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Modified Folin–Ciocalteu Antioxidant Capacity Assay for Measuring Lipophilic Antioxidants

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ABSTRACT: The Folin–Ciocalteu (FC) method of performing a total phenolics assay, originally developed for protein determination, has recently evolved as a total antioxidant capacity assay but was found to be incapable of measuring lipophilic antioxidants due to the high affinity of the FC chromophore, that is, multivalent-charged phospho-tungsto-molybdate(V), toward water. Thus, the FC method was modified and standardized so as to enable simultaneous measurement of lipophilic and hydrophilic antioxidants in NaOH-added isobutanol–water medium. Optimal conditions were as follows: dilution ratio of aqueous FC reagent with iso-BuOH (1:2, v/v), final NaOH concentration of 3.5×10^{-2} M, reaction time of 20 min, and maximum absorption wavelength of 665 nm. The modified procedure was successfully applied to the total antioxidant capacity assay of trolox, quercetin, ascorbic acid, gallic acid, catechin, caffeic acid, ferulic acid, rosmarinic acid, glutathione, and cysteine, as well as of lipophilic antioxidants such as α-tocopherol (vitamin E), butylated hydroxyanisole, butylated hydroxytoluene, *tertiary* butylhydroquinone, lauryl gallate, and β-carotene. The modified FC method reliably quantified ascorbic acid, whereas the conventional method could not. The modified method was reproducible and additive in terms of total antioxidant capacity values of constituents of complex mixtures such as olive oil extract and herbal tea infusion. The trolox equivalent antioxidant capacities of the tested antioxidant compounds correlated well with those found by the Cupric Reducing Antioxidant Capacity reference method.

KEYWORDS: *modified Folin–Ciocalteu assay, total antioxidant capacity (TAC), lipophilic antioxidants: vitamin E, butylated hydroxytoluene, β-carotene*

INTRODUCTION

Antioxidants are health-beneficial substances that can remove or quench excessive amounts of reactive oxygen and nitrogen species (ROS/RNS) under oxidative stress conditions, thereby preventing related diseases such as coronary heart failure, Alzheimer disease, and cancer.[1−3] Thus, the measurement of total antioxidant capacity (TAC) of pure substances, food extracts, and biological fluids is important.

TAC assays may be broadly classified under two groups: electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays.[4] Molecular spectroscopic ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes absorbance or fluorescence when reduced, whereas HAT-based reactions are relatively independent from solvent and pH effects, and are completed in a short time.[5] ET-based assays essentially include 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/trolox equivalent antioxidant capacity (TEAC),[6,7] 2,2-diphenyl-1-picrylhydrazyl (DPPH),[8] Folin–Ciocalteu,[9] ferric reducing antioxidant power (FRAP),[10−12] cupric ion reducing antioxidant capacity (CUPRAC),[13−15] cerium(IV) ion reducing antioxidant capacity (CERAC),[16] ferricyanide/Prussian Blue,[17] and ferrozine[18] methods.

The (Folin–Ciocalteu) FC method was initially intended for the analysis of proteins, taking advantage of the reagent's activity toward protein tyrosine (containing a phenol group) residue.[9] Much later, Singleton et al. extended this assay to the analysis of total phenols in wine.[4,19] The FC assay has certain advantages over some other TAC assays in that it is simple, fast, robust, and does not require specialized equipment, and the long-wavelength absorption of the chromophere minimizes interference from the sample matrix. However, a drawback of the FC assay is that reducing agents such as ascorbic acid or certain amino-acids can interfere with the analysis and thus overestimate the content of phenolic compounds. It is routinely practiced in antioxidant research laboratories testing food and plant extracts. Fundamentally, the Folin–Ciocalteu (FC) assay is based on the oxidation of phenol compounds in alkaline (carbonate) solution with a molybdotungstophosphate heteropolyanion reagent ($3H_2O$-P_2O_5-$13WO_3$-$5MoO_3$-$10H_2O$), yielding a colored product with an absorbance maximum (λ_{max}) at 765 nm. Since most phenolic compounds are in dissociated form (as conjugate bases, mainly phenolate anions) at the working pH of the assay (pH ~10), they can be more easily oxidized with the FC reagent, possibly giving rise to an overestimated TAC value.[4,5] The molybdenum center in the complex reagent is reduced from Mo(VI) to Mo(V) with an e^- donated by an antioxidant to produce a blue color.[4] Among the currently used ET-based TAC assays in literature, only ABTS, CUPRAC, and ferricyanide/Prussian Blue methods have reagents that can effectively dissolve in both hydrophilic and hydrophobic solvents. It is known that the DPPH reagent has a high affinity toward lipophilic antioxidants but not as much for

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hydrophilic ones.[5,20,21] The FC chromophore, the molybdotungstophosphate heteropolyanion ($PMoW_{11}O_{40}^{4-}$), does not have an affinity toward organic solvents owing to its quadruple negative charge[22] giving rise to strong ion−dipole interactions with solvent water molecules. Therefore, once formed, the anion cannot be easily extracted into organic solvents, though extraction using quaternary ammonium-type (i.e., methyltrialkyl (C_8−C_{10}) ammonium chloride) cationic surfactant from aqueous carbonate solution into chloroform was tried with limited success, as very low absorbances had to be magnified with thermal lens spectrometry.[23] Thus, the conventional FC assay is mainly carried out in aqueous phase and is inapplicable in its current form to lipophilic antioxidants.[4] In a most widely cited review work of Huang et al.,[4] the authors stated that they have actually "attempted but have been unable to measure the total phenols of the lipid soluble fraction of bee pollen as the sample did not have sufficient water solubility". Although a wide range of antioxidant compounds (comprising phenols and nonphenols) were tested for their response to the FC assay by Singleton et al.[19] and various food samples were subjected to the same assay by Vinson et al.,[24] none of those had lipophilic character. In this respect, there is an urgent need for a modified FC method applicable to TAC determination of lipophilic antioxidants in food, constituting the basic motivation of this work.

Thus in this study, the FC method has been successfully adapted to the assessment of lipophilic antioxidants in isobutanol medium. The modification of the FC assay was performed by using an isobutanol-diluted version of the FC reagent and providing an alkaline medium with aqueous NaOH such that both organic and aqueous phases necessary for lipophilic and hydrophilic antioxidants, respectively, were supplied simultaneously. In this modified FC method, the reaction time was decreased to 20 min, and the original reagent mixture was simplified (i.e., separate preparation of Lowry A, Lowry B, and Lowry C is no longer required).

