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Novel optical sensor-based method for determining total tocopherol content in serum

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Abstract: Lipid-soluble endogenous antioxidants, such as tocopherols, protect biological structures from oxidative damage through their chain-breaking ability and thus the development of a simple method for tocopherol determination is important. In this study, a novel optical sensor-based method was proposed for determining total tocopherol content (TTC) of serum, with selectivity for α-tocopherol over β-carotene. The proposed method is based on the use of a copper(II)-neocuproine complex (Cu(II)-Nc) reagent immobilized onto a Nafion membrane, and the absorbance changes associated with the formation of Cu(I)-Nc chelate on the membrane strip as a result of reaction with serum tocopherols were measured at 450 nm. The calibration graph of α-tocopherol was linear with a slope of 1.96×10^4 L mol^{-1} cm^{-1}. The limit of detection and limit of quantification for tocopherol in the proposed method were found as 0.50 and 1.67 µM, respectively. The TTC value of synthetic serum samples was determined with the use of the proposed method, and the obtained results were comparable to those of the CUPRAC spectrophotometric method. The proposed method enables simple, rapid, and in situ determination of total tocopherol content of serum by taking advantage of optical sensors.

Key words: Optical sensor, tocopherol, serum, antioxidants, CUPRAC assay

1. Introduction

Under oxidative stress conditions, biological systems are exposed to oxidative damage resulting in serious problems such as cancer, cardiovascular diseases, and neurodegenerative disorders.[1,2] Vitamin E, an important lipid-soluble antioxidant vitamin, defends against oxidative injury and is therefore believed to provide protection against various related diseases.[3,4] Vitamin E is present in at least eight structural forms in nature, including four tocopherols (α, β, γ, and δ) and four tocotrienols (α, β, γ, and δ). In vivo, α-tocopherol is the most prevalent type of vitamin E and the most biologically active ingredient.[5,6] α-Tocopherol is capable of breaking the chain reaction of lipid peroxidation by scavenging lipid peroxyl radicals. Low levels of tocopherol were reported to be possibly associated with some vital disease states such as some types of cancer, coronary heart disease, other degenerative and neurological disorders.[7–10] On the other hand, high levels of tocopherol may also cause toxic effects.[11–13]

There are several methods in the literature for the identification of tocopherols alone or in combination with other antioxidants, many of which are based on chromatography.[14–17] These high-cost and sophisticated techniques are not available in many conventional laboratories. For this reason, simple spectroscopic methods utilizing UV-Vis spectrophotometers, though less selective, can be found in almost every laboratory and are

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frequently preferred. The Emmerie–Engel reaction was used for the spectrophotometric estimation of serum tocopherol levels.[18] This colorimetric method is based on the measurement of the red color of the α,α'-dipyridyl-iron(II) complex $Fe^{II}(bipy)_3$ formed from the reduction of iron(III) chloride by tocopherols in the presence of bipy ligand. The Emmerie–Engel method was modified to increase the sensitivity using reagents such as 2,2',2''-tripyridine, 2,4,6-tripyridyl-s-triazine, and 4,7-diphenyl-1,10-phenanthroline instead of the bipyridyl ligand used to form the colored complex with iron.[19–22] In some studies, the tocopherol compound was separated from other antioxidants and interferents before analysis using techniques such as saponification, extraction, and thin layer chromatography.[23,24]

It has been known for some situations that the serum level of cholesterol can be related to the level of total tocopherol in serum. Since vitamin E supplementation shows both benefit and toxicity, vitamin E status as a serum tocopherol concentration should be controlled. Therefore, simple, practical, and low-cost methods are needed to measure the total tocopherol level in human serum.

