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Novel Optical Fiber Reflectometric CUPRAC Sensor for Total Antioxidant Capacity Measurement of Food Extracts and Biological Samples

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ABSTRACT: A novel fiber optic sensor was developed for screening the total antioxidant capacity (TAC) based on the use of cupric−neocuproine (Cu(II)−Nc) immobilized onto a Nafion cation-exchange membrane with reflectance spectrometric measurement. The reflectance change associated with the formation of the highly colored Cu(I)−Nc chelate on the membrane as a result of reaction with antioxidants was measured at 530 nm by using a miniature reflectance spectrometer. The calibration graph of trolox (TR) was linear with a slope of 3.40×10^3 L mol^{-1} mm^{-1}. The limit of detection (LOD) and limit of quantification (LOQ) for TR in the reflectometric cupric reducing antioxidant capacity (CUPRAC) method were found as 0.53 and 1.76 μM, respectively. The trolox equivalent antioxidant capacities (TEAC) of various antioxidant compounds using the proposed method were comparable to those of the main CUPRAC assay. This assay was validated through linearity, additivity, precision, and recovery. The developed reflectance sensor was used to screen the TAC of some commercial fruit juices and mice tissue homogenates without preliminary treatment. The method is rapid, inexpensive, versatile, and nonlaborious, uses stable reagents on the sensor, and enables the in situ estimation of antioxidant capacity of food extracts and biological samples.

KEYWORDS: *total antioxidant capacity (TAC), cupric reducing antioxidant capacity (CUPRAC) assay, optical sensor, reflectance spectroscopy, fruit juices, mouse tissues*

INTRODUCTION

"Oxidative stress" conditions emerge as a result of the generation of an unbalanced excess of reactive oxygen and nitrogen species (ROS/RNS) concomitant with a change in cellular redox status, in which biological macromolecules (proteins, lipids, and nucleic acids) can suffer oxidative damage, causing tissue injury leading to various diseases.[1,2] Because intrinsic and extrinsic antioxidants are natural combat agents against oxidative stress, development of selective and sensitive techniques for rapid sensing of the total antioxidant capacity (TAC) of complex samples is important for food, biochemical, and biomedical scientists.

Different antioxidant capacity/activity assays applicable to food and biological solutions are generally based on two different principles such as electron transfer (ET) and hydrogen atom transfer (HAT),[3] although in some cases these two mechanisms may not be clearly distinguished. HAT-based assays include inhibition of induced low-density lipoprotein autoxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays.[3,4] HAT-based assays have essentially not found use in sensing applications on solid substrates.

Spectrophotometric ET-based assays determine the capacity of an antioxidant in the reduction of an oxidant that changes color when reduced.[3,4] The degree of color 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. The most widely used ET-based assays include ABTS/TEAC,[5] DPPH,[6] Folin−Ciocalteu (FCR),[7,8] FRAP,[9] and CUPRAC,[10] having different chromogenic oxidizing reagents with various formal redox potentials.

The chromogenic oxidizing reagent used for the CUPRAC assay originally developed in our laboratory[10] is the bis-(neocuproine) copper(II) cation (Cu(II)−Nc) acting as an outer-sphere electron transfer agent, and the CUPRAC chromophore showing maximum absorption at 450 nm is bis(neocuproine) copper(I) cation (Cu(I)−Nc), formed by chemical reduction of Cu(II)−Nc with antioxidants. The CUPRAC method has been successfully applied to TAC measurements in food plants,[11−15] natural dyes,[16] and human serum,[17,18] and finally this assay in aqueous alcoholic solutions was integrated to a reflectometric CUPRAC sensor in the present study.

Optical sensors have recently attracted remarkable attention because of their advantages in biomedical sensing, environmental monitoring, and industrial process control applications.[19] Compared to classical spectrophotometric methods, optical sensors have many advantages such as flexibility, miniaturization, and inexpensive use outside the laboratory. Optical chemical sensors use optical transduction techniques (absorbance, luminescence, or reflectance) for evaluating analytical information. Nowadays, these sensors exploit fiber optic technology because of its specific advantages. A type of these sensors, reagent-mediated sensors (optrodes), is suitable for rapid and low-cost applications with high sensitivity and selectivity and can be available in the form of inexpensive test kits and strips for practical assays.[20,21] Although a great many optical sensors have been developed over the years for a wide

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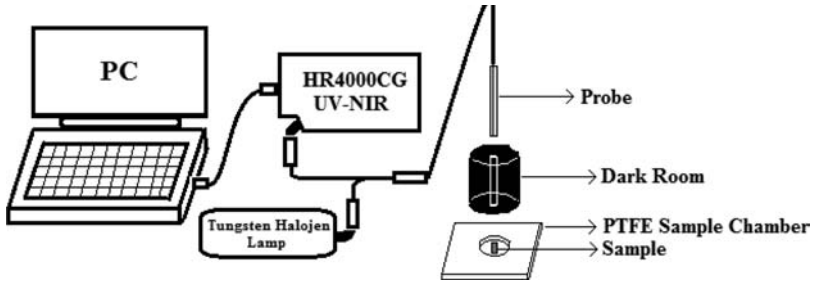

Figure 1. Shematic of the instrumentation used for reflectance measurements.

range of analyte samples, only a few possess the combined quality characteristics of sensitivity and selectivity. Nafion is a perfluorosulfonate-type cation-exchange membrane having R−$\{-O-CF_2-CF(CF_3)-\}_x-O-(CF_2)_2SO_3H$ functional groups.[22,23] Because of their numerous favorable properties such as thermal stability, mechanical strength, and high transparency in the UV−vis−NIR region, Nafion ionomers can be used as polymeric support for redox-active spectroscopic sensors using ET reagents.

