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ORIGINAL PAPER

# Determination of Total Antioxidant Capacity by a New Spectrofluorometric Method Based on Ce(IV) Reduction: Ce(III) Fluorescence Probe for CERAC Assay

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**Abstract** A Ce(IV)-based reducing capacity (CERAC) assay was developed to measure the total antioxidant capacity (TAC) of foods, in which Ce(IV) would selectively oxidize antioxidant compounds but not citric acid and reducing sugars which are not classified as antioxidants. The method is based on the electron-transfer (ET) reaction between Ce(IV) ion and antioxidants in optimized acidic sulphate medium (*i.e.*, 0.3 M $H_2SO_4$ and 0.7 M $Na_2SO_4$) and subsequent determination of the produced Ce(III) ions by a fluorometric method. The fluorescent product, Ce(III), exhibited strong fluorescence at 360 nm with an excitation wavelength of 256 nm, the fluorescence intensity being correlated to antioxidant power of the original sample. The linear concentration range for most antioxidants was quite wide, *e.g.*, $5.0\times10^{-7}$–$1.0\times10^{-5}$ M for quercetin. The developed procedure was successfully applied to the TAC assay of antioxidant compounds such as trolox, quercetin, gallic acid, ascorbic acid, catechin, naringin, naringenin, caffeic acid, ferulic acid, glutathione, and cysteine. The proposed method was reproducible, additive in terms of TAC values of constituents of complex mixtures, and the trolox equivalent antioxidant capacities (TEAC coefficients) of the tested antioxidant compounds gave good correlations with those found by reference methods such as ABTS and CUPRAC.

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## Introduction

The exposure of living organisms to reactive oxygen species (ROS) is unavoidable in aerobic life since the generation of ATP from molecular oxygen demands electrons, leading to transient intermediates in the $O_2\rightarrow O^{2-}$ pathway. ROS are either radicals containing unpaired electrons (*e.g.*, $^{\cdot}OH$, $O_2^{\cdot-}$), or molecular species capable of extracting electrons from other molecules ($H_2O_2$, HOCl). These species may damage biomolecules directly, or initiate radicalic chain reactions in which ROS are passed from one molecule to another, resulting in extensive damage to cell structures such as membranes and proteins. Antioxidants may either retard or obstruct these undesired oxidation reactions, thereby preventing related diseases such as cardiovascular, neurodegenerative diseases and cancer. Antioxidants break chain reactions leading to lipid radicals, thus prevent the oxidative degradation of lipids forming an essential part of diet, and thereby preserve food quality [1, 2]. The beneficial influence of many foodstuffs and beverages including fruits, vegetables, tea, red wine, coffee, and cacao on human health has been recently recognized to originate from their natural antioxidant constituents.

Antioxidants have been traditionally divided into two classes; primary or chain-breaking antioxidants, and secondary or preventive antioxidants. The chemical diversity of antioxidants makes it difficult to separate and quantify antioxidants from the vegetable matrix. Therefore it is desirable to establish methods that can measure the total antioxidant capacity (TAC) level directly from vegetable

and fruit extracts [3]. TAC is an integrated parameter reflecting the cumulative action of all antioxidants in a complex sample. Several analytical methods have been developed to measure total antioxidant capacity [4] (based on reaction thermodynamics or conversion efficiency) and total antioxidant activity (based on reaction kinetics) [5]. A mechanistic classification of total antioxidant capacity assays is the type of reaction: Electron transfer (ET)- and hydrogen atom transfer (HAT)- based assays [6]. ET-based assays include ABTS/TEAC (trolox equivalent antioxidant capacity) [7, 8], DPPH [9], FCR (Folin-Ciocalteau reagent) assay [10, 11], FRAP (ferric ion reducing antioxidant power) [12–14], CERAC (cerium (IV) ions reducing antioxidant capacity) [15] and CUPRAC (Cupric Reducing Antioxidant Capacity) [16–18]. Generally, ET-based assays find wider practical use than HAT-based ones, because they are cheaper and less laborious, more flexible, and thus more suitable to routine testing of natural products. However, ET-based assays may be less reproducible due to strong dependency on the protocol, *e.g.*, reagent concentrations, pH, solvent, and time of incubation. The problem can be likely solved by standardization of protocols.

Due to the importance of antioxidants as contributors to beneficial health effects of fruit and vegetable consumption, the purpose of this work was to develop and validate a rapid, simple and reliable fluorometric method as an alternative approach to the widely used spectrophotometric methods [7, 13, 19–23] for the determination of total antioxidant capacity in food. Fluorescence method is particularly appealing for this purpose, because it combines very simple and relatively low-cost instrumentation with high sensitivity and selectivity.