The CUPRAC (cupric ion reducing antioxidant capacity) method has been successfully applied to serum for evaluating antioxidant capacity.[25] Lipophilic antioxidants (α-tocopherol and β-carotene) were measured in total with the use of the CUPRAC method. In this study, a CUPRAC sensor was applied to measure the total tocopherol content of serum samples. In the CUPRAC sensing method, the chromogenic oxidizing reagent, i.e. bis(neocuproine)copper(II) complex (Cu(II)-Nc), was immobilized onto a Nafion membrane, and the absorbance changes associated with the formation of the highly colored Cu(I)-Nc chelate on the membrane as a result of reaction with antioxidants were measured at 450 nm. Lipophilic antioxidants were extracted from an ethanolic solution of serum by using n-hexane subjected to centrifugation. The total tocopherol content was determined as α-tocopherol equivalent by applying the CUPRAC sensor to the obtained n-hexane extract of serum. This method has superiority over the conventional CUPRAC assay (which measures the cumulative action of all serum antioxidants), because serum tocopherol content is determined separately. Although β-carotene coexisting with tocopherol in the lipophilic fraction of serum would normally interfere with the conventional CUPRAC assay carried out in bulk solution, this compound does not adversely affect the individual determination of tocopherol by the CUPRAC sensor. The proposed sensing method has the advantage of being a fast and simple analytical tool for on-site analysis.

2. Results and discussion

The optical sensor-based method was proposed to determine total tocopherol content (TTC) of serum samples. The proposed method enables simple and inexpensive in situ determination of tocopherol. Absorbance measurement of the colored Nafion membrane layer can be used to evaluate the TTC of a serum sample. The selectivity of the sensor for tocopherol is realized by the solvent (excluding hydrophilic serum antioxidants) and by the sensor itself (excluding the β-carotene constituent of the lipophilic fraction of serum). Figure 1 illustrates the redox reaction between the chromogenic oxidizing reagent used for the CUPRAC assay and tocopherols in hexane extract of serum. This reagent was useful at physiological pH (pH 7.0), and the absorbance of the Cu(I)-chelate formed as a result of redox reaction with Cu(II)-reducing tocopherols was measured at 450 nm.

The absorbance recorded with the proposed method was linearly dependent on the concentration of tocopherol. The calibration curve of tocopherol was obtained by using the proposed method, and the results were expressed as tocopherol equivalent (Figure 2). The linear equation for the calibration graph of tocopherol drawn with respect to the proposed method at the wavelength of 450 nm was $A_{450} = 1.96 \times 10^4\ C_{tocopherol} +$

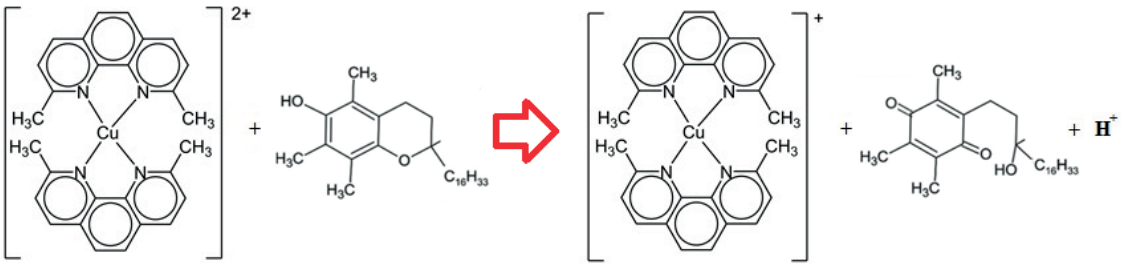

Figure 1. The reaction of the CUPRAC reagent (Cu(II)-Nc) with tocopherols.

0.0219, where C symbolizes the molar concentration and slope expressed the molar absorptivity ($\varepsilon = 1.96 \times 10^4$ L mol^{-1} cm^{-1}). The limit of detection and limit of quantification values for tocopherol obtained by the proposed method were 0.50 and 1.67 µM, respectively. Absorbances of tocopherol were linear in the range of 2.15–64.65 µM as final concentrations in solution, and the method showed high linearity (r = 0.9990) over a wide concentration range. Both the width of the linear concentration range and the closeness of the correlation coefficient (r) to unity were unusual for optical sensors.

