocuproine chelate complex. As an ET-based method, similar to the FRAP method, the CUPRAC method can react with a leading thiol-group antioxidant, GSH, as opposed to the non-responsive FRAP method [13]. In a further differentiation from the FRAP method employed at pH 3.6 and the Folin method [20] at pH 10, the CUPRAC method measures the antioxidant capacity at nearly physiological pH (i.e. pH 7), so it better simulates the physiological action of these antioxidants. The univalent-charged CUPRAC chromophore ($Cu(Nc)_2^+$) is soluble in both aqueous and organic solvents, enabling the simultaneous determination of hydrophilic and lipophilic antioxidants. This article focuses on the CUPRAC assay for TAC measurement [14], which forms the nucleus of further projects to establish an inventory of foodstuffs with respect to antioxidant content in the food sector, and to develop special (antioxidant-rich) diets for risk groups for public health protection. It has been demonstrated to be useful for the determination of plasma antioxidants and protein thiols, and, with more specific research, it may show good prospects for applicability in medicinal chemistry involving the diagnosis, treatment, and follow-up of oxidative stress-linked diseases.

2. The main CUPRAC method

The CUPRAC method was first developed as a spectrophotometric TAC assay and its scope has been expanded with some modifications. Since the cupric-ion-reducing abilities of polyphenols and plasma antioxidants are measured, we introduced this method as the "cupric ion reducing antioxidant capacity" (CUPRAC) method. The selected chromogenic redox reagent is easily accessible, stable, selective, and capable of responding to all types of biologically important antioxidants (e.g., ascorbic acid, α-tocopherol, β-carotene, reduced GSH, uric acid, and bilirubin) and all types of food antioxidants (e.g., flavonoids, simple phenolic and hydroxycinnamic acids) regardless of chemical type or hydrophilicity. The redox reaction concerned is relatively rapid (because cupric neocuproine is an outer-sphere electron-transfer agent) and the resulting color is stable for a reasonable period of time. The chromogenic redox reagent used for the CUPRAC assay is bis(neocuproine)copper(II) chelate. This reagent is useful at pH 7, and the absorbance of the Cu(I)-chelate formed as a result of redox reaction with reducing polyphenols is measured at 450 nm. The color is due to the Cu(I)-Nc chelate formed (see Fig. 1 for the CUPRAC reaction and the chromophore). The reaction conditions (e.g., reagent concentration, pH, and oxidation time at room and elevated temperatures) were optimized [14,18]. In addition to these parameters, the solvent effect for selected antioxidants using CUPRAC method was investigated [21] and it was shown that only CUPRAC and ABTS/persulfate methods (but not FRAP) can assay TAC of lipophilic antioxidants in hydrophobic media, and the antioxidant behavior of phenolic compounds show variations based on solvent type and polarity, reaction mechanism, solubility parameters as well as an essential structural property (i.e. electron-transfer capability). CUPRAC proved to be relatively independent of solvent effects in alcohol–water mixtures of varying compositions [21].

The chromogenic oxidizing reagent of the CUPRAC method developed [i.e. bis(neocuproine)copper(II) chloride (Cu(II)-Nc)] reacts with n-electron-reductant antioxidants (AOX) in the following manner:

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

In this reaction, the reactive Ar-OH groups of polyphenolic antioxidants (AOX) are usually oxidized to the corresponding quinones (Ar=O), and Cu(II)-Nc is reduced to the highly-colored $Cu(Nc)_2^+$ chelate showing maximum light absorption at 450 nm. Although the concentration of Cu^{2+} ions is in stoichiometric excess of that of neocuproine in the CUPRAC reagent for driving the redox equilibrium reaction to the right, the oxidant is the $Cu(Nc)_2^{2+}$ species and not just Cu^{2+}, because the standard redox potential of the Cu(II/I)-neocuproine complex is 0.6 V, much higher that of the single Cu^{2+}/Cu^+ couple (0.17 V) [22]. As a result, polyphenols are oxidized much more rapidly and efficiently with Cu(II)-Nc than with Cu^{2+}, and the amount of colored product [i.e. Cu(I)-Nc chelate] emerging at the end of the redox reaction is equivalent to that of reacted Cu(II)-Nc. The liberated protons are buffered in ammonium-acetate medium. The CUPRAC reagent can oxidize phenolic –OH groups that are suitably situated to the corresponding quinones as long as the corresponding conditional quinone-phenol potential is less than, or close to, that of cupric/cuprous-neocuproine in neutral medium.

Essentially, the method is applied by mixing aqueous $CuCl_2$, ammonium acetate buffer (at pH 7) and alcoholic neocuproine (Nc) solutions, adding a suitable aliquot of the unknown antioxidant solution to this mixture, and reading the absorbance at 450 nm against a reagent blank after 30 min. The color development as a result of

oxidation of polyphenol coupled to reduction of the reagent was rapid for ascorbic acid, gallic acid, and quercetin, but slow for naringin and naringenin.

In the normal CUPRAC method ($CUPRAC_N$), the oxidation reactions were essentially complete within 30 min at room temperature. Flavonoid glycosides required acid hydrolysis to their corresponding aglycons to exhibit their antioxidant potency fully [$CUPRAC_{Hydrolyzed\ (H)}$; medium: 1:1 (v/v) MeOH–H_2O containing 1.2 M HCl]. Slow-reacting antioxidants may need slightly elevated temperature incubation so as to complete their oxidation with the CUPRAC reagent ($CUPRAC_{Incubated\ (I)}$; medium: aqueous solution with varying solvent) [14,17,18,23]; nevertheless, the normal CUPRAC procedure employed at room temperature for 30 min gives satisfactory results for almost all biologically-relevant antioxidants. Special precautions to exclude oxygen from the freshly prepared, analyzed solutions of pure antioxidants were unnecessary, since oxidation reactions with the CUPRAC reagent were much more rapid than with dissolved O_2 (i.e. the latter would not appreciably occur during the period of the CUPRAC protocol, since there is a spin restriction for the ground-state triplet of the dioxygen molecule to participate in fast reactions).

