JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Novel Total Antioxidant Capacity Index for Dietary Polyphenols and Vitamins C and E, Using Their Cupric Ion Reducing Capability in the Presence of Neocuproine: CUPRAC Method

REŞAT APAK,* KUBILAY GÜÇLÜ, MUSTAFA ÖZYÜREK, AND SALIHA ESIN KARADEMIR

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

The chemical diversity of antioxidants makes it difficult to separate and quantify antioxidants from the vegetable matrix. Therefore, it is desirable to establish a method that can measure the total antioxidant activity level directly from vegetable extracts. The current literature clearly states that there is no "total antioxidant" as a nutritional index available for food labeling because of the lack of standard quantitation methods. Thus, this work reports the development of a simple, widely applicable antioxidant capacity index for dietary polyphenols and vitamins C and E, utilizing the copper(II)−neocuproine [Cu(II)-Nc] reagent as the chromogenic oxidizing agent. Because the copper(II) (or cupric) ion reducing ability of polyphenols is measured, the method is named by our research group "cupric reducing antioxidant capacity" abbreviated as the CUPRAC method. This method should be advantageous over the ferric reducing antioxidant power (FRAP) method because the redox chemistry of copper(II)—as opposed to that of ferric ion—involves faster kinetics. The method comprises mixing of the antioxidant solution (directly or after acid hydrolysis) with a copper(II) chloride solution, a neocuproine alcoholic solution, and an ammonium acetate aqueous buffer at pH 7 and subsequent measurement of the developed absorbance at 450 nm after 30 min. Because the color development is fast for compounds such as ascorbic acid, gallic acid, and quercetin but slow for naringin and naringenin, the latter compounds were assayed after incubation at 50 °C on a water bath for 20 min [after Cu(II)-Nc reagent addition] so as to force the oxidation reaction to reach completion. The flavonoid glycosides were hydrolyzed to their corresponding aglycons by refluxing in 1.2 M HCl-containing 50% MeOH so as to exert maximal reducing power toward Cu(II)-Nc. Certain compounds also needed incubation after acid hydrolysis to fully exhibit their reducing capability. The CUPRAC antioxidant capacities of synthetic mixtures of antioxidants were experimentally measured as Trolox equivalents and compared to those theoretically found by making use of the principle of additivity of absorbances assuming no chemical interaction between the mixture constituents. Because ascorbic acid is not resistant to elevated temperature incubation, it should be assayed initially by measuring the absorbance (at 450 nm) difference of original and ascorbate oxidase-added mixture solutions at the end of 1 min of Cu(II)-Nc reagent addition. Thus, the total CUPRAC antioxidant capacity of a mixture containing various antioxidants should be that finally measured after a suitable combination of hydrolysis and incubation procedures, added to the initially measured capacity due to ascorbate. The antioxidant polyphenolic compounds tested demonstrate that the highest capacities in the CUPRAC method were observed for epicatechin gallate, epigallocatechin gallate, quercetin, fisetin, epigallocatechin, catechin, and caffeic 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. The antioxidant potency of flavonoids is nearly proportional to the total number of −OH groups and is positively affected by the presence of an *o*-dihydroxy moiety in the B-ring. β-Carotene, which did not react with the CUPRAC reagent in alcoholic aqueous medium, could be assayed in dichloromethane solvent. Linear calibration curves for ascorbic acid and flavonoids were redrawn in synthetic solutions containing a mixture of antioxidants, and also in real matrices such as grape and orange juices, green tea, and blackberry tea, showing an initial nonzero absorbance with the CUPRAC reagent. The parallellism of the linear calibration curves of pure compounds in a given complex matrix effectively demonstrated that there were no interferent chemical interactions among the solution constituents and that the antioxidant capacities of the tested antioxidants were additive. The CUPRAC reagent is reasonably selective, stable, easily accessible, and sensitive toward thiol-type oxidants, unlike the FRAP method. The reaction is carried out at nearly physiological pH as opposed to the unrealistic acidic pH of FRAP.

KEYWORDS: Antioxidant activity; cupric reducing antioxidant capacity (CUPRAC); dietary antioxidants; polyphenols; flavonoids; copper(II)−neocuproine reagent

INTRODUCTION

Oxygen free radicals that emerge as a result of the respirative cycle of oxidative phosphorylation may attack biological macromolecules such as cellular DNA, giving rise to single- and double-strand breaks that may eventually cause cell aging, cardiovascular diseases, mutagenic changes, and cancerous tumor growth. When the natural defenses of the organism (of enzymatic, nonenzymatic, or dietary origin) are overwhelmed

* Author to whom correspondence should be addressed (telephone +90.212-4737028; fax +90-212-591-1997 e-mail rapak@istanbul.edu.tr.

10.1021/jf048741x CCC: $27.50
Published on Web 11/25/2004

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*). Consumption of foods naturally bearing antioxidant activity is the most efficient way of combating such tissue injuries, undesired transformations, and health risks. The chemical diversity of antioxidants makes it difficult to separate and quantify antioxidants from the vegetable matrix. Therefore, it is desirable to establish a method that can measure the total antioxidant activity level directly from vegetable extracts (*3*). Currently in the literature, enzymatic methods, which are too much dependent on possible inhibitors coming from various sources, as well as enzyme-free but lengthy chemiluminescence methods have been applied as screening procedures for measuring the antioxidant activity of food products. Among these chemiluminescence methods, the cobalt(II)/EDTA/luminol/hydrogen peroxide method has been applied to aqueous and aqueous−alcoholic media, and the iron(III), cobalt(II)/peroxalate/imidazole, and the 8-hydroxyquinoline system has been applied to nonaqueous media. The common disadvantage of the latter two methods is their length as well as their dependence on numerous parameters such as the presence of transition metals, chelators, and solvents and the pH (*4*).

Antioxidant activity assay methods existing in the 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 (*5*) such as 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) (*6*) and 2,2′-diphenyl-1-picrylhydrazyl (DPPH) (*7*). 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. Measurement of oxygen radical scavengers with methods such as the ORAC test of antioxidant capacity (*8*) may be interfered with hydroxyl radical scavengers such as benzoate, which are not true antioxidants. On the other hand, the ferric reducing ability (FRAP) assay of antioxidants (*9*), which is based on ferric-to-ferrous reduction in the presence of an Fe(II)-stabilizing ligand such as tripyridyltriazine, 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 blind to thiol-type antioxidants such as glutathione. The hydrophilic and lipophilic reagents used for antioxidant assay are applicable only to a certain restricted class of compounds and sample matrices. Currently, there are at least five subclasses of methodologies for antioxidant assays in foodstuffs and plasma, as classified by Llesuy et al. in a comprehensive review (*10*):

- The consumption of a stable free radical is measured in the presence of the tested compound. Generally, the radicals involved in these procedures may be far from those relevant under oxidative stress conditions, and the kinetics of the process may be complex.
- The time required to consume all of the antioxidants contained in a complex sample is measured, also known as the total reactive antioxidant potential (TRAP) assay. However, the measured quantity (e.g., induction time) is not always directly proportional to antioxidant concentration.
- The rate of a given free radical process is observed, and the way this rate decreases after addition of the antioxidant sample is evaluated. These procedures are strongly dependent upon the radical being trapped. In complex systems, it is very hard to determine which radical(s) is (are) being trapped. For example, the improved oxygen radical absorbance capacity (ORAC) assay measures only the antioxidant activity against peroxyl and hydroxyl radicals, not that against all reactive oxygen species (e.g., superoxides and singlet oxygen). The fluorescent probe of the ORAC method, B-phycoerythrin, may show inconsistency from lot to lot and photoinstability (*8*).
- Some methodologies are based on equating the total amount of antioxidants to the reducing capacity of samples (reducing capacity tested by chemical methods or cyclic voltametry). These methods (e.g., FRAP) generally do not measure all antioxidants in a complex matrix, and those antioxidants (reductants) giving slow reactions with the reagent may not be totally oxidized within the recommended time protocol of the method.
- Other procedures do not conform to the previously described ones.

The common drawback of the existing antioxidant activity assay methods for foodstuffs is that the measured variable is either the quantity or reactivity (or both) of antioxidant compounds, thereby posing a debate as to the objectivity of such procedures as high- and low-reactivity compounds produce a wide range of stoichiometric factors compared to a reference compound such as Trolox. The range of tests used for antioxidant activity measurement is also a testimony to the uncertainty surrounding the chemistry of phenolic compounds. Thus, for example, in tests when free radical oxidation is induced by a metal ion such as Cu(II) or Fe(III), it is uncertain whether the test measures the ability of the phenolic to interact with a free radical or its ability to bind the metal ion (*11*). The 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 (*3*). As a result, the antioxidant activities of common vegetables (total sample size = 927) collected from the U.S. market, analyzed using the ORAC and FRAP procedures, did not correlate well (*3*). To briefly summarize the current situation, there is no single, widely acceptable assay method applicable to a reasonable variety of compounds and food matrices. Due to this difficulty, various food products may not be classified with respect to their antioxidant activity index (AAI), and, therefore, the public cannot easily understand and compare the antioxidant content of foodstuffs and beverages (e.g., like the hardness index of potable water products, which is generally understood by everyone). Thus, the aim of this work is to develop a simple, widely applicable antioxidant capacity index for dietary polyphenols and vitamins C and E, utilizing the copper(II)−neocuproine [Cu(II)-Nc] reagent as the chromogenic oxidizing agent. Because the copper(II) (or cupric) ion reducing ability of polyphenols is measured, the method is designated by our research group the "copper reducing antioxidant capacity", abbreviated as the CUPRAC method. This method should be advantageous over ferric reducing antioxidant power (FRAP) because the redox chemistry of copper(II)—as opposed to that of ferric ion—should involve faster kinetics.