Ce(IV) sulphate solutions in sulphuric acid medium are known to be stable over prolonged periods, but hydrolysis complexes of the type $Ce(OH)_n^{4-n}$ form in solutions of reduced acidities [15, 24–27]. Czappa [26] showed that the presence of complexing ions like sulphate may reduce the complexing ability of Ce(IV) with organic substrates, and may therefore retard the oxidation of organic compounds with Ce(IV), since Ce(IV)–organic substrate complexation is a prerequisite for fast inner-sphere electron transfer [25]. Moreover, the Ce(IV)/Ce(III) reduction potential is decreased in the presence of appreciable concentrations of $SO_4^{2-}$ ions [27], preferentially stabilizing the higher oxidation state. This work aims to find optimal conditions (*i.e.*, optimal sulfuric acid and sodium sulfate concentrations) for Ce(IV) oxidation of antioxidants but not of citric acid and reducing sugars, with the aid of the fluorescence measurement of Ce(III) emerging as the reduction product of Ce(IV) with antioxidants. The fluorescence method was preferred to absorptimetric assay[15, 28] due to its sensitivity, narrowness of emission bands, and lack of fluorescence of either Ce(IV) reagent or antioxidant analyte at the analytical wavelength. Furthermore, it is expected that the possible interferences of plant pigments known to exist at the optimal absorption wavelength of the spectrophotometric method [15, 28] will not pose problems in the fluorometric assay. The proposed method was tested for known antioxidants, their synthetic mixtures, and herbal plant extracts, and was validated against established ET-based methods of antioxidant characterization such as CUPRAC, optimized ferricyanide/Prussian blue, and Folin-Ciocalteau.

## Experimental

### Chemicals and Solutions

Quercetin was purchased from Sigma (Steinheim, Germany); cerium(IV) sulfate tetrahydrate ($Ce(SO_4)_2.4H_2O$), cerium (III) nitrate hexahydrate ($Ce(NO_3)_3.6H_2O$), Folin-Ciocalteau phenol reagent, $FeCl_3.6H_2O$, $K_3[Fe(CN)_6]$, $Na_2CO_3$, $Na_2SO_4$ $NaKC_4H_4O_6$, $CuCl_2$, $CuSO_4$, $H_2SO_4$, HCl, $CH_3COONH_4$, $C_2H_5OH$ (96%, by wt.) were purchased from E. Merck (Darmstadt, Germany); NaOH, $CH_3COOH$ were supplied from Riedel-de Haën (Steinheim, Germany). Neocuproine (2,9-dimethyl-1,10-phenanthroline) and TCA (trichloroacetic acid) were purchased from Sigma-Aldrich (St Louis, MO, USA). The chemicals were of analytical reagent grade, and double-distilled water was used throughout. All polyphenolic compounds and vitamin solutions were freshly prepared in 96% ethanol at 1 mM concentration.

A mass of 0.0404 g $Ce(SO_4)_2.4\ H_2O$ was transfered to a 100-mL flask with some water. After the addition of 17 mL of 98% (by wt.) $H_2SO_4$, the flask was diluted to line with water. A mass of 0.0868 g $Ce(NO_3)_3.6H_2O$ was dissolved in approximately 25 mL water, and diluted to line in a 100-mL flask with water. Sodium sulfate solution contained 35.50 g $Na_2SO_4$ in 250 mL water. Standard antioxidant solutions were prepared in absolute EtOH at $1.0\times10^{-3}$ M concentration.

Copper(II) chloride stock solution ($1.0\times10^{-2}$ M) was prepared by dissolving 0.4262 g dihydrate salt in distilled water, and diluting to a final volume of 250 ml. Ammonium acetate ($NH_4Ac$) buffer at pH 7 was prepared by dissolving 19.27 g $NH_4Ac$ in water and diluting to 250 ml. Neocuproine solution ($7.5\times10^{-3}$ M) was freshly prepared by dissolving 0.039 g neocuproine free base in 96% ethanol, and diluting to 25 ml with the same solvent [16–18].

Potassium ferricyanide solution (1%, w/v) was prepared daily by dissolving 1.0 g $K_3[Fe(CN)_6]$ in 1 mL of 1 M HCl and some water, and diluting to 100 mL with water. Ferric chloride solution (0.2%, w/v) was prepared daily by dissolving 0.2 g of $FeCl_3{\bullet}6H_2O$ in 1 mL of 1 M HCl and some water, and diluting to 100 mL with water. Sodium dodecyl sulfate solution (SDS: 1%, w/v) was prepared by

dissolving 1.0 g of SDS in water and diluting to 100 mL with water [3].