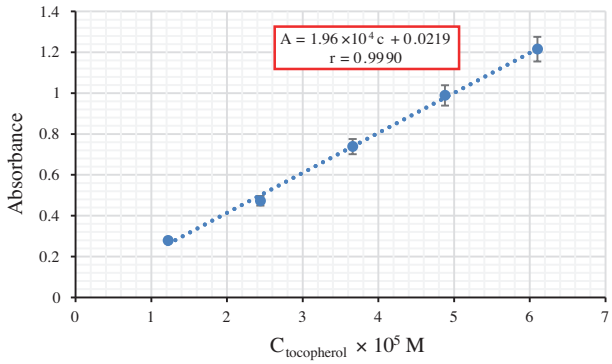

Figure 2. Calibration curve with varying concentrations of tocopherol for the proposed optical sensor-based method.

Known amounts of tocopherol were spiked to serum extract, and TTC determination with the optical sensor was performed in these solutions to investigate the precision and recovery of the method (Table 1). The relative standard deviation of the absorbance measurements at the concentration level of the experiments was representative of the precision of the method, where the highest value for the proposed optical sensor was found to be 8.16%. The percentage recovery values of the added antioxidants were found to vary from 95.49% to 106.8% by the proposed method. The TTC of the serum extract (i.e. without spike) is shown in Table 1 in µM concentration of added tocopherol standard.

2.1. Interference effect of β-carotene on the proposed sensing method

Among the serum antioxidants, only the lipophilic constituents, namely α-tocopherol and β-carotene, can pass to the organic phase after serum extraction with n-hexane. Although the molar absorptivity is very high for β-carotene with the conventional CUPRAC method selected as the reference method ($\varepsilon = 5.6 \times 10^4$ L mol^{-1} cm^{-1}), the observed molar absorptivity was very low with the proposed optical sensor-based method ($\varepsilon = 3.5 \times 10^3$ L mol^{-1} cm^{-1}). This 16-fold decrease in molar absorptivity compared to the bulk solution-based

Table 1. Precision and recovery of the proposed method (n = 3).

		Concentration
1.22 µM of tocopherol addition to serum extract 1	Mean (µM)[a]	1.28
	SD[b]	0.09
	RSD, %[c]	7.03
	REC, %[d]	104.91
2.44 µM of tocopherol addition to serum extract 1	Mean (µM)[a]	2.33
	SD[b]	0.19
	RSD, %[c]	8.16
	REC, %[d]	95.49
3.66 µM of tocopherol addition to serum extract 1	Mean (µM)[a]	3.91
	SD[b]	0.24
	RSD, %[c]	6.14
	REC, %[d]	106.83

TTC of unspiked (but appropriately diluted) serum extract was 3.84 µM as tocopherol equivalents. Mean and recovery (%) were calculated on the basis of concentration of added antioxidant.
[a]Mean. [b]Standard deviation. [c]Relative standard deviation. [d]Recovery.

method possibly arose from the large molecular size of β-carotene being an important parameter in the Nafion membrane response to an analyte to be sensed.[26] The average β-carotene and α-tocopherol concentrations in the serum of a healthy human adult are 0.54 and 26.64 µM, respectively.[27] For the measurement of interference that can originate from β-carotene by the proposed method, we added 0.54 µM of β-carotene to synthetic serum samples and found that this concentration was equivalent to 0.2 µM α-tocopherol, corresponding to less than 1% of the average tocopherol value. As a result, it may be concluded that the low sensitivity of the optical sensor toward β-carotene may enable an almost interference-free determination of tocopherol levels of sera with about 0.75% positive error arising from β-carotene.

2.2. TTC measurement in real samples

In Table 2, the optical sensor-based and solution-based TTC values of serum samples were reported as tocopherol equivalents (µM tocopherol). The results obtained by both methods were compatible with each other at 95% confidence level ($P = 0.05$, $F_{exp} = 7.38$, $F_{crit(table)} = 7.71$, $F_{exp} < F_{crit(table)}$). Although a similar order was obtained by the conventional CUPRAC method (i.e. carried out in bulk solution), its TTC findings were somewhat higher than those of the proposed method. The most important reason for this behavior may be the high sensitivity of the conventional CUPRAC method toward β-carotene, and, in fact, the measured CUPRAC-TAC value of the lipophilic fraction of serum is the sum of the equivalent concentrations of tocopherols and β-carotene. As a result, the data obtained by the proposed sensing method were more realistic in the context of determination of tocopherols.