However, plant extracts should be purged with N_2 to drive off O_2, and should be kept in a refrigerator if not analyzed on the day of extraction, since complex catalyzed reactions with unpredictable kinetics may take place in real systems. Also, the oxidation of ascorbic acid with dissolved oxygen may take place more rapidly than that of polyphenolics, especially in the presence of transition-metal salts.

Assuming the additivity of absorbances of individual antioxidants, the experimental TACs (as trolox-equivalents) of synthetic mixtures of antioxidants were measured, and compared to the theoretically computed TAC values to yield coherent results. When the trolox equivalent antioxidant capacities (as TEAC coefficients) of polyphenols were compared, it was observed that TEAC values varied as a function of the total number and the position of hydroxyl groups in the phenolic compound as well as the overall conjugation degree of the molecule (e.g., quercetin yielded the highest $TEAC_{CUPRAC}$ value among flavonoids of similar structure) [14,18]. As a typical ET-based assay, CUPRAC yielded TEAC coefficients for polyphenols that also correlated with their reduction potentials.

As a distinct advantage over other electron-transfer-based TAC assays (e.g., ABTS, DPPH, FRAP and Folin), CUPRAC is superior for its realistic pH close to the physiological pH, favorable redox potential, accessibility and stability of reagents, flexibility, simplicity, low cost, and applicability to lipophilic antioxidants as well as hydrophilic ones [17,23]. CUPRAC gives additive responses for the contributions of antioxidants to TAC, and perfectly linear calibration curves (of absorbance *versus* concentration) over a relatively wide concentration range of antioxidants.

2.1. Modified CUPRAC methods

It is worthy of notice that the CUPRAC assay does not merely measure the TAC of an antioxidant sample, but gives rise to many other modified assays of radical scavenging or activity/capacity measurement that may be useful for antioxidant research [18,24–32]. In this regard, CUPRAC should be perceived as a series of antioxidant-measurement methods in varying media, one evolving from the other.

2.1.1. TAC assay of human serum. The TAC measurement of biological fluids may be useful in providing an estimate for the capability of an organism to counteract ROS, resist oxidative damage and combat oxidative stress-related diseases (e.g., atherosclerosis, cardiovascular diseases, diabetes, liver disorders, neurodegenerative diseases, and inflammatory rheumatic diseases). The original CUPRAC method was applied to a complete series of compounds known as non-enzymatic plasma antioxidants (e.g., GSH, bilirubin, and uric acid), and the TEAC coefficients were evaluated in comparison to the findings of reference methods (e.g., ABTS/persulfate) (Table 1) [18]. The molar absorptivities, linear ranges, and TEAC coefficients of plasma antioxidants were established with respect to the CUPRAC spectrophotometric method. The highest molar absorptivities were obtained for bilirubin ($\varepsilon = 5.3 \times 10^4$ and 8.0×10^4 L/(mol cm) at room temperature and in 50°C-incubated aqueous solutions, respectively) and β-carotene ($\varepsilon = 5.6 \times 10^4$ L/(mol cm) in dichloromethane), although an absorbance correction may be required for the 2-e oxidation product of bilirubin (i.e. biliverdin) at high concentrations. The TEAC coefficients for plasma antioxidants were generally in accordance with those found by ABTS/persulfate and ORAC methods, except for bilirubin and β-carotene, where the CUPRAC method scored higher than both reference tests, and for GSH, where the CUPRAC assay scored lower than the ABTS test. A distinct advantage of the CUPRAC assay was that it proved to be efficient for GSH and thiol-type antioxidants, for which the FRAP (ferric-reducing antioxidant potency) test was basically non-responsive. For lipophilic and hydrophilic antioxidants of human-serum samples extracted with different solvents, the findings of the CUPRAC method developed agreed with those of ABTS/persulfate for the lipophilic phase (first extracted with hexane, and subsequent color development performed in dichloromethane). As for the hydrophilic phase, a curvilinear correlation existed between the CUPRAC and ABTS findings for 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 method developed, as the relevant literature reports that

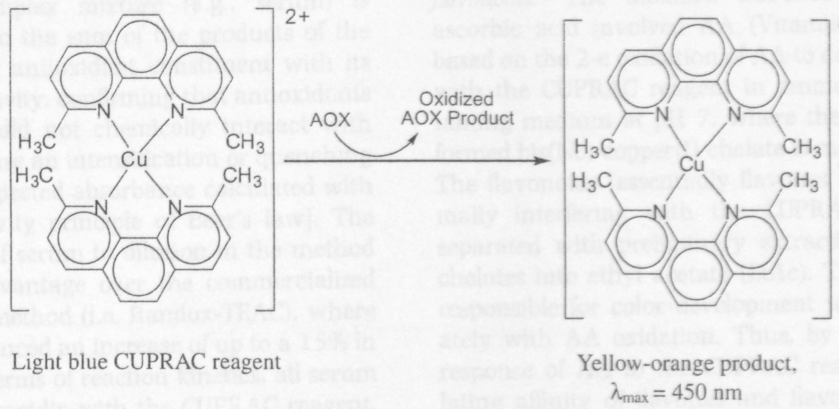

Figure 1. The CUPRAC reaction and chromophore: bis(neocuproine)copper(I) chelate cation. (Protons liberated in the reaction are neutralized by the NH_4Ac buffer).

