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Mechanism of antioxidant capacity assays and the CUPRAC (cupric ion reducing antioxidant capacity) assay

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Abstract. We report on the application of a simple and versatile antioxidant capacity assay for dietary polyphenols, vitamin C and vitamin E utilizing the copper(II)-neocuproine (Cu(II)-Nc) reagent as the chromogenic oxidant, which we term the CUPRAC (cupric reducing antioxidant capacity) method. It involves mixing the antioxidant solution (directly or after acid hydrolysis) with solutions of $CuCl_2$, neocuproine, and ammonium acetate at pH 7, and measuring the absorbance at 450 nm after 30 min. Slowly reacting antioxidants required an incubation at 50 °C for 20 min for color development. The flavonoid glycosides were hydrolyzed to their corresponding aglycones by refluxing in 1.2 M HCl-containing 50% MeOH for fully exhibiting their antioxidant potencies. Certain compounds also needed incubation after acid hydrolysis for color development. The CUPRAC absorbances of mixture constituents were additive, indicating lack of chemical deviations from Beer's law. The CUPRAC antioxidant capacities of a wide range of polyphenolics are reported in this work and compared to those found by ABTS/persulfate and Folin assays. The trolox-equivalent capacities of the antioxidants were linearly correlated ($r = 0.8$) to those found by ABTS but not to those of Folin. The highest antioxidant capacities in the CUPRAC method were observed for epicatechin gallate, epigallocatechin gallate, quercetin, fisetin, epigallocatechin, catechin, caffeic acid, epicatechin, gallic acid, rutin, and chlorogenic acid in this order, in accordance with theoretical expectations. The experiences of other CUPRAC users also are summarized.

Keywords: CUPRAC antioxidant capacity; dietary flavonoids; polyphenols; copper(II)-neocuproine; spectrophotometry

Reactive oxygen species (ROS) that emerge as a result of the respirative cycle of oxidative phosphorylation may attack biological macromolecules like cellular DNA and proteins. Excessive ROS may give rise to single- and double-strand DNA breaks that may eventually cause cell ageing, cardiovascular diseases, mutagenic changes and cancerous tumor growth [1, 2]. Consumption of foods naturally bearing antioxidant power is the most efficient way of combating such undesired transformations and health risks. Consequently, the opportunity for improving health by improving diet is great [3].

The chemical diversity of antioxidants makes it difficult to separate and quantify individual antioxidants (i.e., parent compounds, glycosides, polymers, and many isomers) from the vegetable matrix. Moreover, the total antioxidant power is often more meaningful to evaluate health beneficial effects because of the cooperative action of antioxidants. Therefore it is desirable to establish a method that can measure the total anti-

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oxidant capacity level directly from vegetable extracts [4]. Antioxidant capacity assays may be broadly classified as electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays [5, 6].

The majority of HAT-based assays involve a reaction scheme in which antioxidant and substrate compete for thermally generated peroxyl radicals through the decomposition of azo compounds. These assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays [5, 6]. Most of these assays are kinetic-based, meaning that they are more concerned with the rate rather than thermodynamic conversion efficiency of the radical reaction with the antioxidant. An exceptional assay is ORAC [7] which deals with both kinetic and thermodynamic aspects of the reaction and reports results based on the net area under curve (AUC) of the fluorescence decay/time curve of the probe in the presence and absence of antioxidants.

On the other hand, ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced [5, 6]. The degree of colour change is correlated to the concentration of antioxidants in the sample. These assays generally set a fixed time for the concerned redox reaction, and measure thermodynamic conversion during that period. ET-based assays include ABTS/TEAC [8], DPPH [9], Folin-Ciocalteu (FCR) [10, 11], and FRAP [12], using different chromogenic redox reagents with different standard potentials. Although the reducing capacity of a sample is not directly related to its radical scavenging capability, it is a very important parameter of antioxidants.

