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Correlation of Total Antioxidant Capacity with Reactive Oxygen Species (ROS) Consumption Measured by Oxidative Conversion

Sema Demirci Çekiç, Aydan Çetinkaya, Aslı Neslihan Avan, and Reşat Apak*

Department of Chemistry, Faculty of Engineering, Istanbul University, 34320 Istanbul, Turkey

ABSTRACT: Although both antioxidant capacity and oxidative conversion (hazard) are important in food and bioanalytical chemistry, there is considerable confusion in the literature between the results of these two types of assays. After the generation of ROS in the medium via Fe(III)−H_2O_2 reaction, attenuation of total oxidative conversion (TOC; as measured by thiobarbituric acid-reactive substances (TBARS) and *N,N*-dimethyl-*p*-phenylenediamine (DMPD) assays) was tested for possible correlation with the total antioxidant capacity (TAC; as measured by cupric reducing antioxidant capacity (CUPRAC) and trolox equivalent antioxidant capacity (ABTS/TEAC) assays) of the introduced antioxidant sample. The inverse relationship between oxidative conversion and antioxidant capacity was processed to establish a curvilinear relationship between the absolute values of TAC increments and TOC decrements as a function of added antioxidant concentration. This simple relationship may form a bridge between the two diverse disciplines of medical biochemistry and food analytical chemistry mainly using TOC and TAC results, respectively.

KEYWORDS: *total antioxidant capacity, reactive oxygen species, N,N-dimethyl-p-phenylenediamine, CUPRAC, ABTS/persulfate, TBARS*

INTRODUCTION

Reactive oxygen and nitrogen species (ROS/RNS) that emerge as a result of the respirative cycle of oxidative phosphorylation may attack biological macromolecules (e.g., cellular DNA), giving rise to single-strand and double-strand breaks that may eventually cause cell aging, cardiovascular diseases, mutagenic changes, and cancerous tumor growth.[1] ROS is a collective term often used to include oxygen radicals [superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$), and alkoxyl ($RO^{\bullet}$)] and certain nonradicals that either are oxidizing agents and/or are easily converted into radicals, such as HOCl, ozone (O_3), peroxynitrite ($ONOO^-$), singlet oxygen ($^1O_2$), and H_2O_2. On the one hand, ROS and RNS have been shown to possess many characteristics of carcinogens.[2] On the other hand, ROS at physiological concentrations may be required for normal cell functioning[3] and are thought to have played a major role in the evolution of plants and animals.[4] Environmental or behavioral stressors (pollution, sunlight exposure, cigarette smoking, excessive alcohol consumption, etc.) or simply a malfunction of antioxidant production may lead to a free radical excess, known as "oxidative stress",[5] in which the dynamic redox balance between oxidants and antioxidants is intensely shifted toward oxidative potentials.[6] Antioxidants are defined as "any substance that delays, prevents or removes oxidative damage to a target molecule" by Halliwell and Gutteridge.[7] All cells possess elaborate antioxidant defense systems consisting of low and high molecular weight components to defend against ROS attack. These protective systems are both endogenous (produced in the body) and exogenous (supplied through diet). Biologically important antioxidative compounds within cells, cell membranes, and extracellular fluids can be up-regulated and mobilized to neutralize excessive and inappropriate ROS formation.[8] Diets rich in food plants such as fruits, grains, and vegetables help to maintain human health via restoration of the antioxidant/prooxidant balance, and usually a variety of antioxidants rather than one or two "unique antioxidant" compounds serve such a purpose.[9] Due to the cooperation between antioxidants, the total antioxidant capacity (TAC) is believed to give more reliable biological information than that obtained from measuring concentrations of individual antioxidants. Measuring plasma antioxidant capacity (AC) may help in the evaluation of physiological, environmental, and nutritional factors of the redox status in humans. Determining plasma AC may help to identify conditions affecting oxidative status in vivo (e.g., exposure to ROS and antioxidant supplementation). Moreover, changes in the plasma AC after supplementation with galenic antioxidants or with antioxidant-rich foods may provide information on the absorption and bioavailability of nutritional compounds.[10] Measuring the antioxidant activity/capacity levels of food and biological fluids (e.g., human serum) is carried out for the meaningful comparison of the antioxidant content of foodstuffs and for the diagnosis and treatment of oxidative stress-associated diseases in clinical biochemistry.[11] As an example, there exists a consistent difference in the level of antioxidants between the tumoral sample and its corresponding peritumoral tissue, independent of the tumoral type.[12] In another study, plasma TAC levels were found to be significantly lower and plasma malondialdehyde (MDA) concentrations higher in rheumatoid arthritis (RA) patients than in osteoarthritis (OA) and control groups.[13] The higher serum uric acid concentration was reported to be associated with elevated total serum antioxidant capacity among individuals with atherosclerosis.[14]

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Because free radical generation is directly related with oxidation in foods and biological systems, the search for methods to determine free radical scavenging is important. The scavenging of the radical cation 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonate) ($ABTS^{\bullet+}$) using the trolox equivalent antioxidant capacity (TEAC) method[15] or of the radical cation *N*,*N*-dimethyl-*p*-phenylenediamine ($DMPD^{\bullet+}$)[16] can be mentioned among the well-known measurement methods of free radical scavenging action, although both colored radicals are physiologically irrelevant. The DMPD method can also be used for measuring oxidative damage or conversion under the attack of reactive species. The thiobarbituric acid-reactive substances (TBARS) method[17] measures levels of MDA and other reactive substances (such as final products of lipid peroxidation) as an indicator of oxidative status, but has been largely criticized for being unspecific. The cupric reducing antioxidant capacity (CUPRAC) assay is an electron transfer (ET)-based TAC method[18,19] applicable to both food and biological matrices, working on the principle of Cu(II)→Cu(I) reduction by antioxidants in a 2,9-dimethyl-1,10-phenanthroline (neocuproine) ligand environment.

