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Solvent effects on the antioxidant capacity of lipophilic and hydrophilic antioxidants measured by CUPRAC, ABTS/persulphate and FRAP methods

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ABSTRACT

Antioxidants are health beneficial compounds that can protect cells and macromolecules (e.g., fats, lipids, proteins, and DNA) from the damage of reactive oxygen species (ROS). Solvent effect is a crucial parameter on the chemical behaviour of antioxidant compounds but there has been limited information regarding its role on antioxidant capacity and its assays. Therefore, the present study was undertaken to investigate the total antioxidant capacity (TAC) of some certain lipophilic and hydrophilic antioxidants, measured in different solvent media such as ethanol (EtOH) (100%), methanol (MeOH) (100%), methanol/water (4:1, v/v), methanol/water (1:1, v/v), dichloromethane (DCM)/EtOH (9:1, v/v). The cupric reducing antioxidant capacity (CUPRAC) values of selected antioxidants were experimentally reported in this work as trolox equivalent antioxidant capacity (TEAC), and compared to those found by reference TAC assays, *i.e.*, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)/persulphate (ABTS/persulphate) and ferric reducing antioxidant power (FRAP) methods. The TAC values of synthetic mixtures of antioxidants were experimentally measured as trolox equivalents and compared to those theoretically found by making use of the principle of additivity of absorbances assuming no chemical interaction between the mixture constituents. Possible synergistic (e.g., BHT and BHA in DCM/EtOH) or antagonistic behaviours of these synthetic mixtures were investigated in relation to solvent selection.

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1. Introduction

Reactive oxygen species (ROS) and the related oxidative stress have been proposed to play several roles in the pathogenesis of chronic-degenerative conditions, such as neurodegenerative diseases, cancer, arteriosclerosis, malaria, rheumatoid arthritis, some forms of anemia, auto-immune diseases, ageing, and diabetes. The nutritional protection that fruits, vegetables and various foodstuffs provide against several oxidative stress-based diseases has been attributed to various antioxidants including vitamin C, phenolic compounds including flavonoids, carotenoids, anthocyanins and phenolic acids, α-tocopherol (TOC), and antioxidant food additives (e.g., butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA)) [1].

The chemical diversity of antioxidants makes it difficult to separate and quantify individual antioxidants (*i.e.*, parent compounds, glycosides, and many isomers) from the plant based food matrix. Moreover, the total antioxidant power as an 'integrated parameter of antioxidants present in a complex sample' [2] is often more meaningful to evaluate health beneficial effects (*i.e.*, prevention of oxidative stress-related diseases) because of the cooperative action of antioxidants. Therefore, a variety of assays were developed to determine total antioxidant capacity of food and vegetable extracts, beverages and biological fluids. These antioxidant capacity assays may be broadly classified as electron-transfer (ET)-based assays (CUPRAC, Folin, ABTS/TEAC, FRAP) and hydrogen atom transfer (HAT)-based assays (ORAC, TRAP) [3]. These assays are frequently used to determine the antioxidant capacity of several food extracts obtained with different extraction systems, such as ethanol, ethanol/water, acetone/water, methanol/water, acidic methanol/water followed by acetone/water. Some researchers have examined the influence of the extraction solvent on the CUPRAC [4], ORAC [5,6], DPPH [6,7], and ABTS [6,8] assays. On the other hand, there has been no conclusive study describing the solvent effects on the antioxidant capacity of mixture solutions including several antioxidants such as phenolic acids, flavonoids, synthetic antioxidants, vitamins, and hydroxycinnamic acids measured by widely used spectrophotometric total antioxidant capacity (TAC) assays.

Solvent effect is an essential parameter on the chemical behaviour of antioxidant compounds. The choice of extracting solvents with different polarities can have a significant effect on the performance of HAT- and ET-based antioxidant reactions [8,9], especially on the latter with respect to solvent dependence of ET kinetics [10]. It has been claimed that HAT-based reactions involving competition kinetics are relatively solvent and pH-independent

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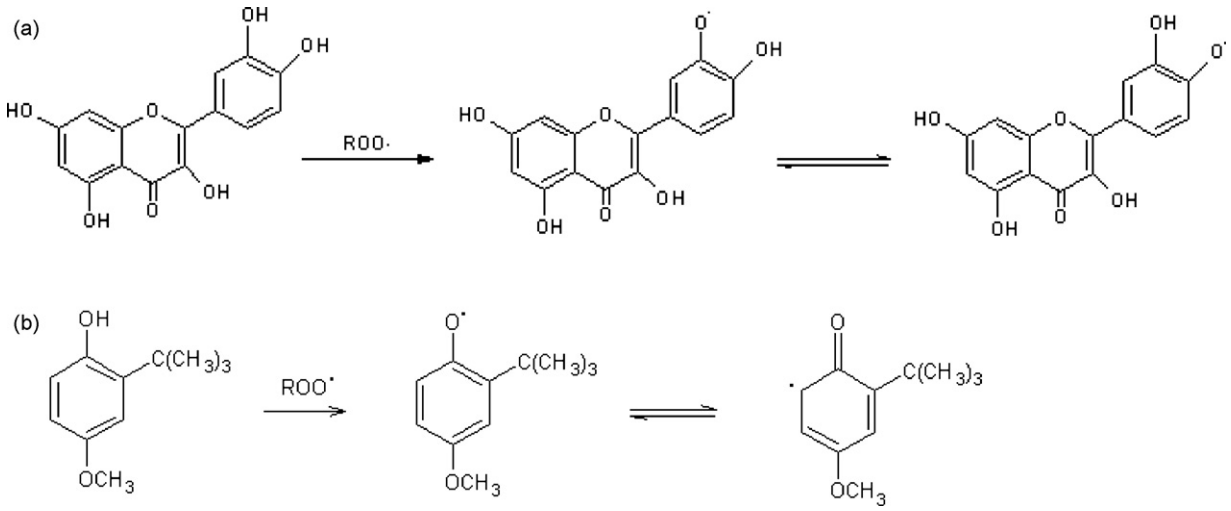

Fig. 1. (a) Possible resonance stabilization of the aryloxy radicals formed from 1-e oxidation of the *o*-catechol moiety in the B ring of quercetin by intramolecular H-bonding. (b) Possible resonance stabilization of the aryloxy radicals formed from 1-e oxidation of butylated hydroxyanisole (BHA).

and are quite rapid, usually completed in seconds to minutes [3]. Hydrogen bonding in polar solvents may induce dramatic changes in the H-atom donor activities of phenolic antioxidants and consequently affect the measured reducing antioxidant capacity [7,11].

As a brief outline of the theory for solvent effects, it can be resumed that the rates of phenol oxidation reactions (*i.e.*, *via* hydrogen (H)-atom or proton-coupled electron (e)-transfer) by reactive species (e.g., free radicals) are profoundly influenced by H-bond-accepting (HBA) and anion solvation abilities of solvents, as well as by the nature and position of phenol ring substituents [12]. Kinetic solvent effects (KSE) on antioxidant activity (AOA) of phenolic compounds can be investigated under two categories:

(i) KSE on AOA of phenolic molecules;
(ii) KSE on the rate of H-atom or electron (e) transfer of the probe molecule used for measuring AOA.

KSE on H-atom abstractions from phenolic hydroxyl groups (Ar–OH) are independent of the nature of the abstracting radical [13].