The usage of optical sensors with linear absorbance response over a relatively wide concentration range is very rare in antioxidant research, and our group has introduced such an absorptimetric sensor of cupric neocuproine for the first time[14] to screen the TAC of some commercial fruit juices without preliminary treatment. It can theoretically be envisaged that development of copper-based TAC sensors is more likely than the development of iron-based ones, because, as opposed to the distorted tetrahedral geometry of tetracoordinate copper complexes adsorbed on a planar membrane surface, hexacoordinate octahedral iron complexes usually adsorb by partial loss (dissociation) of iron due to enforced geometry imposed by reagent−substrate interactions reducing the coordination number.[24] Iron(III)-based TAC assays such as FRAP or tris(1,10-phenanthroline)iron(III) have not yet been adapted to a solid sensor most probably because the hexacoordinate Fe(II) complex chromophores having 2:1 (mol mol^{-1}) TPTZ-to-Fe(II) or 3:1 phen-to-Fe(II) stoichiometry that are expected to form upon chemical reduction by antioxidants may not efficiently form on the solid substrate surface used for TAC sensing. Choudhary et al. estimated lycopene concentration in watermelon and tomato puree by fiber optic reflectance spectrometry with the use of two monochromatic light sources at 565 and 700 nm.[25] The use of chromogenic radicals, that is, 2,2-diphenyl-1-picrylhydrazyl ($DPPH^•$) and galvinoxyl radical ($GV^•$), immobilized on plasticized PVC matrices for antioxidant (i.e., radical scavenging) sensing, was described by Steinberg and Milardović.[26] Lee et al. reported antioxidant screening by using a colored thin film containing DPPH immobilized onto polyvinyl alcohol.[27] A chemiluminescence (CL) biosensing system, bearing luminol and hematin co-immobilized on a cellulose membrane disk, was used to sense antioxidants.[28] Recently, the original cerium(IV)-based TAC (CERAC) assay[29] was adapted to an optical sensor version,[30] where filter paper disks were soaked in nanoceria (CeO_2) suspension, dried, and used for antioxidant sensing. The direct quantification of colored species retained on the surface of a solid support (i.e., filter paper, polymer) with diffuse reflectance spectroscopy has attracted great interest in analytical chemistry[31] because of the difficulty in utilizing light-impermeable structures for which the classical transmittance spectrophotometric methods cannot be implemented.[32]

The aim of this study is to develop a simple and sensitive optical fiber reflectometric sensor using cupric reducing antioxidant capacity (CUPRAC) methodology for determining the TAC of food extracts and biological samples. The miniature fiber optic spectrometer setup used for reflectometric CUPRAC measurements is schematically shown in Figure 1. The developed method was successfully applied to solutions containing pure antioxidants, synthetic antioxidant mixtures, and real samples of fruit juices and mouse tissues. Due to the adsorbability of the CUPRAC reagent on a wide range of low-cost materials having relatively inhomogeneous surfaces suitable for fiber optic reflectance measurements, the proposed reflectometric CUPRAC sensor may open the way to novel commercial products for TAC sensing in food analytical chemistry.

MATERIALS AND METHODS

Reagents, Materials, and Apparatus. The following chemical substances of analytical reagent grade were supplied from the corresponding sources: neocuproine (2,9-dimethyl-1,10-phenanthroline), morin (MR), quercetin (QR), rutin (RT), L-glutathione reduced (GSH), naringenin (NG), gallic acid (GA), and uric acid (UA), Sigma (Steinheim, Germany); trolox (TR), rosmarinic acid (RA), α-tocopherol (TOC), L-ascorbic acid (AA), naringin (N), and Nafion 115 perfluorinated membrane (thickness = 0.005 in.), Aldrich (Steinheim, Germany); copper(II) chloride dihydrate, ammonium acetate (NH_4Ac), ethanol (EtOH), and bilirubin (BIL), Merck (Darmstadt, Germany); (+)-catechin (CT), myricetin (MYR), L-cysteine (CYS), DL-homocysteine (HCYS), fisetin (FS), and kaempferol (K), Fluka (Buchs, Switzerland).

Lipton green tea (*Camellia sinensis*) was purchased from Unilever San. Tic. Turk AS (Istanbul, Turkey), and orange, cherry, apricot, and peach juices were purchased from Coca-Cola Icecek AS (Istanbul, Turkey).

The visible spectra and absorption measurements were recorded in matched quartz cuvettes (using a Varian CARY Bio 100 UV−vis spectrophotometer (Mulgrave, Victoria, Australia)). The optical thickness of the cuvettes was 1 cm for solution phase and 1 mm for Nafion solid membrane sensor measurements.

The reflectance spectra and reflectance measurements were recorded using a miniature fiber optic-based spectrometer (Ocean Optics Inc., HR4000CG-UV-NIR), which utilizes a small tungsten halogen lamp (Ocean Optics Inc., LS-1) as the light source, micrometer reflection probe (Ocean Optics Inc., R400-7-SR), and a charge-coupled device (CCD)-based detector. Light reflected from the reflection probe was transmitted by a bundle of optical fibers to the fiber optic spectrophotometer, which was connected to a PC (Toshiba Satellite C660D), and Spectra Suite software (Ocean Optics Inc.) was used for evaluating data. A dark room apparatus was used for optical isolation of the flow cell and the detector to minimize any interference from ambient light. Sample reflectance measurement was carried out on a sample chamber that was made of PTFE. The experimental setup of reflectance measurement is shown in Figure 1.

Preparation of Solutions. $CuCl_2$ solution, 1.0×10^{-2} M, was prepared by dissolving 0.4262 g of $CuCl_2 \cdot 2H_2O$ in water and diluting to 250 mL. Ammonium acetate buffer at pH 7.0, 1.0 M, was prepared by dissolving 19.27 g of 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 of Nc in absolute ethanol and diluting to 25 mL with EtOH.

All phenolic compounds were freshly prepared in EtOH at 1 mM concentration and AA, CYS, and HCYS in water at the same concentration. Uric acid (1 mM) and bilirubin (0.5 mM) were prepared in 0.01 M NaOH, and the excess base was neutralized with 0.01 M HCl. The phenolics stock solutions were stored at 4 °C in a refrigerator prior to analysis.

The green tea bag (2 g) was dipped into and pulled out of a beaker containing 250 mL freshly boiled water for the first 2 min and allowed to steep for the remaining 3 min in the covered beaker (total steeping time was 5 min). The bag was removed, and the partly turbid solution was filtered through a black-band Whatman quantitative filter paper after cooling to room temperature.

Preparation of Tissue Homogenates. CD-1 mice were obtained from the experimental animal facility of the Faculty of Veterinary Medicine of Istanbul University. The mice were housed in polycarbonate cages (450 cm^2 area per animal), acclimatized under laboratory conditions (23 ± 2 °C, humidity 50−60%, 12 h light/dark cycles), and fed standard mouse food. Liver and kidney tissues were isolated from mice after sacrifice by decapitation. The tissue samples were washed with 0.9% NaCl, weighed (10%, w/v), and homogenized by adding cold 1.15% KCl solution in a glass homogenizer. Homogenates were immediately frozen in liquid nitrogen and kept at −80 °C until analysis.[33] Homogenates were separated from proteins by adding EtOH, centrifuging (4000 rpm, 5 min), and filtering through a 0.45 μm membrane filter before analysis.