Total antioxidant capacity/activity assays try to answer two basic questions: how much does the antioxidant reduce the rate of oxidation, and how long does it suppress or retard this oxidation? Fixed-time TAC assays measure only the efficiency of antioxidant action (i.e., to what extent a colored or fluorescent probe has been reduced by antioxidants, such as CUPRAC,[1,11,18] ABTS/TEAC with persulfate,[15] and oxygen radical absorbance capacity (ORAC) assays[20,21]) within a prespecified time period. On the other hand, total oxidative conversion (TOC) assays are in a way markers of oxidative stress, measuring oxidative damage on biological model macromolecules such as lipids, proteins, and DNA via quantification of oxidation products (such as protein carbonyl,[22] DMPD,[16] and deoxyribose/TBARS[17] methods). Usually, there is a gap between these two types of tests, hindering the equal availability of results to both food and biological scientists. As a rare example from the literature to fill this gap, it has been theorized that with an increase in the level of free radicals (such as in rabbit bladder tissue subjected to ischemia/reperfusion), the level of protective antioxidants (as measured by CUPRAC and ferric reducing antioxidant power (FRAP) tests) should decrease, but only CUPRAC was experimentally capable of detecting this decrement in the antioxidant stock.[23] Often, there is an inverse relationship between oxidative conversion and antioxidant capacity, and a simple relationship between them is required to reach a consensus in food and bioanalytical chemistry. The aim of this work is to establish such a relationship that is believed to start the construction of a bridge between medical/biological analytical chemistry mainly relying on TOC results and food analytical chemistry often using TAC results, because these two disciplines are known to frequently use quite different languages.

MATERIALS AND METHODS

Chemicals and Apparatus. The following chemical substances of analytical reagent grade were supplied from the corresponding sources: iron(III) chloride hexahydrate, hydrogen peroxide (30% by mass), copper(II) chloride dihydrate, ammonium iron(II) sulfate hexahydrate, trichloroacetic acid (TCA), glutathione (reduced) (GSH) from E. Merck; *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride, (±)-6-hydroxy-2,5,7,8-teramethylchroman (trolox), *N*-acetyl-L-cysteine, uric acid from Fluka; 2-deoxy-D-ribose, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), quercetin, caffeic acid, (±)-catechin hydrate, (−)-epicatechin, rutin hydrate, *p*-coumaric acid (*trans*-4-hydroxycinnamic acid), L-cysteine from Sigma; ammonium acetate from Riedel de Haen; 2-thiobarbituric acid (TBA) from Sigma-Aldrich; neocuproine (2,9-dimethyl-1,10-phenanthroline) hydrochloride (Nc) from Sigma Chemical Co.

Preparation of Antioxidant Solutions. The standard solutions of trolox (TR), quercetin (QR), catechin (CAT), epicatechin (EC), caffeic acid (CFA), *p*-coumaric acid (*p*-CUM), naringin (NG), rutin (RT), cysteine (CYS), reduced glutathione (GSH), homocysteine (HCYS), and ascorbic acid (AA) were prepared at 10.0 mM concentrations. Uric acid (UA) was at 1.0 mM concentration. GSH and AA solutions were prepared in distilled water. Suitable weights of CYS and HCYS were dissolved with 0.5 mL of 1.0 M HCl solution and diluted to 25 mL with distilled water. UA was dissolved using 1.0 mL of 0.1 M NaOH, excess base neutralized with 0.1 M HCl, and finally diluted to 25 mL with distilled water. The other antioxidants were dissolved with ethanol (EtOH).

***N*,*N*-Dimethyl-*p*-phenylenediamine (DPMD) Method Solutions.** To prepare 10 mM Fe(III) stock solution, 0.0676 g of $FeCl_3{\cdot}6H_2O$ was dissolved with 1.0 mL of 0.5 M H_2SO_4 and diluted to 25 mL with distilled water. To obtain 1.0×10^{-7} M Fe(III) working solution, this solution was appropriately diluted to 1.0×10^{-4} M intermediary stock solution and then diluted 10^3 times with 10 mM H_2SO_4 to avoid hydrolysis. The pH 5.7 buffer solution was prepared by mixing 45.25 mL of 2.0 M sodium acetate (NaAc) with 4.75 mL of 2.0 M acetic acid (HAc) solution. DMPD solution at 2.4×10^{-2} M concentration was prepared by dissolving 0.125 g of DMPD in water, followed by the addition of 0.25 mL of 0.5 M H_2SO_4 and final dilution to 25 mL with distilled water.[16] The 3.0% H_2O_2 solution was prepared by diluting 30% commercial hydrogen peroxide with water.

Ten healthy volunteers, six female and four male individuals between the ages of 25 and 55 who regularly undergo annual health examinations, were selected from the staff of the Analytical Chemistry Division of Istanbul University for donating blood samples for the analyses. The blood samples were also examined for total cholesterol and triglyceride levels in a private hospital's laboratory. Serum samples were prepared from blood as previously mentioned by Cekic et al.[24] Human serum was used to test its ROS scavenging activity in the experimental design. Serum sample was diluted at different ratios, namely, 5 times for CUPRAC and TBARS, 50 times for ABTS, and 20 times for DMPD methods. Serum samples were tested alone and also in the presence of CYS and GSH (i.e., with standard additions).

DMPD Method. The proposed method for the determination of ROS in the reaction medium was adapted with some modifications from the method described earlier by Hirayama and Unohara[16] for catalytic determination of ultratrace amounts of iron(III). To a test tube were added 0.25 mL of $FeCl_3$, 0.5 mL of acetate buffer solution (pH 5.7), 0.5 mL of H_2O_2 (3%), and *x* mL of sample solution, and the volume was completed to 9 mL with distilled water. The mixture was shaken after each addition and then allowed to stand on a water bath at 25 °C for 5 min. After the addition of 4.8×10^{-3} M DMPD solution (DMPD solution at 2.4×10^{-2} M described in the original method was diluted 5 times with distilled water to get a final absorbance of ∼0.9−1.0 in the absence of scavenger sample solution), the mixture was kept on the water bath for an additional 20 min, and the absorbance in the absence or presence of sample was recorded against distilled water at 514 nm. The decrease in absorbance in the presence of sample linearly correlated with antioxidant concentration over a reasonable range.