MATERIALS AND METHODS

Guide to Methods for Total Antioxidant Capacity Assay. The method comprises the mixing of the antioxidant solution (directly or after acid hydrolysis) with a copper(II) chloride solution, a neocuproine alcoholic solution, and an ammonium acetate aqueous buffer at pH 7 and subsequent measurement of the developed absorbance at 450 nm after 30−60 min (normal measurement). Because the color development is fast for compounds such as ascorbic acid, gallic acid, and quercetin but slow for naringin and naringenin, the latter compounds were assayed

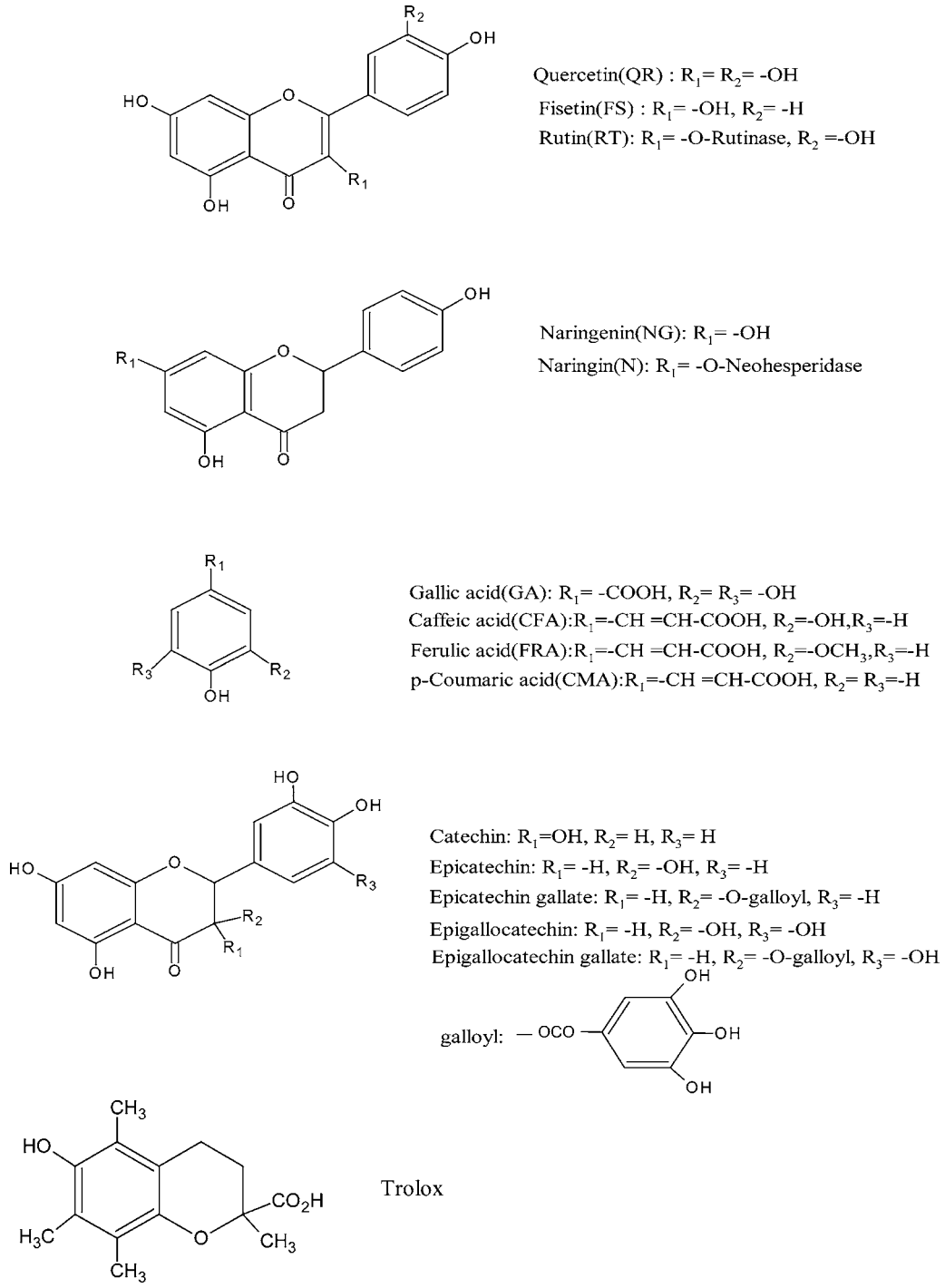

Figure 1. Structural formulas of the tested antioxidants.

after incubation at 50 °C on a water bath for 20 min [after Cu(II)-Nc reagent addition] so as to enable complete oxidation (incubated measurement). The flavonoid glycosides were hydrolyzed to their corresponding aglycons by refluxing in 1.2 M HCl-containing 50% MeOH so as to exert maximal reducing power toward Cu(II)-Nc (hydrolyzed measurement). Certain compounds also needed incubation after acid hydrolysis to fully exhibit their reducing capability (hydrolyzed and incubated measurement). The CUPRAC antioxidant capacities of synthetic mixtures of antioxidants (i.e., polyphenols and vitamins C and E) were experimentally measured as Trolox equivalents and compared to those theoretically found by making use of the principle of additivity of absorbances, assuming no chemical interaction between the mixture constituents. Because ascorbic acid is not resistant to elevated temperature incubation, it may be assayed initially by measuring the absorbance (at 450 nm) difference of simple and ascorbate oxidase-added mixture solutions at the end of 1 min of Cu(II)-Nc reagent addition, similar to the route followed in the improved FRAP method (*12*). Thus, the total CUPRAC antioxidant capacity of a mixture containing various antioxidants should be that finally measured after a suitable combination of hydrolysis and incubation procedures so as to obtain maximum absorbance at 450 nm, added to the initially measured capacity due to ascorbate, if hydrolysis and/or incubation was exercised.

Standards, Samples, and Reagents. The flavonoids fisetin, quercetin, rutin, naringin, naringenin, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate were purchased from Sigma Chemical Co. and (−)-catechin and gallic acid were from Fluka Chemicals. Ferulic acid, coumaric acid, caffeic acid, ascorbic acid, and Trolox were supplied from Aldrich Chemical Co. α-Tocopherol, ammonium acetate, copper(II) chloride, and 96% EtOH were

Table 1. Trolox Equivalent Antioxidant Capacities of Various Antioxidant Compounds Calculated with Respect to the Original ABTS Method, FRAP Method, and the Developed (CUPRAC) Method

compound	$TEAC_{orig}$	$TEAC_{FRAP}$	$TEAC_{CUPRAC}$
quercetin (QR)	4.7	2.7	4.38
gallic acid (GA)	3.0	1.9	2.62
naringin (N)	0.2		0.13
naringenin (NG)	1.5		(2.28) [3.03]
caffeic acid (CFA)	1.3	2.0	2.8 (2.96) [3.22]
ferulic acid (FRA)	1.9	0.83	1.2 (1.23) [1.34]
p-coumaric acid (CMA)	2.2		0.55 (1.00) [1.15]
catechin (CT)	2.4	0.9	3.09 (3.56)
rutin (RT)	2.4	1.0	[2.56, 3.80]
ascorbic acid (AA)	1.0	0.9	0.96
fisetin (FS)			3.9 (4.18)
α-tocopherol (TP)	1.0		1.11 (1.02)
epicatechin (EC)	2.2	2.5	2.77 (2.89)
epigallocatechin (EGC)	3.82		3.34 (3.60)
epigallocatechin gallate (EGCG)	4.75		4.88 (5.49)
epicatechin gallate (ECG)	5.0	4.93	(5.30, 5.64)
β-carotene (BC)[a]	2.4		3.3

[a] β-Carotene did not react with the CUPRAC reagent in aqueous EtOH as described under Normal Sample Measurement, but was attacked by Cu(II)–neocuproine in dichloromethane.

from E. Merck. Neocuproine (2,9-dimethyl-1,10-phenanthroline) was from Sigma Chemical Co. The real matrix media containing a mixture of antioxidants were the following: Commercial samples of tea bags were supplied from the Turkish food market, namely, as green tea from Doga Bitki Urunleri Sanayi, blackberry tea from DIASA, grape juice from Kavaklidere Sanayi, and orange juice freshly obtained from the fruit at the time of measurement.

Preparation of Solutions. 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 of Nc in 96% EtOH and diluting to 25 mL with ethanol. All polyphenolic compounds and vitamin solutions were freshly prepared in 96% EtOH at 1 mM (1.0×10^{-3} M) concentration prior to measurement.

Normal Sample Measurement. To a test tube were added 1 mL each of Cu(II), Nc, and NH_4Ac buffer solutions. Antioxidant sample (or standard) solution (x mL) and H_2O [$(1.1 - x)$ mL] were added to the initial mixture so as to make the final volume 4.1 mL. The tubes were stoppered, and after 1 h, the absorbance at 450 nm (A_{450}) was recorded against a reagent blank. The UV−vis spectrophotometer used was a Varian Cary 1E, equipped with quartz cuvettes. The standard calibration curve of each antioxidant compound was constructed in this manner as absorbance versus concentration, and the molar absorptivitiy of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned. The scheme for normal measurement is summarized as follows:

1 mL of Cu(II) + 1 mL of Nc + 1 mL of buffer + x mL of antioxidant solution + $(1.1 - x)$ mL of H_2O; total volume = 4.1 mL, measure A_{450} against a reagent blank after 1 h of reagent addition

Normal Sample Measurement in Dichloromethane (DCM). Cu(II), Nc, and buffer were added in the same order and amount; x mL of 2.0×10^{-5} M β-carotene solution in DCM and $(5 - x)$ mL of DCM were added and agitated. The phases were separated after 1 min, and absorbance at 450 nm of the lower (DCM) phase was measured. β-Carotene could be assayed only in DCM medium.

Incubated Sample Measurement. The mixture solutions containing sample and reagents were prepared as described under Normal Measurement; the tubes were stoppered and incubated for 20 min in a water bath at a temperature of 50 °C. The tubes were cooled to room temperature under running water, and their A_{450} values were measured.