Main CUPRAC Method. The main CUPRAC method, as described by Apak et al.,[10] is based on the reduction of a cupric neocuproine complex (Cu(II)−Nc) by antioxidants to the cuprous form (Cu(I)−Nc). To a test tube were added 1 mL each of Cu(II), Nc, and NH_4Ac buffer solutions. Antioxidant 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 30 min, the absorbance at 450 nm (A_{450}) was recorded against a reagent blank solution. The scheme for normal measurement of antioxidants is summarized as follows:

1 mL of Cu(II) + 1 mL of Nc + 1 mL of buffer + x mL of antioxidant soln + ($1.1 - x$) mL of H_2O; total volume = 4.1 mL; measure A_{450} against a reagent blank after 30 min of reagent addition.

Fiber Optic Reflectometric CUPRAC Sensor Method. To develop the fiber optic reflectometric sensor, Cu(II)−Nc complex was immobilized on the Nafion membrane. The membrane was prepared in 2.0 × 1.0 cm sizes, put into the CUPRAC solutions containing 2 mL of 2.0×10^{-2} M Cu(II) + 2 mL of 1.5×10^{-2} M Nc + 2 mL of 1 M NH_4Ac + 2.2 mL of H_2O, and mixed for 30 min in a rotator. The reagent-impregnated membrane (Nafion−Cu(II)−Nc) was taken out and placed in a tube containing 8.2 mL of ethanolic antioxidant solutions. The tube was placed in a rotator and mixed for 30 min so as to enable color development (from light blue to yellow orange color). The colored membrane was taken out and placed on a sample chamber, and its reflectance (R_f) (%) at 530 nm was read against a blank membrane prepared under identical conditions excluding analyte.

The calibration curves (reflectance vs concentration graphs) of each antioxidant were constructed under the described conditions, and their trolox equivalent antioxidant capacities (TEAC coefficients, found as the ratio of the slope of the calibration curve of each compound to that of trolox in the fiber optic reflectometric CUPRAC sensor method) were calculated.

Standard Addition of AA, QR, and TOC to Green Tea Extract. A 20 μL aliquot of green tea infusion and 20 μL of 1 mM QR, 100 μL of 1 mM AA, or 50 μL of 1 mM TOC solution were taken into a tube. AA-, QR-, and TOC-added solutions were separately subjected to reflectometric CUPRAC sensor analysis.

Measurement of Synthetic Mixture Solutions. Synthetic mixtures of the antioxidants in EtOH were prepared in suitable volume ratios, and these mixtures were diluted to 8.2 mL with EtOH and subjected to reflectometric CUPRAC sensor analysis. The theoretical trolox equivalent TAC of a synthetic mixture solution (expressed in the units of mM TR) was calculated by multiplying the TEAC coefficient of each antioxidant constituting the mixture with its final concentration (in mM TR units) and summing the products. The experimental trolox equivalent TAC of the same mixture was calculated by dividing the observed reflectance to the slope of TR calibration curve (m_{Trolox} being 3.40×10^3 under the selected conditions).

$$\text{TAC found experimentally} = \frac{\text{reflectance (total)} \pm \text{intercept}}{m_{\text{trolox}}} \times 10^3$$

Statistical Analysis. Descriptive statistical analyses were performed using Excel software (Microsoft Office 2007) for calculating the means and standard errors of the mean. Results were expressed as the mean ± standard deviation (SD). Using SPSS software for Windows (version 13), the data were evaluated by two-way analysis of variance (ANOVA).[34]

RESULTS AND DISCUSSION

The antioxidant capacity of individual antioxidants (quercetin, trolox, cysteine, uric acid, α-tocopherol, glutathione, etc.), synthetic mixtures, fruit juices, and mouse tissue homogenates (kidney and liver) were assessed by the fiber optic reflectometric sensor. A miniature fiber optic-based spectrometer, used in this study, enables practical and inexpensive antioxidant analysis outside the laboratory. Diffuse reflectance measurement from a Nafion membrane layer can be used to deduce the TAC of an unknown antioxidant sample.

The Kubelka−Munk remission function, $F(R)$, is defined as

$$F(R) = (1 - R)^2/(2R)$$

where R is the percent reflectance measured with respect to a standard white. In some literature sources, R is taken as the diffuse reflectance component of the total reflection from a mat surface.[35] $F(R)$ can be related to analyte concentration by

$$F(R) = 2.303\varepsilon C/s$$

where ε is absorptivity, C the concentration of the analyte, and s the scattering coefficient of the sample surface. By assuming the absorptivity and scattering coefficient of the measured surface are constant at a given wavelength, $F(R)$ can therefore be related directly to analyte concentration.[36] The use of the modified versions of the Kubelka−Munk equation has demonstrated a linear or quasi-linear relationship between the intensity of reflectance and the sample concentration.[35]

The chromogenic oxidizing reagent of the CUPRAC method, bis(neocuproine)copper(II) chloride [Cu(II)−Nc] electrostatically attached to the polysulfonate functional groups of the sensor membrane, reacts with polyphenols [$Ar(OH)_n$] to produce the CUPRAC chromophore [Cu(I)−Nc] on the membrane where the liberated protons are buffered by the relatively concentrated NH_4Ac buffer solution (pH 7.0).

$$n\text{Cu(Nc)}_2^{2+} + \underset{\{\text{e.g., Ar(OH)}_n\}}{n\text{-}e \text{ reductant}} \leftrightarrow n\text{Cu(Nc)}_2^{+} + \underset{\{\text{e.g., Ar(=O)}_n\}}{n\text{-}e \text{ oxidized product}} + n\text{H}^+$$

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In this reaction, polyphenols with suitably situated Ar−OH groups are oxidized through semiquinone radicals to the corresponding quinones, and the reduction product, that is, yellow-orange bis(neocuproine)copper(I) chelate, is the active chromophore responsible for absorption or reflection of visible light showing maximum absorption at 450 nm and reflection at 530 nm (see Figure 2 for Cu(I)−Nc reflectance (%) spectra