Deoxyribose/TBARS Method. The original deoxyribose (TBARS) method[17] was modified by Bektaşoğlu et al. for the determination of free radical production/scavenging activity of samples. In the present study, the TBARS method was applied as previously described by Bektasoğlu et al.[25]

ABTS Method. To evaluate the total antioxidant capacity of samples the ABTS/persulfate method[15] was followed. For serum analysis, phosphate-buffered saline (PBS) (100 mM potassium phosphate buffer containing 150 mM NaCl at pH 7.4) was used instead of EtOH in the mentioned procedure.

CUPRAC Method. The CUPRAC assay was first applied to food[26] and then to serum antioxidants.[18] Except serum samples, the original CUPRAC method was applied.[26] For serum samples, the classical pH 7.0 ammonium acetate buffer (which would otherwise cause the precipitation of serum proteins) was replaced with pH 7.0 urea buffer. Urea buffer was prepared as described earlier by Çekiç et al.[27]

Preparation of Synthetic Mixture Solutions of Antioxidants. All four methods (two TAC methods:, CUPRAC and ABTS; and two TOC methods, DMPD and TBARS) were applied to all mentioned antioxidants individually and in binary mixtures. Suitable concentrations and volumes of antioxidant solutions were taken to prepare these mixtures so as to remain within the optimal absorbance range conforming to Beer's law.

Statistical Analysis. Statistical analyses were performed using Excel software (Microsoft Office 2007) for calculating the means and percentage relative standard deviations (RSD, %). Pearson's correlation test was used to determine the presence or lack of correlation for a given sample size and confidence level[28] with the aid of the statistical software package SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). Correlation and regression analyses of one total antioxidant capacity measurement versus another and the antioxidant capacity of a serum or antioxidant sample versus its concentration were also described in detail by Cao and Prior.[21]

RESULTS AND DISCUSSION

In the proposed methodology, ROS (in the form of TOC) was determined by DMPD and TBARS methods, whereas TAC was measured by CUPRAC and ABTS/$K_2S_2O_8$ assays. Hydroxyl radicals were produced along with ROS in the selected oxidant system (i.e., iron + H_2O_2) of this work, and $^{\bullet}OH$, being at the top of the pecking order (having a formal reduction potential at pH 7 of $E^{o\prime}_{pH7}$ = 2.310 V, greater than that of any antioxidant redox couple used), is the most powerful oxidant in biological systems capable of oxidizing all tested species.[29] Among the applied methods, only the DMPD method, adapted from Hirayama and Unohara, was modified. ROS consumption (in the form of a decrease in TOC) was correlated to TAC of added antioxidants. CUPRAC and ABTS are ET-based and mixed mode (ET- and hydrogen atom transfer (HAT-based) TAC assays, respectively,[30] measuring the extent of reduction of a colored probe by the tested antioxidant. Because only these two assays are realistically applicable to biological fluids (including human serum), they were chosen as representative TAC assays, as the other TAC assays are either responsive to a specific type of antioxidant (only hydrophilic or lipophilic) or nonresponsive to GSH and protein thiols. On the other hand, DMPD and TBARS are responsive, although nonselectively, to a wide range of ROS (e.g., hydroxyl and superoxide anion radicals and H_2O_2), and they are simple TOC assays that can be practically and flexibly used for measuring oxidative conversion. Therefore, these assays were chosen for their indicated characteristics. Both TAC assays chosen (ABTS and CUPRAC tests) are not of the direct type of antioxidant assays "assessing the capacity of antioxidant for inhibition of lipid peroxidation by measuring the extent of suppression of lipid peroxidation by the test antioxidant",[31] but they are of the indirect type, measuring the extent of reduction of a colored probe by the tested antioxidant.

Optimization of the DMPD Method. DMPD is a chromogenic compound that has been used in the literature for measuring either antioxidant potential or oxidative conversion of natural products, depending on the experimental design. In the literature, there are different applications of the DMPD method. It is used as a TAC assay by some researchers; for example, the radical cation may be preformed as in the ABTS/TEAC method and then added to the test solution.[32] On the other hand, DMPD can be used for free radical determination; for example, Kubo et al.[33] developed a method for the determination of free radicals and peroxides. In the presence of trace Fe(III) as catalyst, DMPD is converted by ROS to the highly colored $DMPD^{\bullet+}$ radical cation. In a recent paper, the authors have devised a method for measuring the oxidant potential of plasma utilizing its oxidizing effect on DMPD so as to produce a stable pink color useful for colorimetric assay and demonstrated its reproducibility. However, DMPD in the presence of antioxidants itself produces free radicals, and the intensity of color gradually enhances with time. In the presence of Fe^{3+}, DMPD is converted to $DMPD^{\bullet+}$ radical cation, which is scavenged by antioxidant molecules present in test samples, and therefore it can indirectly measure the antioxidant potential of a sample.[34] The absorbance at 505 nm of a DMPD solution in the presence of plasma is assumed to be proportional to the amount of hydroperoxyl compounds,[35] but other physiologically relevant oxidizing agents (e.g., H_2O_2, Cu^{2+}, HOCl, and Fe^{3+}) also yield colored radicals of varying stability and kinetics, and both sensitivity and reproducibility are dramatically decreased in the presence of hydrophobic antioxidants and methanol solvent.[36] Thus, it depends on the researcher's point of view whether oxidant or antioxidant potential is measured by DMPD, and there is not a uniquely established assay in this regard. Therefore, optimization of this methodology to fit a specific aim is required.