MATERIALS AND METHODS

Instrumentation and Chemicals. The chemical substances used in the experiments were all of analytical reagent grade: the Folin−Ciocalteu phenol reagent, sodium hydroxide, sodium carbonate, sodium potassium tartarate, isobutyl alcohol, copper(II) sulfate, absolute ethyl alcohol, pure acetone, methanol, and cysteine (CYS) were from E. Merck (Darmstadt, Germany); rutin (RT), quercetin (QR), reduced glutathione (GSH), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2carboxylic acid, TR), ascorbic acid (ASC), ferulic acid (FA), caffeic acid (CF), butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), *tert*-butyl hydroquinone (TBHQ), β-carotene (CAR), and rosmarinic acid (RA) were purchased from Sigma (Steinheim, Germany); vitamin E (α-tocopherol (TOC)), gallic acid (GA), and lauryl gallate (LG) were supplied by Fluka. All polyphenolic compounds and vitamin solutions were freshly prepared in pure acetone apart from ascorbic acid, cysteine, and gluthatione (water), at required concentration values. Commercial olive oil and tea bags were all purchased from a local market in Istanbul, Turkey. Green tea bags (*Camellia sinensis*) and sage herbal tea bags (*Salvia officinalis*) were used for the preperation of infusion solutions.

The visible spectra and absorption measurements were recorded in matched quartz cuvettes using a Varian Cary 100 UV−vis spectrophotometer (Mulgrave, Victoria, Australia). All of the prepared solutions were homogenized with the aid of a Heidolph vortex stirrer (Nuremberg, Germany). Sample solutions were centrifuged using an MSE Mistral 2000 centrifuger (Sanyo Gallenkap PLC, Middlesex, United Kingdom) before the analysis procedure. Liquid sampling at 5−50 μL and 200−500 μL was performed with Genex Beta-type (Torquay, Devon, United Kingdom) variable and Brand Transferpette-type fixed-volume micropipets (Essex, Connecticut, USA), respectively.

Original Folin−Ciocalteu Method of the Total Phenolics Assay. *Preparation of Solutions.* Folin−Ciocalteu's phenol reagent was diluted at a volume ratio of 1:2 with distilled water (1 volume Folin−Ciocalteu's phenol reagent + 2 volumes distilled water) prior to use. Lowry A solution was prepared from sodium carbonate such that the weight percentage of Na_2CO_3 in 0.1 M NaOH solution was 2.0% (w/v). Lowry B solution was prepared from copper(II) sulfate such that the weight percentage of $CuSO_4$ in 1.0% sodium potassium tartrate ($NaKC_4H_4O_6$) solution was 0.5% (w/v). Lowry C solution was prepared by mixing 50 mL of Lowry A with 1 mL of Lowry B.[19] Stock solutions of antioxidant compounds were prepared in pure acetone medium. Standard solutions of each antioxidant were prepared at increasing concentration values after appropriate dilutions were made. A fixed volume of solution (200 μL) was taken for the procedure.

Procedure. A volume of 1.8 mL of H_2O was added to 200 μL of antioxidant sample solution (in pure acetone medium at different concentration values). It should be noted that in this slight modification, sample or standard solution was prepared in 200 μL of acetone, replacing the water in the original FC method, due to the requirement of testing both hydrophilic and lipophilic antioxidants in the same solvent medium. An aliquot of 2.5 mL of Lowry C solution was added, and the mixture was allowed to stand for 10 min. At the end of this period, 250 μL of Folin reagent was added, and 30 min were allowed for stabilization of the blue color formed. Reagent blank solution was prepared with the same procedure using only acetone instead of phenolic sample solution. The absorbance against a reagent blank was read at 750 nm.[19]

Modified Folin−Ciocalteu Method of the Total Phenolics (Hydrophobic and Hydrophilic Antioxidants) Assay. *Preparation of Solutions.* Folin−Ciocalteu's phenol reagent was diluted at a volume ratio of 1:2 with isobutyl alcohol prior to use (i.e., 1 volume of Folin−Ciocalteu's phenol reagent + 2 volumes of iso-BuOH). The necessary alkalinity in the determinations was achieved with 0.1 M aqueous NaOH solution (as tetrabutylammonium hydroxide caused the precipitation of molybdotungstophosphate heteropolyanion having (4−) charge).

Recommended Procedure for Modified FC Assay. To 300 μL of (1:2 diluted) Folin−Ciocalteu's phenol reagent were added 200 μL of antioxidant sample solution (prepared in pure, peroxide-free acetone), followed by 3.5 mL of 0.1 M aqueous NaOH, and the necessary amount of H_2O for dilution to 10 mL of total volume (for dilute antioxidant samples, more than 200 μL of acetone solution can be taken for analysis; however, increase in sample volume up to 800 μL caused turbidity formation, whereas contact of excessive acetone with iso-BuOH extract of the Folin reagent caused the appearance of the blue color without antioxidant, requiring the restriction of the sample volume to 200 μL of acetone solution in the recommended procedure).

After incubation at room temperature for 20 min, the optical absorbance of the final solution was recorded at 665 nm against a reagent blank prepared with the same procedure using acetone instead of sample solution, and absorbance was correlated to antioxidant concentration.

Preparation of Real Samples for Analysis. A volume of 25 mL of olive oil sample was mixed with 25 mL of methanol in a beaker and homogeneously shaken with a mechanical shaker at 450 rpm for 30 min. The homogeneous mixture was kept at −25 °C for 4 h. The liquid was decantated and centrifuged for 5 min at 2500 rpm. The supernatant phase was used for the analysis. Commercial herbal tea bags 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. The herbal tea solution was allowed to cool to room temperature and filtered through a Whatman black-band paper for removing particulates.