Table 1. The trolox equivalent antioxidant capacities (TEACs) of various antioxidant compounds in different solvent media calculated with respect to the CUPRAC method

Antioxidants	EtOH 100%[14,21]	H_2O [14,18]	MeOH 100% [21]	MeOH/H_2O (1:1, v/v) [21]	Ac 100% [24]	DCM 100%[18]	DCM/EtOH (9:1, v/v) [21]
Polyphenols							
Quercetin	4.38		5.54	5.78	5.77		3.38
Catechin	3.09		3.70	3.46	3.08		3.30
Naringenin[a]	0.05 (2.28)		0.57 (3.04)	0.36 (2.11)			1.07
Ferulic Acid	1.20		1.38	1.30	1.47		1.11
Vitamins							
Ascorbic Acid		0.96	1.12	1.04	1.03		
α-tocopherol			1.02		1.02	1.10	1.11
Thiols							
Glutathione		0.57	0.56	0.54	0.64		
Cysteine		0.39					
Plasma antioxidants							
Uric acid		0.96					
Bilirubin		3.18					
β-carotene						3.35	
Synthetics							
Butylated Hydroxy Anisole	1.40		1.48	1.45	1.57		0.97
Tert-Butyl Hydroquinone	0.94		0.99	1.10	1.02		0.95

[a]The second value ($TEAC_{CUPRAC}$) in the column shows the $TEAC_{Incubated\ (I)}$ of Naringenin.

serum ORAC and serum FRAP do not correlate with serum TEAC. The higher serum (hydrophilic) antioxidant capacity values reported in the ABTS method could be ascribed to the contribution of thiols of varying origin, for which the ABTS method yielded a higher TEAC coefficient than the CUPRAC method (i.e. the TEAC coefficients for GSH were 0.57 *versus* 1.51 for the CUPRAC and ABTS methods, respectively). The TEAC coefficients obtained with CUPRAC for biologically important thiols (e.g., cysteine and GSH) were close to 0.5, which corresponded with the reversible 1-e oxidation of these thiols under physiological "oxidative stress" conditions to the corresponding disulfides, whereas various modifications of the ABTS test possibly oxidize thiols to higher oxidation levels (e.g., sulfenic and sulfinic acids), which is less likely *in vivo* [33] and therefore score higher TEAC values [18]. Absorbance *versus* concentration data at different dilutions and upon standard additions of model antioxidant compounds (ascorbic acid, α-tocopherol, β-carotene, GSH, uric acid, and bilirubin) to serum showed that the absorbances (at 450 nm of the CUPRAC method) due to different

antioxidant compounds in serum were additive [i.e. the absorbance of a complex mixture (e.g., serum) is approximately equal to the sum of the products of the concentration of each antioxidant constituent with its corresponding absorptivity, confirming that antioxidants in the CUPRAC test did not chemically interact with each other so as to cause an intensification or quenching of the theoretically expected absorbance calculated with the use of the additivity principle of Beer's law]. The relative insensitivity of serum to dilution in the method developed was an advantage over the commercialized version of the ABTS method (i.e. Randox-TEAC), where dilution of serum produced an increase of up to a 15% in the TEAC values. In terms of reaction kinetics, all serum antioxidants reacted rapidly with the CUPRAC reagent, except for bilirubin and uric acid, whose absorbances gradually increased during the first 15 min and finally reached saturation within 30 min at room temperature, whereas a 20-min incubation at 50°C caused a further increase in bilirubin absorbance.

2.1.2. Simultaneous measurement of lipophilic and hydrophilic antioxidants. A novel modified CUPRAC assay was developed for the simultaneous capacity measurement of both lipophilic (i.e. β-carotene, vitamin E, and oil-soluble synthetic) antioxidants and hydrophilic (i.e. vitamin C, flavonoids and phenolic acids) antioxidants in the same solution containing 2% methyl-β-cyclodextrin (M-β-CD) in H_2O-acetone (1:9, v/v) as 'host-guest' inclusion complexes [24]. The M-β-CD concentration was optimized at a low level so as not to cause a decrease in the TACs of certain lipophilic compounds.

If the oligosaccharide content is selected at a high level (e.g., 8% M-β-CD in water-acetone solvent of modified ORAC), then the inclusion-complexation equilibria of antioxidants may predominate over those of their oxidation, adversely affecting the accuracy of the results. Thus, the order of antioxidant potency of various compounds in CUPRAC irrespective of their lipophilicity could be established in the same solvent medium.

The proposed assay was validated through linearity, additivity, precision, and recovery in comparison with the ABTS/HRP assay [34]. It was shown that only the modified CUPRAC and ABTS assays could measure carotenoids together with hydrophilic antioxidants in the optimized system. The TEAC coefficients of various antioxidant compounds as well as the TACs of their mixtures were reported using the proposed method. This method eliminates the wide variability in apparent antioxidant capabilities arising from different levels of accumulation of oil-soluble and water-soluble antioxidants at emulsion interfaces (i.e. the origin of the "antioxidant paradox"), and assigns to each antioxidant an objective TEAC value that depends only on its chemical character (i.e. electron-donating ability).

2.1.3. Determination of ascorbic acid in the presence of flavonoids. The modified CUPRAC method [25] for ascorbic acid involved AA (Vitamin C) determination based on the 2-e oxidation of AA to dehydroascorbic acid with the CUPRAC reagent in ammonium acetate-containing medium at pH 7, where the absorbance of the formed bis(Nc)-copper(I) chelate is measured at 450 nm. The flavonoids (essentially flavones and flavonols) normally interfering with the CUPRAC procedure were separated with preliminary extraction as their La(III) chelates into ethyl acetate (EtAc). The Cu(I)-Nc chelate responsible for color development was formed immediately with AA oxidation. Thus, by combining the fast response of AA to the CUPRAC reagent with the chelating affinity of flavones and flavonols toward La(III) and solubility of their chelates in EtAc, it was possible to determine AA alone in mixtures with flavonoids (and with other La(III)-complexing polyphenols).