The optimal pH interval for the modified DMPD method was investigated. A series of buffer solutions at pH between 3.5 and 5.7 were prepared by mixing appropriate volumes of 2.0 M acetic acid and sodium acetate solutions. In the absence of scavenger solution, absorbances were very close to each other in the pH range of 4.0−5.7, whereas no significant absorbance decrease was noted in the presence of a scavenger sample at pH <5.5. The greatest absorbance difference in the absence and presence of a scavenger was seen at pH 5.7. Although in the original method absorbance was read exactly 5 min after DMPD addition, in the proposed method absorbance was recorded (against water) every 5 min following DMPD addition. Absorbance reached its maximal value and stayed nearly constant within the time period of 20−35 min, so after the addition of DMPD, the reaction mixture was kept in a thermostated bath for 20 min. The optimal concentration of Fe(III) to generate an effective concentration of ROS that can be quenched by the tested antioxidants was also determined. For this purpose, different volumes ranging between 0.1 and 1.0 mL of 1.0×10^{-7} M Fe(III) solution (in 10 mM H_2SO_4) were added to the reaction mixture. As the absorbances were very close to each other at volumes >0.25 mL, this amount of Fe(III) solution was chosen for the determination. Finally, under the experimental conditions, various volumes of 3% (w/w) H_2O_2 in the range of 0.1−1.0 mL produced no significant absorbance difference, and therefore an amount of 0.5 mL was chosen for further experiments.

Responses of the Four Methods to Individual Antioxidant Compounds. The tested antioxidant samples can be divided into three groups: (i) polyphenols, including QR and RT (flavonols), NG (flavanone), CFA, *p*-CUM (phenolic or hydroxycinnamic acids), CAT, EC (flavanols), and TR as important food antioxidants; (ii) CYS, GSH, AA, HCYS, and UA as human serum antioxidants; and (iii) human serum. For all selected antioxidant samples, calibration curves were established, and the corresponding equations together with r^2

Table 1. Calibration Equations of the Tested Antioxidants (AOx) as Absorbance (A) for CUPRAC or Absorbance Difference (ΔA) for ABTS, DMPD, and TBARS against AOx Molar Concentration

AOx	CUPRAC	ABTS	DMPD	TBARS
TR	$A = 1.509 \times 10^4C - 0.0008$ $R^2 = 0.9983$	$\Delta A = 2.780 \times 10^4C + 0.426$ $R^2 = 0.9813$	$\Delta A = 3.367 \times 10^4C + 0.0411$ $R^2 = 0.9936$ (linear)	$\Delta A = 2.631 \times 10^4C + 0.4591$ $R^2 = 0.9782$ (linear) $\Delta A = -9 \times 10^8C^2 + 3.758 \times 10^4C + 0.4328$ $R^2 = 0.9852$ (polynomial, 2nd order)
CFA	$A = 5.067 \times 10^4C - 0.0498$ $R^2 = 0.9947$	$\Delta A = 3.435 \times 10^4C + 0.3914$ $R^2 = 0.9894$	$\Delta A = 7.246 \times 10^4C + 0.2685$ $R^2 = 0.8702$ (linear) $\Delta A = -2 \times 10^{10}C^2 + 2.130 \times 10^5C + 0.1045$ $R^2 = 0.9975$ (polynomial, 2nd order)	$\Delta A = 8.699 \times 10^4C + 0.404$ $R^2 = 0.9362$ (linear) $\Delta A = -2 \times 10^{10}C^2 + 2.007 \times 10^5C + 0.2713$ $R^2 = 0.9984$ (polynomial, 2nd order)
CAT	$A = 5.660 \times 10^4C + 0.0017$ $R^2 = 0.9958$	$\Delta A = 5.94 \times 10^4C + 0.3016$ $R^2 = 0.9916$	$\Delta A = 4.198 \times 10^4C + 0.2627$ $R^2 = 0.9645$ (linear) $\Delta A = -1 \times 10^{10}C^2 + 7.490 \times 10^4C + 0.243$ $R^2 = 0.9893$ (polynomial, 2nd order)	$\Delta A = 9.777 \times 10^4C + 0.5126$ $R^2 = 0.9122$ (linear) $\Delta A = -5 \times 10^{10}C^2 + 2.447 \times 10^5C + 0.4244$ $R^2 = 0.9981$ (polynomial, 2nd order)
EC	$A = 6.772 \times 10^4C + 0.0125$ $R^2 = 0.9839$	$\Delta A = 5.820 \times 10^4C + 0.4336$ $R^2 = 0.9839$	$\Delta A = 7.263 \times 10^4C + 0.176$ $R^2 = 0.9617$ (linear) $\Delta A = -1 \times 10^9C^2 + 8.142 \times 10^4C + 0.1658$ $R^2 = 0.9622$ (polynomial, 2nd order)	$\Delta A = 6.261 \times 10^4C + 0.5274$ $R^2 = 0.8482$ (linear) $\Delta A = -2 \times 10^{10}C^2 + 1.951 \times 10^5C + 0.3729$ $R^2 = 0.9959$ (polynomial, 2nd order)
RT	$A = 4.509 \times 10^4C + 0.1012$ $R^2 = 0.9984$	$\Delta A = 2.445 \times 10^4C + 0.3742$ $R^2 = 0.9908$	$\Delta A = 2.238 \times 10^4C - 0.0493$ $R^2 = 0.9941$ (linear)	$\Delta A = 1.943 \times 10^4C + 0.4519$ $R^2 = 0.975$ (linear) $\Delta A = -5 \times 10^8C^2 + 3.415 \times 10^4C + 0.366$ $R^2 = 0.9968$ (polynomial, 2nd order)
QR	$A = 8.40 \times 10^4C + 0.0015$ $R^2 = 0.9993$	$\Delta A = 7.85 \times 10^4C - 0.0179$ $R^2 = 0.9921$	$\Delta A = 1.298 \times 10^5C + 0.1667$ $R^2 = 0.967$ (linear) $\Delta A = -1 \times 10^{10}C^2 + 2.145 \times 10^5C + 0.0678$ $R^2 = 0.9831$ (polynomial, 2nd order)	$\Delta A = 5.233 \times 10^4C + 0.4499$ $R^2 = 0.9161$ (linear) $\Delta A = -1 \times 10^{10}C^2 + 1.297 \times 10^5C + 0.3596$ $R^2 = 0.994$ (polynomial, 2nd order)
p-CUM	$A = 8.344 \times 10^3C + 0.0557$ $R^2 = 0.9965$	$\Delta A = 1.230 \times 10^4C + 0.1466$ $R^2 = 0.9928$	$\Delta A = 3.231 \times 10^3C + 0.1864$ $R^2 = 0.9636$ (linear) $\Delta A = -5 \times 10^7C^2 + 6.347 \times 10^3C + 0.1501$ $R^2 = 0.9984$ (polynomial, 2nd order)	$\Delta A = 6.747 \times 10^3C + 0.4762$ $R^2 = 0.9477$ (linear) $\Delta A = -1 \times 10^8C^2 + 1.449 \times 10^4C + 0.3858$ $R^2 = 0.9962$ (polynomial, 2nd order)
NG	$A = 479C + 0.0224$ $R^2 = 0.9986$	$\Delta A = 1.159 \times 10^4C + 0.1097$ $R^2 = 0.9997$	$\Delta A = 7.401 \times 10^3C + 0.2426$ $R^2 = 0.8905$ (linear) $\Delta A = -2 \times 10^8C^2 + 2.027 \times 10^4C + 0.0925$ $R^2 = 0.9952$ (polynomial, 2nd order)	$\Delta A = 1.546 \times 10^3C + 0.6553$ $R^2 = 0.9679$ (linear) $\Delta A = 7 \times 10^6C^2 + 1.143 \times 10^3C + 0.66$ $R^2 = 0.9705$ (polynomial, 2nd order)
GSH	$A = 8.597 \times 10^3C - 0.0426$ $R^2 = 0.9929$	$\Delta A = 2.016 \times 10^4C + 0.393$ $R^2 = 0.9957$	$\Delta A = 1.303 \times 10^4C + 0.3313$ $R^2 = 0.9126$ (linear) $\Delta A = -3 \times 10^8C^2 + 3.121 \times 10^4C + 0.1192$ $R^2 = 0.9817$ (polynomial, 2nd order)	$\Delta A = 2.081 \times 10^3C + 0.2341$ $R^2 = 0.9506$ (linear) $\Delta A = 3 \times 10^7C^2 + 234C + 0.2556$ $R^2 = 0.9798$ (polynomial, 2nd order)
CYS	$A = 7.728 \times 10^3C - 0.0054$ $R^2 = 0.9991$	$\Delta A = 1.469 \times 10^4C + 0.4625$ $R^2 = 0.9941$	$\Delta A = 1.197 \times 10^4C - 0.0115$ $R^2 = 0.9883$ (linear) $\Delta A = 9 \times 10^7C^2 + 6.792 \times 10^3C + 0.0489$ $R^2 = 0.9955$ (polynomial, 2nd order)	$\Delta A = 3.164 \times 10^3C - 0.0334$ $R^2 = 0.8347$ (linear) $\Delta A = 1 \times 10^8C^2 - 3.573 \times 10^3C + 0.0452$ $R^2 = 0.9818$ (polynomial, 2nd order)
AA	$A = 1.463 \times 10^4C - 0.0443$ $R^2 = 0\ .9984$		$\Delta A = 5.351 \times 10^3C + 0.0681$ $R^2 = 0.9767$ (linear) $\Delta A = -3 \times 10^7C^2 + 9.193 \times 10^3C - 0.0215$ $R^2 = 0.9963$ (polynomial, 2nd order)	$\Delta A = 764C - 0.0148$ $R^2 = 0.9007$ (linear) $\Delta A = 1 \times 10^7C^2 - 434C + 0.0132$ $R^2 = 0.9869$ (polynomial, 2nd order)