Folin-Ciocalteu's phenol reagent was diluted at a volume ratio of 1:3 with 96% EtOH prior to use. Lowry A solution was prepared from sodium carbonate such that the strength of $Na_2CO_3$ in 0.1 M NaOH solution was 2% (w/v). Lowry B solution was prepared from copper(II) sulfate such that the strength of $CuSO_4$ in 1% sodium potassium tartrate ($NaKC_4H_4O_6$) solution was 0.5% (w/v). Lowry C solution was prepared by mixing 50 ml Lowry A with 1 ml Lowry B at the time of measurement [10, 11].

## Instrumentation

A Varian Cary Eclipse fluorescence (Mulgrave, Victoria, Australia) spectrophotometer and a Varian CARY 1E UV–vis spectrophotometer (Mulgrave, Victoria, Australia) were used for emission and absorbance readings, respectively, using a pair of matched quartz cuvettes (Hellma). The pH measurements were made with a Knick pH-meter equipped with a combined glass electrode. Liquid sampling at 5–50 μL and 200–500 μL were made with Genex Beta-type (Torquay, Devon, United Kingdom) variable and Brand Transferpette-type fixed-volume micropipettes (Essex, Connecticut, USA), respectively. Equilibration of solutions was made using a Vortex mini-stirrer.

## Preparation of Real Samples

The real samples of tea bags and herbal teas were supplied from the the local food market in Istanbul-Turkey, namely as green tea bags (*Camellia sinensis*) and nettle herbal tea bags (*Urtica dioica*/urens) from Doga Company Co., linden tea bags (*Tilia*), mint tea bags (*Mentha spicata*) and sage herbal tea bags (*Salvia officinalis*) from Dogadan Company Co., and chamomile (*Matricaria chamomilla* L.) from Malatya Pazari A.S.

Preparation of tea bag infusions; each of the commercial tea bags and herbal teas were 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 steeping time of 5 min. The herbal tea solution (infusion) was allowed to cool to room temperature, and filtered through a Whatman black-band filter paper for removing particulates.

## CERAC Method of TAC Measurement by Spectrofluorometric Titrimetry

### *General*

Spectrofluorometric titration can be performed in two different ways. When increasing amounts of antioxidant solution are added to a fixed concentration of Ce(IV), antioxidants are oxidized with Ce(IV). As a result, the fluorescence signal of the produced Ce(III) reaches a peak value at the time of complete consumption of Ce(IV), and beyond this value, the signal reaches a plateau region. When the opposite procedure is applied (*i.e.*, increasing amounts of Ce(IV) are added to a fixed concentration of antioxidant), excessive Ce(IV) beyond the equivalence point shows a quenching effect on Ce(III) fluorescence, and a maximum in fluorescence signal is observed. In both cases, the end point is taken as the limiting or maximum of Ce(III) emission, at which the amount of reacted antioxidant (corresponding to TAC) is equivalent to Ce(IV). After investigating possible effects on the excitation and emission spectral properties of Ce(III), the reaction conditions (*i.e.*, 0.3 M $H_2SO_4$ and 0.7 M $Na_2SO_4$) were maintained as in the modifed CERAC procedure [28], enabling selective oxidation of antioxidants. The trolox-equivalent antioxidant capacity (TEAC value) of an antioxidant is defined as the mM concentration of a trolox solution having equal reducing power to that of 1 mM of the antioxidant solution under investigation (TEAC is unitless). This definition practically reduces to the ratio of the slope of the spectrofluorometric titration curve of the tested antioxidant to that of trolox obtained under identical conditions.

### *Procedures*

A- Ce(IV) titration with antioxidant

In this method, increasing amounts of antioxidant were added to a fixed amount of Ce(IV). For this purpose, 1 mL of $1.0\times10^{-3}$ M Ce(IV) solution +7 mL of 1 M $Na_2SO_4$ solution +x mL (increasing variable) antioxidant solution were placed in a 20-mL test tube, sufficient sulphuric acid was added to yield a final acid concentration of 0.3 M $H_2SO_4$, and diluted to 10 mL with distilled water. After standing for 30 min at room temperature, the fluorescence intensity of the 256 nm−excited solution was read at 360 nm. The titration curve was constructed as signal intensity *vs* antioxidant concentration.

B- Antioxidant titration with Ce(IV)

In this method, increasing amounts of Ce(IV) were added to a fixed amount of antioxidant. For this purpose, 1 mL of $1.0\times10^{-4}$ M quercetin solution + 7 mL 1 M $Na_2SO_4$ solution + x mL (increasing variable) Ce(IV) solution were placed in a 20-mL test tube, sufficient sulphuric acid was added to yield a final acid concentration of 0.3 M $H_2SO_4$, and diluted to 10 mL with distilled water. After standing for 30 min at room temperature, the fluorescence intensity of the 256 nm−excited solution was

read at 360 nm. The titration curve was constructed as signal intensity *vs* Ce(IV) concentration.