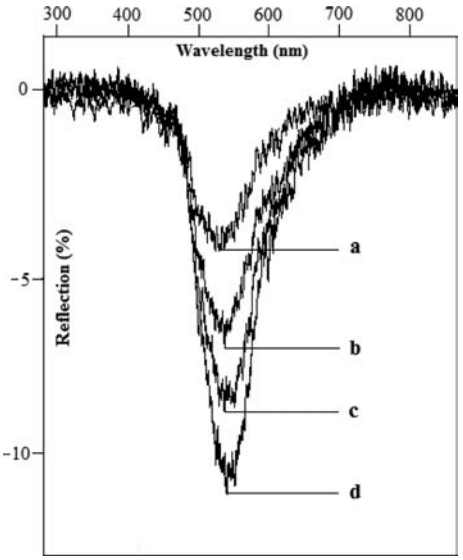

Figure 2. Reflectance (%) spectra of Cu(I)−Nc chelate produced on the sensor membrane as a result of sensor-attached Cu(II)−Nc reaction with various concentrations of trolox (final concentration): (a) 7.9 μM; (b) 15.8 μM; (c) 23.7 μM; (d) 31.6 μM.

obtained by reacting various concentrations of TR with the CUPRAC reagent). It should be noted that not all phenolic −OH are reduced to the corresponding quinones, and the efficiency of this reduction depends on the number and position of the phenolic −OH groups, as well as on the overall conjugation level of the polyphenolic molecule. In addition, the geometry of the CUPRAC oxidant, Cu(II)−Nc, attached to the membrane layer may not be identical with that in solution.

Analytical Figures of Merit. Table 1 summarizes the precision and recovery of the fiber optic reflectometric CUPRAC sensor method using AA, QR, and TOC as representative antioxidant compounds. The precision, which is expressed as the relative standard deviation (RSD, %) in reflectance measurement within the tested concentration range, was approximately 5.0%. The recovery of the method varied from 91.5 to 93.4% within individual batches covering vitamins, simple phenolic antioxidants, and flavonoids. The recoveries for the individual antioxidant compounds were calculated by means of a CUPRAC calibration curve (as reflectance vs concentration) for the specific antioxidant of concern.

The linear equation for the calibration graph of TR drawn at the wavelength of 530 nm with respect to the reflectometric CUPRAC sensor method is

$$R_{f(530)} = 3.40 \times 10^3 C_{TR} + 0.004 \ (r = 0.991)$$

and the slope of TR calibration curve is $m = 3.40 \times 10^3$ L mol^{-1} mm^{-1}. The limit of detection (LOD) and limit of quantification (LOQ) for TR in the fiber optic reflectometric CUPRAC method were calculated using the equations LOD = $3s_{bl}/m$ and LOQ = $10s_{bl}/m$, respectively (where s_{bl} is the standard

Table 1. Precision and Recovery of the Fiber Optical Sensor Based-CUPRAC Assay

		concn
quercetin addition to green tea extract	added concn (μM)	2.44
	mean (μM)	2.28
	SD[a]	0.05
	% RSD[b]	2.21
	% REC[c]	93.4
ascorbic acid addition to green tea extract	added concn (μM)	12.2
	mean (μM)	11.2
	SD[a]	0.56
	% RSD[b]	4.97
	% REC[c]	91.8
α-tocopherol addition to green tea extract	added concn (μM)	6.10
	mean (μM)	5.58
	SD[a]	0.26
	% RSD[b]	4.58
	% REC[c]	91.5

[a]Standard deviation. [b]Relative standard deviation. [c]Recovery (N = 3).

deviation of a blank and m is the slope of the calibration line). The LOD and LOQ for TR were found to be 0.53 and 1.76 μM, respectively. The precision, which is expressed as the RSD % in the tested concentration range, was approximately 4.80%. CUPRAC reflectances of TR were linear or quasi-linear within the concentration range 1.6−57.6 μM (as final concentrations in solution), and the method showed relatively high linearity (r = 0.991) over a broad concentration range of analyte, a condition not frequently experienced in solid sensors.

The reagent-loaded (single use) sensor was tested for stability and was shown to lose only 3% signal intensity after 15 days of storage in distilled water kept in a desiccator at room temperature.

The correlation between the reflectance (R_f) for a given antioxidant compound (ascorbic acid, α-tocopherol, glutathione, uric acid, bilirubin, and other phenolic antioxidants) and its molar concentration (C) was evaluated using 19 compounds. Table 2 summarizes the linear equations ($R_f = mC + n$), the coefficient of determination (r), and linear concentration ranges of these pure compounds. As can be seen from Table 2, all antioxidants could be assayed with the fiber optic reflectometric CUPRAC sensor method (TEAC coefficients (significantly different) and (P = 0.05, F_{exp}= 3.554, $F_{crit(table)}$= 4.414, $F_{exp} < F_{crit(table)}$)). The TEAC coefficients found by the fiber optic reflectometric CUPRAC sensor method correlated well (r = 0.884) with those of the main CUPRAC method (Table 2).[10,37] The redox reactions between antioxidants and Cu(II)−Nc attached to the membrane initially required the diffusion of the analytes to the membrane surface, so the reaction rate is expected to decrease with respect to that in bulk solution. The steric hindrance caused by the bulky substituents of the polyphenol and the methyl groups of the immobilized oxidizing reagent, Cu(II)−Nc, is also a slowing factor for electron transfer, whereas the local enrichment of the oxidizing reagent for antioxidant molecules diffused to the membrane surface is a rate-enhancing factor compared to that in solution.[14] In addition, the planar structure of the functionalized membrane may impart a somewhat distorted geometry relative to the ideal one[24] such as a distorted square-planar character, to the originally tetrahedral $Cu(Nc)_2^{2+}$ chelate