This work reports the application of a simple, versatile, and low-cost antioxidant capacity assay for dietary polyphenols, vitamins C and E, utilizing the copper(II)-neocuproine (Cu(II)-Nc) reagent as the chromogenic oxidizing agent, named by our research group as the CUPRAC (cupric ion reducing antioxidant capacity) method [13, 14]. Another aim is to tabulate the CUPRAC antioxidant capacities of a wide range of polyphenolics and flavonoids as trolox equivalents (TEAC), and compare the results to those found by reference methods, ABTS/persulfate [15] and Folin [11] antioxidant assays. The advantages of the CUPRAC method over similar ET-based methods were summarized, and the experiences of other CUPRAC users shared.

Experimental

Instrumentation

All spectrophotometric measurements were made with a pair of matched quartz cuvettes using a CARY 1E UV-Vis spectrophotometer. The pH measurements were made with the aid of a E512 Metrohm Herisau pH-meter using a glass electrode.

Reagents

The flavonoids, fisetin, quercetin, rutin, naringin, naringenin, (−)epicatechin, (−)epigallocatechin, (−)epicatechin gallate, (−)epigallocatechin gallate, morin, neocuproine (2,9-dimethyl-1,10-phenanthroline), vanilic acid, hesperetin, chlorogenic acid, syringic acid, Folin-Ciocalteu reagent were purchased from Sigma Chemical Co.; (−)catechin, gallic acid, hesperidin, ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) from Fluka Chemicals; ferulic acid, coumaric acid, caffeic acid, ascorbic acid and trolox were supplied from Aldrich Chemicals Co. (the above chemicals are currently available from www.sigmaaldrich.com). α-Tocopherol, ammonium acetate, copper(II) chloride, sodium hydroxide, sodium carbonate, copper(II) sulfate, potassium persulfate, methanol, sodium potassium tartarate and 96% EtOH were from E. Merck (www.merck.de).

Preparation of solutions

$CuCl_2$ solution, 1.0×10^{-2} M, was prepared by dissolving 0.4262 g $CuCl_2 \cdot 2H_2O$ in water, and diluting to 250 mL. Ammonium acetate buffer at pH = 7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in water and diluting to 250 mL. Neocuproine (Nc) solution, 7.5×10^{-3} M, was prepared daily by dissolving 0.039 g Nc in 96% ethanol, and diluting to 25 mL with ethanol. Trolox, 1.0×10^{-3} M, was prepared in 96% ethanol. The chromogenic radical reagent ABTS, at 7.0 mM concentration, was prepared by dissolving 0.1920 g of the compound in water, and diluting to 50 mL. To this solution was added 0.0331 g $K_2S_2O_8$ such that the final persulfate concentration in the mixture be 2.45 mM. The resulting ABTS radical cation solution was left to mature at room temperature in the dark for 12–16 h, and then used for TEAC assays. The solutions used in the Folin assay of polyphenolics were prepared as follows: Lowry A: 2% aqueous Na_2CO_3 in 0.1 M NaOH; Lowry B: 0.5% $CuSO_4$ aqueous solution in 1% $NaKC_4H_4O_6$ solution; Lowry C: prepared freshly as mixture (50 mL Lowry A + 1 mL Lowry B); Folin-Ciocalteau reagent was diluted with H_2O at a volume ratio of 1:3 prior to use. All percentages are given as (w/v), and distilled and deaerated (N_2-bubbled) water was used throughout.

CUPRAC assay of total antioxidant capacity

The CUPRAC method was applied as four interrelated procedures, i.e., normal (N), incubated (I), hydrolyzed (H), and hydrolyzed & incubated (H&I) versions of the assay, depending on the nature of sample [13]. The standard procedure that has to be applied for completing all procedures during the development of final color is summarized below:

Add 1 mL 10^{-2} M Cu^{2+} + 1 mL 7.5×10^{-3} M neocuproine + 1 mL 1 M NH_4Ac + x mL 10^{-3} M antioxidant neutral solution + (1.1 − x) H_2O; $V_T = 4.1$ mL; measure final absorbance at 450 nm

The experimental conditions of each procedure are briefly described in Table 1.