In conclusion, it was possible to determine tocopherol in serum samples with a simple, rapid, sensitive, and selective assay by using the proposed optical sensor-based method without interference from β-carotene. The proposed sensor was validated through linearity, additivity, precision, and recovery to demonstrate the robustness and reliability of the proposed method. The proposed sensor shows the potential for conversion to an easy-to-use kit format for in situ TTC determination of serum samples.

Table 2. TTCs of some synthetic serum samples as assayed by the sensor and CUPRAC methods (n = 3).

Synthetic serum samples	TTC_{Sensor} (µM tocopherol)	TTC_{CUPRAC} (µM tocopherol)
Serum sample 1	38.23 ± 1.93	40.21 ± 1.11
Serum sample 2	22.89 ± 0.89	24.08 ± 1.49
Serum sample 3	28.41 ± 3.01	30.57 ± 2.14
Serum sample 4	33.58 ± 2.71	34.14 ± 2.87
Serum sample 5	39.24 ± 3.47	40.46 ± 2.99

P = 0.05, F_{exp} = 7.38, $F_{crit(table)}$ = 7.71, F_{exp} <$F_{crit(table)}$.

3. Experimental

3.1. Chemicals and samples

Copper(II) chloride dihydrate and ammonium acetate (NH_4Ac) were from Merck (Darmstadt, Germany). Fetal bovine serum (synthetic serum sample 1), β-carotene, 2,9-dimethyl-1,10-phenanthroline (known as neocuproine (Nc)), α-tocopherol, Nafion 115 membrane (thickness: 0.127 mm), n-hexane, dichloromethane (DCM), and ethanol were from Sigma-Aldrich (Steinheim, Germany). Tocopherol standards at different final concentration (5, 10, 15, 20 µM) were added to twice diluted fetal bovine serum sample (synthetic serum sample 1) for the preparation of synthetic serum samples. Serum samples were stored at –80 °C in deep freeze just prior to analysis.

3.2. Instrumentation

A Varian CARY Bio 100 UV-Vis spectrophotometer (Mulgrave, Australia) was used for absorbance measurements. An Elektro-Mag vortex apparatus (İstanbul, Turkey), Elektro-Mag centrifuge apparatus (İstanbul, Turkey), and programmable rotator-mixer Biosan Bulti Bio RS-24 (Riga, Latvia) were the general laboratory equipment utilized. Pure water used to prepare and dilute all solutions was obtained from the Millipore Simpak1 Synergy185 ultrapure water system (Waltham, MA, USA).

3.3. Preparation of solutions

In distilled water, 1.0×10^{-2} M of $CuCl_2$ solution and 1 M of NH_4Ac were prepared. Furthermore, 7.5×10^{-3} M of Nc solution and a suitable concentration of β-carotene were prepared in ethanol. A suitable concentration of α-tocopherol was prepared in n-hexane.

The hexane extracts of serum samples were prepared according to the procedure described by Aebischer et al. with some modifications.[28] One milliliter of sample was transferred to a centrifuge tube; 2 mL of 96% ethanol and 1 mL of distilled water were added and mixed well. The tube containing the mixture was allowed to stand for a few minutes until phase separation. The mixture was centrifuged at 5000 rpm for 5 min. The organic phase was transferred to a dark tube. This hexane extract was used for TTC determination by using the proposed optical sensor-based method.

For the assay of lipophilic antioxidants using the CUPRAC spectrophotometric method, the hexane extract of serum sample was dried under N_2 flow, and the residue was dissolved and diluted with 4 mL of DCM.

For standard additions study, an aliquot of 1.0 mL of the 10-times diluted hexane extract of synthetic serum 1 and standard 1.22, 2.44, or 3.66 µM α-tocopherol solutions at the final concentrations were taken into tubes separately. Sample solutions, to which different standard additions of α-tocopherol were made, were separately analyzed with the proposed optical sensor method.