RESULTS

Optimization of Modified Folin−Ciocalteu (FC) Assay Parameters. *UV−Vis Spectra and Maximum Absorption Wavelength.* In order to obtain UV−vis spectra and maximum absorption wavelength for the proposed method, a synthetic antioxidant BHA (representative of lipophilic antioxidants) was used. The sample solutions were prepared for analysis according to the proposed method, the modified FC assay. The absorption spectra of the final solutions in the 200−900 nm range are shown in Figure 1, with maximum absorption

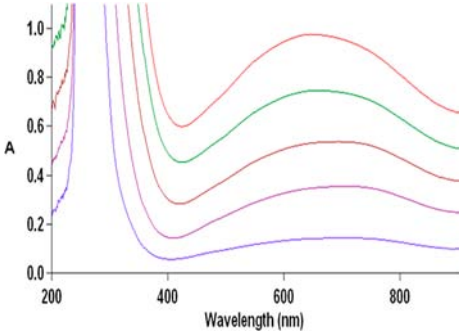

Figure 1. Absorption spectra of the final solutions comprising 300 μL, taken from the iso-BuOH phase, of Folin−Ciocalteu's modified phenol reagent (prepared by mixing one volume of commercially available Folin−Ciocalteu's phenol reagent with 2 volumes of isobutyl alcohol) + 200 μL BHA solutions in the concentration range (2.0×10^{-5}−6.0×10^{-5} M) + 3.5 mL of 0.1 M NaOH solution + distilled water of dilution to a total volume of 10.0 mL.

wavelength recorded at 665 nm. Since the original FC method was modified, the maximum absorption wavelength (λ_{max}) due to the molybdenum blue heteropoly species, known to be sensitive to the conditions of formation, did not coincide with that (i.e., 750 nm) of the classical method.[19] By similar reasoning, Box[25] found that among the FC method variations using different alkaline constituents, λ_{max} with Na_2CO_3 was slightly longer than that with NaOH in the wavelength range 752−765 nm.

Amount of Modified Folin−Ciocalteu's Reagent. For the optimization of the amount of modified FC reagent in routine analyses, increasing volumes were taken from the iso-BuOH extract of the 1:2 diluted commercial FC reagent and mixed with a fixed amount of quercetin, aqueous NaOH, and water of dilution. The order of reagent addition is presumably important, e.g., NaOH should be added after the FC reagent to minimize reagent degradation and spontaneous air oxidation of polyphenols. According to Singleton et al.,[19] "the fact that FC reagent is not stable under alkaline conditions emphasizes the importance of having sufficient excess present to react with all the phenolics before it is destroyed." Absorbance values at 665 nm of solutions versus increasing volumes of Folin reagent were recorded (Figure 2).

As seen in Figure 2, the optimal volume of 300 μL of modified FC reagent was chosen for future studies. Higher amounts of reagent only diluted the reaction medium but did not react with antioxidant molecules, resulting in diminution of the absorbance values.

Amount of NaOH Solution. During preliminary experiments, it was observed that alkalinity had direct effects on both precipitation and color formation in the reaction medium. For optimizing NaOH concentration, 300 μL of the extracted iso-

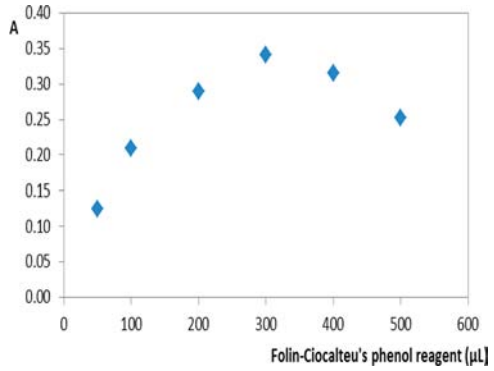

Figure 2. Effect of reagent volume. Absorbance values of the final solutions prepared by adding increasing volumes of modified Folin−Ciocalteu's phenol reagent (taken from the iso-BuOH extract of 1:2 diluted commercial FC solution) in the range of 50−500 μL + 200 μL of 5.0×10^{-4} M quercetin solution (in acetone) + 3.5 mL of 0.1 M aqueous NaOH solution + distilled water of dilution to a total volume of 10.0 mL.

BuOH phase of the modified FC reagent and increasing volumes of 0.1 M aqueous NaOH were reacted (in 10 mL total volume with the water of dilution) with 200 μL of either 5.0×10^{-4} M quercetin solution or 2.0×10^{-3} M BHT solution (such that the final sodium hydroxide concentration would lie between 5.0×10^{-3} and 5.0×10^{-2} M). Dependence of absorbance on NaOH alkalinity (Figure 3) showed that a final volume of 3.5 mL of 0.1 M NaOH solution should be chosen as optimal for further studies (providing an experimental pH value of about 12).

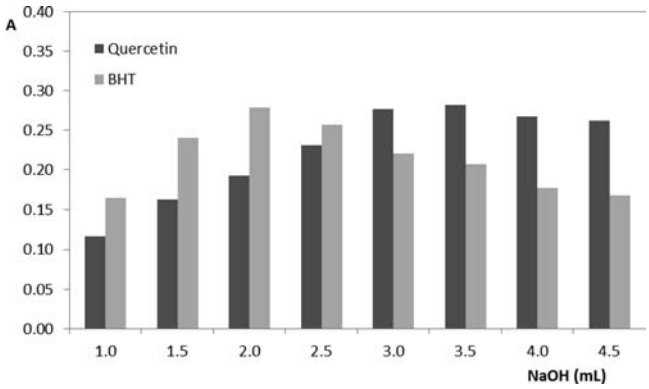

Figure 3. Effect of NaOH alkalinity. The absorbance values of the final solutions comprising 300 μL of modified FC reagent + 200 μL of 2.0×10^{-3} M BHA or 200 μL of 5.0×10^{-4} M QR solutions + increasing volumes of 0.1 M aqueous NaOH solution to achieve a final concentration range between 1.0×10^{-2}−4.5×10^{-2} M + distilled water of dilution to a total volume of 10.0 mL.

Crouch and Malmstadt[26] had reported that *ortho*-phosphoric acid initially forms a 12-molybdophosphoric acid (12-MPA) complex with molybdate(VI) according to the following equilibrium:

$$H_3PO_4 + 6Mo(VI) \rightarrow (12\text{-MPA}) + 9H^+ \quad (1)$$

The reduction of 12-MPA to phosphomolybdenum blue (PMB) may be expressed as follows:

$$(12\text{-MPA}) + n\text{Red} \rightarrow \text{PMB} + n\text{Ox} \quad (2)$$

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where Red and Ox represent the reducing agent and the corresponding oxidized species, respectively.[26] It is apparent from the above equilibria that 12-MPA formation would be incomplete with increasing acidity. The phenolic dissociation reactions to the more easily oxidizable phenolate conjugate bases also require sufficient alkalinity. Thus, optimal amount of NaOH should be carefully adjusted, as less alkalinity would not result in quantitative oxidation of phenolics (which require complete ionization before oxidation), whereas excessive alkalinity would adversely affect the stability of the FC reagent.[19] According to Singleton et al.,[19] "it is important to have enough but not excessive alkalinity." A similar decrease of absorbance with increasing NaOH concentration beyond a certain limit was also noted by Box[25] who reported an optimal pH between pH 10−11.5.