Table 1. The reaction conditions of normal, incubated, hydrolyzed and (hydrolyzed & incubated) CUPRAC procedures

Method	Medium	Temp. (°C)	Time (min)	Operation before standard procedure
Normal (N)	aq. solution with varying % alcohol	25	30	no operation needed
Incubated (I)	aq. solution with varying % alcohol	50	20	cool to 25 °C
Hydrolyzed (H)	1:1 (v/v) MeOH–H_2O contg. 1.2 M HCl	80	120	neutralize and cool to 25 °C
Hydrolyzed and incubated (H&I)	1:1 (v/v) MeOH–H_2O contg. 1.2 M HCl	80	120	incubate, neutralize and cool

Calculation of antioxidant capacity of phenolic antioxidants as trolox equivalents (TEAC values) in the CUPRAC method

Sample calculation:
ε_{TR}: $1.67 \times 10^4\,L\,mol^{-1}\,cm^{-1}$
ε_{QR}: $7.3 \times 10^4\,L\,mol^{-1}\,cm^{-1}$
$TEAC_{QR} = \varepsilon_{QR}/\varepsilon_{TR} = 7.3 \times 10^4/1.67 \times 10^4 = 4.38$

ABTS assay of total antioxidant capacity

The matured ABTS radical solution of blue-green colour 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 1 mL of the radical cation solution, 4 mL of ethanol were added, and the absorbance at 734 nm was read at the end of the first and sixth minute. The procedure was repeated for the unknown extract by adding 1 mL of the radical cation solution to (x) mL of antioxidant solution and $(4.0 - x)$ mL of ethanol, and recording the absorbance readings at the end of first and sixth minutes. The absorbance difference (ΔA) was found by subtracting the extract absorbance from that of the reagent blank (pure radical solution). This was correlated to trolox equivalent antioxidant concentration with the aid of a linear calibration curve (usually the absorbance decrease at the 6th minute was used for calculations).

Folin-Ciocalteu reagent (FCR) assay of total phenolics

To (x) mL of the antioxidant solution was added $(2 - x)$ mL H_2O. An aliquot of 2.5 mL of Lowry C solution was added, and the mixture was let to stand for 10 min. At the end of this period, 0.25 mL of Folin reagent was added, and 30 more min was allowed for stabilization of the blue colour formed. The absorbance against a reagent blank was measured at 750 nm.

Results and discussion

The chromogenic redox reagent used for the CUPRAC assay was bis(neocuproine) copper(II) chelate. This reagent was useful at pH 7, and the absorbance of the Cu(I)-chelate formed as a result of redox reaction with reducing polyphenols was measured at 450 nm. The color was due to the Cu(I)-Nc chelate formed. The reaction conditions such as reagent concentration, pH, and oxidation time at room and elevated temperatures were optimized as shown elsewhere [13, 14].

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

$$\mathrm{nCu(Nc)_2^{2+}} + \text{n-electron reductant (AO)} \rightarrow \mathrm{nCu(Nc)_2^{+}} + \text{n-electron oxidized product} + \mathrm{nH^+}$$

In this reaction, the reactive Ar-OH groups of polyphenolic antioxidants are oxidized to the corresponding quinones (ascorbic acid is oxidized to dehydroascorbic acid) and Cu(II)-Nc is reduced to the highly colored Cu(I)-Nc chelate showing maximum absorption at 450 nm. Although the concentration of Cu^{2+} ions was in stoichiometric excess of that of neocuproine in the CUPRAC reagent for driving the above redox equilibrium reaction to the right, the actual oxidant was the $Cu(Nc)_2^{2+}$ species and not the sole Cu^{2+}, because the standard redox potential of the Cu(II/I)-neocuproine was 0.6 V, much higher that that of Cu^{2+}/Cu^{+} couple (0.17 V) [14]. As a result, polyphenols were oxidized much more rapidly and efficiently with Cu(II)-Nc than with Cu^{2+}, and the chromogen (i.e., $Cu(Nc)_2^{+}$) emerging at the end of the redox reaction was equivalent to the reacted Cu(II)-Nc. The liberated protons are buffered in NH_4Ac medium. In the normal (N) CUPRAC method, the oxidation reactions were essentially complete within 30 min. Flavonoid glycosides needed to be hydrolyzed to their corresponding aglycons for fully exhibiting their antioxidant potency. Slow reacting antioxidants needed elevated temperature incubation so as to complete their oxidation with the CUPRAC reagent [13, 14]. Special precautions to exclude oxygen from the freshly prepared and analyzed solutions of pure antioxidants were not necessary since oxidation reactions with the CUPRAC reagent were much more rapid than with dissolved O_2 (i.e., the latter would not appreciably occur unless suitable catalysts were present). 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 make take place in real systems (results not shown).