Table 1. continued

AOx	CUPRAC	ABTS	DMPD	TBARS
UA	$A = 1.955 \times 10^4 C - 0.0136$ $R^2 = 0.9991$	$\Delta A = 3.479 \times 10^4 C + 0.3283$ $R^2 = 0.9998$	$\Delta A = 2.263 \times 10^3 C + 0.0601$ $R^2 = 0.8452$ (linear) $\Delta A = -2 \times 10^8 C^2 + 6.478 \times 10^3 C + 0.0453$ $R^2 = 0.9591$ (polynomial, 2nd order)	

values (for both linear and nonlinear quadratic calibrations) are shown in Table 1. The calibration curves were drawn using absorbance (A) versus molar concentration only for the CUPRAC method, and for all of the other three assays, $\Delta A = (A_0 - A_f)$ measurements were used, where A_0 is the absorbance of the reference (i.e., in the absence of antioxidant) and A_f is the absorbance in the presence of scavenger antioxidant sample.

Becaue two TAC and two TOC/ROS (altogether four) methods were applied to these samples, acceptable common concentration intervals of antioxidant compounds were determined, enabling the exposition of all absorbance changes in a single figure for a given antioxidant. To investigate the relationship between ROS consumption (i.e., TOC decrease) and TAC, ROS was synthetically produced in the reaction medium. For TBARS method, ROS was produced via Fe(II)−H_2O_2 reaction, whereas for DMPD measurements, ROS was produced by the reaction of Fe(III) with H_2O_2. For both DMPD and TBARS methods, the absorbance of generated ROS was separately measured as described under Materials and Methods. Then a series of selected antioxidants were added to the reaction medium in different amounts, and the resulting ROS consumption was measured as a decrease in TOC (Figure 1).