### CUPric Reducing Antioxidant Capacity (CUPRAC) Assay of Total Antioxidant Capacity

To a test tube were added 1 ml $CuCl_2$ solution ($1.0\times10^{-2}$ M), 1 ml neocuproine alcoholic solution ($7.5\times10^{-3}$ M) and 1 mL 1 M $NH_4Ac$ buffer solution, followed by mixing; (x) ml antioxidant followed by (1.1-x) ml $H_2O$ were then added (total volume, 4.1 ml) and mixed well. Absorbance against a reagent blank was measured at 450 nm after 30 min [16–18].

### Optimized Ferricyanide/Prussian Blue Assay of Reducing Power

To (x) mL antioxidant solution were added (1−x) mL EtOH (96%), 6.3 mL $H_2O$, 0.2 mL of 1 M HCl, 1.5 mL ferricyanide solution (1%), 0.5 mL SDS (1%), and finally 0.5 mL $FeCl_3{\cdot}6H_2O$ (0.2%) so that the final volume would be 10 mL. Absorbance against a reagent blank was measured at 750 nm after 30 min standing at room temperature [3].

### Folin-Ciocalteau Method of Total Phenolics Assay

To (x) ml phenolic sample solution was added (2-x) ml $H_2O$. An aliquot of 2.5 ml Lowry C solution was added, and the mixture was let to stand for 10 min. At the end of this period, 0.25 ml Folin reagent was added, and 30 more min was allowed for stabilization of the blue colour formed. The absorbance against a reagent blank was read at 750 nm [10, 11].

## Results and Discussion

### Ce(III) Fluorescence Spectra and Calibration Curve

The fluorescence spectrum of $1.0\times10^{-4}$ M Ce(III) solution in the 200–450 nm range is shown in Fig. 1, with maximal excitation and emission wavelengths being 256 and 360 nm, respectively. Ce(IV) did not show fluorescence under the selected conditions.

The calibration curve of Ce(III) in the concentration range $5.0\times10^{-6}$ M–$2.0\times10^{-4}$ M (Fig. 2) was shown to be linear, enabling quantitative determination of Ce(III), and indirectly of antioxidants reducing the Ce(IV) reagent to Ce(III).

### Effect of $H_2SO_4$ and $Na_2SO_4$ Concentrations on Ce(III) Fluorescence Spectra

The fluorescence spectra (not given) of $2.0\times10^{-4}$ M Ce(III) solutions in 0.08–0.4 M $H_2SO_4$ media recorded between

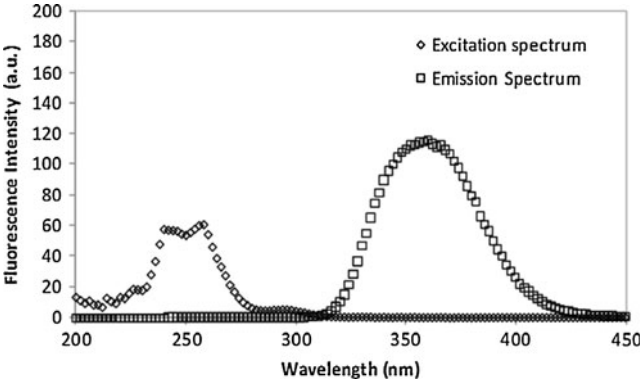


**Fig. 1** Fluorescence spectrum of $1.0\times10^{-4}$ M Ce(III) solution ($\lambda_{max(ex)}$ = 256 nm, $\lambda_{max(em)}$ = 360 nm)

200 and 500 nm wavelengths showed that Ce(III) fluorescence intensity at a fixed wavelength of $\lambda_{max(em)}$=360 nm did not appreciably change above a limiting sulfuric acid concentration of 0.08 M preventing hydrolysis. To remain on the safe side for Ce(IV) oxidation reactions monitored by Ce(III) fluorescence, an optimal sulphuric acid concentration of 0.3 M was chosen for further studies, in accordance with previous findings [28]. It was also necessary to observe the dependence on sulfate concentration, as Ce(IV)/Ce(III) potential should be diminished by sulfate complexation of Ce(IV) to render the reagent a more selective oxidant for true antioxidants (and not for citric acid or reducing sugars). At a fixed $H_2SO_4$ concentration of 0.3 M, increasing sulfate concentrations in the range 0.05–0.9 M caused a slight increase in $\lambda_{max(em)}$ (spectra not shown), however both the variations in the wavelength shift and maximal fluorescence intensity (at 360 nm) were negligible.