Hydrolyzed Sample Measurement. A suitable mass of the polyphenol or vitamin standard was weighed such that the final antioxidant concentration of the methanolic solution would be 1 mM. Each standard was dissolved in a suitable volume of 50% MeOH. In a 100 mL flask, 5 mL of 1.2 M HCl (aq) was added to each solution and diluted to the mark with 50% MeOH. This solution was decanted to a distillation flask into which a few pieces of boiling stone were added and refluxed at 80 °C for 2 h. The flask was cooled to room temperature under running tap water. The hydrolysate was neutralized with ∼6.5 mL of 1 M NaOH. The neutralized hydrolysate was then subjected to "normal measurement".

Hydrolyzed and Incubated Sample Measurement. The neutralized hydrolysate was subjected to incubation at 50 °C in a water bath for 20 min. The A_{450} of running water-cooled samples were normally measured.

Measurement of Ternary Synthetic Solutions. Individual 1 mM solutions of the antioxidant compounds were prepared in 96% EtOH. Ternary mixtures of the antioxidants were prepared in suitable volume ratios such that the final absorbance of the mixture did not exceed 0.80 using the CUPRAC method. To the mixtures were added 1 mL each of Cu(II), Nc, and NH_4Ac buffer in this order. Water was added for dilution to a final volume of 4.1 mL. The ternary mixture solutions were subjected to both normal and incubated measurement so as to test the hypothesis of the additivity of absorbances due to each

Table 2. Molar Absorptivities and Linear Ranges of Various Antioxidant Compounds with the CUPRAC Method[a]

name	ε, L mol^{-1} cm^{-1}				linear range, M
	ϵ_N	ϵ_I	ϵ_H	$\epsilon_{H\&I}$	
quercetin	7.33×10^4				5.19×10^{-7}–1.90×10^{-5}
catechin	5.16×10^4	6.60×10^4	5.14×10^4	6.49×10^4	3.20×10^{-7}–2.50×10^{-5} (3.50×10^{-8}–1.90×10^{-5})
rutin	4.27×10^4	4.77×10^4	4.16×10^4	7.06×10^4	1.16×10^{-7}–3.16×10^{-5} (7.78×10^{-7}–2.80×10^{-5})
gallic acid	4.39×10^4				1.57×10^{-6}–3.23×10^{-5}
fisetin	6.50×10^4	7.78×10^4			7.02×10^{-7}–2.15×10^{-5} (4.50×10^{-7}–1.70×10^{-5})
naringin	3.40×10^2	2.17×10^3	3.41×10^2	2.20×10^3	1.47×10^{-4}–3.98×10^{-3} (4.60×10^{-6}–5.94×10^{-4})
naringenin		4.23×10^4		5.63×10^4	(8.29×10^{-7}–3.28×10^{-5})
ascorbic acid	1.59×10^4				5.6×10^{-6}–8.5×10^{-5}
ferulic acid	2.00×10^4	2.28×10^4	1.97×10^4	2.42×10^4	2.46×10^{-6}–6.74×10^{-5} (2.29×10^{-6}–5.94×10^{-5})
coumaric acid	9.20×10^3	1.85×10^4	8.88×10^3	2.14×10^4	4.63×10^{-6}–1.51×10^{-4} (2.90×10^{-6}–7.60×10^{-5})
caffeic acid	4.83×10^4	5.51×10^4	4.80×10^4	5.98×10^4	1.24×10^{-6}–2.81×10^{-5} (1.42×10^{-6}–2.50×10^{-5})
α-tocopherol	1.83×10^4	1.91×10^4	1.65×10^4	1.61×10^4	6.13×10^{-6}–7.73×10^{-5} (2.6×10^{-6}–7.00×10^{-5})
epicatechin	4.63×10^4	5.37×10^4			1.51×10^{-7}–2.80×10^{-5} (1.86×10^{-7}–2.35×10^{-5})
epicatechin gallate	8.88×10^4	1.05×10^5			9.34×10^{-7}–1.50×10^{-5} (2.82×10^{-7}–1.21×10^{-5})
epigallocatechin	5.59×10^4	6.68×10^4			7.64×10^{-7}–2.40×10^{-5} (2.10×10^{-7}–1.97×10^{-5})
epigallocatechin gallate	8.17×10^4	1.02×10^5			5.25×10^{-7}–1.58×10^{-5} (4.16×10^{-7}–1.27×10^{-5})
β-carotene[b]	5.57×10^4				3.23×10^{-7}–2.24×10^{-5}

[a] N, normal; I, incubated; H, hydrolyzed; H&I, hydrolyzed and incubated sample measurements. [b] Measured in dichloromethane.

Table 3. Comparison of Expected and Found CUPRAC Antioxidant Capacities of Synthetic Mixture Solutions (as Millimolar Trolox Equivalents

measurement method	composition of mixture	capacity expected[a]	capacity found (exptl)	measurement method	composition of mixture	capacity expected[a]	capacity found (exptl)
normal	10 μL of 1 mM QR 10 μL of 1 mM CT 10 μL of 1 mM RT	2.45×10^{-2}	2.55×10^{-2}	normal	20 μL of 1 mM FRA 50 μL of 1 mM CMA 50 μL of 1 mM TR	2.46×10^{-2}	2.43×10^{-2}
incubated	10 μL of 1 mM QR 10 μL of 1 mM CT 10 μL of 1 mM RT	2.56×10^{-2}	2.97×10^{-2}	incubated	20 μL of 1 mM FRA 50 μL of 1 mM CMA 50 μL of 1 mM TR	3.04×10^{-2}	2.85×10^{-2}
normal	30 μL of 1 mM GA 20 μL of 1 mM CFA 50 μL of 1 mM CMA	3.99×10^{-2}	3.96×10^{-2}	normal	20 μL of 1 mM FRA 20 μL of 1 mM CFA 50 μL of 1 mM TR	3.21×10^{-2}	3.21×10^{-2}
incubated	30 μL of 1 mM GA 20 μL of 1 mM CFA 50 μL of 1 mM CMA	4.58×10^{-2}	4.25×10^{-2}	incubated	20 μL of 1 mM FRA 20 μL of 1 mM CFA 50 μL of 1 mM TR	3.26×10^{-2}	3.28×10^{-2}
normal	20 μL of 1 mM GA 20 μL of 1 mM FRA 20 μL of 1 mM CFA	3.27×10^{-2}	3.09×10^{-2}	normal	50 μL of 1 mM CMA 20 μL of 1 mM CFA 50 μL of 1 mM TR	3.30×10^{-2}	3.43×10^{-2}
incubated	20 μL of 1 mM GA 20 μL of 1 mM FRA 20 μL of 1 mM CFA	3.32×10^{-2}	3.11×10^{-2}	incubated	50 μL of 1 mM CMA 20 μL of 1 mM CFA 50 μL of 1 mM TR	3.80×10^{-2}	3.70×10^{-2}
normal	20 μL of 1 mM FRA 50 μL of 1 mM CMA 20 μL of 1 mM CFA	2.66×10^{-2}	2.62×10^{-2}	normal	20 μL of 1 mM FRA 50 μL of 1 mM CMA 60 μL of 1 mM AA	2.64×10^{-2}	2.46×10^{-2}
incubated	20 μL of 1 mM FRA 50 μL of 1 mM CMA 20 μL of 1 mM CFA	3.26×10^{-2}	3.10×10^{-2}	incubated	0.4 mL of 1 mM N 20 μL of 1 mM NG 20 μL of 1 mM GA	3.56×10^{-2}	3.72×10^{-2}
normal	20 μL of 1 mM FRA 50 μL of 1 mM CMA 20 μL of 1 mM GA	2.53×10^{-2}	2.45×10^{-2}	normal	50 μL of 1 mM TP 10 μL of 1 mM QR 50 μL of 1 mM TR	3.63×10^{-2}	3.48×10^{-2}
incubated	20 μL of 1 mM FRA 50 μL of 1 mM CMA 20 μL of 1 mM GA	3.09×10^{-2}	3.21×10^{-2}	incubated	50 μL of 1 mM TP 10 μL of 1 mM QR 50 μL of 1 mM TR	3.52×10^{-2}	3.47×10^{-2}
normal	10 μL of 1 mM QR 10 μL of 1 mM CT 50 μL of 1 mM TR	3.04×10^{-2}	3.08×10^{-2}	normal	50 μL of 1 mM TP 10 μL of 1 mM QR 60 μL of 1 mM AA	3.79×10^{-2}	3.59×10^{-2}
incubated	10 μL of 1 mM QR 10 μL of 1 mM CT 50 μL of 1 mM TR	3.15×10^{-2}	3.09×10^{-2}	normal	20 μL of 1 mM TP 20 μL of 1 mM GA 60 μL of 1 mM AA	4.00×10^{-2}	3.71×10^{-2}
normal	10 μL of 1 mM QR 10 μL of 1 mM RT 60 μL of 1 mM AA	3.10×10^{-2}	3.25×10^{-2}	normal	50 μL of 1 mM TP 20 μL of 1 mM GA 10 μL of 1 mM QR	3.70×10^{-2}	3.57×10^{-2}
normal	10 μL of 1 mM RT 20 μL of 1 mM CT 50 μL of 1 mM TR	3.35×10^{-2}	3.48×10^{-2}	incubated	50 μL of 1 mM TP 20 μL of 1 mM GA 10 μL of 1 mM QR	3.59×10^{-2}	3.43×10^{-2}
incubated	10 μL of 1 mM RT 20 μL of 1 mM CT 50 μL of 1 mM TR	3.58×10^{-2}	3.38×10^{-2}	normal	20 μL of 1 mM FRA 50 μL of 1 mM TP 20 μL of 1 mM CFA	3.34×10^{-2}	3.20×10^{-2}
normal	10 μL of 1 mM RT 20 μL of 1 mM CT 60 μL of 1 mM AA	3.54×10^{-2}	3.42×10^{-2}	incubated	20 μL of 1 mM FRA 50 μL of 1 mM TP 20 μL of 1 mM CFA	3.29×10^{-2}	3.13×10^{-2}

[a] Found by means of the following equation: $\text{capacity}_{\text{mixture}} = \text{TEAC}_1 \cdot C_1 + \text{TEAC}_2 \cdot C_2 + \text{TEAC}_3 \cdot C_3$.

antioxidant, and the theoretically computed CUPRAC antioxidant capacities of the mixtures were compared to those experimentally found.