When antioxidants are added to the test system of interest, effective absorbance (either A or ΔA) of the chromogenic probe (CUPRAC or ABTS) undergoing e-reduction or H-atom transfer increases, whereas oxidative damage on the oxidative stress marker probe (DMPD or TBARS) decreases (Figure 1). Thus, it is more instructive to show absorbance increments (of CUPRAC and ABTS) and absorbance decrements (of DMPD and TBARS) on the same graph for a given antioxidant added at various concentrations, to effectively demonstrate the aimed correlation (of TAC vs TOC) of this work. However, conforming to standard reporting formats for three representative antioxidants, namely, caffeic acid, epicatechin, and naringin, micromolar trolox equivalent TAC values calculated from raw absorbances against the molar concentration of added antioxidant were also recorded (Figure 2).

As can be seen from Figure 1, there is a linear relationship between antioxidant molar concentration and absorbance changes in the CUPRAC and ABTS methods of TAC measurement; that is, A absorbance (or ΔA) increases linearly with antioxidant concentration. The highest linear correlation coefficients ($r^2 > 0.99$) comprising all tested antioxidants were obtained for the CUPRAC method. On the other hand, there is a distinct decrease of ROS (measured as TOC) with increasing amounts of antioxidants, as reflected by the decreasing absorbances of DMPD and TBARS methods in response to antioxidant addition. This decrease was almost linear or curvilinear for DMPD and pseudolinear or basically nonlinear for TBARS. For the TBARS method, the highest correlation coefficients were achieved for RT and TR ($r^2 \sim 0.97-0.98$), then for QR, CAT, CFA, and p-CUM ($r^2 \sim 0.92-0.95$) despite the high blank values (intercepts ~0.4−0.5), whereas the results were basically nonlinear for NG, AA, CYS, GSH, and UA, with very little change of absorbance with respect to concentration for the last four antioxidants (Figure 1). Aside from the instability of MDA in the presence of millimolar concentrations of H_2O_2,[37] increase in serum antioxidant capacity was previously shown to correlate poorly ($r^2 = 0.45$) to improvement in lipid peroxidation status measured by the TBARS assay,[38] probably due to the lack of specificity of this assay, as TBA reacts with diverse compounds, such as sugars, amino acids, a variety of aldehydes, and bilirubin, producing interference with colorimetric measurements of MDA.[39] For the DMPD method, the results were curvilinear for most antioxidants ($r^2 \geq 0.95$ or higher) with the exception of RT ($r^2 \sim 0.87$) and UA (nonlinear), showing relatively low blank values (intercepts ~ 0.1−0.25). The curvilinear and essentially nonlinear relationships could be better fitted to second-order polynomial (quadratic) curves where appropriate (e.g., Figure 2) with $r^2 \approx 0.97-0.99$. DMPD absorbances exhibited a uniform decrease with concentration for all antioxidants but UA, for which very little response was obtained (Figure 1k). The concentration of UA was selected at the same order of magnitude as that in human serum so as to simulate real-life conditions as closely as possible, because UA in serum is responsible for the observed TAC value at 19.3, 45.4, and 61.7%, in terms of estimated contribution to the antioxidant capacity found by ABTS/TEAC, ORAC (applied to perchloric acid-treated serum), and FRAP methods, respectively.[21] Nevertheless, as diverse antioxidants were tested in this work, it should be added that the obtained results reflect only the chemical responsivity under the specific conditions of the assays such as concentration levels of antioxidants.

This trend noted for individual antioxidant compounds was also observed for serum samples when they were added to ROS-generated media. As increasing concentrations of antioxidants caused an increase in TAC and a decrease in ROS (and indirectly in TOC) concentrations, simultaneous display of the results of the four methods on one figure would be a better way to express the main correlation between TAC and TOC values. Unfortunately, for AA and UA, there was no optimal concentration interval for showing the responses of all four methods in the same figure. When studying AA, the common suitable range for DMPD, TBARS, and CUPRAC methods (i.e., $2.0 \times 10^{-5}-1.0 \times 10^{-4}$ M) was too concentrated for ABTS (Figure 1i). In UA measurements, CUPRAC, ABTS, and DMPD methods were applied in the $3.0 \times 10^{-6}-1.6 \times 10^{-5}$ M concentration interval (Figure 1k), but no significant absorbance difference between reference and UA samples was observed for the TBARS method in this range.

Study of Synthetic Mixtures. The absorbances of individual antioxidants as well of their binary mixtures prepared by maintaining their original concentrations are depicted in Table 2 using the four methods. Using the principle of additivity of absorbances due to individual compounds constituting a mixture, the theoretically calculated (expected)