In general, the aryloxy radicals formed from the oxidation of catechol (*o*-dihydroxy phenol) moieties of phenolic compounds are stabilized in non- or weak hydrogen (H)-bonding solvents by intramolecular H-bonding (Fig. 1(a and b)), through the interaction of two adjacent substituents on catechol, *i.e.*, –C(O$^{\cdot}$)═(HO)C–. Such an intramolecular H-bonding stabilization will lower the standard redox potential of the aryloxy radical/catechol couple, making the phenolic compound a stronger antioxidant. For example, the rate constant of autoxidation inhibition: k_{inh} (of methyl linoleate) for quercetin and epicatechin was 20-fold ($k_{inh} = 4 \times 10^5\ M^{-1}\ s^{-1}$) in chlorobenzene (non-H-bonding solvent) than in *t*-butanol (H-bond accepting (HBA) solvent) with a rate constant of $k_{inh} = 2 \times 10^4\ M^{-1}\ s^{-1}$ [11]. The excellent AOA of catechol or pyrogallol moieties of polyphenols has been largely attributed to the intramolecular H-bonding stabilization of aryloxy radicals emerging as 1-e oxidation products of these moieties [14]. However, it can be generalized that intermolecular H-bonding of phenolic hydroxyl groups with HBA solvent molecules lowers AOA. Thus, in evaluating H-atom transfer kinetics of polyphenols, a distinction must be made between a linear H-bond (with a solvent (S) molecule, in the form of ArOH. . .S) and a non-linear H-bond (intramolecular H-bond), because only the former prevents H-atom abstraction from a phenolic compound by a free radical [15]. On the other hand, catechol can form only one linear H-bond with the free –OH in a HBA solvent, and thus may still react with peroxyl (ROO$^{\cdot}$) radicals by the intramolecularly bonded –OH [16].

AOA of polyphenols in preventing lipid oxidation is either based on H-atom abstraction from ArOH to peroxyl radicals, as shown by the reaction:

$$ROO^{\cdot} + ArOH \leftrightarrow (ROO^{\cdot} \ldots HOAr)_{solvent\ cage} \rightarrow (ROOH \ldots {}^{\cdot}OAr)_{solvent\ cage} \rightarrow \text{diffusion}$$

or an accompanying e^-/H^+ transfer, as symbolized by:

$$ROO^{\cdot} + HOAr \rightarrow (ROO^- \ldots {}^{+\cdot}HOAr)_{solvent\ cage} \rightarrow \text{products}$$

where the abstracted proton is transferred to either ROO$^-$ (making it ROOH) or to HBA solvent [16]. In this context, polar solvents may have a double-edged effect on the rate of this whole process. On one hand, they can make e-transfer easy by stabilizing the ion pair and thereby accelerate the overall e-transfer. On the other hand, they can hinder the formation of a complex between ArOH and ROO$^{\cdot}$ by preferential formation of a H-bonded complex between ArOH (acting as H-bond donor: HBD) and a molecule of solvent (HBA) in the form of (ArOH. . .S). As an example of e-transfer acceleration in ionizing solvents, abnormally enhanced rate constants were observed for the oxidation of phenols with DPPH$^{\cdot}$ (diphenylpicrylhydrazyl radical) not only in alcohols but also, for phenols with low pK_a values, in non-hydroxylic, polar solvents like di-*n*-butyl ether, acetonitrile, THF and DMSO, as a result of acidic ionization of phenol into phenoxide anion (ArO$^-$) followed by fast e-transfer from ArO$^-$ to DPPH$^{\cdot}$ [17]. Likewise, the abnormal solvent effects on the AOA of curcumin and isoeugenol exerted by MeOH, EtOH, dioxane, and ethyl acetate having equal HBA abilities have been resolved by detecting faster (by an order-of-magnitude) proton-coupled e-transfer of the phenoxide anion to DPPH$^{\cdot}$ in ionizing alcoholic solvents [18]. As a compromise between the two contradictory effects of polar solvents (*i.e.*, accelerating proton-coupled e-transfer by enhanced phenol ionization and inhibiting H-atom transfer by intermolecular H-bonding of phenols with the solvent), the prevailing effect is usually the hindrance of AOA with increasing HBA ability of the solvent. For example, solvent (S)-induced lowering of the rate constants, *i.e.*, k^S_{inh} (ROO$^{\cdot}$ + ArOH), was proportional to the HBA ability of the solvent; inhibition rate constants of catechol-bearing phenols at the order of $(3–15) \times 10^5\ M^{-1}\ s^{-1}$ in cyclohexane dramatically declined to quite low (even non-detectable) values in a strong HBA solvent like *t*-butanol [16].

Solvent effects may also be considered from the standpoint of choosing the reagents encountered in common AOA assays. If the AOA assay reagent is a coordinatively saturated metal complex species (involving different oxidation states of a given metal ion in the same ligand environment such as bis(neocuproine)copper(II,I), tris(1,10-phenanthroline)iron(III,II), hexacyanoferrate(III,II)) capable of outer-sphere e-transfer with the polyphenol [19], then ligand addition or removal is out of question, and a negligible reorientation of the already existing ligands around the central metal ion may expected in the formation of the transient intermediate during e-transfer, and consequently, the rate of e-transfer may only be affected to a limited extent by solvent polarity. However, inner-sphere e-transfer reactions of the assay reagent (e.g., $Fe(H_2O)_6^{3+}$) with the phenolic compound will naturally be affected by the H-bonding behaviour of the solvent due to stabilization or inhibition of the intermediary state formed during e-transfer. When other factors are not considered or assumed to remain constant, AOA assay methods based on H-atom donation (e.g., ORAC, TRAP, and ABTS assays) from a phenolic compound are generally affected to a greater extent by the solvent behaviour (polarity, HBA, *etc.*) than methods based on outer-sphere e-transfer (e.g., CUPRAC, ferricyanide, and FRAP).

Perez-Jimenez and Saura-Calixto [8] have noted that the $TEAC_{ABTS}$ value for the mixture of catechin and gallic acid was 40% lower in methanol/water (1:1, v/v) than in water, and the measured $TEAC_{ABTS}$ values increased with increasing water content of the solvent. In the same work, no difference was observed between the $TEAC_{FRAP}$ values in methanol/water (1:1, v/v) and in water.

The solvent effect in TAC measurement of complex food matrices and synthetic antioxidant mixtures may show some differences. The differences observed for different solvents would be greater if the analyzed food sample had a complex matrix in which different compounds may exhibit different interactions among themselves, other matrix components, and solvent molecules. Non-antioxidant food constituents such as amino acids may also show interfering effects on antioxidant assays depending on the nature of solvent [8]. Therefore, when making comparisons of antioxidant capacities, these have to be measured in extracts or solutions obtained with the same solvents [8].

As far as we know, there is limited work in literature dealing with the effect of the solvent on the antioxidant properties of dietary flavonoids, phenolics, and synthetic antioxidants in complex mixtures. The significantly different antioxidant capacities of synthetic antioxidants (especially of BHT) in mixtures prepared by varying solvent composition is an unresolved matter in antioxidant research. The ability of certain phenolic compounds to resist oxidative cleavage and polymerize, leading to an improvement in the overall antioxidant activity of plant foods, has been reported to be highly associated with their structure and solvent characteristics [7]. Thus, this work aims to investigate the solvent effect for selected antioxidants using CUPRAC and other TAC assays. Therefore, ethanol, methanol/water mixtures of differing compositions (containing 100, 80, and 50 volume per cent of methanol), and dichloromethane (DCM)/EtOH mixture (9:1, v/v) were selected as solvents. Finally, green tea extracts prepared in these solvent media were analyzed for antioxidant capacity by the CUPRAC [20], ABTS/persulphate [21], and FRAP [22] assays.