2.1.4. Hydroxyl-radical scavenging antioxidant activity of water-soluble antioxidants. Oxidative attack on essential cell components by ROS (e.g., hydroxyl radicals) has been associated with several human diseases. This assay includes the development of a novel "test-tube" method for hydroxyl-radical scavenging antioxidant activity assay of water-soluble antioxidants by modifying the CUPRAC method of TAC measurement originally applied to human serum and plant extracts [26]. Although the measurement of aromatic hydroxylation with ESR or HPLC/electrochemical detection is more specific than the low-yield thiobarbituric-acid reactive substances (TBARS) test [35], it requires sophisticated instrumentation. The TBARS test is the most widely used method for screening the hydroxyl-radical activity of plant extracts, biological fluids and pharmaceuticals. However, there are also criticisms to the classical TBARS method in that it is rather unspecific, is of low yield (i.e. only a small percentage of the carbohydrate deoxyribose is converted to TBA-reactive substances), and cannot properly assay the $^{\cdot}OH$ scavenging power of phenolic antioxidants that may show pro-oxidant activity in the Fenton reaction system *via* iron recycling [36]. As a more convenient and less costly alternative, we used *p*-aminobenzoate, 2,4-dimethoxybenzoate and 3,5-dimethoxybenzoate probes for detecting hydroxyl radicals generated from an equivalent mixture of Fe(II) + EDTA with hydrogen peroxide (see Fig. 2 for the mechanism of this modified CUPRAC method).

The hydroxyl radicals produced attacked both probe and water-soluble antioxidants (or reductants) in 37°C-incubated solutions for 2 h. The CUPRAC absorbance of the ethyl-acetate extract due to the reduction of Cu(II)-neocuproine reagent by the hydroxylated probe decreased in the presence of $^{\cdot}OH$ scavengers, the difference being proportional to the scavenging ability of the

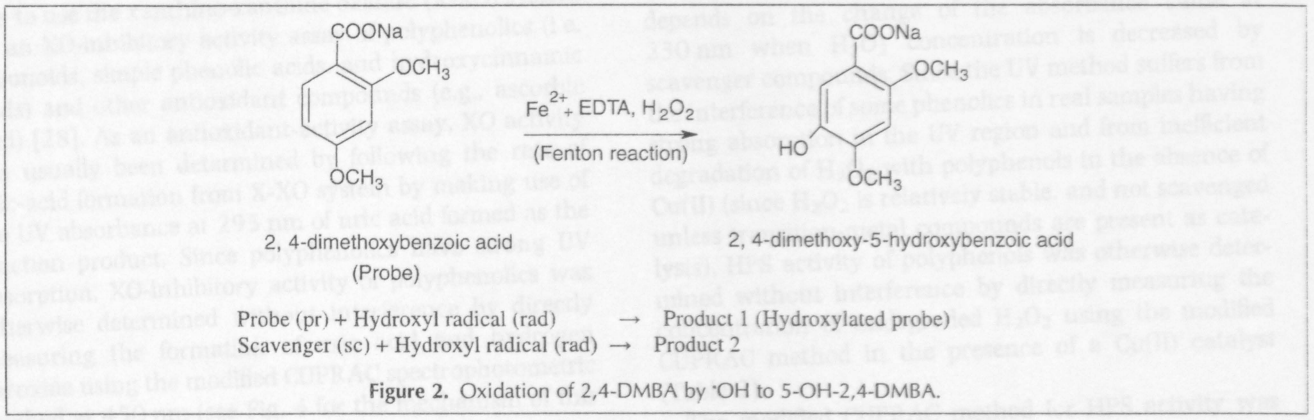

Figure 2. Oxidation of 2,4-DMBA by ·OH to 5-OH-2,4-DMBA.

compound tested. A rate constant for the reaction of the scavenger with hydroxyl radical can be deduced with the aid of competition kinetics from the inhibition of color formation. Iodide, metabisulfite, hexacyanoferrate(II), thiourea, formate, and dimethylsulfoxide were shown by the modified CUPRAC assay to be more effective scavengers than mannitol, glucose, lysine, and simple alcohols, as in the TBARS assay.

The method developed is less lengthy, more specific, and of a higher yield than the classical TBARS assay. The hydroxyl-radical scavenging rate constants of ascorbic acid, formate, and hexacyanoferrate(II) that caused interference in other assays could easily be found with the proposed procedure.

2.1.5. Hydroxyl-radical scavenging antioxidant activity of phenolics and flavonoids. Detection of hydroxyl radicals and measurement of hydroxyl-radical scavenging activity is very important in food and bioanalytical chemistry in regard to anti-radical and antioxidant activity of foodstuffs and antioxidant therapy. The widely-used TBARS colorimetric assay has been greatly criticized for its low specificity and low yield, while the HPLC procedure, though much more reliable, is lengthy and costly, and requires sophisticated instrumentation and trained personnel. The proposed CUPRAC/salicylate assay for ·OH detection is much more efficient than the conventional TBARS assay, as the conversion ratio of the probe is much higher [27] (see Fig. 3 for the mechanism of this modified CUPRAC method).

Since one of the most effective, sensitive indicators of hydroxyl-radical formation in biological fluids is a salicylate probe, separation of the dihydroxybenzoate (DHBA) isomers {major hydroxylation products: 2,3-DHBA, 2,4-DHBA, 2,5-DHBA and 2,6-DHBA) formed from salicylate [37]} was performed with HPLC. Salicylate was preferred as the ·OH-trapping agent in the colorimetric assay developed, because it had a very low CUPRAC absorbance and its definitely known, stable hydroxylation products (DHBAs) had relatively high CUPRAC absorbances. The most important contribution of the assay developed was stopping the Fenton reaction in 10 min with catalase degradation of hydrogen peroxide so that the remaining H_2O_2 would not give a CUPRAC absorbance or be involved in redox cycling of phenolic antioxidants. This enabled rapid, precise measurement of ·OH scavenging rate constants of polyphenolics that cannot be found by most other techniques (Table 2).