### The Effect of of $Na_2SO_4$ Concentration on the Interaction of Ce(IV) with Citric Acid and Quercetin in Sulphuric Acidic Medium

To $2.0\times10^{-4}$ M Ce(IV) solutions containing a fixed concentration of 0.3 M sulphuric acid, sodium sulfate

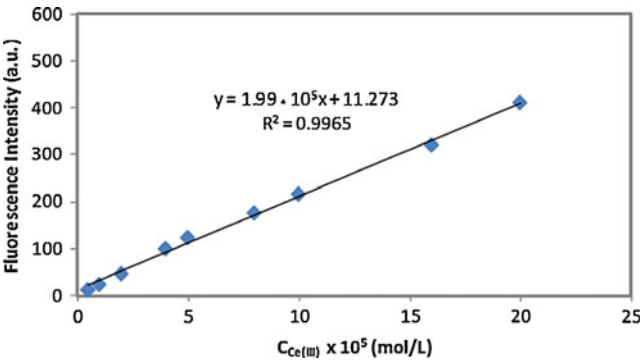


**Fig. 2** The calibration curve of Ce(III) in the concentration range $5.0\times10^{-6}$ M–$2.0\times10^{-4}$ M

solution at concentration varying between 0.1 M and 0.8 M were added, and reacted with $1.0\times10^{-4}$ M citric acid. As the sulfate concentration was increased, Ce(III) formation as a result of citric acid oxidation decreased because Ce(IV) became increasingly unable to oxidize citric acid due to sulfate complexation (spectra not given). This was a desired property for the reagent to bring about selective oxidation of antioxidants but not of citric acid which is normally not classified under this class of compounds. Since 0.7 M $Na_2SO_4$ (in the presence of 0.3 M sulphuric acid) caused ≤5% oxidation of citric acid, this combination of acid and salt was maintained in further studies with antioxidants. On the other hand, under these selected optimal conditions, $1.5\times10^{-5}$ M quercetin (a typical antioxidant of the flavonoid class) quantitatively reacted with Ce(IV), and Ce(III) formation –as measured by fluorescence − as a result of quercetin oxidation was basically not affected by sulfate complexation of Ce(IV) up to 0.8 M concentrations of $Na_2SO_4$ (spectra not shown).

## Spectrofluorometric Titration

When increasing amounts of antioxidant were added to a fixed amount of Ce(IV), the titration curves were constructed as signal intensity *vs* antioxidant concentration (Fig. 3). The TAC can be calculated from the point of intersection of tangents drawn to the curve at the intensity rise and plateau regions. This procedure was modified with the addition of increasing amounts of Ce(IV) to a fixed amount of antioxidant, and the titration curve was constructed as signal intensity *vs* Ce(IV) concentration, the maximum point being used for TAC calculation (Fig. 4). The maximum can be precisely found from the point of intersection of the tangents drawn to both arms of the Λ-type curve.

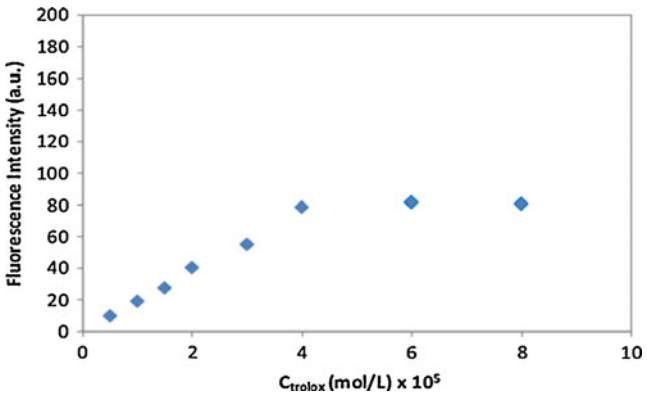


**Fig. 3** Ce(IV) titration with trolox antioxidant (curve as intensity *vs* antioxidant concentration) by spectroflourometric CERAC method (Ce(IV) concentration: constant)