2. Experimental

2.1. Reagents and apparatus

The following chemical substances of analytical reagent grade were supplied from the corresponding sources: neocuproine (2,9-dimethyl-1,10-phenanthroline) (Nc), quercetin (QR), α-tocopherol (TOC), naringenin (NG), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), *tert*-butyl hydroquinone (TBHQ), catechin, glutathione (reduced, GSH), and dichloromethane (DCM): Sigma (Steinheim, Germany); ferulic acid (FRA), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (TR), and L-ascorbic acid (AA): Aldrich (Steinheim, Germany); copper(II) chloride dihydrate, ammonium acetate (NH_4Ac), absolute ethyl alcohol, potassium persulphate, iron(III) chloride hexahydrate, hydrochloric acid, glacial acetic acid, sodium acetate trihydrate, methanol: Merck (Darmstadt, Germany); ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) (kept at +4 °C), 2,4,2-tri(2-pyridyl)-s-triazine (TPTZ), and lauryl gallate (LG): Fluka (Buchs, Switzerland). The reagents were 'analytical reagent' grade unless otherwise stated. Green tea (*Camellia sinensis*) was purchased from Malatya Pazari AS (Istanbul, Turkey).

The spectra and absorption measurements were recorded in matched quartz cuvettes using a Varian CARY Bio 100 UV–vis spectrophotometer (Mulgrave, Victoria, Australia). Other related apparatus and accessories were a J.P. Selecta water bath (Barcelona, Spain), E521 model Metrohm pH-meter equipped with glass electrodes (Herisau, Switzerland), Ultra-Turrax CAT X-620 model homogenizer apparatus (Staufen, Germany) and Elektromag vortex stirrer (Istanbul, Turkey).

2.2. Preparation of solutions

For the CUPRAC test of TAC, the following solutions were prepared: $CuCl_2$ solution, 10 mM, was prepared by dissolving 0.4262 g $CuCl_2{\cdot}2H_2O$ in water, and diluting to 250 mL. Ammonium acetate buffer at pH = 7.0, 1.0 M, was prepared by dissolving 19.27 g NH_4Ac in water and diluting to 250 mL. Neocuproine (Nc) solution, 7.5 mM, was prepared daily by dissolving 0.039 g Nc in absolute ethanol, and diluting to 25 mL with ethanol. For the ABTS test of TAC, the chromogenic radical reagent ABTS, at 7.0 mM concentration, was prepared by dissolving this compound in water and adding $K_2S_2O_8$ to this solution such that the final persulphate concentration in the mixture is 2.45 mM. The resulting ABTS radical cation solution was left to mature at room temperature in the dark for 12–16 h, and then used for ABTS/TEAC assays. The reagent solution was diluted with EtOH at a volume ratio of 1:10 prior to use. For the FRAP test of TAC, the following solutions were prepared: a suitable mass of $FeCl_3{\cdot}6H_2O$ was weighed so that the final concentration of Fe(III) in solution would be 20 mM; 1 mL of 1 M HCl solution was added, dissolved in some water and diluted to 50 mL with H_2O. A suitable mass of TPTZ was weighed such that its final concentration would be 10 mM, dissolved in absolute EtOH, and diluted to 50 mL. In order to prepare 0.3 M CH_3COOH/CH_3COONa buffer solution at pH 3.6, 3.1 g of $CH_3COONa{\cdot}3H_2O$ was weighed and 16 mL glacial acetic acid was added, diluted with water to 1 L. The FRAP reagent was prepared daily as follows: the pH 3.6 acetic acid buffer, 10 mM TPTZ solution, and 20 mM $FeCl_3{\cdot}6H_2O$ solution were mixed in this order at a volume ratio of 10:1:1.

The standard solutions at 1.0×10^{-3} M concentration of antioxidant compounds were all prepared in 100% EtOH, 100% MeOH, MeOH/water (4:1, v/v), MeOH/water (1:1, v/v), DCM/EtOH (9:1, v/v). The α-tocopherol standard solutions were not prepared in 80% and 50% MeOH due to low solubility. All working solutions of antioxidant compounds were freshly prepared.

2.3. Solvent extraction of plant materials

The dry plant specimens were crushed in a mill, and 2-g samples were taken for each plant species. These samples were soaked in 80% MeOH overnight, and homogenized in an Ultra-Turrax apparatus by gradually increasing the number of cycles per unit time. The obtained extracts were transferred to centrifuge tubes, centrifuged

for 10 min (5000 rpm), and subsequently filtered through a filter paper into 100-mL flasks. The same procedure was repeated 3 times with 25 mL portions of 80% MeOH on the remaining part of the plants. All filtered extracts were combined, and diluted to 100 mL using the same solvent. Each extraction was run thrice in parallel [23]. The obtained extracts could be analyzed for their antioxidant capacities on the next day after preserving the N_2-bubbled and stoppered extracts in a refrigerator at +4 °C.

2.4. CUPRAC assay of total antioxidant capacity

2.4.1. Normal sample measurement

The CUPRAC method, as described by Apak et al. [20], is based on the reduction of a cupric neocuproine complex (Cu(II)–Nc) by antioxidants to the cuprous form (Cu(I)–Nc). To a test tube were added 1 mL each of Cu(II), Nc, and NH_4Ac buffer solutions. Antioxidant standard solution (x mL) and H_2O $(1.1 - x)$ mL were added to the initial mixture so as to make the final volume: 4.1 mL. The tubes were stoppered, and after 1/2 h, the absorbance at 450 nm (A_{450}) was recorded against a reagent blank. The standard calibration curves of each antioxidant compound was constructed in this manner as absorbance *vs*. concentration, and the molar absorptivity of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned. The scheme for normal measurement of antioxidants is summarized as:

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

2.4.2. Incubated sample measurement

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

2.4.3. CUPRAC-DCM method

The CUPRAC-DCM method was developed for the measurement of lipophilic antioxidant capacity of synthetic or real samples [24]. To a test tube were added 1 mL of copper(II) chloride solution, 1 mL of neocuproine solution, and 1 mL of NH_4Ac buffer solution in this order. Antioxidant standard solution (x mL) and DCM $(4 - x)$ mL were added to the initial mixture so as to make the final volume: 7 mL, shaken, and the organic phase was separated from the aqueous phase. Absorbance reading was made against a reagent blank at 450 nm. Since the boiling temperature of DCM was low, the DCM used in the procedure was cooled to an initial temperature of +4 °C to prevent evaporation losses. No elevated temperature incubation tests (as applied to hydrophilic antioxidants in the aqueous phase) were carried out with the organic extract.

The scheme for normal CUPRAC-DCM measurement was:

1 mL Cu(II) + 1 mL Nc + 1 mL buffer + x mL antioxidant soln. in DCM + $(4 - x)$ mL DCM; total volume = 7 mL, measure A_{450} against a reagent blank after 30 min of reagent addition.