2.1.6. Measurement of xanthine-oxidase inhibition activity of phenolics and flavonoids. The idea in this modification

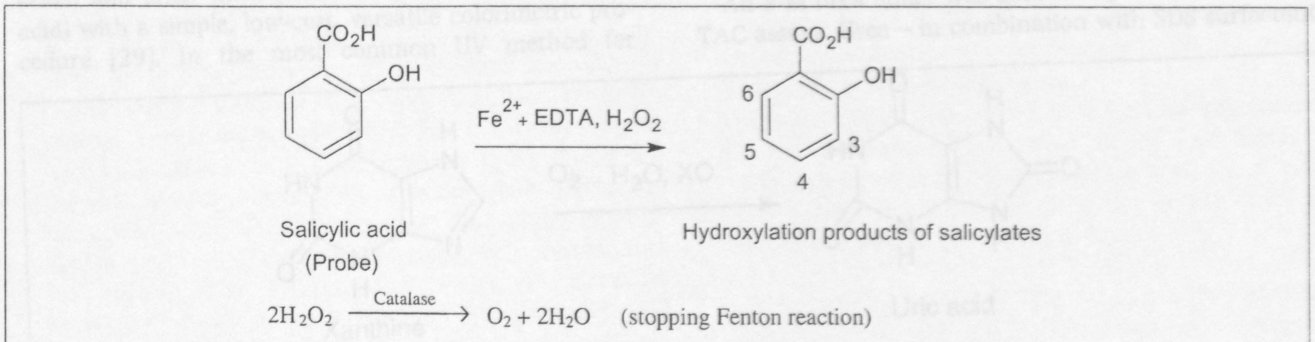

Figure 3. Major hydroxylation products formed from a salicylic-acid probe upon the attack of ·OH radicals: 3-OH: 2,3-DHBA; 4-OH: 2,4-DHBA; 5-OH: 2,5-DHBA; 6-OH: 2,6-DHBA.

was to use the xanthine-xanthine oxidase (X-XO) system for an XO-inhibitory activity assay of polyphenolics (i.e. flavonoids, simple phenolic acids, and hydroxycinnamic acids) and other antioxidant compounds (e.g., ascorbic acid) [28]. As an antioxidant-activity assay, XO activity has usually been determined by following the rate of uric-acid formation from X-XO system by making use of the UV absorbance at 295 nm of uric acid formed as the reaction product. Since polyphenolics have strong UV absorption, XO-inhibitory activity of polyphenolics was otherwise determined without interference by directly measuring the formation of uric acid and hydrogen peroxide using the modified CUPRAC spectrophotometric method at 450 nm (see Fig. 4 for the mechanism of this modified CUPRAC method).

The CUPRAC absorbance of the incubation solution due to the reduction of Cu(II)-neocuproine reagent by the products of the X-XO system decreased in the presence of polyphenolics, the difference being proportional to the XO-inhibition ability of the compound being tested. The assay developed was validated against classical UV and HPLC assays of uric acid, and was applied to the measurement of XO-inhibitory activity of herbal extracts. The proposed spectrophotometric method was practical, low cost, rapid, and less open to interferences by UV-absorbing substances, and could reliably assay uric acid and hydrogen peroxide in the presence of polyphenols (flavonoids, simple phenolic acids and hydroxycinnamic acids). The fact that the analyzed real samples (such as plant extracts) normally react with the CUPRAC reagent at elevated concentrations was not important for the proposed method, as long as sufficiently small amounts of these samples were taken to measure XO inhibition (see Table 2 for 50% inhibitive concentrations: IC_{50} of antioxidants).

2.1.7. Hydrogen-peroxide scavenging antioxidant activity of phenolics and flavonoids. Measurement of hydrogen-peroxide scavenging (HPS) activity is considered to be important in food and bioanalytical chemistry. It was aimed to measure the HPS activity of polyphenols (i.e. flavonoids, simple phenolic acids, and hydroxycinnamic acids) and other antioxidant compounds (e.g. ascorbic acid) with a simple, low-cost, versatile colorimetric procedure [29]. In the most common UV method for determination of HPS activity, scavenging ability depends on the change of the absorbance value at 230 nm when H_2O_2 concentration is decreased by scavenger compounds. Since the UV method suffers from the interference of some phenolics in real samples having strong absorption in the UV region and from inefficient degradation of H_2O_2 with polyphenols in the absence of Cu(II) (since H_2O_2 is relatively stable, and not scavenged unless transition-metal compounds are present as catalysts), HPS activity of polyphenols was otherwise determined without interference by directly measuring the concentration of undegraded H_2O_2 using the modified CUPRAC method in the presence of a Cu(II) catalyst (Table 2).

The modified CUPRAC method for HPS activity was based on the incubation of a scavenger with H_2O_2 and analyzing the reaction mixture for the loss of H_2O_2. The proposed methodology is also superior to the rather non-specific horseradish peroxidase (HRP)-based assays. Some H_2O_2 scavengers also interacted with HRP, an enzyme that is expensive and unstable in solution.

The proposed modified CUPRAC method can be considered as a low-cost, precise and accurate alternative for measuring the HPS activity of polyphenols and natural antioxidants in plant extracts. The method was validated against a HPLC-ECD and GSH-Px-DTNB (GSH peroxidase-5,5′-dithio-bis(2-nitrobenzoic acid)) spectrophotometric method of H_2O_2 measurement.

2.1.8. Antioxidant-capacity assay of thiol-containing proteins. In most assays measuring TAC, proteins are not taken into account (e.g., in assays carried out in the hydrophilic fraction of human serum) and remain in the precipitate (e.g., obtained by using perchloric acid, trichloroacetic acid, and ammonium sulfate). The modified CUPRAC assay for proteins [30] has verified that the contribution of proteins, especially thiol-containing proteins, to the observed TAC is by no means negligible. Various protein fractions (egg white, whey proteins, gelatin) and peptides (e.g., GSH) may either respond to TAC assays directly, *via* their free –SH groups, or indirectly, after protein denaturation through their exposed (originally buried) thiol groups.