Application of the Method to Real Mixtures. One tea bag of each of the commercial green tea and blackberry tea was dipped separately into 250 mL of freshly boiled water in a beaker, occasionally shaken for 2 min, and allowed to stand in the same solution for an additional 3 min, enabling a total stewing time of 5 min. The tea solution was allowed to cool to room temperature, and filtration was applied to the green tea sample using a Whatman black-band filter paper for removing particulates. Blackberry tea was directly used as decantate.

A commercial orange sample was freshly supplied from the market, pressed in a suitable apparatus, and the extracted juice was filtered three times through a black-band filter paper. The commercial grape juice kept in a glass bottle was used as such.

These tea infusions and fruit juices were appropriately diluted with water such that their original CUPRAC absorbances at 450 nm would lie between 0.2 and 0.4 absorbance units. The suitable dilution ratios selected were 1:10 for grape and fresh orange juices and blackberry tea infusion and 1:15 for the green tea infusion. The standard calibration curves of some selected pure antioxidants were redrawn in these real solutions so as to observe the parallelism between the calibration lines (of ascorbic acid, Trolox, and quercetin, individually) in water and in real solution.

RESULTS AND DISCUSSION

The copper(II)−neocuproine (2,9-dimethyl-1,10-phenanthroline) reagent, introduced for various reducing agents as a mild oxidant (*13*), was previously used by our research team to determine the biochemically important reductants such as cysteine (*14*) and vitamin E (*15*). It has recently been used for ascorbic acid assay in foods and beverages (*16*), and a novel

spectrophotometric method we developed for protein assay using this reagent is now on the way. The present method correctly reports lower antioxidant activity for singly hydroxy-substituted flavanones such as naringenin than for 3′,4′-dihydroxy-substituted flavonoids with conjugated structure such as quercetin, as the theory predicts (*11*). Likewise, the developed method reports lower antioxidant activity for glycosides such as naringin than for the corresponding aglycon, naringenin, again in accord with theoretical expectations (*11*). The Trolox equivalent antioxidant capacities ($TEAC_{CUPRAC}$) for gallic acid and quercetin were close to 3 and 4, respectively, although the capacity of quercetin was higher than that of fisetin, a one −OH group analogue. Structural formulas of some of the tested antioxidant polyphenols are shown in **Figure 1**.

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 ($TEAC_{orig}$) (*6*, *17*), FRAP method ($TEAC_{FRAP}$) (*9*, *18*), and calculated with respect to the developed CUPRAC method ($TEAC_{CUPRAC}$) are listed in **Table 1**. The $TEAC_{CUPRAC}$ values were 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 1.86×10^4 L mol^{-1} cm^{-1}, respectively). The normal TEAC values in **Table 1** were found from the absorbances of solutions allowed to stand for 30 min at room temperature after reagent addition. The values in parentheses were found from the absorbances of solutions incubated at 50 °C , and finally the values in square brackets were computed from the absorbances of acid hydrolyzed solutions that were incubated at 50 °C (see **Table 1**). Quercetin and gallic acid did not show a capacity change after incubation or hydrolysis.

The antioxidant polyphenolic compounds listed in **Table 1** demonstrate that the highest capacities in the CUPRAC method were observed for epicatechin gallate, epigallocatechin gallate, quercetin, fisetin, epigallocatechin, catechin, and caffeic acid in this order. This is 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 (*19*). The antioxidant potency of flavonoids of similar conjugation level is roughly proportional to the total number of −OH groups and is positively affected by the presence of an *o*-dihydroxy moiety in the B-ring (*11*). The decreasing order of antioxidant capacities using the CUPRAC method were observed for epicatechin gallate [7 OH; 5.30, (5.64)], epigallocatechin gallate [8 OH; 4.88, (5.49)], quercetin (5 OH; 4.38), fisetin [4 OH; 3.9, (4.18)], epigallocatechin [6 OH; 3.34, (3.60)], catechin [5 OH; 3.09, (3.56)], caffeic acid {2 OH, −CH═CH−COOH; 2.8, (2.96), [3.22]}, epicatechin [5 OH; 2.77, (2.89)], gallic acid (3 OH; 2.62), and rutin {4 OH, −O-rutinose; 2.56, [3.8]}, the values in parentheses showing the number of hydroxyl groups in the molecule; the normal, incubated, hydrolyzed, and incubated $TEAC_{CUPRAC}$ coefficients of these compounds, respectively, whichever is applicable (See **Materials and Methods** for the definitions of these terms).

Differences in the activity of cinnamic acids may be ascribed to variations in hydroxylation and methylation patterns of the aromatic ring (*19*). For example, caffeic acid (CFA) is one hydroxyl group richer than the other two cinnamic acids (FRA and CMA). Ferulic acid has the electron-donating $-OCH_3$ group in the ortho-position to the phenolic −OH,

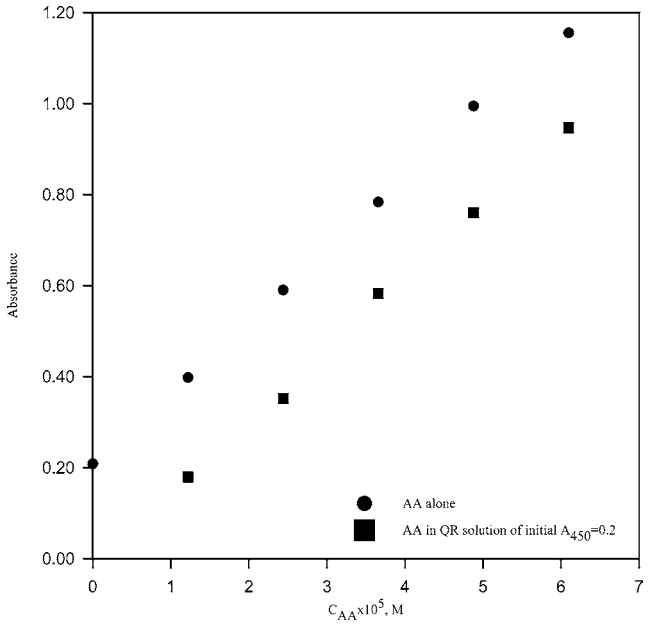

Figure 2. Calibration curve of ascorbic acid (AA) in quercetin (QR).

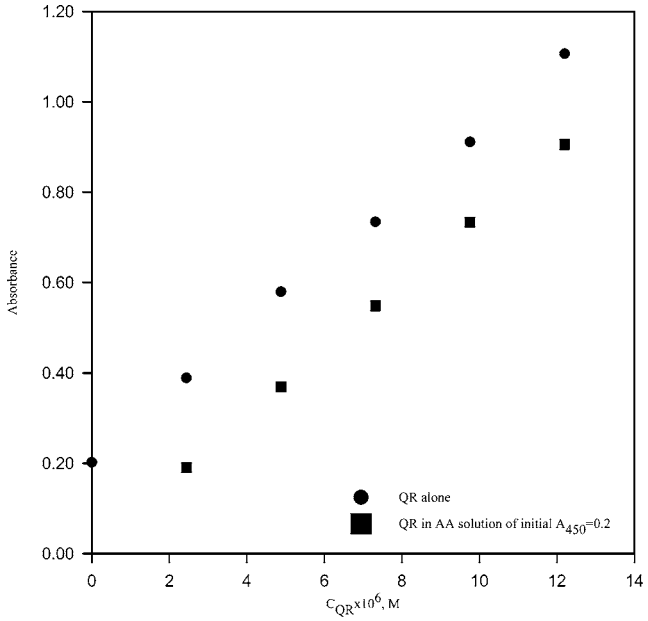

Figure 3. Calibration curve of quercetin (QR) in ascorbic acid (AA).

whereas *p*-coumaric acid lacks such a group. As opposed to the successful results of the developed CUPRAC method on cinnamic acids, caffeic acid and ferulic acid have been reported to give rise to color intensification with the FRAP reagent after several hours of reaction time, indicating incomplete oxidation (*20*).

The glycoside rutin (RT) initially having a TEAC of 2.56 was effectively hydrolyzed to the aglycon, presumably QR, and eventually showed a $TEAC_{CUPRAC}$ value of 3.80 (close to that of QR). Such hydrolysis reactions already take place enzymatically in the human organism, and the glycosides demonstrate full antioxidant potential in vivo. Thus, the sugar bond is broken so that the parent flavonoid may exert full antioxidant potency, nearly approaching that of the corresponding aglycon. The acid hydrolysis conditions in methanolic solution chosen for this task were as mentioned by other researchers (*21*, *22*). This method of hydrolysis of glycosides of flavones and flavonols was also recommended in the literature

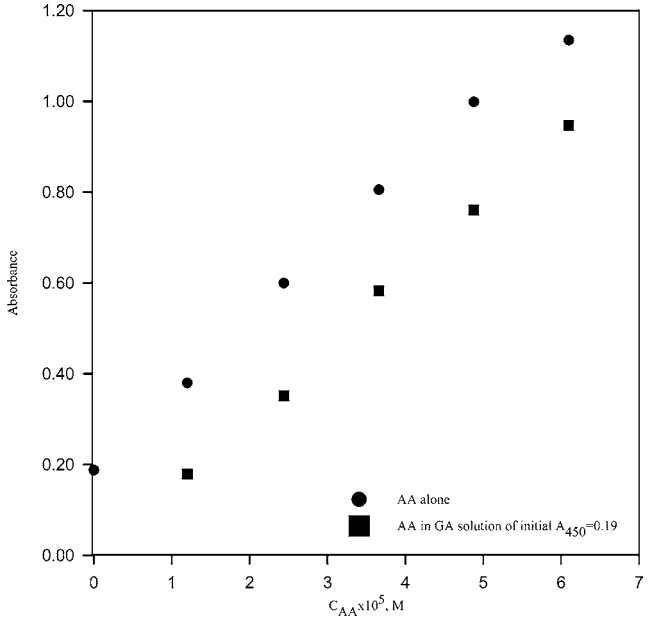

Figure 4. Calibration curve of ascorbic acid (AA) in gallic acid (GA).