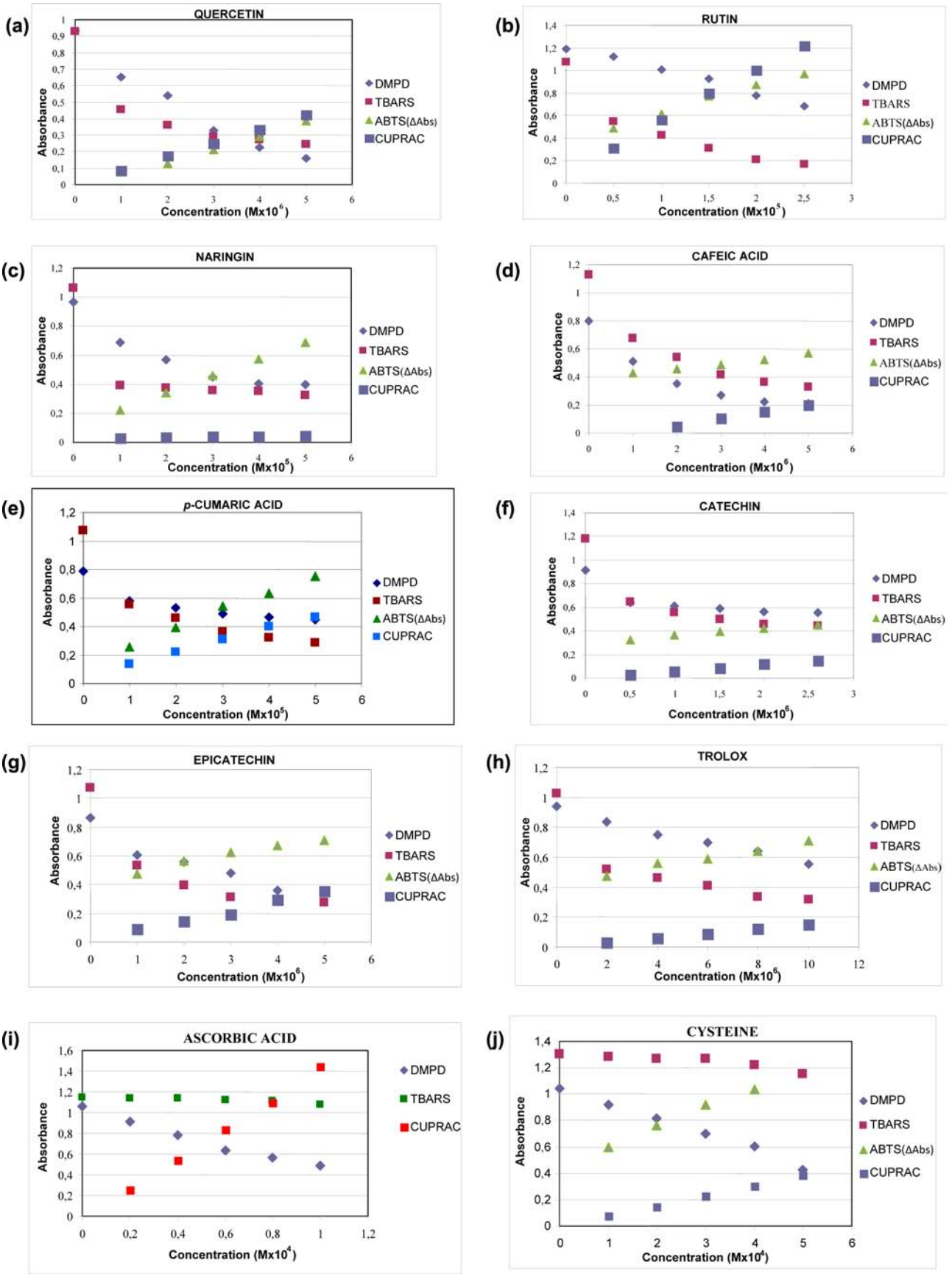

Figure 1. continued

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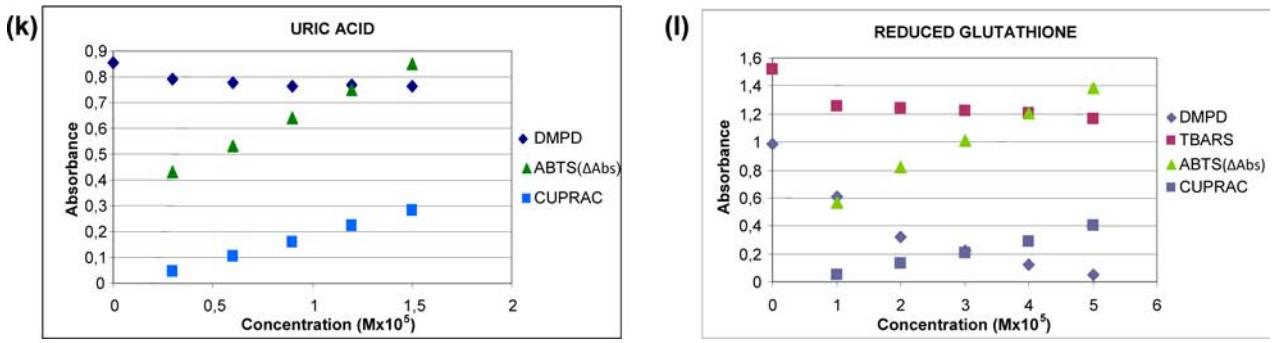

Figure 1. Absorbance versus concentration (mol L^{-1}) curves of selected antioxidants for TAC (determined by CUPRAC and ABTS assays) and ROS consumption measurements (found as TOC with the use of DMPD and TBARS methods).

absorbance values of mixtures are listed along with experimental measurements. The absorbances of mixtures were found to be perfectly additive for CUPRAC and almost additive for ABTS (Table 2) methods, as reported in many other literature sources of antioxidant research. However, the expected results somewhat deviated for DMPD and significantly deviated for TBARS (Table 2) from the experimental ones. All tested antioxidant mixtures showed greater consumption of ROS than individual antioxidants at the same concentration, but not necessarily in an additive manner. These data regarding the lack of additivity for TOC methods are in accordance with the partly linear and curvilinear character of the corresponding (absorbance versus concentration) curves of DMPD and TBARS methods, respectively (Figure 1), because additivity of absorbances of antioxidants in mixtures requires that Beer's law be obeyed over a reasonable concentration range for the concerned individual antioxidants.

Human Serum Experiments. Human serum samples were diluted at different ratios with distilled water, and 0.2 mL volumes of diluted sera were taken for DMPD and TBARS measurements to observe the ROS quenching effects. ROS consumption by serum antioxidants (as measured by the TOC tests of DMPD and TBARS) increased with decreasing dilution ratio of sera in a nonlinear manner (Table 3). For $N = 10$ serum samples with various lipid contents, it was shown by preliminary experiments that there was no relationship between the initial lipid (or cholesterol) levels of serum samples and either their TAC or TOC values (data not shown). For a sample size of 10 data pairs, the related correlation coefficients were <0.564 (i.e., the minimum value of Pearson's correlation coefficient that is significantly different from zero at the 0.05 significance level for a given sample size).[28] Although TBARS is known as a lipid peroxidation test, the correlation between lipid content and TBARS color intensity is not straightforward: the aldehydes (like MDA) that react with TBA to form the colored TBARS products are derived from peroxides and polyunsaturated fatty acids during the test procedure,[40] so the fatty acid composition of lipids is important, and furthermore, the test is unspecific for lipid peroxidation. When diluted serum was spiked with various concentrations of CYS or GSH such that the original TAC was increased up to 5-fold for CUPRAC and up to 2-fold for other methods (i.e., ABTS, DMPD, and TBARS), the relative standard error (RSE) of results varied between −1.5 and −6.0% for CUPRAC as opposed to much greater errors with other methods ($-15 \le$ RSE $\le -6\%$ for DMPD, $-38 \le$ RSE $\le -18\%$ for ABTS, and $-28 \le$ RSE $\le +15\%$ for TBARS; data not shown). This means that tolerance of the measured TAC values to both dilution and possible thiol interactions of serum constituents was better in CUPRAC than in other methods. Moreover, TAC measurement of five replicate samples of 1:5 diluted sera using the four methods showed that CUPRAC had the highest precision (i.e., lowest RSD of 1.94%, Table 3).