2.4.4. Measurement of synthetic mixture solutions

Synthetic mixtures of antioxidants in different solvent media were prepared in suitable volume ratios such that the final absorbance of the mixture did not exceed 1.20 using the CUPRAC method. To the mixtures were added 1 mL each of Cu(II), Nc, and NH_4Ac buffer in this order and distilled water was added for dilution to a final volume of 4.1 mL. The theoretical trolox equivalent antioxidant capacity of a synthetic mixture solution (expressed in the units of mM TR) was calculated by multiplying the TEAC coefficient of each antioxidant constituting the mixture with its final concentration, and summing up the products (e.g., 10 μM TOC having a TEAC coefficient of 1.10 would count as 11.0 μM TR in such a mixture, where the expected total antioxidant capacity (TAC) of the mixture should be equal to the sum of the TACs of its constituents, considering the validity of Beer's law for this modified CUPRAC assay). The experimental trolox equivalent TAC of the same mixture was calculated by dividing the observed absorbance (A_{450}) to the molar absorptivity of trolox (ε_{TR} being 1.62×10^4 L mol^{-1} cm^{-1} under the specified conditions). Then the theoretically found TACs were compared to the experimentally observed ones to test the applicability of Beer's law (*i.e*., the principle of additivity of individual absorbances of constituents making up a mixture). Validity of Beer's law for a mixture implies that the observed absorbance is the sum of the individual absorbances of the constituents not chemically interacting among each other.

$$\text{TAC expected} = \text{TEAC}_1\ \text{concn.}_1 + \text{TEAC}_2\ \text{concn.}_2 + \text{TEAC}_3\ \text{concn.}_3 + \cdots + \text{TEAC}_5\ \text{concn.}_5 \quad (2.1)$$

$$\text{TAC found experimentally} = \frac{\text{absorbance (total)} \pm \text{intercept}}{\varepsilon_{\text{trolox}}} \times 10^3 \quad (2.2)$$

2.4.5. Standard addition of BHT to green tea extract

A 4-mL aliquot of 1:20 diluted green tea extract (in 80% MeOH) and 1.0 mL of 1 mM BHT (in 80% MeOH) or 1 mL 80% MeOH solution were taken into a tube. Green tea extract and BHT-added solutions were separately subjected to CUPRAC spectrophotometric analysis.

2.4.6. Measurement of green tea extracts in different solvent media

A 2-mL aliquot of the 80% methanolic green tea extract was withdrawn and freeze-dried in a flask for 1 h. The residue remaining in the flask was dissolved separately with 2 mL of 100% EtOH, 100% MeOH, 1:1 (v/v) MeOH/H_2O, and 4:1 (v/v) MeOH/H_2O. These final solutions were subjected to both CUPRAC and reference spectrophotometric assays.

2.5. ABTS/persulphate assay of total antioxidant capacity

The ABTS/persulphate method [21] was followed. Briefly, the volumes of $(4 - x)$ mL EtOH and x mL sample solution were taken. The reagent blank was prepared with 4 mL EtOH. One milliliter amount of 1:10 diluted ABTS radical cation solution was added to each mixture at 15 s intervals, and well mixed (total volume = 5.0 mL). The absorbance of the reagent blank (A_0) diminished in the presence of antioxidants, the absorbance decrease (ΔA) being proportional to antioxidant concentration. The decrease in absorbance (ΔA) caused by antioxidants, recorded at 734 nm against ethanol at the end of 6th min, reflected the $ABTS^{\bullet+}$ radical cation scavenging capacity and was plotted against the concentration of the antioxidant. The $TEAC_{ABTS}$ value of a given antioxidant represents the ratio of the slope of the ΔA *vs*. concentration line of that antioxidant to that of trolox measured under the same conditions of the ABTS decolorization assay. The TEAC coefficient, being a slope ratio, is unitless.

2.6. FRAP assay of total antioxidant capacity

FRAP assay was carried out by the method of Benzie and Strain [22] with minor modifications. The method is based on the reduction of a ferric 2,4,6-tripyridyl-s-triazine complex (Fe^{3+}-TPTZ) by antioxidants to the ferrous form (Fe^{2+}-TPTZ). To 3 mL of the FRAP

Table 1
The trolox equivalent antioxidant capacities of various antioxidant compounds in different solvent media calculated with respect to the ABTS, FRAP, and CUPRAC methods.

Antioxidant	$TEAC_{CUPRAC}$					$TEAC_{ABTS\text{-}TEAC}$/persulphate				$TEAC_{FRAP}$			
	EtOH 100%	MeOH 100%	MeOH/H_2O (4:1, v/v)	MeOH/H_2O (1:1, v/v)	DCM/EtOH (9:1, v/v)	EtOH 100%	MeOH 100%	MeOH/H_2O (4:1, v/v)	MeOH/H_2O (1:1, v/v)	DCM/EtOH (9:1, v/v)	EtOH 100%	MeOH 100%	MeOH/H_2O (4:1, v/v)	MeOH/H_2O (1:1, v/v)
Trolox (TR)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Quercetin (QR)	4.38	5.54	5.22	5.78	3.38	2.77	2.85	2.60	2.54	2.00	2.79	2.85	2.70	3.27
Ferulic acid (FRA)	1.20	1.38	1.29	1.30	1.11	2.16	2.11	2.10	2.10	1.45	0.88	1.09	0.85	0.96
Catechin (CT)	3.09	3.70	3.31	3.46	3.30	3.15	2.95	2.91	2.59	1.26	0.99	1.02	0.99	1.12
Ascorbic acid (AA)	0.96	1.12	1.11	1.04	–	1.08	0.86	0.82	1.10	–	1.01	1.03	0.93	1.13
Naringenin[a] (NG)	0.05 (2.28)	0.57 (3.04)	0.71 (3.74)	0.36 (2.11)	1.07	0.64	–	–	–	2.36	ND	ND	ND	ND
Glutathione (GSH)	0.57	0.56	0.54	–	–	1.56	1.22	1.18	1.49	–	ND	ND	ND	ND
α-Tocopherol (TOC)	1.10	1.02	–	–	1.11	1.02	1.00	–	–	0.89	0.96	1.02	–	–
Lauryl gallate (LG)	1.63	1.68	1.86	1.92	2.42	2.45	2.26	2.64	2.54	2.34	0.55	0.56	0.57	0.57
Butylated hydroxy anisole (BHA)	1.40	1.48	1.50	1.45	0.97	1.02	1.00	1.14	0.99	1.14	1.31	1.28	1.11	1.28
Butylated hydroxy toluene[b] (BHT)	1.15	1.56	2.07	1.92	0.07 0.95[b]	0.47	0.50	0.49	0.60	0.16 1.04[b]	0.14	0.20	0.14	0.17
Tert-Butyl hydroquinone (TBHQ)	0.94	0.99	1.08	1.10	0.95	1.00	0.84	1.12	1.02	1.21	0.98	0.97	1.01	1.22

[a] The second value ($TEAC_{CUPRAC}$) in the column shows the $TEAC_{INC}$ of NG.
[b] $TEAC_{CUPRAC}$ and $TEAC_{ABTS/persulphate}$ values (in DCM) for BHT were measured in the presence of BHA as an internal standard.

reagent was added $(0.4 - x)$ mL H_2O. Antioxidant solution (x mL) and $(0.4 - x)$ mL H_2O were added to 3 mL of the FRAP reagent (final volume 3.4 mL), and the increase in absorbance (ΔA) at 595 nm was measured after 6 min. The $TEAC_{FRAP}$ value of a given antioxidant represents the ratio of the slope of the ΔA *vs.* concentration line of that antioxidant to that of trolox measured under the same conditions of the FRAP assay.

2.7. Statistical analyses

Descriptive statistical analyses were performed using Excel software (Microsoft Office 2002) for calculating the means and the standard error of the mean. Results were expressed as the mean ± standard deviation (SD). Using SPSS software for Windows (version 13), the data were evaluated by two-way ANalysis Of VAriance (ANOVA) [25].

3. Results and discussion

Antioxidants can deactivate reactive species by two major mechanisms, namely electron-transfer (ET) and hydrogen atom transfer (HAT), though in some cases, these two mechanisms may not be distinguished with distinct boundaries [26]. These two mechanisms are affected by antioxidant structure and properties, solubility and partition coefficient, and solvent system. Bond dissociation energy (BDE) and ionization potential (IP) are two major factors that determine the mechanism and the efficacy of antioxidants. There is often confusion in the literature and mistaken attribution of reaction mechanisms in regard to antioxidant action. Therefore it is desirable to establish and standardize methods that can measure more than one property because phenolics have multiple activities, and the dominant activity depends on the medium and substrate of testing [27].