Optimal Reaction Time. In order to find optimal reaction time, 6.0×10^{-5} M quercetin, ferulic acid, *p*-coumaric acid, and naringenin solutions were tested with the modified FC assay (Figure 4). In order to see the effect of antioxidant concentration on reaction time, quercetin solutions in the concentration range 1.0×10^{-5} M−6.0×10^{-5} M were chosen (Figure 5). The absorbance values of the prepared solutions were measured at 665 nm for 60 min by allowing the reaction mixture to stand within the cuvette, as a result of which optimal reaction time was set as 20 min for further studies.

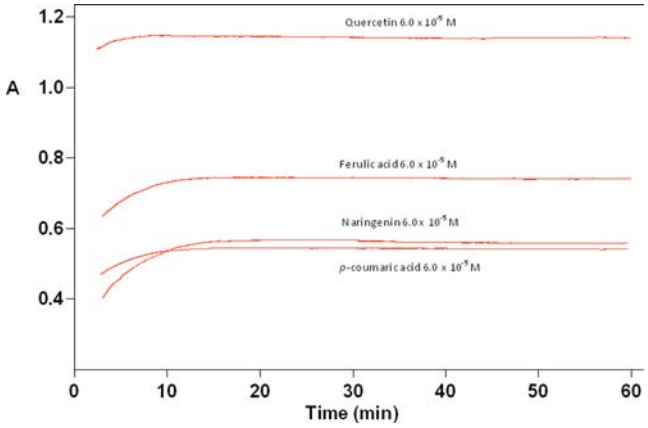

Figure 4. Optimization of reaction time tested with 6.0×10^{-5} M antioxidant (quercetin, ferulic acid, naringenin, and *p*-coumaric acid) solution + 300 μL of modified FC reagent + 3.5 mL of 0.1 M NaOH solution + distilled water of dilution to a total volume of 10.0 mL.

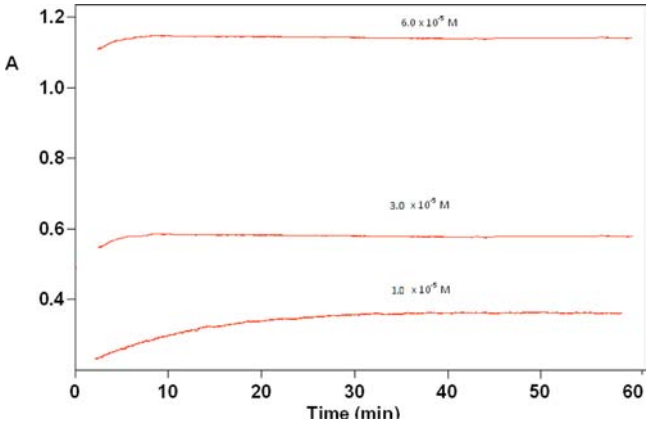

Figure 5. Optimization of reaction time tested with (50, 150, and 300) μL of 2.0×10^{-3} M quercetin solution + 300 μL of modified FC reagent + 3.5 mL of 0.1 M NaOH solution + distilled water of dilution to a total volume of 10.0 mL.

Linear Concentration Range, Molar Absorption, and TEAC Coefficients of Antioxidants Using the Conventional FC Method with Acetone Dissolution of Samples. As reported by previous researchers, the FC assay could only be applied to hydrophilic antioxidants and water-soluble food components because the method in its originally developed form is inapplicable to hydrophobic phenols and antioxidants.[4] In this study, both the tested lipophilic and hydrophilic antioxidants were dissolved in pure acetone medium, and linear concentration ranges, trolox-equivalent antioxidant capacities (TEAC coefficients), calibration equations, linear regression coefficients, and molar absorptivities were calculated with respect to the original FC method, as given in Table 1. The tabulated data (Table 1) are believed to be different from those in the literature (even for the conventional FC assay) because of the use of acetone as solvent replacing the routinely used water or water−alcohol mixtures. The characteristic results presented in Table 1 include relatively low correlation coefficients for the calibration equations of a number of phenolics such as gallic acid, catechin, and especially synthetic antioxidants (BHT, TBHQ, and LG), and nonquantitative response to ascorbic acid. Other researchers experienced similar problems in ascorbic acid determination with the conventional FC assay, where vitamin C present in the water washing eluate from the solid phase extraction cartridge had to be destroyed by heating and thus colorimetrically deduced from the FC absorbance.[27] The FC application with acetone dissolution of samples in this work proved neither useful for ascorbic acid (for which a linear response could not be produced) nor for olive oil polyphenols (due to insufficient solubility). Although ascorbic acid is 2-e oxidized to dehydroascorbic acid and neatly determined by the usual TAC assays,[5] it gives erratic results with the FC assay possibly because dehydroascorbic acid is enolic and can also react with the FC reagent (i.e., dehydroascorbic at 100 mg/L was shown to give FC values in heated flow automatic analysis equivalent to 45 mg of gallic acid per liter).[19] To overcome the mentioned difficulties, the development of a modified FC assay capable of measuring lipophilic antioxidants (including synthetic antioxidants) along with hydrophilic ones was necessary.