The TEAC coefficients (i.e., the reducing potency – in trolox mM equivalents – of 1 mM antioxidant solution under investigation) of various antioxidant compounds found with the developed CUPRAC method and compared to those measured with the reference methods: ABTS/persulfate and Folin (FCR) are tabulated in Table 2. The linear calibration curves of the tested antioxidants as absorbance *vs* concentration with respect to the CUPRAC method (figures not shown) generally gave correlation coefficients close to 1, i.e., $r \geq 0.999$, within the absorbance range of 0.1–1.2. The results obtained with the Folin method were generally higher than with others, because the essential component of the FCR reagent, i.e., molybdo-phospho-tungstate heteropoly acid, had a much higher redox potential than those of the other reagents which lie in the range of $E^0 = 0.6$–0.7 V. The highest antioxidant capacities in the CUPRAC method were observed for epicatechin gallate, epigallocatechin gallate, quercetin, fisetin, epigallocatechin, catechin, caffeic acid, epicatechin, gallic acid, rutin, and chlorogenic acid in this order, in accordance with theoretical expectations, because the number and position of the hydroxyl groups as well as the degree of conjugation of the whole molecule are important for electron transfer.

A novel antioxidant capacity assay that is expected to find serious use in method standardization has to pay attention to some structural requirements of antioxidant potency. For example, the presence of 5-hydroxy-4-keto group in A & C rings in flavonols, the 2,3-double bond connecting the two ring systems of flavonol *via* conjugation, and the 3′,4′-dihydroxy substitution of the B ring are considered as important structural characteristics for antioxidant potency [16], all three of which are combined in quercetin. As a result, the TEAC coefficient in the CUPRAC assay was highest among flavonols for quercetin. Fisetin had one –OH group less than quercetin, and therefore gave the lower TEAC value (Table 2). Rutin, having an O-rutinase substituent instead of –OH in the 3-position, showed the lower capacity. In general, when flavonoid glycosides were hydrolyzed to the corresponding aglycons (i.e., O-sugar substituent being converted to –OH), their CUPRAC antioxidant capacities significantly improved. As for hydroxycinnamic acids which are almost the most abundant phenolic components in the citrus family and in some other fruits, the TEAC coefficients with respect to the CUPRAC method (and with respect to the ABTS assay, as shown in parantheses) were as follows: caffeic

Table 2. The trolox equivalent antioxidant capacities of various antioxidant compounds calculated with respect to the ABTS/persulphate method, FOLIN method and the developed (CUPRAC) method

Antioxidant	$TEAC_{CUPRAC}$				$TEAC_{ABTS}$		$TEAC_{FOLIN}$
	$TEAC_N$	$TEAC_I$	$TEAC_H$	$TEAC_{H\&I}$	$TEAC_{1\,min}$	$TEAC_{6\,min}$	
Quercetin (QR)	4.38				2.75	2.77	5.17
Catechin (CT)	3.09	3.56	3.08	3.49	3.05	3.14	4.09
Rutin (RT)	2.56			3.80	1.00	1.15	6.75
Gallic acid(GA)	2.62				3.65	3.48	1.23
Fisetin (FS)	3.90	4.18			2.59	2.62	3.90
Naringin (N)	0.02	0.13			0.50	0.62	1.12
Naringenin (NG)		2.28		3.03	0.56	0.64	5.52
Ascorbic acid (AA)	0.96				1.02	1.03	
Ferulic acid (FRA)	1.20	1.23	1.18	1.34	2.01	2.16	3.49
p-Coumaric acid (CMA)	0.55	1.00	0.53	1.15	1.75	1.63	2.54
Caffeic acid (CFA)	2.89	2.96	2.87	3.22	1.33	1.39	3.27
Chlorogenic acid (CGA)	2.47	2.72	1.20	1.42	1.22	1.21	2.84
α-Tocopherol (TP)	1.10	1.02	0.99	0.87	1.00	1.02	
Epicatechin (EC)	2.77	2.89			2.98	2.69	3.22
Epicatechin gallate (ECG)	5.32	5.65			2.95	3.51	4.35
Epigallocatechin (EGC)	3.35	3.60					
Epigallocatechin gallate (EGCG)	4.89	5.49			2.72	3.15	2.78
Hesperidin (HD)	0.97	1.11	0.79	0.95	1.05	1.40	3.29
Hesperetin (HT)	0.99	1.05	0.85	0.98	1.01	1.11	4.50
Morin (MR)	1.88	3.32			1.28	1.79	3.37
Syringic acid (SA)	1.12	1.64	1.13	1.67	1.38	1.50	2.49
Vanilic acid (VA)	1.24	1.52	1.32	1.57	1.03	1.25	3.05