In this study, it is aimed to evaluate the effect of solvent on the antioxidant behavior of phenolic compounds. The TAC measurements in different solvent media of other ET-based assays (*i.e.*, ABTS/persulphate, FRAP) were compared to those of the CUPRAC assay. Ethanol, methanol, 4:1 (v/v) MeOH/H_2O, 1:1 (v/v) MeOH/H_2O, and 9:1 (v/v) DCM/EtOH solvent media were chosen as variable solvent environments. The antioxidant compounds of different representative classes used in this work are quercetin (flavonol), catechin (flavanol), naringenin (flavanon), ferulic acid (hydroxycinnamic acid), glutathione (thiol-group antioxidant), Vitamin C and E (vitamins), lauryl gallate, BHA, BHT, and TBHQ (synthetic antioxidants).

3.1. Solvent effect on CUPRAC and other TAC assays

The TEAC coefficients of antioxidants show some variation with solvent polarity. The TEAC coefficients (*i.e.*, the reducing potency – in trolox mM equivalents – of 1 mM antioxidant solution under investigation) of various antioxidant compounds found with ET-based CUPRAC, ABTS/persulphate and FRAP methods are shown in Table 1. In the CUPRAC assay results, TEAC coefficients were higher in methanolic solutions than in ethanol medium. The $TEAC_{CUPRAC}$ values of QR, CT and BHT were higher in pure MeOH than in pure EtOH (Table 1), probably due to facilitated e-transfer in ionizing solvents capable of anion (phenolate) solvation, because MeOH is the alcohol that best supports ionization [17]. Naringenin (NG) which gives a slow reaction needs incubation for complete oxidation in 100% EtOH, as the normal and incubated measurements yielded 0.05 and 2.28 TEAC values, respectively. The corresponding values for NG in 4:1 (v/v) MeOH/H_2O solvent medium increased to 0.71 and 3.74, respectively. The $TEAC_{CUPRAC}$ of NG in DCM solvent was 1.07 to reflect a higher capacity. The antioxidant capacity of NG clearly depended on the solvent type and temperature, and higher

Table 2
Equations and regression coefficients for trolox calibration curves obtained with CUPRAC, ABTS/persulphate and FRAP assays in different solvents.

Solvent	CUPRAC		ABTS/persulphate		FRAP	
	Linear equation	R^2	Linear equation	R^2	Linear equation	R^2
100% EtOH	$y = 1.67 \times 10^4 c - 0.033$	0.9999	$y = 2.60 \times 10^4 c + 0.002$	0.9996	$y = 4.20 \times 10^4 c + 0.014$	0.9995
100% MeOH	$y = 1.58 \times 10^4 c - 0.01$	0.9995	$y = 2.61 \times 10^4 c - 0.009$	0.9991	$y = 4.32 \times 10^4 c - 0.005$	1.00
4:1 (v/v) MeOH/H_2O	$y = 1.50 \times 10^4 c - 0.002$	0.9999	$y = 2.65 \times 10^4 c + 0.018$	0.9991	$y = 4.34 \times 10^4 c + 0.001$	0.9998
1:1 (v/v) MeOH/H_2O	$y = 1.57 \times 10^4 c - 0.025$	0.9991	$y = 2.56 \times 10^4 c - 0.010$	0.9988	$y = 4.10 \times 10^4 c - 0.0094$	0.9999
9:1(v/v) DCM/EtOH	$y = 1.68 \times 10^4 c - 0.02$	0.9995	$y = 2.33 \times 10^4 c - 0.006$	0.9995	–	–

capacity was exhibited in lipophilic medium. Although NG was responsive to the ABTS/persulphate assay in pure EtOH (Table 1), the experimental data were not reproducible and a distinct linear concentration range could not be defined.

Perez-Jimenez and Sauro-Calixto [8] studied the effect of solvent on the measured antioxidant capacity of foods assayed by the four most widely used antioxidant capacity methods (ABTS, FRAP, DPPH and ORAC). In addition, non-antioxidant food constituents like amino acids and uronic acids showed an interfering effect on these assays. The ranking of the interference effect of solvent and food constituents in these assays was: ORAC > ABTS > DPPH > FRAP. When other factors are not considered or assumed to remain constant, AOA assay methods based on H-atom donation (e.g., ORAC and ABTS) from a phenolic compound are generally affected to a greater extent by the solvent behaviour (polarity, HBA, *etc.*) than methods based on outer-sphere [19] e-transfer (e.g., CUPRAC and FRAP) by a coordinatively saturated metal complex (e.g., tetrahedral bis(neocuproine)copper(II,I)) involving minimal re-orientation of uniform ligands around the central metal ion in the formation of a transient intermediate during e-transfer.

The highest $TEAC_{CUPRAC}$ value of quercetin was seen in 1:1 (v/v) MeOH/H_2O solvent medium, whereas the highest $TEAC_{ABTS/persulphate}$ was noted in 100% MeOH. But in general, in the ABTS/persulphate assay, antioxidant capacities of antioxidants decreased in 100% MeOH unlike in the CUPRAC assay. The differences with respect to solvent type for FRA, BHA, and GSH were not significant. The TEAC values for GSH with the ABTS/persulphate assay were quite higher than with CUPRAC for all solvent types, as the former assay possibly gave rise to higher oxidation products of GSH (e.g., sulphenic and sulphinic acids) than CUPRAC, the latter of which yielded the physiologically plausible oxidation product of glutathione disulfide: GSSG, corresponding to a reversible 1-e oxidation [24]. 4:1 (v/v) MeOH/H_2O solvent medium provided the highest capacity for BHT (Table 1).

The statistically significant increase in TEAC values measured with the FRAP assay was noted for QR in 1:1 (v/v) MeOH/H_2O medium compared to those in other solvent media (Table 1), as FRAP is basically a hydrophilic antioxidant assay not well responding to lipophilic antioxidants [26]. In the present work, it was shown that both CUPRAC and ABTS assays were successful in antioxidant capacity estimation of both hydrophilic and lipophilic antioxidants in polar and nonpolar solvent media (Table 1), because both assays involve univalent-charged chromophore species (*i.e.*, $ABTS^{\bullet+}$ and $Cu(Nc)_2^{+}$) capable of being solvated by both water and alcohols as well as by less polar solvent mixtures (such as 9:1 (v/v) DCM/EtOH), while the less successful FRAP is associated with a divalent-charged chromophore ($Fe(TPTZ)_2^{2+}$) having greater affinity toward the aqueous phase (due to ion–dipole interactions of the chromophore with the solvent water molecules) [26]. NG and GSH were not detectable by the FRAP assay, as slow reacting antioxidants and thiols generally gave poor responses to the FRAP test [26]. The reason for this may be the half-filled d-orbitals of high-spin Fe(III), attributing to it a chemical inertness, whereas the electronic structure of Cu(II) enables fast kinetics [20,24]. A redox reaction of cysteine with iron(III) has been reported to proceed slowly in the presence of 1,10-phenanthroline, but the reaction has been accelerated in the presence of copper(II) as catalyst [28]. In relation to this, Gorinstein et al. [29] studied the antioxidant activity of raw and processed garlic sample extracts, and showed that FRAP values were significantly lower than CUPRAC values; the obtained data verified the advantage of CUPRAC over FRAP in reflecting thiol antioxidant content of food, because thiol-type antioxidants rich in garlic responded to CUPRAC but not to FRAP assay.