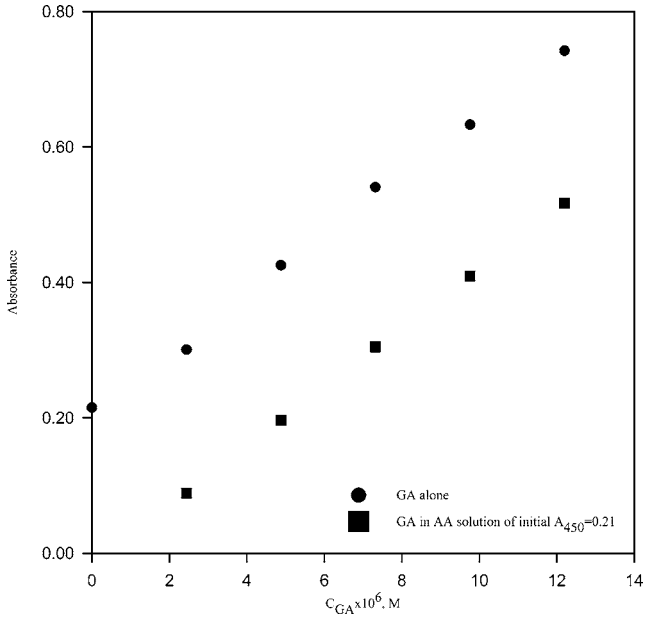

Figure 5. Calibration curve of gallic acid (GA) in ascorbic acid (AA).

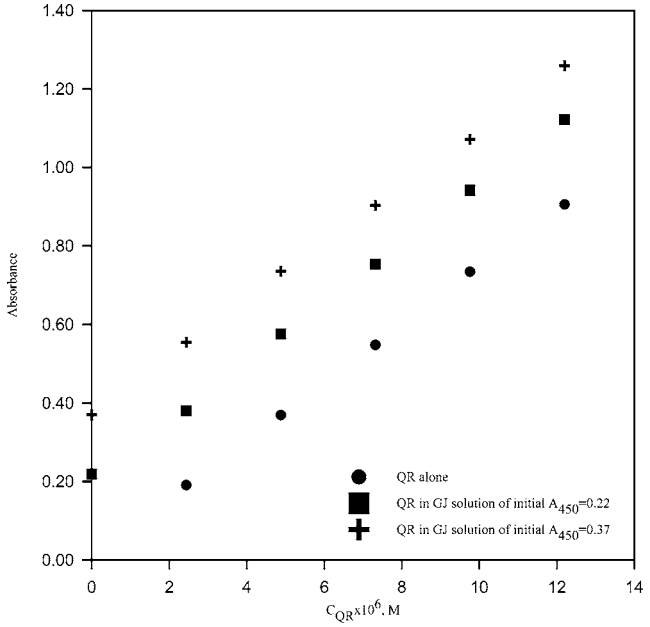

Figure 6. Calibration curve of quercetin (QR) in grape juice (GJ).

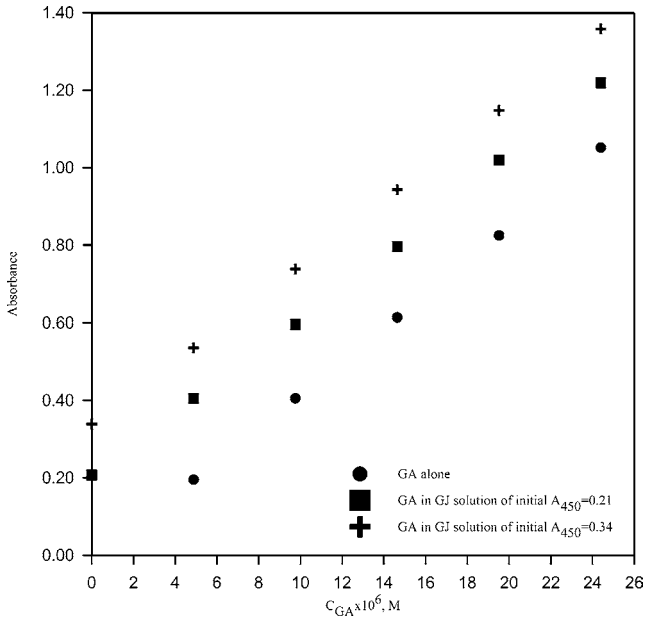

Figure 7. Calibration curve of gallic acid (GA) in grape juice (GJ).

(*23*) in sample preparation for HPLC analysis. Thus, such a hydrolytic procedure may serve the double function of sample preparation for HPLC analysis and for antioxidant capacity assay by the CUPRAC method. Again in accord with theory, naringenin exerts a much higher TEAC value than the corresponding glycoside, naringin. Acid hydrolysis followed by incubation significantly increases the TEAC value of naringenin.

The chromogenic oxidizing reagent of the developed CUPRAC method, that is, bis(neocuproine)copper(II) chloride [Cu(II)-Nc], reacts with polyphenols [$Ar(OH)_n$] in the manner

$$2n\ Cu(Nc)_2^{2+} + Ar(OH)_n = 2n\ Cu(Nc)_2^{+} + Ar(=O)_n + 2n\ H^{+} \quad (1)$$

where the liberated protons may be buffered with the relatively concentrated ammonium acetate buffer solution. In this reaction, the reactive Ar−OH groups of polyphenols are oxidized to the corresponding quinones and Cu(II)-Nc is reduced to the highly colored Cu(I)-Nc chelate showing maximum absorption at 450 nm. In this reaction, each flavonoid (in the aglycon form) having *n* phenolic −OH groups theoretically acts as a 2*n*−e donor. Because Trolox, vitamin E, and vitamin C (ascorbic acid) are 2−e donors toward the Cu(II)-Nc chelate, the TEAC of such polyphenols will theoretically be *n*, and the corresponding molar absorptivity of the polyphenol will be $(7.5-8.5 \times 10^3) \times 2n$ L mol^{-1} cm^{-1}. For example, the molar absorptivities—normally measured upon 30 min of standing at room temperature—of quercetin, fisetin, catechin, caffeic acid, gallic acid, α-tocopherol, and Trolox were 7.33×10^4, 6.50×10^4, 5.16×10^4, 4.83×10^4, 4.39×10^4, 1.83×10^4, and 1.67×10^4, respectively, whereas the (normal−incubated−hydrolyzed & incubated) absorptivities of rutin were $(4.27-4.77-7.06) \times 10^4$ (see **Table 2**). All of the easily oxidized flavonoids exhibited standard reduction potentials of <0.2 V, whereas naringenin, having a potential close to that of the $Cu(Nc)^{2+}-Cu(Nc)_2^{+}$ couple,

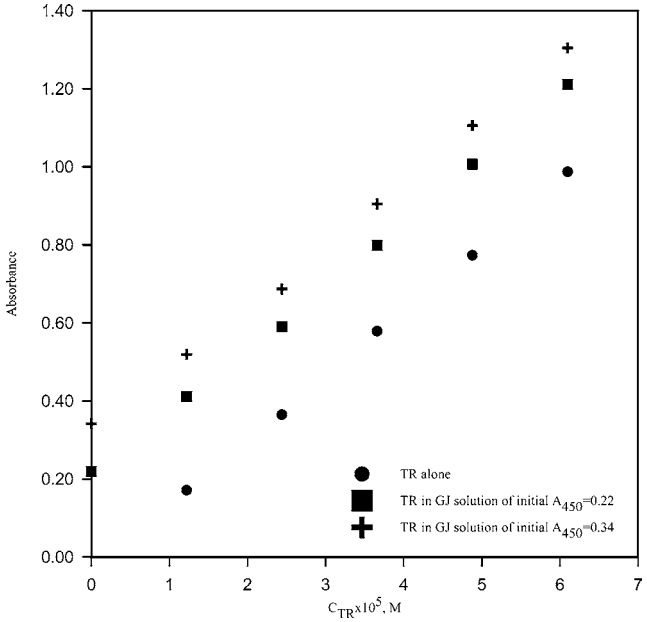

Figure 8. Calibration curve of Trolox (TR) in grape juice (GJ).

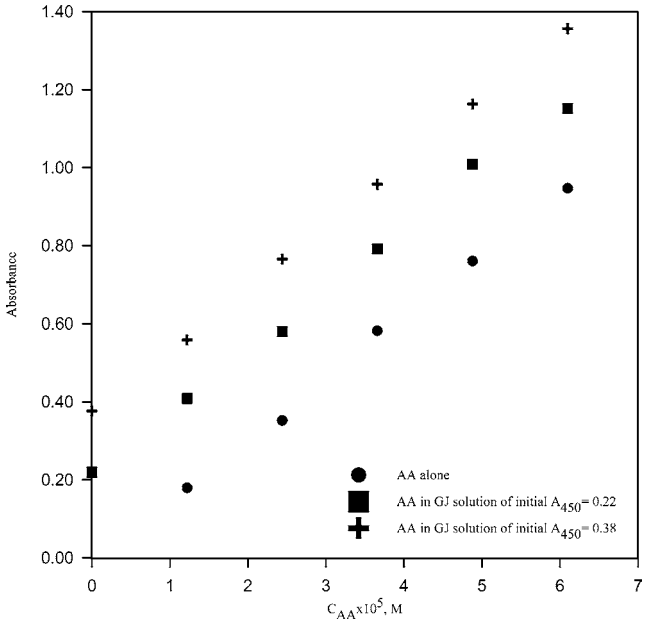

Figure 9. Calibration curve of ascorbic acid (AA) in grape juice (GJ).