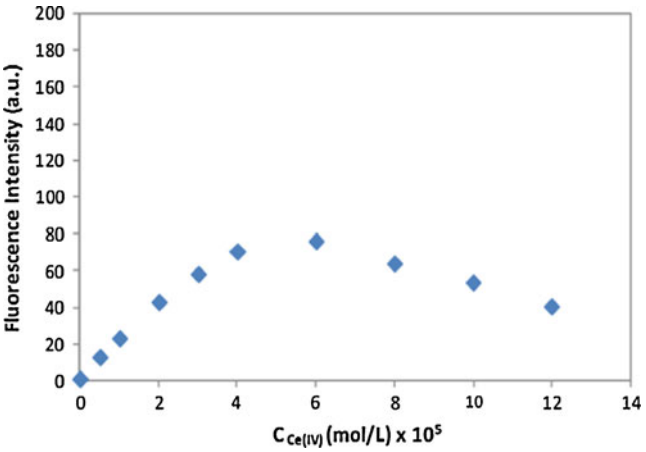


**Fig. 4** Trolox titration with Ce(IV) (curve as intensity *vs* Ce(IV) concentration) by spectroflourometric CERAC method (trolox antioxidant concentration: constant)

## Linear Concentration Range and TEAC Coefficients of Antioxidants Using the Spectrofluorometric CERAC Method

Spectrofluorometric titration curves using the modified CERAC method [28] were obtained for certain antioxidant compounds, namely trolox, quercetin, rutin, gallic acid, ascorbic acid (vitamin C), catechin, naringin, naringenin, caffeic acid, ferulic acid, glutathione, and cysteine. From these curves, the linear ranges (of fluorescence signal *vs* concentration, Table 1) and TEAC coefficients, defined as the ratio of the slope of the spectrofluorometric titration curve of the tested antioxidant to that of trolox (Table 2), were calculated (with intra- and inter-day variability of 3.1% and 5.2% relative standard deviation, respectively, showing that the proposed method was reproducible), and compared to those found by reference antioxidant assays. It is notewothy that the TEAC values found with spectroflu-

**Table 1** Linear concentration ranges (LCR) of the tested antioxidants

| Antioxidant compound | LCR ($\times10^5$ M) |
|---|---|
| Quercetin | 0.05–1.00 |
| Rutin | 0.10–2.00 |
| Catechin | 0.20–6.00 |
| Trolox | 1.00–12.00 |
| Ascorbic acid | 0.40–10.00 |
| Gallic acid | 0.20–5.00 |
| Naringenin | 0.40–6.00 |
| Naringin | 0.40–6.00 |
| Caffeic acid | 0.10–6.00 |
| Ferulic acid | 0.10–6.00 |
| Glutathione | 0.50–5.00 |
| Cysteine | 0.20–3.00 |

**Table 2** The TEAC coefficients of the tested antioxidants using the spectrofluorometric CERAC method and reference TAC assays

| Antioxidant compound | $TEAC_{spectrofluor.CERAC}$ (Ce(IV) titration with antioxidant) | $TEAC_{spectrofluor.CERAC}$ (Antioxidant titration with Ce(IV)) | $TEAC_{spectrofluor.CERAC}$ (Mean value) | $TEAC_{modif.CERAC}$ (Absorptimetric) | $TEAC_{ABTS}$ | $TEAC_{CUPRAC}$ |
|---|---|---|---|---|---|---|
| Trolox | – | – | – | – | – | – |
| Quercetin | 4.58 | 4.71 | 4.65 | 7.05 | 4.7 | 4.4 |
| Rutin | 4.97 | 5.51 | 5.24 | 3.84 | 2.4 | 2.6 |
| Gallic acid | 2.07 | 1.98 | 2.02 | 3.27 | 3.0 | 2.6 |
| Ascorbic acid | 0.99 | 1.05 | 1.02 | 1.00 | 1.0 | 1.0 |
| Catechin | 2.67 | 2.22 | 2.44 | 2.45 | 2.4 | 3.1 |
| Naringin | 1.46 | 1.39 | 1.42 | 1.93 | 0.2 | 0.2 |
| Naringenin | 1.32 | 1.38 | 1.35 | 1.95 | 1.5 | 2.3 |
| Caffeic acid | 1.54 | 1.33 | 1.44 | 2.28 | 1.4 | 2.9 |
| Ferulic acid | 1.06 | 1.31 | 1.18 | 2.18 | 2.1 | 1.2 |
| Glutathione | 0.46 | 0.68 | 0.57 | – | 1.51 | 0.57 |
| Cysteine | 1.23 | 0.90 | 1.07 | – | – | 0.54 |

orometric CERAC generally correlated well with those found by ABTS and CUPRAC methods with the exception of the value for naringin (Table 2). This compound should have hydrolyzed to naringenin in the strongly acidic medium of CERAC and therefore exerted its true antioxidant power, whereas ABTS and CUPRAC methods did not have this capability.

## TAC Determination of Synthetic Mixtures of Antioxidants

Binary, ternary, and quaternary synthetic mixtures of antioxidants were analyzed with spectrofluorometric CERAC, and the observed overall (indirect) emission was found to be the sum of the individual emissions of constituents (Table 3), demonstrating the additivity of TAC values; the expected and found arbitrary fluorescence values (F) agreed within ±5%.