acid 2.9 (1.4), chlorogenic acid 2.5 (1.2), ferulic acid 1.2 (2.2), and p-coumaric acid 0.6 (1.6). The trolox equivalent capacity order for these phenolic acids was just the opposite of that of the most widely used ABTS assay [16]. Structural properties of phenolic (hydroxycinnamic) acids should normally dictate that two –OH bearing caffeic and chlorogenic acids should exhibit higher TEAC coefficients than monophenolic (one –OH bearing) ferulic and p-coumaric acids. Furthermore, ferulic acid having an electron-donating methoxy group in ortho-position to the phenolic –OH, thereby allowing increased stabilization of the resulting aryloxyl radical through electron delocalization after H-atom donation by the hydroxyl group, should show a higher TEAC coefficient than p-coumaric acid which lacks such a group. Thus structural requirements dictate that hydroxycinnamic acids should have a TEAC order as measured by the CUPRAC and not by the ABTS assay. Moreover, the order of peroxyl radical scavenging ability of hydroxycinnamic acids, and thus the order for their ability to enhance the resistance of LDL to oxidation, was measured as caffeic acid > chlorogenic acid > ferulic acid > p-coumaric acid [16–18], again entirely consistent with the results of the CUPRAC method. Gallic acid had one more –OH group than chlorogenic acid, and therefore it showed the higher capacity. The catechin group, also known as ''tea antioxidants'', gave a capacity order in accord with the number and position of their –OH groups, together with the overall extent of conjugation in the molecule. Inspection of Table 2 reveals that all these structural requirements for antioxidant potency are met by the TEAC results of the CUPRAC assay. The TEAC values found with CUPRAC correlated linearly ($r = 0.8$) to those of ABTS but the correlation of both assays to Folin were poor, because both CUPRAC and ABTS were similar ET-based antioxidant assays with close reduction potentials while the exact potential of the Folin reagent with the presumably higher potential is not definitely known. As a result, the Folin assay originally thought to be specific for phenolics [11] should oxidize the tested samples to a greater extent than either CUPRAC or ABTS.

Experiences of other CUPRAC users is believed to reveal some of the advantages of the method

In a comprehensive review by Prior et al. [6], the authors classify CUPRAC as one of the electron transfer-based methods, and summarize the superiorities of the CUPRAC method over other antioxidant assays. They state 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. [19] acknowledges that the highest capacities of polyphenolic compounds measured with CUPRAC were noted for catechin, caffeic acid, and gallic acid, in accordance with the capacity order of ABTS. CUPRAC and ABTS/TEAC antioxidant capacities for the raw and boiled garlic extracts were similar with a linear correlation. The authors state that ''as an advantage to other electron transfer-based assays as ABTS and Folin, CUPRAC values were acceptable in regard to its realistic pH close to the physiological pH'' [19]. The same research group in a different publication [20] correctly acknowledge that the CUPRAC and total polyphenols measurement results in the extracts of kiwifruit (that underwent ethylene treatment) correlated very well ($r^2 = 0.81$), better than with other total antioxidant capacity (TAC) assays (such as ABTS/TEAC). The low correlation the authors observed between CUPRAC results and flavonoids content [20] was due to the nature of measurement technique. The $AlCl_3$ test for flavonoids does not measure those flavonoids that do not bear the characteristic chelating functional groups for Al binding. Essentially flavones (e.g., chrysin, apigenin, luteolin, etc.) and flavonols (e.g., quercetin, myricetin, morin, rutin, etc.) react with Al(III), while flavanones and flavanonols do not complex to the same extent. Fruhwirth et al. [21] acknowledge that the CUPRAC assay, being applicable at pH 7.0 and responsive to thiol-type antioxidants, is a significant improvement over the conventional FRAP assay. The authors note that among the hydroxycinnamic acids, the conventional ABTS/TEAC method gave a much higher TEAC coefficient for ferulic acid than for caffeic acid, while their ''αPROX'' anti-protein fluorescence screening assay results were in accordance with theory and with the findings of CUPRAC. The authors also think that the conventional ABTS and FRAP methods dramatically overestimate the TEAC value of gallic acid, while their measurement was in accord with that of CUPRAC [21]. Mazor et al. [22] measured the trolox (TEAC)-, CUPRAC-, and Fe(II)-equivalents (FRAP) of some antioxidants, i.e., bucillamine (BUC), N-acetyl cysteine (NAC), glutathione (GSH), ascorbic acid, and trolox. The reduction yield of the CUPRAC reagent (Cu(II)-neocuproine) was proportional to the