Linear Concentration Range, Molar Absorption and TEAC Coefficients, and Limits of Detection and Quantification (LOD and LOQ) of Antioxidants Using the Modified FC Method with Acetone Dissolution of Samples. Calibration curves using the modified FC assay were obtained for certain antioxidant compounds, namely, trolox, quercetin, rutin, gallic acid, caffeic acid, ferulic acid, rosmarinic acid, glutathione, cysteine, ascorbic acid, vitamin E, BHA, BHT, LG, TBHQ, and β-carotene. The linear concentration ranges, linear calibration equations (of absorbance versus concentration), regression coefficients, and molar absorption coefficients were calculated for each antioxidant compound and are given in Table 2. Limit of detection (LOD) and limit of quantification (LOQ) values for each antioxidant molecule were calculated by taking 3 and 10 times the standard deviation of a blank, respectively, and dividing by the slope of the calibration line (i.e., molar absorption coefficient). The modified FC method was validated through analytical figures of merit including LOD, LOQ, recovery (%), and relative standard deviation (RSD, %), found by standard additions of vitamin E to olive oil and trolox to

Table 1. Analytical Figures of Merit Found by the Conventional Folin−Ciocalteu Method for Samples Containing both Hydrophilic and Lipophilic Antioxidants

antioxidants	linear calibration equation	regression coefficient (N = 3)	molar absorption coefficient (L mol^{-1} cm^{-1})	linear concn range (M)	TEAC
trolox	$A = -7.70 \times 10^{-3} + 2.02 \times 10^{3} C_{TR}$	0.9829	2.02×10^{3}	2.1×10^{-5}–1.7×10^{-4}	1.00
quercetin	$A = -0.80 \times 10^{-2} + 3.64 \times 10^{3} C_{QR}$	0.9858	3.64×10^{3}	2.1×10^{-6}–2.5×10^{-5}	1.80
gallic acid	$A = -4.92 \times 10^{-1} + 5.14 \times 10^{3} C_{GA}$	0.9485	5.14×10^{3}	8.4×10^{-5}–3.4×10^{-4}	2.54
ferulic acid	$A = 1.00 \times 10^{-1} + 1.13 \times 10^{4} C_{FA}$	0.9972	1.13×10^{4}	1.0×10^{-5}–8.4×10^{-5}	5.59
caffeic acid	$A = -1.80 \times 10^{-2} + 1.12 \times 10^{4} C_{CA}$	0.9985	1.12×10^{4}	1.1×10^{-5}–8.7×10^{-5}	5.54
catechin	$A = -1.24 \times 10^{-1} + 2.15 \times 10^{4} C_{CAT}$	0.9742	2.15×10^{4}	1.1×10^{-5}–5.4×10^{-5}	10.6
vitamin E	$A = -1.00 \times 10^{-2} + 4.12 \times 10^{3} C_{VITE}$	0.9963	4.12×10^{3}	4.0×10^{-5}–1.5×10^{-4}	2.04
BHT	$A = 5.93 \times 10^{-2} + 5.89 \times 10^{3} C_{BHT}$	0.9270	5.89×10^{3}	4.2×10^{-5}–1.7×10^{-4}	2.92
BHA	$A = 6.24 \times 10^{-2} + 6.38 \times 10^{3} C_{BHA}$	0.9973	6.38×10^{3}	4.2×10^{-5}–1.5×10^{-4}	3.16
TBHQ	$A = -4.09 \times 10^{-2} + 2.79 \times 10^{3} C_{TBHQ}$	0.9672	2.79×10^{3}	6.3×10^{-5}–5.0×10^{-4}	1.38
LG	$A = -1.57 \times 10^{-2} + 9.18 \times 10^{3} C_{LG}$	0.9840	9.18×10^{3}	2.1×10^{-5}–1.0×10^{-4}	4.54
ascorbic acid					
β-carotene	$A = 2.20 \times 10^{-3} + 1.47 \times 10^{3} C_{CAR}$	0.9952	1.47×10^{3}	4.2×10^{-5}–2.1×10^{-4}	0.72
rosmarinic acid	$A = -7.40 \times 10^{-3} + 2.13 \times 10^{4} C_{RA}$	0.9922	2.13×10^{4}	5.3×10^{-6}–5.3×10^{-5}	10.5
glutathione	$A = -7.06 \times 10^{-2} + 1.22 \times 10^{3} C_{GSH}$	0.9981	1.22×10^{3}	1.2×10^{-4}–1.2×10^{-3}	0.60
cysteine	$A = -1.68 \times 10^{-1} + 1.65 \times 10^{3} C_{CYS}$	0.9876	1.65×10^{3}	1.0×10^{-4}–8.4×10^{-4}	0.82

Table 2. Analytical Figures of Merit Found by the Modified Folin−Ciocalteu Method for Samples Containing both Hydrophilic and Lipophilic Antioxidants

antioxidants	linear calibration equation	regression coefficient (N = 3)	molar absorption coefficient (L mol^{-1} cm^{-1})	LOD (M) (N = 8)	LOQ (M) (N = 8)	linear concn range (M)	TEAC
trolox	$A = 5.77 \times 10^{-2} + 5.51 \times 10^{3} C_{TR}$	0.9961	5.51×10^{3}	0.91×10^{-6}	3.00×10^{-6}	2.00×10^{-4}–1.00×10^{-3}	1.00
quercetin	$A = 1.06 \times 10^{-1} + 1.53 \times 10^{4} C_{QR}$	0.9981	1.53×10^{4}	0.33×10^{-6}	1.91×10^{-6}	2.00×10^{-5}–1.00×10^{-4}	2.78
gallic acid	$A = 9.86 \times 10^{-2} + 9.82 \times 10^{3} C_{GA}$	0.9905	9.82×10^{3}	0.51×10^{-6}	1.70×10^{-6}	4.00×10^{-5}–2.00×10^{-4}	1.78
ferulic acid	$A = 4.65 \times 10^{-2} + 1.04 \times 10^{4} C_{FA}$	0.9998	1.04×10^{4}	0.48×10^{-6}	1.61×10^{-6}	1.00×10^{-5}–8.00×10^{-5}	1.88
caffeic acid	$A = 3.97 \times 10^{-2} + 1.44 \times 10^{4} C_{CF}$	0.9971	1.44×10^{4}	0.35×10^{-6}	1.16×10^{-6}	1.00×10^{-5}–8.00×10^{-5}	2.61
catechin	$A = 1.96 \times 10^{-2} + 1.78 \times 10^{4} C_{CAT}$	0.9940	1.78×10^{4}	0.28×10^{-6}	0.94×10^{-6}	5.00×10^{-6}–6.00×10^{-5}	3.23
vitamin E	$A = 6.35 \times 10^{-2} + 2.15 \times 10^{3} C_{TOC}$	0.9884	2.15×10^{3}	2.33×10^{-6}	7.76×10^{-6}	2.80×10^{-5}–2.80×10^{-4}	0.39
BHT	$A = 3.14 \times 10^{-2} + 4.50 \times 10^{3} C_{BHT}$	0.9980	4.50×10^{3}	1.11×10^{-6}	3.71×10^{-6}	2.00×10^{-5}–1.60×10^{-4}	0.82
BHA	$A = 5.13 \times 10^{-2} + 5.45 \times 10^{3} C_{BHA}$	0.9977	5.45×10^{3}	0.92×10^{-6}	3.06×10^{-6}	2.00×10^{-5}–1.00×10^{-4}	0.99
TBHQ	$A = 2.98 \times 10^{-2} + 1.05 \times 10^{4} C_{TBHQ}$	0.9929	1.05×10^{4}	0.48×10^{-6}	1.59×10^{-6}	5.00×10^{-6}–6.00×10^{-5}	1.90
LG	$A = 7.59 \times 10^{-2} + 7.17 \times 10^{3} C_{LG}$	0.9881	7.17×10^{3}	0.69×10^{-6}	2.32×10^{-6}	2.00×10^{-5}–8.00×10^{-5}	1.30
ascorbic acid	$A = 2.53 \times 10^{-2} + 8.79 \times 10^{3} C_{ASC}$	0.9975	8.79×10^{3}	0.56×10^{-6}	1.89×10^{-6}	2.00×10^{-5}–1.00×10^{-4}	1.60
β-carotene	$A = 3.85 \times 10^{-2} + 1.87 \times 10^{3} C_{CAR}$	0.9874	1.87×10^{3}	2.68×10^{-6}	8.93×10^{-6}	4.00×10^{-5}–2.00×10^{-4}	0.34
rosmarinic acid	$A = 6.81 \times 10^{-2} + 2.25 \times 10^{4} C_{RA}$	0.9943	2.25×10^{4}	2.23×10^{-6}	7.42×10^{-6}	5.00×10^{-6}–6.00×10^{-5}	4.08
glutathione	$A = 1.07 \times 10^{-1} + 5.65 \times 10^{3} C_{GSH}$	0.9417	5.65×10^{3}	0.89×10^{-6}	2.96×10^{-6}	2.00×10^{-5}–1.00×10^{-4}	1.02
cysteine	$A = 5.56 \times 10^{-2} + 3.63 \times 10^{3} C_{cys}$	0.9931	3.63×10^{3}	1.38×10^{-6}	4.60×10^{-6}	4.00×10^{-5}–2.00×10^{-4}	0.66