## Additivity Testing of Total Antioxidant Capacity (TAC) Using the Method of Standard Additions

Additivity of antioxidant capacities of individual antioxidants constituting a mixture is an important aspect by definition of TAC, as the measured capacity should be the sum of individual capacities [19], and a novel TAC assay should conform to this rule. Using the standard addition method, the calibration curves of trolox in pure aqueous solution and in three selected herbal tea (green tea, sage, and linden) infusions were shown to be parallel to each other (Fig. 5), basically indicating lack of interference in the spectrofluorometric assay that would be expected to arise from deviations from Beer's law. Thus, the principle of additivity of TAC values in the proposed method (shown to be valid for synthetic mixtures) was confirmed by applying the procedure of standard antioxidant additions to complex solutions (herbal tea infusions). The calibration lines of trolox (as fluorescence *vs* added antioxidant concentration) in pure and complex solutions always had the same slope (*i.e.*, $(1.86\pm0.02) \times 10^6$), as shown in Fig. 5.

**Table 3** TAC determination (in the units of arbitrary fluorescence intensity due to Ce(III) produced: F) of synthetic mixtures with the spectrofluorometric CERAC method

| Synthetic mixture | $F_{expected}$ | $F_{found}$ | Deviation (%) |
|---|---|---|---|
| $1.0\times10^{-2}$ mM Cysteine<br>$1.0\times10^{-2}$ mM Ascorbic acid | 95.7 | 96.6 | 0.94 |
| $5.0\times10^{-3}$ mM Caffeic acid<br>$5.0\times10^{-3}$ mM Rutin<br>$1.0\times10^{-2}$ mM Trolox | 82.6 | 78.8 | −4.6 |
| $4.0\times10^{-3}$ mM Catechin<br>$5.0\times10^{-3}$ mM Naringin<br>$1.0\times10^{-2}$ mM Gallic acid | 138.9 | 131 | −5.6 |
| $5.0\times10^{-3}$ mM Glutathione<br>$1.0\times10^{-2}$ mM Gallic acid<br>$2.0\times10^{-3}$ mM Quercetin | 133.2 | 127.5 | −4.5 |
| $5.0\times10^{-3}$ mM Glutathione<br>$1.0\times10^{-2}$ mM Cysteine<br>$5.0\times10^{-4}$ mM Catechin<br>$5.0\times10^{-4}$ mM Caffeic acid | 64.8 | 63.5 | −2.0 |
| $5.0\times10^{-3}$ mM Glutathione<br>$1.0\times10^{-3}$ mM Ascorbic acid<br>$1.0\times10^{-2}$ mM Gallic acid<br>$2.0\times10^{-3}$ mM Quercetin | 91.3 | 89.2 | −2.3 |

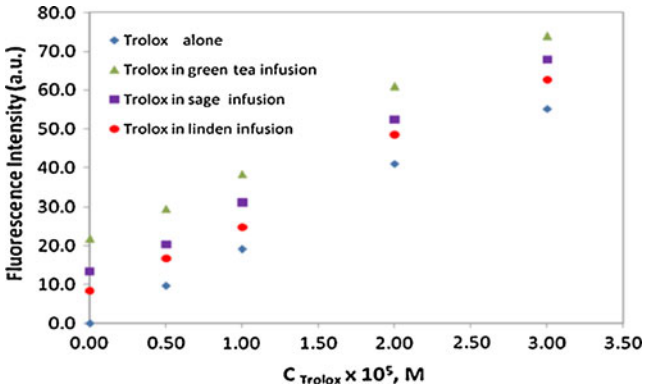


**Fig. 5** The calibration line of trolox (as fluorescence *vs* added antioxidant concentration) in pure aqueous solution, and in the infusions of green tea, sage, and linden, with respect to the developed spectrofluorometric method

## Application of the Spectrofluorometric Assay to Real Samples for Validation

As for validation of the proposed assay by comparison to other similar ET-based assays, the results found with the proposed and reference methods are depicted in Table 4, though it is an established fact that no two antioxidant assays may give the same result for a given antioxidant sample due to differences in the redox potential and mechanism of ET-based method used in measurement. It is noteworthy that the accordance of TAC findings between the proposed method and the CUPRAC assay was similar to the situation observed for TEAC values of individual antioxidants (Table 2). The agreement with CUPRAC results is very important, since the CUPRAC assay having a reasonable redox potential and a working pH close to physiological pH gives a realistically accurate estimate of the true antioxidant capacity of complex samples, neither overestimating (like Folin employed at alkaline pH) nor underestimating (like FRAP performed at acidic pH) the actual TAC value. The infusion of green tea yielded the highest TAC values with respect to all tested methods, in accordance with other literature findings. The fact that the Folin assay results were higher than those of other assays possibly arises from the indefinitely high redox potential of the Folin reagent at the alkaline working pH of the method (pH=10) where phenolic antioxidants lose their protons and become more susceptible to oxidation.