3.4. Analytical methods

3.4.1. The CUPRAC spectrophotometric method for determination TTC of serum

The TTC of organic extract of serum was evaluated by the CUPRAC spectrophotometric method.[25] To a test tube were added 1 mL of $CuCl_2$, 1 mL of Nc, 0.8 mL of DCM extract of serum, and 3.2 mL DCM solution in this order. The mixture was mixed well and allowed to stand for phase separation. Absorbance reading of the DCM phase was done against a reagent blank at 450 nm.

3.4.2. Optical sensor-based method for determining TTC of serum

TTC of hexane extract of serum was determined with the optical sensor-based CUPRAC method[29] with some modifications. The optical sensor measures the reduction of the membrane-held Cu(II)-Nc complex to the Cu(I)-Nc chelate by antioxidants, via measurement of the light absorption of the highly colored Cu(I)-Nc chelate formed on the sensor membrane at 450 nm. For preparation of optical sensor strips, Nafion membrane was cut into pieces of 4.5 × 0.5 cm and immersed in a tube containing the CUPRAC reagent solution (2 mL of $CuCl_2$, 2 mL of NH_4Ac, 2 mL of Nc, and 2.2 mL of distilled water), and rotated for 30 min. The Cu(II)-Nc impregnated membrane was called the blank membrane. The blank sensor was immersed in a tube containing 8.2 mL of sample solution (x mL hexane extract of serum + (8.2 – x) mL ethanol). After agitating for 30 min, the absorbance of the colored membrane was measured at 450 nm against the blank membrane. TTC values of extracts were expressed in α-tocopherol equivalents (µM), based on the calibration curve obtained with α-tocopherol standard. The schematic layout of the proposed method is shown in Figure 3. The assays were carried out in triplicate and the results are expressed as (mean value ± standard deviation).

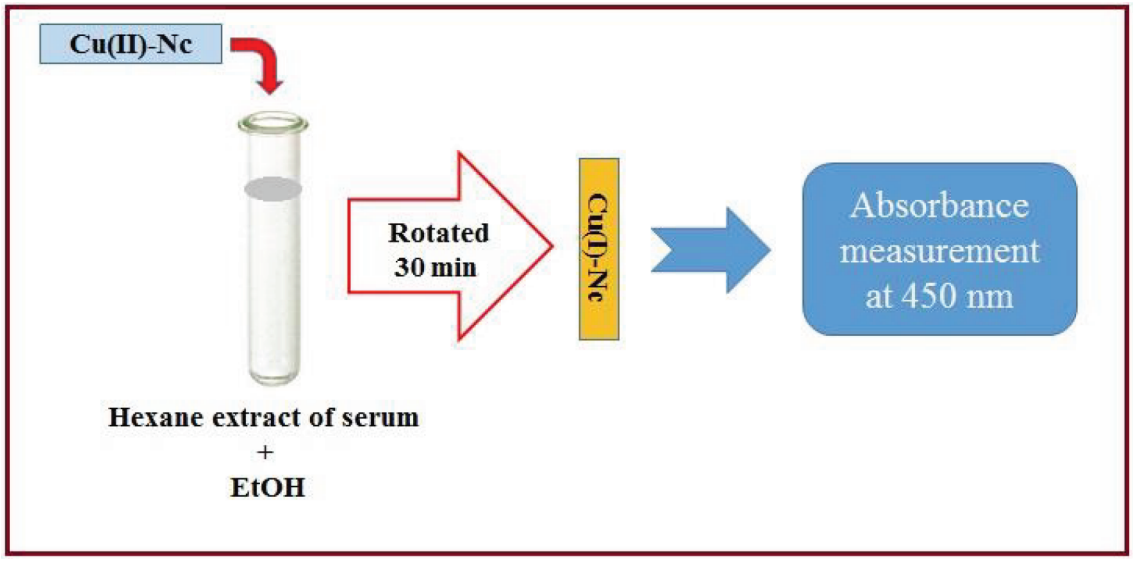

Figure 3. The schematic layout of the proposed optical sensor-based method.

3.5. Statistical analysis

All assays were carried out in triplicate for each sample. Descriptive statistical analyses were performed using Excel software (Microsoft Office 2013) for calculating the mean and the standard error of the mean.

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