green tea infusion (Table 3). The TEAC coefficient is defined as the ratio of the slope of the calibration curve of the tested antioxidant to that of trolox for each assay. The most characteristic feature of the modified FC assay is the significantly improved linear correlation coefficients for phenolics (Table 2), especially for synthetic antioxidants (BHT, BHA, TBHQ, and LG), compared to the corresponding values in Table 1. As a distinct advantage over the conventional FC method, ascorbic acid can be reliably assayed by the proposed FC modification (Table 2). Since the molar absorptivity for trolox showed more than a 2-fold increase with the modified method, the TEAC coefficients for most antioxidants dropped to normal levels, as found by other standard tests (Table 4). Thus, the extremely high TEAC values for rosmarinic acid and catechin, which may originate from the high oxidizing power of the conventional FC reagent,[5,22] were brought to acceptable levels using the proposed method, generally in accordance with the results of standard CUPRAC, ABTS, and FRAP assays (Table 4).

Table 3. Analytical Figures of Merit Found in Complex Food Matrices by the Modified Folin−Ciocalteu Method (N = 5)

validation parameters	3.0×10^{-5} M vitamin E added to olive oil sample	6.0×10^{-5} M trolox added to green tea infusion
LOD (M)	1.51×10^{-5}	5.77×10^{-6}
LOQ (M)	5.05×10^{-5}	1.92×10^{-5}
recovery (%)	103.1	103.3
RSD (%)	7.46	4.04

The correlation equations calculated with the aid of TEAC coefficients given in Table 4 are presented below. According to these results, there is a significant correlation (at 95% confidence level) between the CUPRAC and modified FC methods. However, the conventional FC method did not show an acceptable correlation with the other methods. Modified FC

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Table 4. Comparison of the TEAC Coefficients of the Tested Antioxidants Using Modified and Conventional Folin−Ciocalteu Methods with Those Found by Reference TAC Assays

antioxidants	modified Folin−Ciocalteu method	original Folin−Ciocalteu method	CUPRAC	ABTS	FRAP
trolox	1.00	1.00	1.00	1.00	1.00
quercetin	2.78	1.80	5.77	3.98	2.92
gallic acid	1.78	2.54	3.25	4.17	1.85
ferulic acid	1.88	5.59	1.47	1.70	0.87
caffeic acid	2.61	5.54	2.89	1.39	1.13
catechin	3.23	10.6	3.10	2.40	1.24
vitamin E	0.39	2.04	1.02	1.00	
BHT	0.82	2.92	0.77	0.98	
BHA	0.99	3.16	1.57	1.23	
TBHQ	1.90	1.38	1.02	1.20	
LG	1.30	4.54	1.68	2.26	
ascorbic acid	1.60		1.03	0.99	1.01
β-carotene	0.34	0.72	1.75	2.14	
rosmarinic acid	4.08	10.5	5.2		
glutathione	1.02	1.60	0.64	1.51	
cysteine	0.66	0.82	0.39	1.28	

with CUPRAC: $TEAC_{CUPRAC} = 1.20\ TEAC_{mod.Folin} + 0.051$ (r = 0.802). Conventional FC with CUPRAC: $TEAC_{CUPRAC} = 0.25\ TEAC_{Folin} + 1.24$ (r = 0.493). Modified FC with ABTS: $TEAC_{ABTS} = 0.55\ TEAC_{mod.Folin} + 1.00$ (r = 0.466). Conventional FC with ABTS: $TEAC_{ABTS} = 0.05\ TEAC_{Folin} + 1.67$ (r = 0.134).

TAC Determination of Synthetic Mixtures of Antioxidants. Ternary and quaternary synthetic mixtures of hydrophilic and lipophilic antioxidants (the latter with or without olive oil as a complex sample medium) were analyzed with the modified Folin−Ciocalteu method, and the observed overall TAC values were found to approximate the sum of the individual TAC values of constituents. The results for the theoretically expected and experimentally found TAC values were in agreement within 10%, as shown in Table 5. In addition, the theoretically expected and experimentally found absorbance values of ternary synthetic mixtures of antioxidants in olive oil sample agreed within ±5% (Table 6).