## Potential Interferences

The following potential interferents common in plant food and foodstuffs did not affect the determination of $1.0\times10^{-5}$ M trolox at $5.0\times10^{-4}$ M concentrations: glucose, fructose, mannitol, glycine, serine, valine, proline, and alanine (which caused less than 5% relative error). In the determination of $1.0\times10^{-5}$ M trolox, 20-fold concentration level of potential interferents and 1 mg/ml starch did not affect the results. This confirmed that the proposed spectroflourometric CERAC assay has a favourable redox potential enabling selective oxidation of true antioxidant compounds without affecting other similar substances (*e.g.*, simple sugars, citric acid, and non-thiol amino acids) not belonging to the antioxidant class. This may prove to be a distinct advantage over other similar electron transfer − based assays possibly having a higher redox potential (such as ferric phenanthroline of $E^0=1.06$ V and Folin's molybdato-phospho-tungstate reagent of unknown potential) which have been reported to partly oxidize such substances [19]. On the other hand, the proposed method was capable of oxidizing thiol compounds acting as biologically important antioxidants (such as cysteine and glutathione).

## Conclusions

A novel Ce(IV)-based reducing capacity assay was developed to measure the total antioxidant capacity (TAC) of foods, in which Ce(IV) would selectively oxidize true antioxidant compounds but not citric acid and simple sugars. The reduction product of the reagent, Ce(III), showed fluorescence (at 360 nm upon excitation at

**Table 4** The total antioxidant capacities (TAC) of herbal tea infusions found by the proposed spectrofluorometric, CUPRAC, optimized ferricyanide/Prussian blue, and Folin-Ciocalteau methods (in the units of mmol TR/g)

| Sample | Proposed fluorometric method | CUPRAC method | Optimized ferricyanide/ Prussian blue method | Folin-Ciocalteau method |
|---|---|---|---|---|
| Sage herbal tea, (*Salvia officinalis*) | $6.67\times10^{-1}$ | $9.63\times10^{-1}$ | $4.46\times10^{-1}$ | $9.25\times10^{-1}$ |
| Nettle tea, (*Urtica dioica/urens*) | $5.66\times10^{-1}$ | $6.56\times10^{-1}$ | $2.70\times10^{-1}$ | $5.70\times10^{-1}$ |
| Green tea, *(Camellia sinensis)* | $8.24\times10^{-1}$ | 1.04 | $8.64\times10^{-1}$ | 1.01 |
| Chamomile, (*Matricaria chamomilla L.*) | $2.52\times10^{-1}$ | $1.69\times10^{-1}$ | $1.29\times10^{-1}$ | $2.87\times10^{-1}$ |
| Linden, *(Tilia)* | $2.54\times10^{-1}$ | $2.63\times10^{-1}$ | $1.06\times10^{-1}$ | $3.54\times10^{-1}$ |
| Mint tea, (*Menta spicata*) | $5.28\times10^{-1}$ | $6.69\times10^{-1}$ | $3.20\times10^{-1}$ | $4.20\times10^{-1}$ |

256 nm), the intensity of which indicated the antioxidant power of the sample. The optimal conditions for Ce(IV)–antioxidant reaction was chosen as 0.3 M $H_2SO_4$ and 0.7 M $Na_2SO_4$. The developed procedure was successfully applied to the TAC assay of phenolic acid-, flavonoid-, and thiol-type antioxidant compounds such as trolox, quercetin, gallic acid, ascorbic acid, catechin, naringin, naringenin, caffeic acid, ferulic acid, glutathione, and cysteine. The proposed method was reproducible, additive in terms of TAC values of constituents of complex mixtures, and the trolox equivalent antioxidant capacities (TEAC coefficients) of the tested antioxidant compounds gave good correlations with those found by reference methods such as ABTS/TEAC, ferricyanide/Prussian blue, and CUPRAC. The hierarchic order for TAC of herbal infusion samples was: green tea > sage > nettle > mint > linden > chamomile. Aside from being simple, flexible, rapid and low-cost, the proposed procedure was environmentally friendly without requiring the use of toxic organic solvents. Certain plant pigments absorbing light at the characteristic wavelength of 310 nm of the absorptimetric CERAC method did not interfere with the proposed fluorometric assay, as only the Ce(III) species − formed from Ce(IV) by reduction with antioxidants − showed fluorescence under the chosen conditions.

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