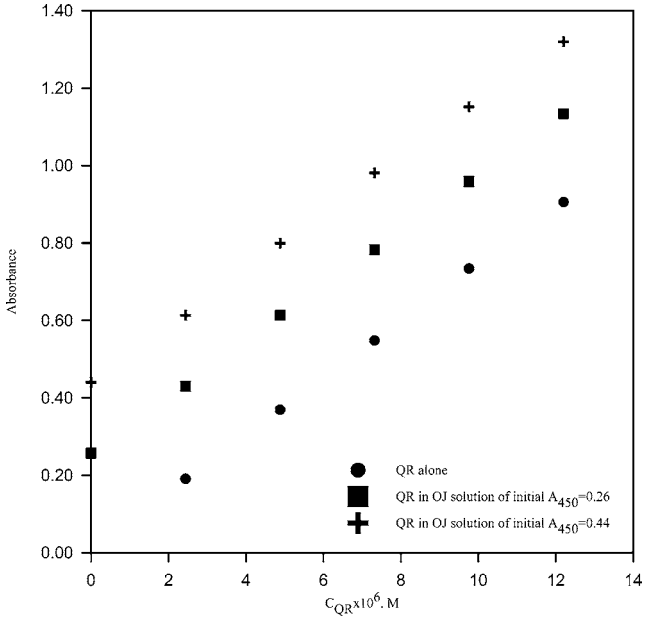

Figure 10. Calibration curve of quercetin (QR) in orange juice (OJ).

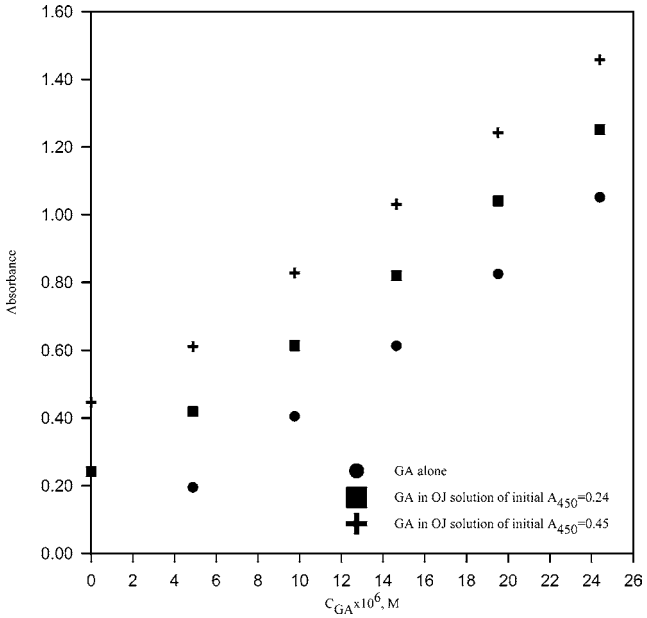

Figure 11. Calibration curve of gallic acid (GA) in orange juice (OJ).

underwent a slow reaction with the reagent. Naringenin oxidation could only be forced to completion after 50 °C incubation ($\epsilon = 4.23 \times 10^4$), and hydrolysis followed by incubation yielded $\epsilon = 5.63 \times 10^4$.

Possible ternary mixtures of the antioxidants (QR, CT, RT, GA, CFA, FRA, CMA, AA, NG, and TR) were synthetically prepared, and the suitably diluted solutions were analyzed for antioxidant capacity using the CUPRAC method. The experimentally measured capacities were generally within (±)5% interval of the theoretically computed values using the formula

$$\text{capacity}_{\text{total}} = \text{TEAC}_1\ \text{concn}_1 + \text{TEAC}_2\ \text{concn}_2 + \text{TEAC}_3\ \text{concn}_3 + \ldots \quad (2)$$

where 1, 2, ..., *i* denote the corresponding constituents of the synthetic mixture. Comparisons of expected (using eq 2) and experimentally found antioxidant capacities of synthetic mixture solutions (as mM Trolox equiv) were made and are given in **Table 3**. The expected and experimentally found CUPRAC capacities were generally in accord with each other. The slight discrepancies observed in some of the ascorbic acid-containing mixtures were further explored by inspection of linear calibration curves of AA in QR and QR in AA (see **Figures 2**−**5**). The accordance of theoretical and experimental findings, combined with the parallellism of the linear calibration curves of each antioxidant compound tested in the presence of the other compound, effectively demonstrated that there were no chemical interactions of intereferent nature among the synthetic solution constituents and that the antioxidant capacities of the tested antioxidants were additive. This reasoning was also applied to grape and orange juices, green tea, and blackberry tea as real complex mixtures, and standard calibration curves of four selected antioxidant compounds (QR, GA, TR, and AA) were redrawn in a solution of grape juice (GJ), orange juice (OJ), green tea (GT), and blackberry tea (BT), showing good parallelism of linear curves in pure aqueous solution and in real

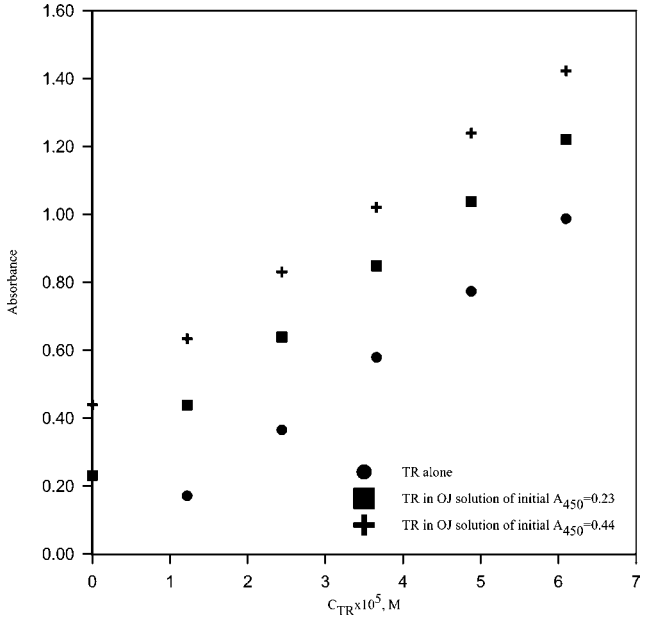

Figure 12. Calibration curve of Trolox (TR) in orange juice (OJ).

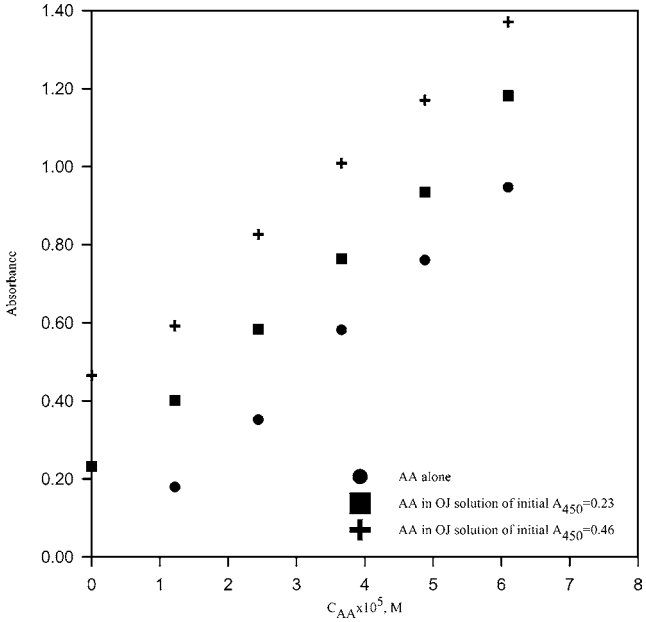

Figure 13. Calibration curve of ascorbic acid (AA) in orange juice (OJ).

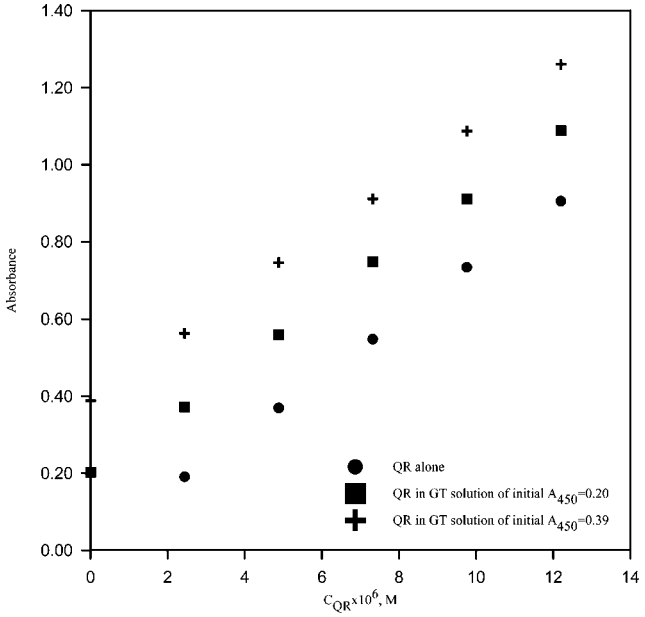

Figure 14. Calibration curve of quercetin (QR) in green tea (GT).