An 8-M urea buffer was used to expose these thiols to TAC assays. Urea – in combination with SDS surfactant

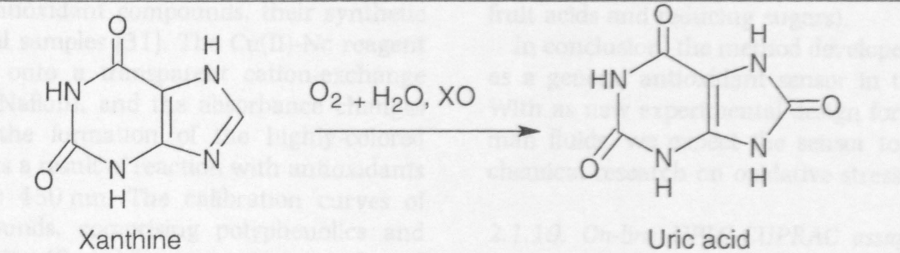

Figure 4. Uric-acid formation from xanthine-xanthine oxidase system.

Table 2. Antioxidant activities of various antioxidant compounds (•OH and H_2O_2 scavenging, XO activity) found by the related modified CUPRAC methods

Antioxidants	Rate const. with respect to CUPRAC Method (M^{-1} s^{-1}) [27]	IC_{50} value with respect to CUPRAC Method (μM) [28]	HPS value (%) with respect to HPS-CUPRAC Method [29]
Simple phenolic acids			
Gallic acid	3.55×10^{12}	1.84	62.8 ± 0.9
Hydroxycinnamic acids			
Rosmarinic acid	3.66×10^{12}	2.05	26.0 ± 0.5
p-Coumaric acid	2.61×10^{12}	1.71	10.6 ± 0.3
Flavan-3-ols			
(-) Epigallocatechin gallate	3.23×10^{12}	2.33	42.9 ± 0.5
(+) Catechin	2.78×10^{12}	1.96	14.6 ± 1.9
Flavons			
Apigenin	3.87×10^{12}	1.46	2.5 ± 0.1
Flavonols			
Quercetin	5.52×10^{12}	1.69	47.1 ± 2.0
Kaempferol	2.78×10^{12}	1.87	31.7 ± 0.4
Flavanons			
Naringin	-	18.00	N.D.*
Vitamins			
Ascorbic acid	0.27×10^{8}	N.D.*	17.5 ± 0.5

*N.D.: HPS and XO activity at the level of studied concentrations could not be detected.

– maximized the reactivity of thiols and disulfides that may be buried within the protein matrix. Urea partly denaturated proteins and significantly lowered the reduction potential of disulfide/thiol couples in peptides facilitating thiol oxidizability.

Among the tested TAC assays, only CUPRAC and ABTS/H_2O_2/HRP possessed the property of optical-absorbance additivity for protein thiols (i.e. obeying Beer's law). This study [30] reports for the first time the measurement of the TAC of thiol-containing proteins in admixture with phenolic antioxidants after taking up the protein fractions with a suitable buffer that does not cause precipitation of proteins or interfere with the selected antioxidant assay (specifically, the CUPRAC assay). We expect this approach to be useful for estimating the TAC values and hence food quality of dairy products and other protein-containing foods in further studies.

2.1.9. CUPRAC antioxidant sensor. A low-cost optical sensor was developed, using an immobilized chromogenic reagent, copper(II)-neocuproine (Cu(II)-Nc) complex, for the assessment of antioxidant capacity of non-enzymatic antioxidant compounds, their synthetic mixtures, and real samples [31]. The Cu(II)-Nc reagent was immobilized onto a transparent cation-exchange polymer matrix (Nafion), and the absorbance changes associated with the formation of the highly-colored Cu(I)-Nc chelate as a result of reaction with antioxidants was measured at 450 nm. The calibration curves of individual compounds, comprising polyphenolics and antioxidant vitamins (C and E), were constructed, and their molar absorptivities and linear concentration ranges determined. The CUPRAC sensor gave a linear response over a relatively wide range of antioxidant concentrations, unlike many electrochemical sensors that yielded non-linear responses. The immobilized reagent retained its reactivity towards antioxidants, and the measured TEAC values of various antioxidants suggested that the reactivity of the immobilized CUPRAC reagent was comparable to that of the standard solution-based reagent.

Testing of synthetic ternary mixtures of antioxidants yielded the theoretically expected CUPRAC antioxidant capacities, considering the additivity of absorbances of constituents following Beer's law. This assay was validated through linearity, additivity, precision and recovery, demonstrating that it was reliable and robust.

The sensor developed was used to screen TACs of some commercial fruit juices (e.g., orange, cherry, peach, and apricot). Experimental results showed that the low-cost optical sensor was an effective tool in measurement of TAC values of food and plant samples without requiring pretreatment. Interference studies proved that the optical-sensor-based CUPRAC assay was not adversely affected by common food ingredients (e.g., citrate, oxalate, fruit acids and reducing sugars).

In conclusion, the method developed has potential use as a general antioxidant sensor in the food industries. With as new experimental design for application to human fluids, we expect the sensor to be useful for biochemical research on oxidative stress.

2.1.10. On-line HPLC-CUPRAC assay with post-column detection. The aim of this modification was design of a post-column derivatization system using CUPRAC assay for extensive, rapid screening of potential sources of

antioxidants [32]. An on-line method (on-line HPLC-CUPRAC) to detect and to quantify polyphenols (flavonoids, simple phenolic and hydroxycinnamic acids) in complex plant extracts was therefore developed as a combination of conventional HPLC separation and post-column reaction with Cu(II)-Nc reagent at neutral pH.

Sample analytes were chromatographed by HPLC, and the post-column formation of Cu(I)-Nc chelate from the CUPRAC reaction of antioxidants in a reactor coil was detected at 450 nm with an on-line UV-vis detector (see Fig. 5 for the chromatogram of TR). An optimized instrumental system was set up using standard polyphenolic compounds, and it was successfully tested with complex plant extracts, including *Camellia sinensis*, *Origanum marjorana* and *Mentha*, where several tea catechins and other polyphenolic compounds were identified.