According to the results of the three assay methods, antioxidant capacities for a given antioxidant varied with working mechanism, antioxidant structure and properties, solubility and partition coefficient, and solvent type. Recent reports by Ingold and coworkers have provided the first quantitative evidence for the role of hydrogen bonding on the H-atom donor activities of phenolic antioxidants. These reports include the studies about the effects of wide range of solvents (protic and nonprotic) on the activities of antioxidants which establish the significant principles for the KSE on phenolic antioxidant activities [30]. Barclay et al. [13] have recently suggested that the main factor controlling the activity of catechols as antioxidants (and therefore also of most flavonoids) is resonance stabilization by intramolecular H-bonding of the aryloxy radical formed after 1-e oxidation by the peroxyl radical (Fig. 1(a and b)). The reason is that once the oxidation equilibrium: $Ar–OH \leftrightarrow Ar–O^{\cdot} + H^{\bullet}$ is shifted to the right by resonance stabilization of the aryloxy radical (Ar-$O^{\bullet}$), the standard reduction potential of the Ar–$O^{\bullet}$/Ar–OH redox couple is decreased to a level rendering Ar–OH a stronger antioxidant.

Solvents with high hydrogen bond-accepting (HBA) ability will interfere with this stabilization through intermolecular H-bonding. Thus, the combined effect of a greater stabilization of the catechol (or flavonoid) due to better solvation and of a destabilization of the aryloxy radical primarily formed is to increase the bond dissociation energy of the polyphenol relative to hydrocarbon solvents. This effect, together with steric hindrance for the approach of the oxidizing (e.g., peroxyl) radical to the solvent-complexed phenol, will reduce the rate constant for H-atom abstraction [11]. This may well explain the weakened antioxidant activity of catechin (measured by ABTS/persulphate) in 1:1 (v/v) MeOH/H_2O compared to that in pure MeOH, however the higher $TEAC_{ABTS}$ values of CT in alcoholic solvents compared to that in DCM/EtOH may be ascribed to enhanced e-transfer from a phenolate anion in alcohols, as ABTS is considered to be a mixed (HAT- and ET-based) assay (Table 1). On the other hand, a previous research in our laboratory showed that possible formation of polycatechin as a result of reduced water activity in high concentration urea-buffered aqueous solution enhanced the CUPRAC antioxidant power of catechin [31]. The enzymatically produced poly(catechin) was reported by Kurisawa et al. [32] to show great improvement in antioxidant activity such as radical scavenging activity against the superoxide anion, inhibition of free radical-induced oxidation of low-density lipoprotein, and inhibition of xanthine oxidase activity, compared with a catechin monomer. The overall conjugation of the catechin momomer is hindered due to the absence of 2,3-double bond connecting the two ring systems of the molecule [26]. Therefore, the plausible lower polymers of catechin (possibly dimers and trimers) in pure alco-

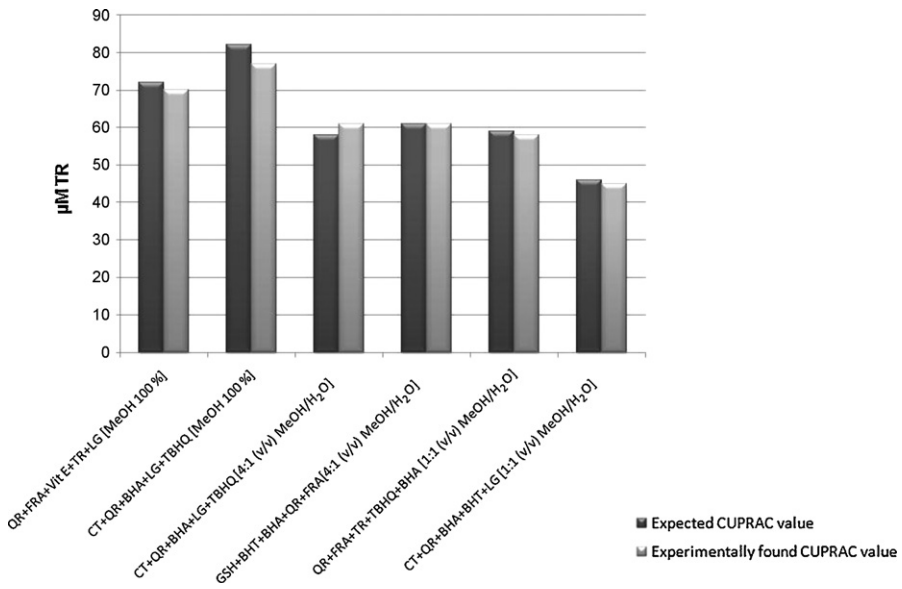

Fig. 2. Comparison of the theoretically expected and experimentally found trolox (TR)-equivalent antioxidant capacities (in μM TR units) of synthetic mixtures of lipophilic and hydrophilic antioxidants in different methanol media (using CUPRAC method) ($F_{exp} = 0.882$, $F_{crit} = 6.608$, $F_{exp} < F_{crit}$ at $P = 0.05$).

hol or alcoholic aqueous solution may increase this conjugation due to the overlap of suitable π-orbitals, resulting in an increase of CUPRAC-TEAC values of catechin from 3.3 (in 4:1 MeOH/H_2O) to 3.7 (in pure MeOH), and a similar increase of ABTS-TEAC values from 2.6 (in 1:1 MeOH/H_2O) to 2.95 (in pure MeOH) (Table 1).

To express the results of CUPRAC, ABTS/persulphate and FRAP as trolox equivalents, TR calibration curves had to be first plotted for each of the solvents tested, where Table 2 summarizes the related linear equations and correlation coefficients. The slopes were high, intercepts low, and R^2 values close to 1 for all the solvents tested, showing a good dose-response curve. However, there are significant differences among the slopes of the three methods, especially in the case of ABTS/persulphate and FRAP, which suggest that trolox, like quercetin, does not behave in the same way in all the tested solvents. Nevertheless, these differences for a given assay were not as strongly emphasized for TR than for some other antioxidants (e.g., QR, CT, NG, and synthetic antioxidants; Table 1), because TR was an easily oxidized antioxidant with no complex kinetics.