Additivity and Interference Effects in TAC Determination. Additivity of antioxidant capacities of individual antioxidants in a mixture is important in defining TAC, and the additivity property of antioxidant capacities can be demonstrated either in synthetic antioxidant mixtures (as in Tables 5 and 6) or in the standard addition of a selected antioxidant to a complex mixture. When applying the standard addition method, the calibration curves of a chosen antioxidant first in standard reaction medium and second in another antioxidant solution or complex matrix such as olive oil/sage/green tea extract were drawn (Figures 6−8). Figures 6−8 indicate the parallelism of the mentioned pair (i.e., the slopes agreed within ±10%) of curves and consequently the lack of interference (in the form of association, dissociation, or interaction with solvent molecules) in the modified FC assay in agreement with Beer's law.

The potential interferents common in food plants and botanicals such as citric acid, glucose, mannitol, serine, lysine, valine, proline, and alanine did not significantly affect the

Table 5. Theoretically Expected and Experimentally Found TAC Values (as mM Trolox-Equivalents) of Synthetic Mixtures Using the Modified Folin−Ciocalteu Method

synthetic mixture	$TAC_{expected}$	TAC_{found}	deviation (%)
2.5×10^{-2} mM ascorbic acid	0.127	0.118	−7.08
5.0×10^{-2} mM cysteine			
1.4×10^{-1} mM vitamin E			
2.5×10^{-2} mM quercetin	0.185	0.178	−3.78
1.4×10^{-1} mM vitamin E			
7.5×10^{-2} mM BHT			
2.0×10^{-2} mM trolox	0.285	0.290	+1.75
2.0×10^{-2} mM gallic acid			
2.0×10^{-2} mM BHA			
2.5×10^{-2} mM quercetin	0.152	0.137	−9.86
2.5×10^{-3} mM rosmarinic acid			
2.5×10^{-2} mM BHA			
2.5×10^{-2} mM TBHQ			
2.5×10^{-1} mM trolox	0.295	0.280	−5.08
2.5×10^{-2} mM BHT			
2.5×10^{-2} mM BHA			
2.5×10^{-2} mM caffeic acid	0.196	0.182	−7.14
2.5×10^{-2} mM quercetin			
7.5×10^{-2} mM BHT			

Table 6. Theoretically Expected and Experimentally Found Absorbance (A) Values of Synthetic Mixtures of Lipophilic Antioxidants in Olive Oil Using the Modified Folin−Ciocalteu Method

synthetic mixture	$A_{expected}$	A_{found}	deviation (%)
3.75×10^{-2} mM BHT	0.948	0.912	−3.83
1.25×10^{-2} mM TBHQ			
6.00×10^{-2} mM trolox			
olive oil			
2.50×10^{-2} mM BHA	0.840	0.868	+3.22
3.75×10^{-2} mM BHT			
1.25×10^{-2} mM TBHQ			
olive oil			
2.50×10^{-2} mM vitamin E	0.745	0.725	−2.57
1.25×10^{-2} mM TBHQ			
1.88×10^{-2} mM BHA			
olive oil			

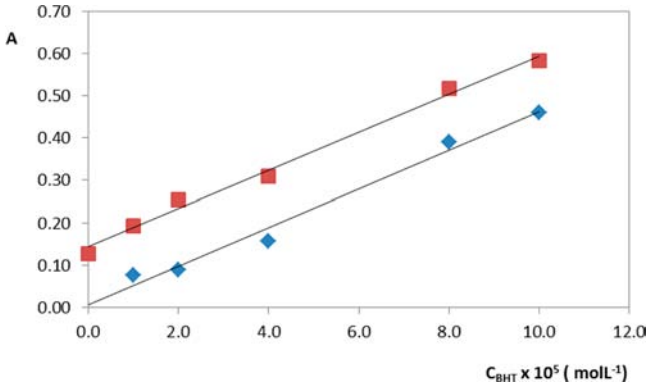

Figure 6. Calibration line of BHT (the regression equations: ◆, $y = 4.55 \times 10^3 x + 0.0068$, $R^2 = 0.9830$, in pure reaction medium, ■, $y = 4.50 \times 10^3 x + 0.1436$, $R^2 = 0.9926$, in BHA solution) with respect to the modified Folin−Ciocalteu method.

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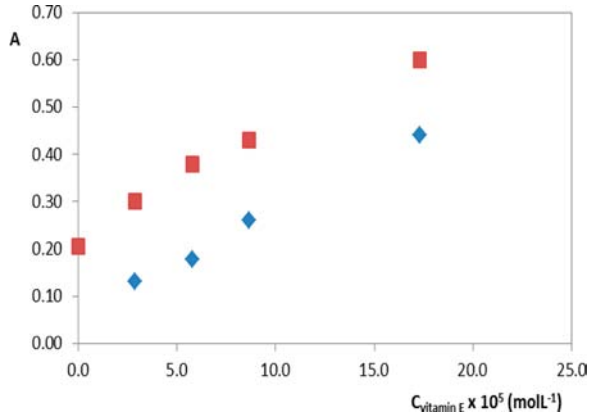

Figure 7. Calibration line of vitamin E (the regression equations: ◆, $y = 2.18 \times 10^3x + 0.0633$, $R^2 = 0.9962$, in pure reaction medium; ■, $y = 2.22 \times 10^3x + 0.2299$, $R^2 = 0.9848$, in olive oil solution) with respect to the modified Folin−Ciocalteu method.

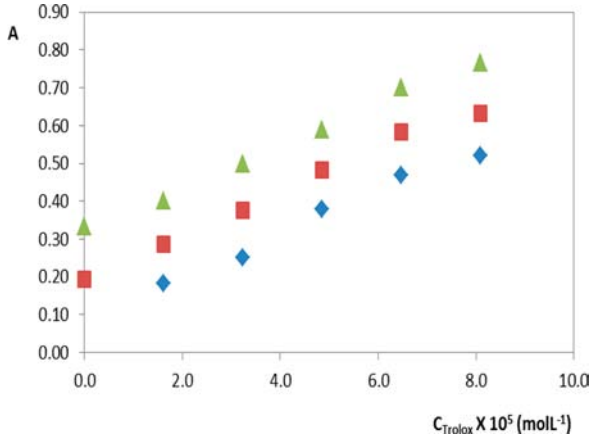

Figure 8. Calibration line of trolox (the regression equations: ◆, $y = 5.52 \times 10^3x + 0.0926$, $R^2 = 0.9813$, in standard reaction medium; ■, $y = 5.65 \times 10^3x + 0.1960$, $R^2 = 0.9928$, in green tea infusion; ▲, $y = 5.56 \times 10^3x + 0.3230$, $R^2 = 0.9957$, in sage infusion) with respect to the modified Folin−Ciocalteu method.