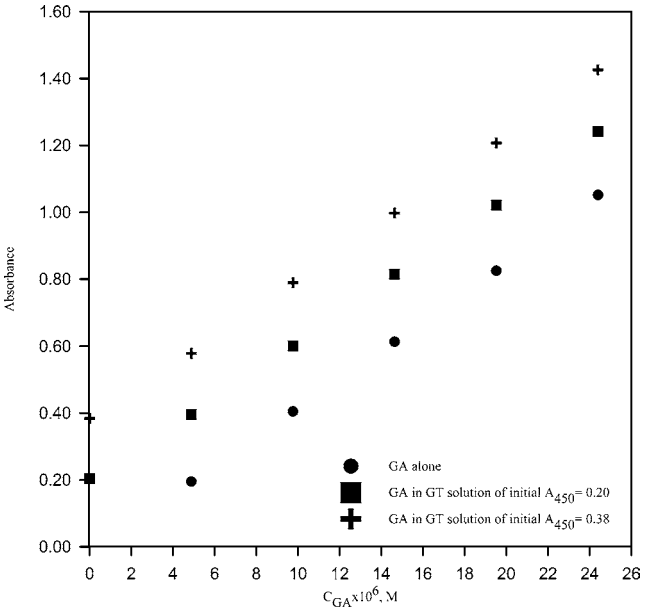

Figure 15. Calibration curve of gallic acid (GA) in green tea (GT).

complex mixtures having an initial nonzero absorbance with the CUPRAC reagent (see **Figures 6**−**21**). Again, this showed that the constituents of a real matrix solution 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 mixtures.

Thus, it is very important to develop a simple, cost-effective, and efficient method capable of being used in conventional laboratories to measure the antioxidant activity of foods and beverages using this developed reagent. Because the color development is relatively fast and the required reagents are relatively stable and cheap, the developed method is much simpler and more widely applicable than the existing methods.

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

• The CUPRAC reagent is fast enough to oxidize thiol-type antioxidants [according to the protocol developed by Benzie et al. (*9*)]; the FRAP method does not measure thiol-type antioxidants such as glutathione (*20*), the major low molecular weight thiol compound of the living plant and animal cell. The reason for this may be the half-filled d orbitals of high-spin Fe(III), attributing to it a chemical inertness, whereas 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 has been accelerated in the presence of copper(II) as catalyst (*24*). On the other hand, glutathione and cysteine are fast one-electron reductants toward the Cu(II)-Nc reagent (*13*, *14*), exhibiting 8.5×10^4 and 7.5×10^4 L mol^{-1} cm^{-1} molar absorptivities, respectively, within 2 min at room temperature.

• The reagent is selective, because it has a lower redox potential than that of the ferric−ferrous couple in the presence of phenanthroline- or tripyridyltriazine-type ligands. Simple sugars and citric acid are not oxidized with the CUPRAC reagent.

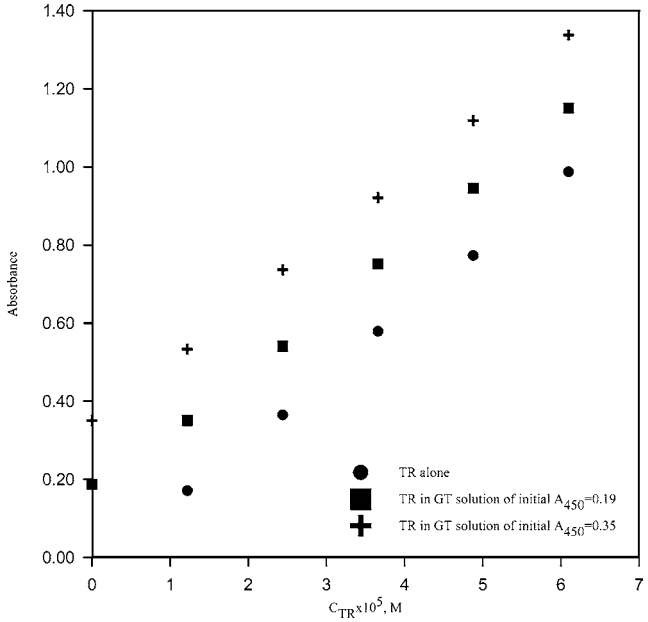

Figure 16. Calibration curve of Trolox (TR) in green tea (GT).

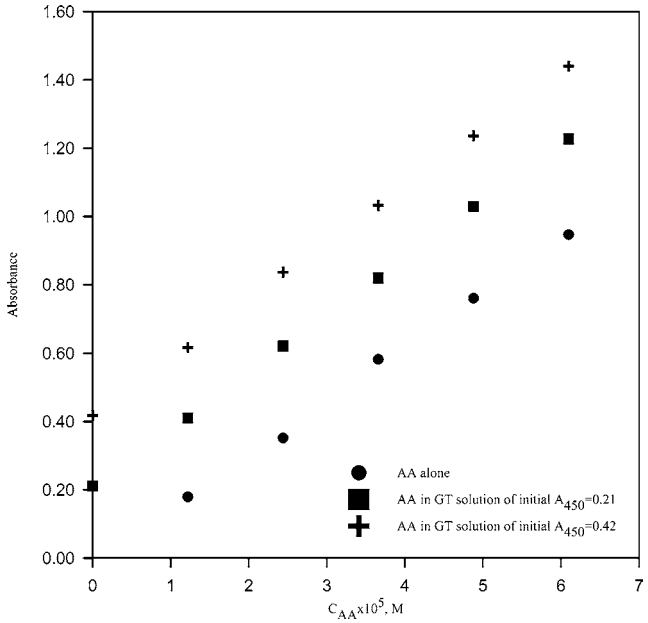

Figure 17. Calibration curve of ascorbic acid (AA) in green tea (GT).

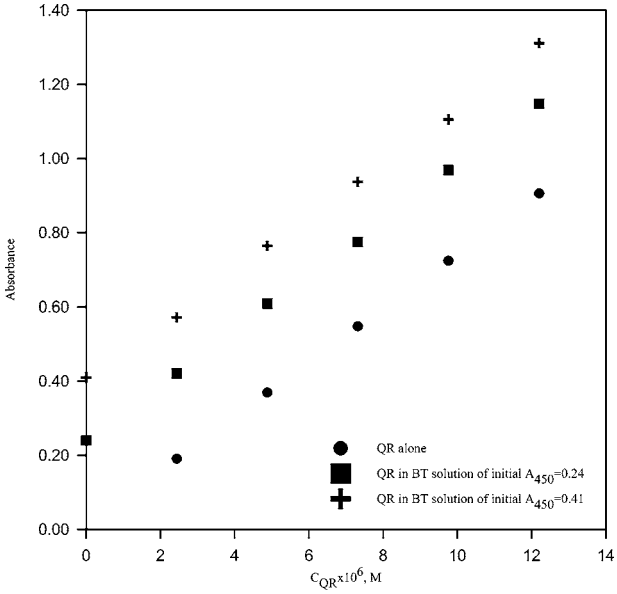

Figure 18. Calibration curve of quercetin (QR) in blackberry tea (BT).

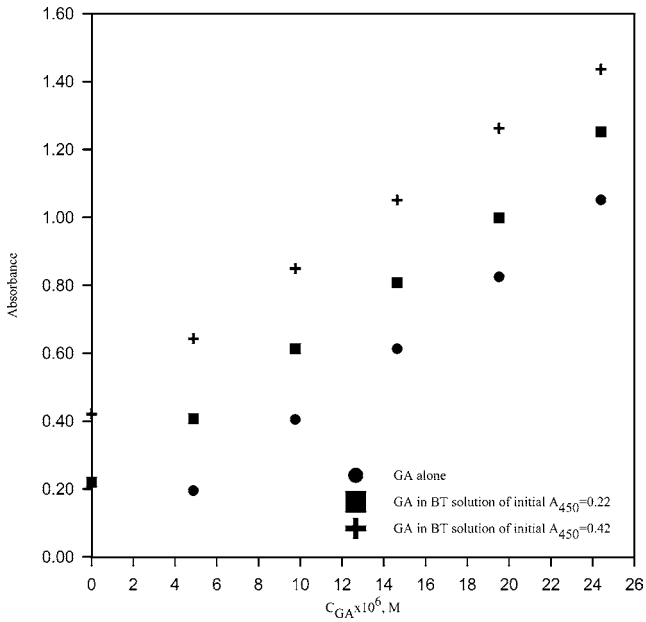

Figure 19. Calibration curve of gallic acid (GA) in blackberry tea (BT).

• The reagent is much more stable and easily accessible than the chromogenic radical reagents (e.g., ABTS and DPPH).

• 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, for example, 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 (e.g., the TEAC of naringenin having a high redox potential is 1.5 with respect to the ABTS method, but is 3.0 in the CUPRAC method using hydrolysis plus incubation, as expected from the three hydroxyl groups of the naringenin molecule. Also, the incompletely oxidized rutin and catechin by the FRAP reagent—as observed from **Table 1**—exert their full antioxidant potency in the CUPRAC method).

• The analytical response (i.e., absorbance) versus concentration curves are perfectly linear in the CUPRAC method over a wide range, unlike those of other methods yielding polynomial curves.

• 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.

• The method can simultaneously measure hydrophilic as well as lipophilic antioxidants (e.g., α-tocopherol).

• The single weakness of the CUPRAC method is that the isolated hydrocarbon double bonds of (Ar−CH═CHCOOH) ferulic and *p*-coumaric acids are probably not attacked by the Cu(II)-Nc reagent. Potential antioxidants with alternating hydrocarbon double bonds (without accompanying −OH groups) such as β-carotene would probably require π-acceptor oxidants forming a π-complex intermediate during electron transfer (e.g., with halogens), whereas the copper(II)−neocuproine reagent may not attack such double bonds due to the lack of coordinating functional groups in the host antioxidant molecule before

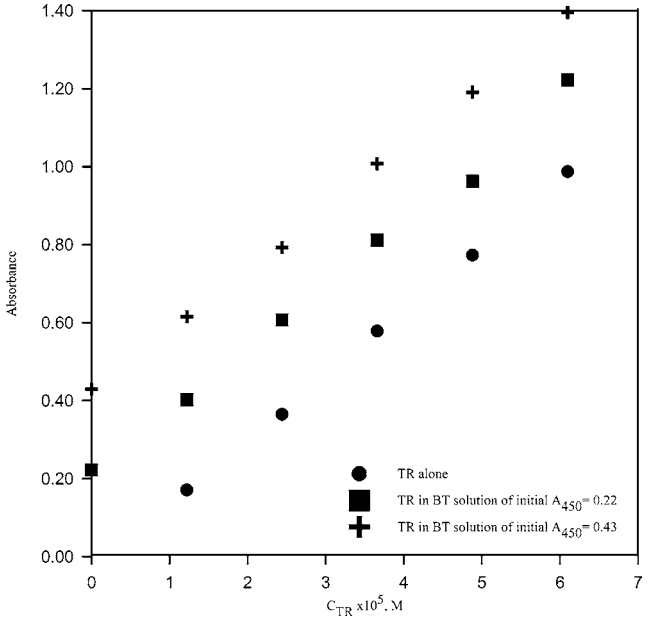

Figure 20. Calibration curve of Trolox (TR) in blackberry tea (BT).