3.2. Investigation of antioxidant capacity of BHT

In order to better observe the effects of polarity and H-bonding ability of the solvent on antioxidant capacity, a nonpolar solvent (DCM) was selected, and used in admixture with EtOH, *i.e.*, 9:1 (v/v) DCM/EtOH. Measurements in this solvent medium could only be performed for the CUPRAC and ABTS/persulphate assays but not for the FRAP assay, because the FRAP chromophore, Fe(II)-TPTZ chelate, had (2+) charge compared to the (1+) charge of CUPRAC and ABTS chromophore cations (*i.e.*, the higher the charge, the stronger ion–dipole interaction would a chromophore have with polar H_2O molecules, and a weaker tendency would be toward nonpolar solvents). It is possible to observe the DCM solvent effect on antioxidant capacity using the modified CUPRAC method. In the tested solvent medium (9:1 (v/v) DCM/EtOH), $TEAC_{CUPRAC}$ and $TEAC_{ABTS}$ values of BHT were found as 0.07 and 0.16, respectively. These TEAC coefficients increased to 0.95 and 1.04 in the presence of an internal standard, BHA (Table 1). This means that in the presence of BHA, antioxidant capacity of BHT increases dramatically. The TEAC values of BHT in polar solvents (EtOH, MeOH) and in their admixtures with water were higher than 1.00, compared to the low value of ~0.10 in nonpolar medium (Table 1). Aryloxy radicals formed from 1-e oxidation of BHT are possibly stabilized by intermolecular H-bonding, so dimerization of BHT molecules (also known as *para–para* coupling reaction) occurs most likely, because during its oxidation with peroxyl radicals, a stoichiometric factor of 2 was reported for BHT [33,34]. In a review study, Heim et al. [35] indicated that polymerization of the flavonoid nuclear structure increases antioxidant activity by affording a more stable flavonoid radical through conjugation and electron delocalization. Plump et al. worked about the effect of polymerization on the antioxidant activity of catechin and anthocyanin, and indicated that antioxidant activity in the lipid phase decreased with polymerization in contrast to antioxidant action in the aqueous phase which increased from monomer to trimer and then decreased from trimer to tetramer [36]. Beyond a certain level of molecular complexity (*i.e.* more than 4 monomer units in polymerized phenols), antioxidant activity would be expected to decrease as a result of steric hindrance [7]. This observation is in accordance with BHT's lower antioxidant activity in nonpolar solvent medium (as in 9:1 (v/v) DCM/EtOH) compared to those in alcoholic media. BHT shows higher activity in aqueous alcoholic solutions possibly due to enhanced e-transfer *via* dimerization, and such a geometric arrangement should have overcome the steric hindrance to e-transfer provided by the *ortho*-tertiary butyl groups in BHT [13]. On the other hand, higher polymeric aggregates should have formed in DCM-containing solvent media, decreasing antioxidant capacity. In this regard, BHA, when used in admixture with BHT, possibly depolymerizes BHT aggregates in DCM solution, enabling BHT to exert its true antioxidant capacity. In addition to BHT, antioxidant capacity of quercetin, catechin and ferulic acid assayed by CUPRAC and ABTS/persulphate methods decreased from polar to nonpolar solvent media (Table 1). As a brief summary, it may be hypothesized that possible interactions of the tested antioxidant molecule with the molecules of solvent or other coexisting antioxidant molecules may lead to associative or dissociative interactions and H-bonding, resulting in enhanced or weakened antioxidant activity of the parent antioxidant depending on the selective stabilization of the aryloxy radical formed during the oxidative process, *i.e.*, if the aryloxy radical is stabilized, the corresponding antioxidant activity is enhanced.

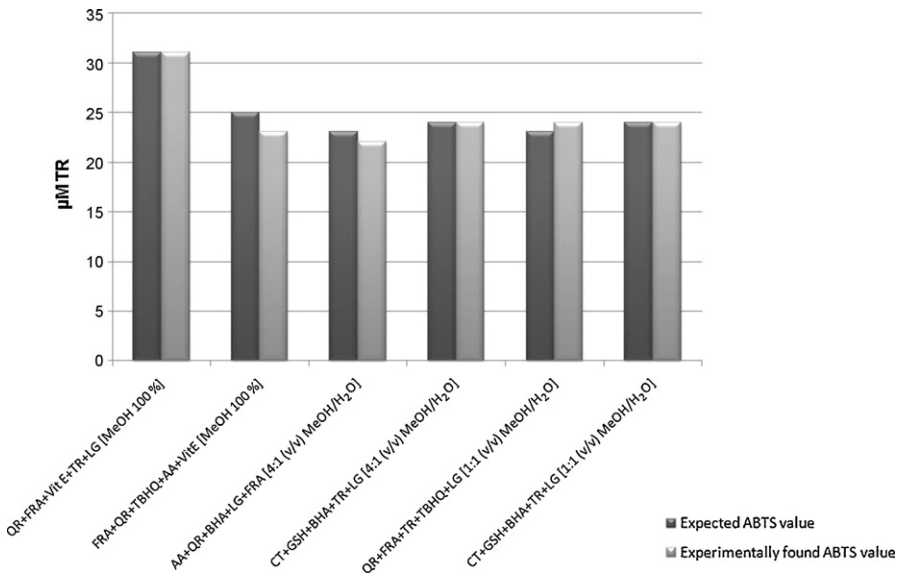

Fig. 3. Comparison of the theoretically expected and experimentally found trolox (TR)-equivalent antioxidant capacities (in μM TR units) of synthetic mixtures of lipophilic and hydrophilic antioxidants in different methanol media (using ABTS/persulphate method) ($F_{exp} = 0.625$, $F_{crit} = 6.608$, $F_{exp} < F_{crit}$ at $P = 0.05$).

3.3. TAC measurement of synthetic mixture solutions in different solvent media

Possible five constituent mixtures of antioxidants were synthetically prepared, and the suitably diluted solutions were analyzed for antioxidant capacity using the CUPRAC, ABTS/persulphate and FRAP assays. Comparison of expected (using Eq. (2.1)) and experimentally found (using Eq. (2.2)) antioxidant capacities of synthetic mixture solutions in different solvents (as μM Trolox equivalent) using these TAC assays are given in Figs. 2–4. The expected and experimentally found capacities were generally in accordance with each other. It was demonstrated that there were no chemical interactions of interferent nature (*i.e.*, violating Beer's law) among the synthetic solution constituents and that the antioxidant capacities of the tested antioxidants were additive (with some exceptions due to solvent variations).

As a specific example, the combination of BHT with NG (in MeOH) and with BHA (in DCM/EtOH) showed antagonistic and synergistic interactions, respectively (Table 3). Synergistic interaction of BHT with BHA in DCM/EtOH medium was noted with both CUPRAC and ABTS/persulphate assays, and can most probably be ascribed to depolymerization and intermolecular H-bonding reactions in a nonpolar environment (FRAP is non-responsive in DCM medium). Another possible reason of synergism may be the easier accessibility of the phenolic–OH (a BHA molecule in close proximity to BHT due to H-bonding will be preferentially oxidized with the probe molecule, e.g., Cu(II)–Nc or $ABTS^{*+}$, rendering the neighboring BHT molecule vulnerable to immediate oxidative attack, which would otherwise be partially closed to such attack by steric hindrance provided by the *ortho*-tertiary butyl groups of BHT) [13]. Calibration curves of BHT alone and in the presence of BHA (internal standard) are given in Fig. 5. Molar absorptivity of BHT alone

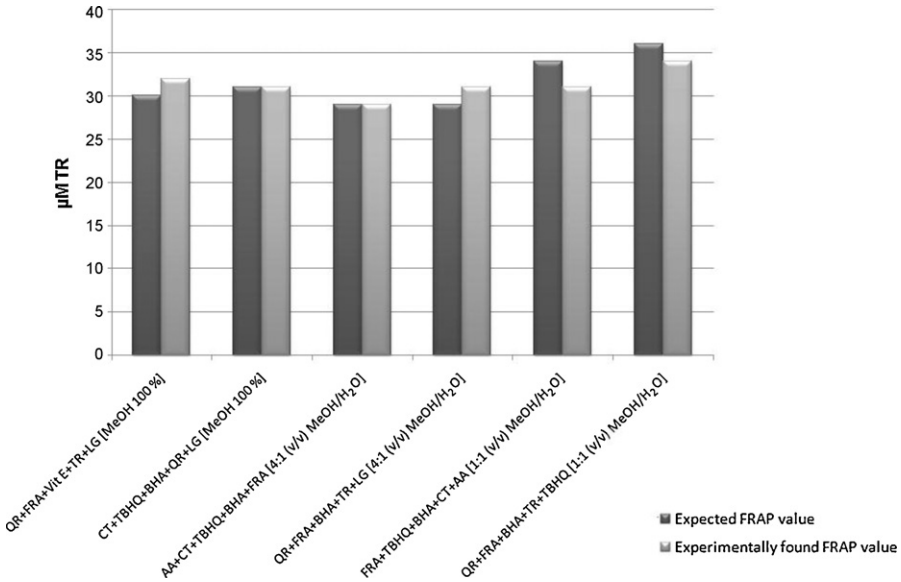

Fig. 4. Comparison of the theoretically expected and experimentally found trolox (TR)-equivalent antioxidant capacities (in μM TR units) of synthetic mixtures of lipophilic and hydrophilic antioxidants in different methanol media (using FRAP method) ($F_{exp} = 0.040$, $F_{crit} = 6.608$, $F_{exp} < F_{crit}$ at $P = 0.05$).