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Table 2. Linear Calibration Equations, Linear Ranges, and TEAC Coefficients of the Tested Antioxidants with Respect to the Fiber Optic Reflectometric CUPRAC Sensor Method[a]

	linear eq and correl coeff	linear range (μM)	TEAC (reflectometric CUPRAC sensor)[b]	TEAC (main CUPRAC)[c10,37]
		Food Antioxidants		
quercetin (QR)	$R_f = 1.29 \times 10^4 c + 0.01$ $r = 0.999$	0.3−14.6	3.79	4.38
catechin (CT)	$R_f = 3.94 \times 10^3 c + 0.006$ $r = 0.999$	2.3−49.2	1.16	3.09
rutin (RT)	$R_f = 7.28 \times 10^3 c + 0.01$ $r = 0.998$	0.5−26.0	2.14	2.56
caffeic acid (CFA)	$R_f = 8.36 \times 10^3 c + 0.007$ $r = 0.991$	0.9−23.1	2.46	2.89
fisetin (FS)	$R_f = 9.19 \times 10^3 c + 0.008$ $r = 0.997$	0.7−20.9	2.70	3.90
morin (MR)	$R_f = 8.09 \times 10^3 c + 0.009$ $r = 0.990$	0.7−23.6	2.38	1.88
gallic acid (GA)	$R_f = 5.99 \times 10^3 c + 0.01$ $r = 0.995$	0.8−31.7	1.76	2.62
naringin (N)	$R_f = 0.81 \times 10^3 c + 0.01$ $r = 0.980$	2.5−231.0	0.24	0.02
naringenin (NG)	$R_f = 1.42 \times 10^3 c + 0.006$ $r = 0.990$	0.1−137.0	0.42	0.05
rosmarinic acid (RA)	$R_f = 9.73 \times 10^3 c + 0.01$ $r = 0.995$	0.5−19.5	2.86	5.30
kaempferol (KA)	$R_f = 3.10 \times 10^3 c + 0.01$ $r = 0.985$	0.3−59.7	0.91	1.58
		Plasma Antioxidants		
ascorbic acid (AA)	$R_f = 1.44 \times 10^3 c + 0.01$ $r = 0.999$	3.5−132	0.42	0.96
uric acid (UA)	$R_f = 3.00 \times 10^3 c + 0.008$ $r = 0.998$	2.3−64.0	0.88	0.96
α-tocopherol (TOC)	$R_f = 3.19 \times 10^3 c + 0.01$ $r = 0.987$	0.3−58.3	0.94	1.10
bilirubin (BIL)	$R_f = 1.15 \times 10^3 c + 0.01$ $r = 0.998$	1.7−163.0	3.38	3.18
glutathione (GSH)	$R_f = 1.89 \times 10^3 c + 0.009$ $r = 0.995$	3.2−101.0	0.56	0.58
cysteine (CYS)	$R_f = 1.35 \times 10^3 c + 0.005$ $r = 0.994$	7.4−144.0	0.40	0.39
homocysteine (HCYS)	$R_f = 1.89 \times 10^3 c + 0.006$ $r = 0.993$	4.8−10.6	0.56	0.47

[a]Samples were analyzed in triplicate. Number of experimental data (n) = 5−7. [b]TEAC coefficients (significantly different); (P = 0.05, F_{exp}= 3.554, $F_{crit(table)}$= 4.414, $F_{exp} < F_{crit(table)}$). [c]$TEAC_{main} = 1.22\ TEAC_{sensor} + 0.08$ (r = 0.884).

and may preferentially increase the reaction affinity of this retained reagent toward fully coplanar polyphenols (such as quercetin having complete conjugation among rings) over noncoplanar ones (such as catechin) devoid of such conjugation.[38] Because of these conflicting factors, there may not be an exact one-to-one correspondence between the TEAC values of antioxidants measured with the main CUPRAC and the reflectometric CUPRAC sensor procedures (Table 2). Nevertheless, the slope of the calibration line between the two methods was close to unity (i.e., $TEAC_{main} = 1.22\ TEAC_{sensor} + 0.08$).

All of the easily oxidized flavonoids exhibited standard 1-e reduction potentials of $\leq$0.2 V, whereas naringenin, having a potential close to that of the $Cu(Nc)_2^{2+}-Cu(Nc)_2^{+}$ couple (i.e., half-wave potential, $E_{1/2}$, of the first oxidation wave of naringenin in pH 7.5 phosphate buffer was measured as 0.59 V),[39] underwent a slow reaction with the reagent having $E^o \approx$ 0.6 V.[10] Naringenin oxidation could be forced to completion only after 50 °C incubation in the main CUPRAC method, the TEAC coefficient for NG being 0.05. In the reflectometric CUPRAC sensor method, NG can be directly assayed without incubation, the corresponding TEAC coefficient being 0.42. The $TEAC_{CUPRAC}$ coefficients of NG in pure EtOH and MeOH were 0.05 and 0.57, respectively,[40] probably due to facilitated electron transfer in ionizing solvents capable of anion (phenolate) solvation, because MeOH is the alcohol that best supports ionization.[41] Compared to aqueous solution, the sensor membrane may also be considered as a less hydrophilic medium, enhancing the rate of e transfer for certain compounds. Similarly, naringin, the glycoside form of naringenin, can be directly assayed with the reflectometric CUPRAC sensor method (unlike the main CUPRAC method) without any incubation or hydrolysis, the corresponding TEAC coefficient being 0.24 (TEAC coefficient with the main CUPRAC method is 0.02). According to the developed method, naringin shows lower antioxidant activity than the

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corresponding aglycon, naringenin, again in accordance with theoretical expectations.[42] In addition, by immobilizing the cationic oxidant (Cu(II)−Nc) onto the water-insoluble Nafion anionic polymer, a molecular aggregate can be formed showing a unique and active redox behavior. Such a confinement of the redox-active complex may increase its local concentration (compared to that in homogeneous solution) in the polymer matrix, thereby increasing the oxidation reaction rate (e.g., for naringenin, naringin). The TEAC coefficients of the tested thiols (GSH, CYS, and HCYS) were close to 0.5, in accordance with their physiological reversible 1-*e* oxidation reactions to the corresponding disulfides.[17,43]

Although the antioxidant power of some antioxidants (naringin, naringenin, etc.) was enhanced in the reflectometric sensor method compared to that in main CUPRAC assay, significantly lower TEAC coefficients were obtained for rosmarinic acid, ascorbic acid, and catechin (i.e., 2-fold reduction of TEAC values for ascorbic acid and rosmarinic acid and 3-fold reduction for catechin) (Table 2). The decreased trolox equivalent antioxidant capacities of both ascorbic acid and rosmarinic acid (having pK_{a1} values of 4.2 and 2.8, respectively) may be attributed to their negative charges at pH 7.0, because both compounds are in the negatively charged anionic conjugate base forms at the working pH of the sensor and should essentially be repelled by the negatively charged free (unbound) sulfonate groups of the Nafion membrane.[43] Despite the four phenolic −OH groups and excellent conjugated structure of rosmarinic acid, the relative decrease in its TEAC coefficient in the reflectometric sensor assay may also arise from its large molecular size (being an important parameter in optical sensor response)[44] as well as from its low pK_a.[45] On the other hand, catechin does not contain the 2,3-double bond conjugated with the 4-oxo group responsible for electron delocalization, which is an important prerequisite for the high antioxidant power of compounds such as quercetin.[46] The excellent antioxidant properties of quercetin result from the formation of a stable aryloxy radical due to C2−C3 double bond and planar geometry,[47] which delocalizes the radical throughout the whole molecule, whereas A and B rings are perpendicular to each other in catechin.[38] When the formed aryloxy radicals resulting from one-electron oxidation of a flavonoid are stabilized by conjugation, the redox potential of the flavonoid is decreased, thereby increasing its antioxidant power. In addition, lack of planarity of catechin is an important obstacle playing a part in its decreased antioxidant power in view of the enforced distorted planar geometry of copper−neocuproine on the sensor membrane.