determination of 6.0×10^{-5} M trolox at 10-fold concentration levels (i.e., caused less than 5% relative error). However, most of these compounds gave rise to more than 10% relative error at 100-fold concentrations, possibly due to the strong oxidizing capability[5,22] of the FC reagent. When tested individually (i.e., without antioxidant) at 6.0×10^{-4} M concentration with the modified FC reagent, these potential interferent compounds gave less than 0.02 absorbance. These findings showed that, aside from the inherent interference susceptibility of the FC method, the modified FC reagent was generally capable of the TAC assay of true antioxidants with reasonable selectivity in sufficiently dilute solutions.

■ DISCUSSION

In this study, the original FC method, which was initially intended for protein analysis[9] and improved for the determination of water-soluble phenolic compounds,[19] was modified for the simultaneous determination of lipophilic and hydrophilic antioxidants in food samples. Among the three recommended methods to be used for TAC assay standardization (in a most cited review by Prior et al.)[22] with the purpose of routine quality control and assessment of antioxidant capacity of dietary supplements and other botanicals, FC was the only ET-based assay found eligible. The possible reasons for this choice are low cost and commercial availability of reagents, simplicity of performance to yield consistent results, long-wavelength maximum minimizing interference from complex sample matrices, routine practice in antioxidant research, and a large body of comparable data produced over the years with this reagent.[4] In spite of the fact that the exact chemistry and redox potential of the FC reagent is unknown and that it may act as a nonspecific oxidizing reagent toward a number of inorganic salts (e.g., ferrous ion, sulfite, and iodide), simple phenols, sugars, amino acids, and citric acid that are not classified under the widely accepted category of antioxidants,[5] the FC reagent is not only a phenol reagent but also an approved TAC reagent[4,22] capable of oxidizing diverse antioxidants. Because phenolics constitute the most abundant antioxidant class in most plants, the FC assay simultaneously gives a rough estimate of the total phenolic content in most cases.[28] Although Singleton et al.[19] specified the assay conditions to minimize variability and eliminate erratic results, very few papers published afterward followed the exact steps of this improved FC method, and hence, continued efforts to standardize the assay were reported to be clearly warranted.[22] The FC method is known to be deficient in responding to lipophilic antioxidants,[4,5] and obviously, the best way to standardize this assay is to increase its scope so as to embrace both hydrophilic and lipophilic antioxidants, forming the subject matter of this article.

The modified method is based on the reaction of antioxidant molecules with Folin−Ciocalteu's phenol reagent (diluted with isobutyl alcohol at a volume ratio of 1:2) in 3.5×10^{-2} M NaOH-containing alkaline medium. The relevant parameters including the iso-BuOH dilution ratio of commercial FC reagent, amount of modified FC reagent, maximum absorption wavelength, final NaOH concentration (i.e., the oxidation of phenolates is much faster than that of corresponding phenols[4,5,22]), and reaction time were optimized. The optimal reaction time of 20 min (at room temperature) of the modified FC assay was less than the 40 min protocol time of the conventional FC method. The modified procedure was successfully applied to the TAC assay of hydrophilic phenolic acids, flavonoids, and thiol-type antioxidant compounds including trolox, quercetin, ascorbic acid, gallic acid, catechin, caffeic acid, ferulic acid, rosmarinic acid, gluthathione, and cysteine. Additionally, lipophilic antioxidants such as vitamin E (α-tocopherol), BHA, BHT, TBHQ, LG, and β-carotene dissolved in acetone solution were also reacted with the modified FC reagent in an iso-BuOH-diluted and NaOH-containing reaction medium. Although the conventional FC reagent also responded to the above-mentioned lipophilic antioxidants dissolved in acetone solution, their linear correlation coefficients were rather low, preventing their precise and accurate quantitative assay. The modified FC assay gave reasonable TEAC coefficients for rosmarinic acid and catechin (i.e., comparable to those found by other reference TAC assays), as opposed to those found by the conventional FC assay yielding exceptionally high values. Unlike the conventional FC assay producing erratic results with ascorbic acid, the proposed FC modification was capable of reliably finding the antioxidant capacity of ascorbic acid with reproducible results, although its TEAC coefficient of 1.60 indicated an oxidation reaction extending further beyond 2-e oxidation provided by reference assays of CUPRAC and ABTS/TEAC (probably due

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to the fact that the FC reagent interacted with dehydroascorbic acid, i.e., the 2-e oxidized product of ascorbic acid). Although it was not among the intentions of the present work to overcome major interferences inherent in the original FC method[28] due to the high redox potential of the reagent[5] enabling partial oxidation of a number of nonphenolic compounds, it was experimentally shown that some common sugars, amino acids, and fruit acids did not interfere with the proposed method at low concentrations (i.e., producing less than 5% absorbance difference at 10-fold concentrations in the determination of 60 μM trolox solution).

The modified method was reproducible and additive in terms of TAC values of constituents of complex mixtures such as olive oil extract and herbal tea infusions. The trolox equivalent antioxidant capacities (TEAC coefficients) of the tested antioxidant compounds of the modified FC method correlated linearly with those found by the reference CUPRAC method, i.e., a correlation coefficient of $r = 0.802$ was found between the results of the two assays tested on $N = 16$ antioxidant compounds (Table 4), reflecting a significant correlation at the 95% confidence level. It should be borne in mind that no two assays, even the results of the same assay under different reaction conditions, may produce the same TEAC value for a given antioxidant compound or sample[4,5] because of the variations in mechanisms, redox potential, thermodynamic efficiency, solvent effects, etc.; for example, total phenolics content assayed by the conventional FC method in blueberries was reported to range from 22 to 4180 mg per 100 g of fresh weight, depending mostly on assay conditions.[22] Hence, a good linear correlation with a reference assay is satisfactory for the reliability of a proposed assay. The developed method was validated through detection limits, relative standard deviations, and recoveries. This improvement is believed to have utility potential to both hydrophilic and lipophilic food samples for which the conventional FC assay had limited applicability.

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