The new method was validated by comparing TAC results of synthetic and real antioxidant mixtures with those of a conventional HPLC method (with UV detection at 280 nm) and the spectrophotometric CUPRAC method. The main advantage of the spectrophotometric CUPRAC method is that antioxidants from a complex sample extract can be qualitatively and quantitatively determined on-line from a chromatogram and the individual antioxidant capacities contribute to the observed TAC, whereas those extract constituents not possessing antioxidant character (e.g., caffeine) do not yield peaks in the "negative trace" chromatogram of post-column HPLC. It is also rapid, reproducible, and uses inexpensive, stable reagents.

In conclusion, the results suggested that the proposed on-line HPLC-CUPRAC method can be a powerful instrumental tool for detection, separation, and characterization of natural antioxidants. Future applications of this methodology may cover authentication of natural dyes and dye plants and phenolic profiling of food-plant extracts, as it enables a complete fingerprinting of antioxidants, regardless of inadequacies in rigorously identifying these constituents with HPLC-DAD techniques.

2.2. Noteworthy experiences of other CUPRAC users

To date, many research groups dealing with antioxidant chemistry have used or evaluated the main or modified CUPRAC methods.

In a comprehensive review, Prior et al. [4] classified CUPRAC as one of the electron-transfer-based methods, and summarized the advantages of the CUPRAC method over other antioxidant assays. They stated that, due to the lower redox potential of the CUPRAC reagent, reducing sugars and citric acid – which are not true antioxidants but oxidizable substrates in other similar assays – are not oxidized with the CUPRAC reagent.

Gorinstein et al. [38] stated, as an advantage over other electron-transfer-based assays (e.g., ABTS and

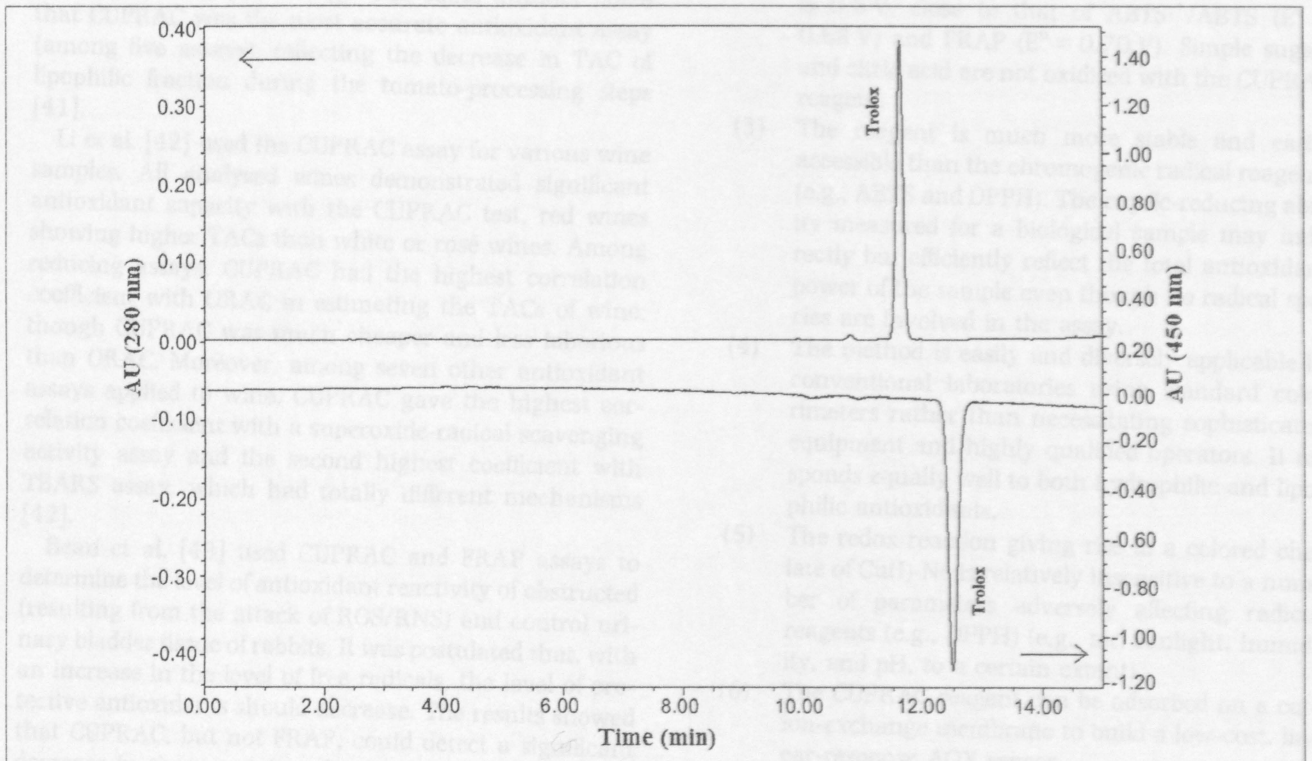

Figure 5. The chromatograms of Trolox at 280 nm (positive trace) by conventional HPLC and at 450 nm (negative trace) by on-line post-column HPLC.

Folin), CUPRAC had values that were acceptable in regard to its realistic pH close to physiological pH. Gorinstein's group generally found higher TAC values with CUPRAC than FRAP for garlic and onion rich in antioxidant thiols.

Min and Ahn [39] applied the CUPRAC TAC assay to various fractions of meat homogenates, and showed that the CUPRAC TAC level of chicken breast was maintained during storage. The CUPRAC assay could measure the higher ratio of TAC in the high-molecular-weight fraction compared with the low-molecular-weight fraction in both meats, meaning that CUPRAC could also effectively measure the TAC of proteins and peptides. By contrast, a large part of the TAC in each fraction could not participate in the reduction of Fe(III) to Fe(II), as assayed by the ferric-ion reducing ferrozine spectrophotometric method [39].