TAC Measurement of Synthetic Mixture Solutions. Possible combinations of ternary mixtures of antioxidants were synthetically prepared, and the suitably diluted mixture solutions were analyzed for TAC in trolox equivalent concentration units (mM TR) using the fiber optic reflectometric CUPRAC sensor method (Table 3). The experimentally measured TAC values of mixtures were generally within ±7% interval of the theoretically computed ones using the formula

$$TAC_{mixture} = TEAC_1 \cdot C_1 + TEAC_2 \cdot C_2 + TEAC_3 \cdot C_3 + \ldots + TEAC_n \cdot C_n$$

where 1, 2, ..., *n* denote the corresponding constituents of the synthetic mixture. This demonstrated additivity of TAC values ensures accurate analysis of antioxidant mixtures in the absence of chemical deviations from Beer's law.

Table 3. Comparison of the Theoretically Expected and Experimentally Found Trolox Equivalent Antioxidant Capacities of Synthetic Mixtures of Antioxidants (with Respect to the Fiber Optic Reflectometric CUPRAC Sensor Method)[a]

no.	composition of mixture	capacity expected (as μM TR equiv)	capacity found experimentally (as μM TR equiv)
1	50 μL of 1 mM TOC 25 μL of 1 mM RA 20 μL of 1 mM QR	23.7	22.0 ± 0.1
2	50 μL of 1 mM UA 25 μL of 2 mM GA 20 μL of 1 mM QR	25.4	24.5 ± 0.1
3	50 μL of 1 mM UA 25 μL of 1 mM BIL 100 μL of 1 mM AA	20.8	20.4 ± 0.3
4	25 μL of 2 mM GA 100 μL of 1 mM AA 25 μL of 1 mM RA	24.6	24.0 ± 0.2

[a]Samples were analyzed in triplicate. $P = 0.05$, $F_{exp} = 9.92$, $F_{crit(table)} = 10.13$, $F_{exp} < F_{crit(table)}$.

The two-way analysis of variance (ANOVA) comparison by the aid of *F* test of the mean-squares of "between-treatments" (i.e., theoretically expected capacity with respect to fiber optic reflectometric CUPRAC sensor method and experimentally found capacities of different mixtures in Table 3) and of residuals[34] for a number of real samples (consisting of synthetic mixtures of antioxidants) enabled us to conclude that there was no significant difference between the population means for a given sample. In other words, the experimentally found and theoretically calculated capacities were alike at the 95% confidence level ($F_{exp} = 9.92$, $F_{crit(table)} = 10.13$, $F_{exp} < F_{crit(table)}$ at $P = 0.05$). Thus, the proposed methodology was validated for synthetic mixtures of antioxidants of differing lipophilicities. On the other hand, there was significant difference between samples with respect to composition of mixtures (i.e., the "residual" mean-square was much greater than the "between-sample" mean-square at the 95% confidence level). This was natural, as these mixtures were deliberately prepared at different total concentrations of trolox equivalents.

TAC Measurement of Fruit Juices. In Figure 3, the TAC values found with the fiber optic reflectometric CUPRAC sensor and main CUPRAC method are reported as trolox equivalents (mM TR). The hierarchy for TAC of fruit juices with respect to the fiber optic reflectometric CUPRAC sensor method was apricot < peach < orange < cherry. Linear regression analysis of capacity data presented in Figure 3 found with the main CUPRAC assay showed that this assay correlated well with the fiber optic reflectometric CUPRAC sensor assay ($P = 0.05$, $F_{exp} = 1.43$, $F_{crit(table)} = 10.13$, $F_{exp} < F_{crit(table)}$).

Application of the Fiber Optic Reflectometric CUPRAC Sensor to Mouse Tissue Homogenates. The fiber optic reflectometric CUPRAC sensor was applied to tissue homogenates, and the results were compared with those obtained by the main CUPRAC method as TAC values (mM TR). Liver homogenates were shown to exhibit higher

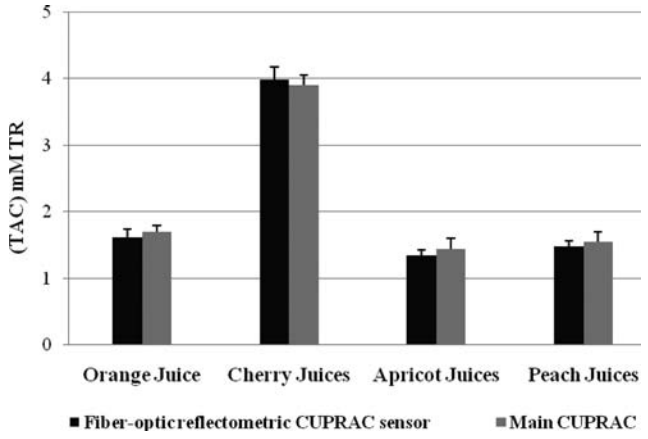

Figure 3. TAC values (mM) of some commercial fruit juices found with the fiber optic reflectometric CUPRAC sensor and main CUPRAC method. Data are presented as the mean ± SD (error bars), $N = 3$.

antioxidant capacity than kidney homogenates. The TAC values of identical tissue homogenates found with fiber optic reflectometric CUPRAC sensor method were very close to those measured with the main CUPRAC method (Figure 4) ($P = 0.05$, $F_{exp} = 0.131$, $F_{crit(table)} = 10.13$, $F_{exp} < F_{crit(table)}$).