antioxidant concentrations, and doubled for the 2-e reducing agents like ascorbic acid, trolox and BUC (the latter containing 2 –SH groups), relative to the 1-e reducing agents, NAC and GSH (containing 1 –SH group). On the other hand, the widely used FRAP method, although being capable of detecting BUC and NAC, was unable to detect the 1 –SH bearing tripeptide GSH [22], which was a distinct inferiority.

Remarks on the advantages of the CUPRAC method [13, 14] over other ET-based assays

The advantages of the CUPRAC method over other similar assays are summarized below:

1) The CUPRAC reagent is fast enough to oxidize thiol-type antioxidants [14, 23], whereas according to the protocol developed by Benzie and Strain [12], the FRAP method does not measure certain thiol-type antioxidants like glutathione. 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 is accelerated in the presence of copper(II) as catalyst.
2) The CUPRAC reagent is selective, because it has a lower redox potential than that of the ferric–ferrous couple in the presence of phenanthroline – or similar ligands. The standard potential of the Cu(II,I)-Nc redox couple is about 0.6 V, close to that of $ABTS^{+}/ABTS$, i.e., 0.68 V. Simple sugars and citric acid, which are not true antioxidants, are not oxidized with the CUPRAC reagent.
3) The reagent is more stable and accessible than other chromogenic reagents (e.g., ABTS, 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.
4) The method is easily and diversely applicable in conventional laboratories using standard colorimeters rather than necessitating sophisticated equipment and qualified operators.
5) The redox reaction giving rise to a colored chelate of Cu(I)-Nc is relatively insensitive to a number of parameters adversely affecting certain reagents such as DPPH, i.e., air, sunlight, humidity, and pH, to a certain extent.
6) The absorbance *vs*. concentration curves are 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 sensitively determine biologically important antioxidants.
7) The TAC values of antioxidants found with CUPRAC are perfectly additive, i.e., the TAC of a mixture is equal to the sum of TAC values of its constituents.
8) The method is suitable for automation.
9) The method proved to correlate well with ABTS or Folin-Ciocalteu assays in herbal [24] and apricot [25] extract samples.
10) The redox reaction producing colored species is carried out at pH 7 buffer as opposed to the acidic conditions (pH 3.6) of FRAP, or basic conditions (pH 10) of the Folin-Ciocalteu 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.
11) The method can simultaneously measure hydrophilic and 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 dichloromethane [14]. As an advantage over the widely used Folin-Ciocalteu reagent, CUPRAC can measure lipophilic antioxidants, while FCR cannot be used for TAC assay of biological fluids.
12) The intra- and inter-assay coefficients of variation (CV) 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 [14]. The CV (RSD) data of CUPRAC were definitely better than kinetic-based assays where even the intra-assay CV may reach up to 8%.
13) 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. The stable Cu(I)-

chelate was previously 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 [26]. Thus, Cu(I) chelated to Nc may not act as a prooxidant toward the tested antioxidants in a Fenton-type reaction in the absence of H_2O_2 or its precursors [27, 28].

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