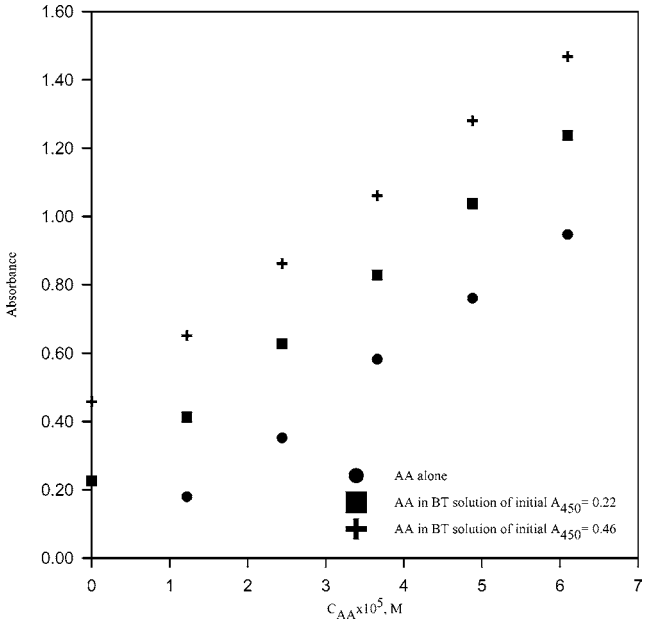

Figure 21. Calibration curve of ascorbic acid (AA) in blackberry tea (BT).

e-transfer takes place. It should be noted that these double bonds are also not attacked by the FRAP reagent. However, β-carotene was indeed attacked by the CUPRAC reagent, not in aqueous ethanol but in dichloromethane solvent medium. In the meantime, it is noteworthy that β-carotene has been reported not to inhibit in vivo and in vitro LDL oxidation (*25*) and not to protect low-density lipoprotein as opposed to the protective effect of vitamin E (*26*), that is, an important feature of dietary antioxidants.

Generally, the various aspects emphasizing the importance of the development of a novel and widely acceptable antioxidant activity index (AAI) can be summarized as follows:

- Simple, efficient, rapid, and cost-effective analytical methods must be developed for the assay of antioxidant compounds (e.g., vitamins C and E, polyphenols, flavonoids, carotenoids, and amino acids bearing thiol groups) present in foodstuffs, and validation of the developed methods with standard reference methods of determination.
- A widely acceptable, practical, and versatile AAI capable of being applied to both water- and oil-soluble antioxidants present in various foodstuffs is needed.
- A wide variety of foodstuffs must be classified with respect to their AAI, and new types of foods and beverages, for which the antioxidant properties were not publicly known before, must be evaluated.
- An inventory of existing and emerging natural food products with respect to their AAI should be prepared.
- Identification and separation of antioxidant-rich fractions of foodstuffs must be performed, and new diet supplements using natural vegetative food material rich in antioxidants should be designed.
- AAI variations of foods processed or stored under different conditions (e.g., of pH, temperature, relative humidity, time, etc.) need to be monitored, and better conditions of storage and food processing that would not adversely alter the AAI of foodstuffs should be proposed.
- Internationally accredited reference laboratories that are responsible for food quality and safety should be organized.
- New health care and diet supplement products (e.g., for the elderly people, children, and possible risk groups that may be adversely affected from antioxidant-poor diets) should be entered in the world food and medical market.
- Research into the prevention and treatment of cardiovascular diseases and cancer through antioxidant-enriched diets should be performed, thereby strengthening public health.

ACKNOWLEDGMENT

Ulker Food Research and Development Division is acknowledged for the donation of some of the polyphenolic compounds used in this work.

LITERATURE CITED

(1) Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*; Oxford University Press: Oxford, U.K., 1989.

(2) Halliwell, B.; Aruoma, O. I. DNA damage by oxygen-derived species: its mechanisms and measurement in mammalian systems. *FEBS Lett.* **1991**, *281*, 9−19.

(3) Ou, B.; Huang, D.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: A comparative study. *J. Agric. Food Chem.* **2002**, *50*, 3122−3128.

(4) Kefalas, P.; Petrakis, C.; Arnous, A.; Codina, C.; Parejo, I.; Antonio, A.; Gotsiou, P. Assessment of antioxidant activity by novel chemiluminescent techniques: Evaluation of wine, honey and olive oil. Presented at the 3rd Aegean Analytical Chemistry Days, international meeting, Lesvos, Greece, Sept 29−Oct 3, 2002; oral presentation IL-07.

(5) Arnao, M. B. Some methodological problems in the determination of antioxidant activity using chromogen radicals: a practical case. *Trends Food Sci. Technol.* **2000**, *11*, 419−421.

(6) Miller, N. J.; Rice-Evans, C. A.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. *Clin. Sci.* **1993**, *84*, 407−412.

(7) Sanchez-Moreno, C.; Larrauri, J. A.; Saura-Calixto, F. A procedure to measure the antiradical efficiency of polyphenols. *J. Sci. Food Agric.* **1998**, *76*, 270−276.

(8) Cao, G.; Verdon, C. P.,; Wu, A. H. B.; Wang, H.; Prior, R. L. Automated oxygen radical absorbance capacity assay using the COBAS FARA II. *Clin. Chem.* **1995**, *41*, 1738−1744.

(9) Benzie, I. F. F.; Strain, J. J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.

(10) Llesuy, S.; Evelson, P.; Campos, A. M.; Lissi, E. Methodologies for evaluation of total antioxidant activities in complex mixtures: A critical review. *Biol. Res. (Santiago)* **2001**, *34* (2) (ISSN 0716-9760 version impresa).

(11) Robards, K.; Prenzler, P. D.; Tucker, G.; Swatsitang, P.; Glover, W. Phenolic compounds and their role in oxidative processes in fruits. *Food Chem.* **1999**, *66*, 401−436.

(12) Benzie, I. F. F.; Strain, J. J. Ferric reducing/antioxidant power assay: direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. *Methods Enzymol.* **1999**, *299*, 15−27.

(13) Tütem, E.; Apak, R.; Baykut, F. Spectrophotometric determination of trace amounts of copper(I) and reducing agents with neocuproine in the presence of copper(II). *Analyst* **1991**, *116*, 89−94.

(14) Tütem, E.; Apak, R. Simultaneous spectrophotometric determination of cystine and cysteine in amino acid mixtures using copper(II)-neocuproine reagent. *Anal. Chim. Acta* **1991**, *255*, 121−125.

(15) Tütem, E.; Apak, R.; Günaydı, E.; Sözgen, K. Spectrophotometric determination of vitamin E (α-tocopherol) by the copper(II)-neocuproine reagent. *Talanta* **1997**, *44*, 249−255.

(16) Güçlü, K.; Sözgen, K.; Tütem, E.; Özyürek, M.; Apak, R. Spectrophotometric determination of ascorbic acid using copper-(II)-neocuproine reagent in beverages and pharmaceuticals. *Talanta* **2004**, in press.

(17) Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. A. Polyphenolic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339−346.

(18) Pulido, R.; Bravo, L.; Saura-Calixto, F. Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.* **2000**, *48*, 3396−3402.

(19) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(20) Ou, B.; Hampsch-Woodill, M.; Prior, R. L. Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe. *J. Agric. Food Chem.* **2001**, *49*, 4619−4626.

(21) Hertog, M. G. L.; Hollman, P. C. H.; Venema, D. P. Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruits. *J. Agric. Food Chem.* **1992**, *40*, 1591−1598.

(22) Crozier, A.; Lean, M. E. J.; McDonald, M. S.; Black, C. Quantitative analysis of the flavonoid content of commercial tomatoes, onions, lettuce, and celery. *J. Agric. Food Chem.* **1997**, *45*, 590−595.

(23) Merken, H. M.; Beecher, G. M. Measurement of food flavonoids by high-performance liquid chromatography: A review. *J. Agric. Food Chem.* **2000**, *48*, 577−599.

(24) Teshima, N.; Katsumata, H.; Kurihara, M.; Sakai, T.; Kawashima, T. Flow-injection determination of copper(II) based on its catalysis on the redox reaction of cysteine with iron(III) in the presence of 1,10-phenanthroline. *Talanta* **1999**, *50*, 41−47.

(25) Gaziano, J. M.; Hatta, A.; Flynn, M.; Johnson, E. J.; Krinsky, N. I.; Ridker, P. M.; Hennekens, C. H.; Frei, B. Supplementation with β-carotene *in vivo* and *in vitro* does not inhibit low-density lipoprotein oxidation. *Atherosclerosis* **1995**, *112*, 187−195.

(26) Reaven, P. D.; Khouw, A.; Beltz, W. F.; Parthasarathy, S.; Witztum, J. L. Effect of dietary antioxidant combinations in humans: protection of LDL by vitamin E but not by β-carotene. *Atheroscler. Thromb.* **1993**, *13*, 590−600.

Received for review July 27, 2004. Accepted October 5, 2004.

JF048741X