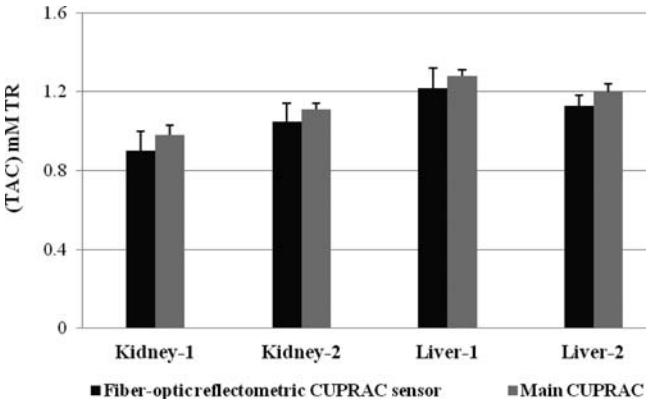

Figure 4. TAC values (mM) of mouse tissues found with the fiber optic reflectometric CUPRAC sensor and main CUPRAC method. Data are presented as the mean ± SD (error bars), $N = 3$.

Performance and Prospects of the CUPRAC Reflectometric Sensor. This work describes the design of a fiber optic reflectometric CUPRAC sensor that is rapid, reasonably sensitive, and selective for the determination of TAC in synthetic mixtures, commercial fruit juices, and biological samples. The Cu(II)−Nc chromogenic reagent was immobilized onto a Nafion membrane, and the reflectance change associated with the formation of the highly colored Cu(I)−Nc chelate as a result of reaction with antioxidants was measured at 530 nm. Reflectance was recorded with a miniature, commercially available fiber optic spectrometer. The reflectometric CUPRAC sensor can be used for quantitative assessment of total antioxidant capacity of complex matrices such as food extracts and biological samples in trolox equivalent units. This assay was validated through linearity, additivity, precision, and recovery, demonstrating its reliability and robustness. Despite the differences in TEAC values of certain antioxidants measured with the main CUPRAC and reflectometric sensor methods, the TAC values of the tested food and biological extracts measured with the two assays were almost alike. The sensor is small and cheap, suitable to fit in a portable instrument for in situ antioxidant analysis. This method can be potentially adapted to a kit format capable of performing simple antioxidant sensing in a complex matrix. As opposed to the more valuable but less frequent transparent membrane sensors, the proposed methodology is believed to give rise to the development of more versatile sensors having optical inhomogeneity, as reflectance rather than absorbance is measured. As a result of this study, the proposed fiber optic reflectometric CUPRAC sensor for practical, versatile, and low-cost TAC measurement is believed to broaden the applicability of the method to antioxidant researchers in all fields comprising food, bioanalytical, and medicinal chemistry.

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REFERENCES

(1) Halliwell, B.; Gutteridge, J. M. C. Free radicals, other reactive species and disease. In *Free Radicals in Biology and Medicine*, 3rd ed.; Halliwell, B., Gutteridge, J. M. C., Eds.; Oxford University Press: Oxford, UK, 1999; pp 617−783.

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

(3) Prior, R. L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. *J. Agric. Food Chem.* **2005**, *53*, 4290−4302.

(4) Huang, D.; Ou, B.; Prior, R. L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* **2005**, *53*, 1841−1856.

(5) Miller, N. J.; Rice-Evans, C.; 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.

(6) 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.

(7) Folin, O.; Ciocalteu, V. On tyrosine and tryptophane determinations in proteins. *J. Biol. Chem.* **1927**, *73*, 627−650.

(8) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152−178.