Table 3
The synergistic and antagonistic interaction between BHT and BHA (or NG) when present in different solvent media using CUPRAC, ABTS/persulphate, and FRAP assays.

Synthetic mixture sample	TAC_{CUPRAC} (μM TR)		$TAC_{ABTS/persulphate}$ (μM TR)		TAC_{FRAP} (μM TR)	
	Capacity expected	Capacity found experimentally	Capacity expected	Capacity found experimentally	Capacity expected	Capacity found experimentally
0.2 mL 0.1 mM NG + 50 μL 1 mM BHT (MeOH 100%)	24 42[b]	22 29[b]	–[a]	–[a]	ND	ND
20 μL 1 mM BHA + 50 μL 1 mM BHT (MeOH 100%)	37	39	9	5	10	16
50 μL 1 mM BHA + 50 μL 1 mM BHT DCM/EtOH (9:1; v/v)	12	24[c]	13	23	ND	ND

ND: not detected.
[a] ABTS/persulphate method is not repeatable for NG at the level of working concentration.
[b] TAC (incubation measurement).
[c] There is a synergistic effect because of the simultaneous presence of BHT and BHA in the mixture.

Table 4
The total antioxidant capacities (TAC) of green tea extracts, as measured by the CUPRAC, FRAP and ABTS/persulphate assays ($N = 3$) in different solvent media.

Solvent	TAC_{CUPRAC} (mmol g^{-1} TR)	$TAC_{ABTS/persulphate}$ (mmol g^{-1} TR)	TAC_{FRAP} (mmol g^{-1} TR)
Green tea in 100% EtOH	1.00 ± 0.026	1.20 ± 0.096	0.44 ± 0.023
Green tea in 100% MeOH	0.88 ± 0.015	1.08 ± 0.087	0.38 ± 0.020
Green tea in 4:1(v/v) MeOH/H_2O	1.19 ± 0.036	1.34 ± 0.036	0.47 ± 0.010
Green tea in 1:1(v/v) MeOH/H_2O	1.01 ± 0.015	1.26 ± 0.055	0.41 ± 0.010

Results were expressed as the average ± standard deviation.

and in the presence of BHA measured by the CUPRAC-DCM assay was found as 1.2×10^3 L mol^{-1} cm^{-1} and 1.6×10^4 L mol^{-1} cm^{-1}, respectively, showing significant enhancement.

3.4. Antioxidant capacity of pure and BHT-added green tea extract

The TAC values of green tea extracts in different solvent media, as measured by the three assays, are tabulated in Table 4. Total antioxidant capacity found by CUPRAC and ABTS/persulphate methods were in the order: 4:1 (v/v) MeOH/H_2O > 1:1 (v/v) MeOH/H_2O > 100% EtOH > 100% MeOH. The FRAP assay results were in the order: 4:1 (v/v) MeOH/H_2O > 100% EtOH > 1:1 (v/v) MeOH/H_2O > 100% MeOH. Antioxidant components in green tea are seemingly more efficient in 4:1 (v/v) MeOH/H_2O, but less efficient in 100% MeOH medium. This may derive from the hydrophilic-lipophilic balance of green tea antioxidants. BHT-added green tea extracts showed the expected TAC values in 4:1 (v/v) MeOH/H_2O medium within reasonable relative error (Table 5). The results showed that in the green tea extract, the tested antioxidants did not chemically interact to cause apparent deviations from Beer's law (Table 5).

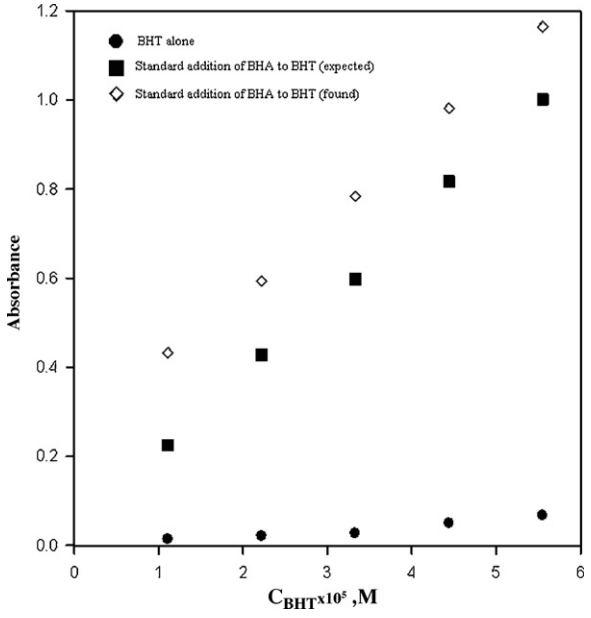

Fig. 5. The calibration curves of BHT alone, and in BHA-added solution with respect to the CUPRAC-DCM method.

Table 5
The total antioxidant capacities (TAC) of standard-added mixtures of green tea extracts, as measured by the CUPRAC, and ABTS/persulphate assays ($N = 3$). (Solvent: 4:1(v/v) MeOH/H_2O).

	TAC_{CUPRAC} (mmol g^{-1} TR)	$TAC_{ABTS/persulphate}$ (mmol g^{-1} TR)
Green tea	1.24 ± 0.03	1.08 ± 0.06
Green tea + BHT	$TAC_{expected}$: 1.75 ± 0.03 TAC_{found}: 1.66 ± 0.04	$TAC_{expected}$: 1.20 ± 0.06 TAC_{found}: 1.26 ± 0.09

4. Conclusion

This work reports the antioxidant assay of selected main lipophilic and hydrophilic antioxidants in different solvent media using CUPRAC, ABTS/persulphate and FRAP methods. The trolox equivalent antioxidant capacities (TEAC coefficients) of hydrophilic antioxidants in EtOH did not differ significantly from those reported in the original CUPRAC method defined for aqueous solutions in EtOH, while TEAC values of lipophilic antioxidants including BHT, BHA, TBHQ in DCM were reported for the first time in the CUPRAC assay. It may be argued that CUPRAC, involving a coordinatively saturated metal complex reagent capable of outer-sphere electron-transfer, is relatively independent of solvent effects in alcohol–water mixtures of varying composition. Synthetic mixtures comprised of lipophilic and hydrophilic antioxidants gave the theoretically expected CUPRAC antioxidant capacities, indicating that chemical deviations from Beer's law were basically absent, and the observed CUPRAC absorbances were additive. Binary mixtures of BHT with BHA or NG showed positive or negative deviations from

Beer's law arising from synergistic or antagonistic interactions, respectively. The effect of solvent type on the measured TAC values was discussed in regard to possible polymerization, depolymerization, and H-bonding interactions of antioxidants among themselves and with solvent molecules. In conclusion, the results obtained from this work demonstrated that the antioxidant behaviour of phenolic compounds show variations based on solvent type and polarity, reaction mechanism, solubility parameters as well as on an essential structural property, *i.e.*, electron-transfer capability.

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