Mazor et al. [40] applied CUPRAC and FRAP simultaneously to a number of –SH compounds, and noted that CUPRAC, but not FRAP, could quantify the one thiol-bearing tripeptide GSH in accordance with the 1-e reductant behavior of GSH.

Capanoglu et al. stressed that, during the processing of tomato fruit to tomato paste, CUPRAC antioxidant capacities were the highest values for lipophilic extracts, indicating that this is a sensitive assay in organic solvents (e.g., CUPRAC values broadly followed the trend of lycopene during processing). The same authors noted that CUPRAC was the most accurate antioxidant assay (among five assays), reflecting the decrease in TAC of lipophilic fraction during the tomato-processing steps [41].

Li et al. [42] used the CUPRAC assay for various wine samples. All analyzed wines demonstrated significant antioxidant capacity with the CUPRAC test, red wines showing higher TACs than white or rosé wines. Among reducing assays, CUPRAC had the highest correlation coefficient with ORAC in estimating the TACs of wine, though CUPRAC was much cheaper and less laborious than ORAC. Moreover, among seven other antioxidant assays applied to wine, CUPRAC gave the highest correlation coefficient with a superoxide-radical scavenging activity assay and the second highest coefficient with TBARS assay, which had totally different mechanisms [42].

Bean et al. [43] used CUPRAC and FRAP assays to determine the level of antioxidant reactivity of obstructed (resulting from the attack of ROS/RNS) and control urinary bladder tissue of rabbits. It was postulated that, with an increase in the level of free radicals, the level of protective antioxidants should decrease. The results showed that CUPRAC, but not FRAP, could detect a significant decrease in the reactivity of antioxidants found within the obstructed bladder tissue as compared to the control bladder tissue in both the muscle and mucosa. The authors concluded that, as the CUPRAC assay was responsive to hydrophilic, lipophilic, and thiol-containing antioxidants at physiological pH, it was a much better tool to analyze the reactivity found within tissues [43].

3. Summary

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

(1) The CUPRAC reagent (an outer-sphere e-transfer agent) is fast enough to oxidize thiol-type antioxidants, whereas the FRAP method may only measure with serious negative error certain thiol-type antioxidants like GSH (i.e. the major low-molecular-weight thiol peptide of the living cell). Since the redox potential of oxidized GSH (GSSG) or GSH is the basic indicator of biological conditions of a cell, and GSH acts as reconstituent of intercellular ascorbic acid from the dehydroascorbic acid, a non-enzymatic antioxidant assay has to measure GSH reliably.

(2) The reagent is selective, because it has a lower redox potential than that of the ferric/ferrous couple in the presence of *ortho*-phenanthroline-type or *batho*-phenanthroline-type ligands. The standard potential of the Cu(II,I)-Nc redox couple is 0.6 V, close to that of $ABTS^{\cdot+}$/ABTS (E° = 0.68 V) and FRAP (E° = 0.70 V). Simple sugars and citric acid are not oxidized with the CUPRAC reagent.

(3) The reagent is much more stable and easily accessible than the chromogenic radical reagents (e.g., ABTS and DPPH). The cupric-reducing ability measured for a biological sample may indirectly but efficiently reflect the total antioxidant power of the sample even though no radical species are involved in the assay.

(4) The method is easily and diversely applicable in conventional laboratories using standard colorimeters rather than necessitating sophisticated equipment and highly qualified operators. It responds equally well to both hydrophilic and lipophilic antioxidants.

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

(6) The CUPRAC reagent can be adsorbed on a cation-exchange membrane to build a low-cost, linear-response AOX sensor.

(7) The CUPRAC absorbance *versus* concentration curves are perfectly linear over a wide concentration range, unlike those of other methods yielding polynomial curves. The molar absorptivity

for n-e reductants, 7.5–9.5x10^3 nL/(mol/cm), is sufficiently high to determine most phenolic antioxidants sensitively.

(8) The TAC values of antioxidants found with CUPRAC are perfectly additive (i.e. the TAC of a phenolic mixture is equal to the sum of individual AOCs of its constituent polyphenols). Additivity in other antioxidant measurements is not strictly valid.

(9) HPLC/post-column CUPRAC is possible (direct methods of antioxidant assay involving ROS/RNS are unsuitable for post-column applications).

(10) 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 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. Thus, excluding the complications arising from the differences in bioavailability, absorption, distribution and metabolism of polyphenolic antioxidants under physiological conditions, CUPRAC gives a better estimate of *in vivo* antioxidant behavior.

(11) Since the Cu(I) ion emerging as a product of the CUPRAC redox reaction is in a 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 ·OH radicals as a result of its reaction with H_2O_2, and thereby cause a "redox cycling" of antioxidants during the assay, subsequently yielding unreliable results. We showed that the stable Cu(I)-chelate did not react with hydrogen peroxide, but the reverse reaction (i.e. oxidation of H_2O_2 with Cu(II)-Nc) is possible.

(12) It should be borne in mind that strong Cu(II)-chelators (e.g., EDTA) and strong reductants (other than antioxidants) should not be present in solutions tested by the CUPRAC method. As possible measures to avoid these interferences, citrate may be added to serum (instead of EDTA) for preservation, and reductants other than ascorbic acid or polyphenolic compounds (e.g., sulfite in sulfur dioxide-fumigated apricot extract) should be removed from solution before testing (e.g., as HSO_3^-, which can be retained on an anion-exchange column at pH 3).

In conclusion, the CUPRAC methodology is evolving into an "antioxidant-measurement package" in biochemistry and food chemistry comprising many assays, and the validated results seem to have shown distinct advantages over certain established methods. By maintaining the CUPRAC reagent and related chemicals in the laboratory, one can measure ROS scavenging activity and TAC of antioxidants.

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