(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) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E. 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. *J. Agric. Food Chem.* **2004**, *52*, 7970−7981.
(11) Güçlü, K.; Altun, M.; Özyürek, M.; Karademir, S. E.; Apak, R. Antioxidant capacity of fresh, sun- and sulfited-dried Malatya apricot (*Prunus armeniaca*) assayed by CUPRAC, ABTS/TEAC and Folin methods. *Int. J. Food Sci. Technol.* **2006**, *41*, 76−85.
(12) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E.; Erçağ, E. The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. *Int. J. Food Sci. Nutr.* **2006**, *57*, 292−304.
(13) Çelik, S. E.; Özyürek, M.; Altun, M.; Bektaşoğlu, B.; Güçlü, K.; Berker, K. I.; Özgökçe, F.; Apak, R. Antioxidant capacities of herbal plants used in the manufacture of Van herby cheese: 'otlu peynir'. *Int. J. Food Prop.* **2008**, *11*, 747−761.
(14) Bener, M.; Özyürek, M.; Güçlü, K.; Apak, R. Development of a low-cost optical sensor for cupric reducing antioxidant capacity measurement of food extracts. *Anal. Chem.* **2010**, *82*, 4252−4258.
(15) Özyürek, M.; Bener, M.; Güçlü, K.; Dönmez, A. A.; Süzgeç-Selçuk, S.; Pırıldar, S.; Apak, R.; Meriçli, A. H. Evaluation of antioxidant activity of *crataegus* species collected from different regions of Turkey. *Rec. Nat. Prod.* **2012**, *6*, 263−277.
(16) Bener, M.; Özyürek, M.; Güçlü, K.; Apak, R. Polyphenolic contents of natural dyes produced from industrial plants assayed by HPLC and novel spectrophotometric methods. *Ind. Crops Prod.* **2010**, *32*, 499−506.
(17) Apak, R.; Güçlü, K.; Özyürek, M.; Karademir, S. E.; Altun, M. Total antioxidant capacity assay of human serum using copper(II)-neocuproine as chromogenic oxidant: the CUPRAC method. *Free Radical Res.* **2005**, *39*, 949−961.
(18) Bekdeşer, B.; Özyürek, M.; Güçlü, K.; Apak, R. Development of a novel spectroscopic sensor for the hydroxyl radical scavenging activity measurement of biological samples. *Talanta* **2012**, *99*, 689−696.
(19) Ojeda, C. B.; Rojas, F. S. Recent development in optical chemical sensors coupling with flow injection analysis. *Sensors* **2006**, *6*, 1245−1307.
(20) McDonagh, C.; MacCraith, B. D.; O'Keefe, G.; Butler, T.; O'Kelly, B.; McGilp, J. Fibre optic chemical sensors based on evanescent wave interactions in sol-gel derived porous coatings. *J. Sol−Gel Sci. Technol.* **1994**, *2*, 661−665.
(21) Wolfbeis, O. S. *Fiber Optic Chemical Sensors and Biosensors*; CRC Press: Boca Raton, FL, 1991.
(22) Silva, V. S.; Ruffmann, B.; Vetter, S.; Boaventura, M.; Mendes, A.; Madeira, L. M.; Nunes, S. P. Mass transport of direct methanol fuel cell species in sulfonated poly(ether ether ketone) membranes. *Electrochim. Acta* **2006**, *51*, 3699−3706.
(23) Zinger, B.; Shier, P. Spectroscopic studies of cationic dyes in Nafion®. Preliminary investigation of a new sensor for hydrophilic contamination in organic solvents. *Sens. Actuators B* **1999**, *56*, 206−214.
(24) Ditder, M. A.; Pierre-Jacques, H.; Harrington, S. A. Immobilization as a mechanism for improving the inherent selectivity of photometric reagents. *Anal. Chem.* **1986**, *58*, 195−200.
(25) Choudhary, R.; Bowser, T. J.; Weckler, P.; Maness, N. O.; McGlynn, W. Rapid estimation of lycopene concentration in watermelon and tomato puree by fiber optic visible reflectance spectroscopy. *Postharvest Biol. Technol.* **2009**, *52*, 103−109.
(26) Steinberg, I. M.; Milardović, S. Chromogenic radical based optical sensor membrane for screening of antioxidant activity. *Talanta* **2007**, *71*, 1782−1787.
(27) Lee, P. M.; Arsad, M. S. M.; Yussoff, A. C.; Hung, L. K. Colored thin film for screening and evaluation of antioxidants. *Sci. Soc. Res. (CSSR)* **2010**, 717−720.
(28) Palaroan, W. S.; Bergantin, J.; Sevilla, F. Optical fiber chemiluminescence biosensor for antioxidants based on an immobilized luminol/hematin reagent phase. *Anal. Lett.* **2000**, *33*, 1797−1810.
(29) Ozyurt, D.; Demirata, B.; Apak, R. Determination of total antioxidant capacity by a new spectrophotometric method based on Ce(IV) reducing capacity measurement. *Talanta* **2007**, *71*, 1155−1165.
(30) Sharpe, E.; Frasco, T.; Andreescu, D.; Andreescu, S. Portable ceria nanoparticle-based assay for rapid detection of food antioxidants (NanoCerac). *Analyst* **2013**, *138*, 249−262.
(31) Moliner-Martinez, Y.; Herraez-Hernandez, R.; Campins-Falco, P. Improved detection limit for ammonium/ammonia achieved by Berthelot's reaction by use of solid-phase extraction coupled to diffuse reflectance spectroscopy. *Anal. Chim. Acta* **2005**, *534*, 327−334.
(32) Andreescu, S.; Avramescu, A.; Bala, C.; Magearu, V.; Marty, J. L. Detection of organophosphorus insecticides with immobilized acetylcholinesterase-comparative study of two enzyme sensors. *Anal. Bioanal. Chem.* **2002**, *374*, 39−45.
(33) Alia, M.; Horcajo, C.; Bravo, L.; Goya, L. Effect of grape antioxidant dietary fiber on the total antioxidant capacity and the activity of liver antioxidant enzymes in rats. *Nutr. Res. (N.Y.)* **2003**, *23*, 1251−1267.
(34) Miller, J. C.; Miller, J. N. *Statistics for Analytical Chemists*, 3rd ed.; Ellis Horwood and Prentice Hall: New York and London, 1993.
(35) Ghauch, A.; Turnar, C.; Fachinger, C.; Rima, J.; Charef, A.; Suptil, J.; Martin-Bouyer, M. Use of diffuse reflectance spectrometry in spot test reactions for quantitative determination of cations in water. *Chemosphere* **2000**, *40*, 1327−1333.
(36) Gazda, D. B.; Fritz, J.; Porter, S. M. D. Determination of nickel(II) as the nickel dimethylglyoxime complex using colorimetric solid phase extraction. *Anal. Chim. Acta* **2004**, *508*, 53−55.
(37) Apak, R.; Güçlü, K.; Demirata, B.; Özyürek, M.; Çelik, S. E.; Bektaşoğlu, B.; Berker, K. I.; Özyurt, D. Comparative evaluation of total antioxidant capacity assays applied to phenolic compounds, and the CUPRAC assay. *Molecules* **2007**, *12*, 1496−1547.
(38) Fukuhara, K.; Nakanishi, I.; Kansui, H.; Sugiyama, E.; Kimura, M.; Shimada, T.; Urano, S.; Yamaguchii, K.; Miyata, N. Enhanced radical-scavenging activity of a planar catechin analogue. *J. Am. Chem. Soc.* **2002**, *124*, 5952−5953.
(39) Yang, B.; Kotani, A.; Arai, K.; Kusu, F. Estimation of the antioxidant activities of flavonoids from their oxidation potentials. *Anal. Sci.* **2001**, *17*, 599−604.
(40) Çelik, S. E.; Özyürek, M.; Güçlü, K.; Apak, R. Solvent effects on the antioxidant capacity of lipophilic and hydrophilic antioxidants measured by CUPRAC, ABTS/persulphate and FRAP methods. *Talanta* **2010**, *81*, 1300−1309.
(41) Litwinienko, G.; Ingold, K. U. Abnormal solvent effects on hydrogen atom abstractions. 1. The reactions of phenols with 2,2-diphenyl-1-picrylhydrazyl (dpph*) in alcohols. *J. Org. Chem.* **2003**, *68*, 3433−3438.
(42) 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.
(43) Furbee, J. W., Jr.; Thomas, C. R.; Kelly, R. S.; Malachowski, M. R. Mediated electrochemical reduction of cytochrome *c* and tyrosinase at perfluorosulfonated ionomer coated electrodes. *Anal. Chem.* **1993**, *65*, 1654−1657.
(44) Zhao, S.; Zhang, K.; Bai, Y.; Yang, W.; Sun, C. Glucose oxidase/colloidal gold nanoparticles immobilized in Nafion film on glassy carbon electrode: direct electron transfer and electrocatalysis. *Bioelectrochemistry* **2006**, *69*, 158−163.
(45) Rein, M. *Copigmentation Reactions and Color Stability of Berry Anthocyanins*. Academic dissertation, University of Helsinki, Department of Applied Chemistry and Microbiology, Food Chemistry Division, Helsinki, Finland, 2005.
(46) Pietta, P.-G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63*, 1035−1042.
(47) 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.