General Evaluation of TAC and TOC Methods. The DMPD assay was mentioned as a method for the determination of both ROS[33] and TAC[41] in different papers with some differences. The common point of all was the spectrophotometric measurement of the production and/or decolorization of reddish-pink radicals produced from DMPD. In two different studies, the production of colored radicals was explained in two different ways. In the first one, Hirayama and Unohara[16] showed that, in the presence of trace amounts of Fe(III), DMPD was oxidized by hydrogen peroxide to form two semiquinone derivatives (called DMPDQ, Figure 3). In the second one, Kubo et al.[33] showed the generation of the same two radicals (called DMPD radicals, identical with DMPDQ) by the reaction between DMPD and free radicals.

Oxidation of DMPD with hydrogen peroxide in the presence of trace amounts of iron(III) proceeded as described by eqs 1−3. One way to generate $^{\bullet}OH$ is by the well-known reactions of hydrogen peroxide with Fe^{2+} and Fe^{3+} salts. Briefly, ferrous ion combined with hydrogen peroxide (Fenton's reagent) react stoichiometrically to give $^{\bullet}OH$.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \quad (1)$$

In the closely related ferric system, Fe^{3+} acts as a catalyst for decomposition of hydrogen peroxide to O_2 and H_2O, during which "steady-state" concentrations of Fe^{2+} (as a source of $^{\bullet}OH$ via eq 1) are generated, as in the following steps:[42]

$$Fe^{3+} + H_2O_2 \rightleftarrows FeO_2H^{2+} + H^+ \quad (2a)$$

and

$$FeO_2H^{2+} \rightleftarrows Fe^{2+} + HO_2^{\bullet} \quad (2b)$$

$$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + H^+ + O_2 \quad (3)$$

As can be seen from Figure 1 and Table 2, increasing TAC values of added antioxidants (as measured by CUPRAC and ABTS tests) resulted in the consumption of ROS (as measured by diminished TOC with DMPD and TBARS), and TAC of added antioxidants showed a curvilinear relationship with TOC decrease. As a real sample, human serum, known to be rich in natural antioxidants, showed good ROS scavenging activity; with increasing dilution of serum samples, their TAC and ROS scavenging activity decreased (Table 3).

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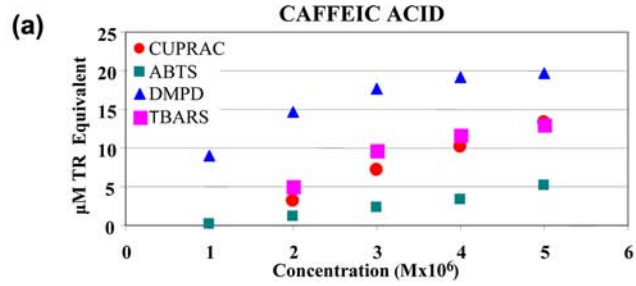
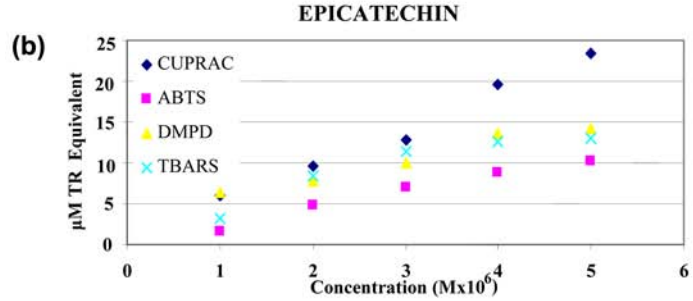
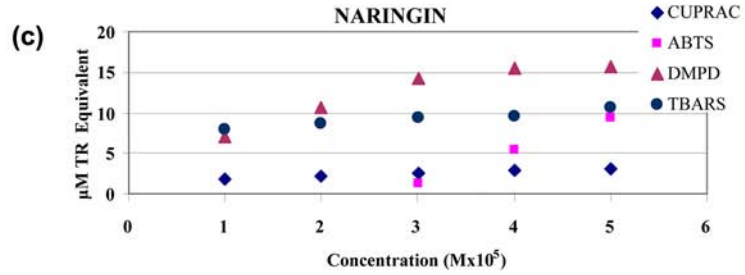


CUPRAC	µM TR = $3.36\times10^{6}C - 3.27$	($R^2 = 0.9949$)
ABTS	µM TR = $1.24\times10^{6}C - 1.28$	($R^2 = 0.9874$)
DMPD	µM TR = $2.60\times10^{6}C + 8.22$	($R^2 = 0.8728$) Linear
	µM TR = $-8\times10^{11}C^2 + 8\times10^{6}C + 2.42$	($R^2 = 0.9969$) Polynomial (2^{nd} degree)
TBARS	µM TR = $2.60\times10^{6}C + 0.7$	($R^2 = 0.9245$) Linear
	µM TR = $-8\times10^{11}C^2 + 8\times10^{6}C - 8.1$	($R^2 = 0.9945$) Polynomial (2^{nd} degree)



CUPRAC	µM TR = $4\times10^{6}C + 0.9$	($R^2 = 0.9840$)
ABTS	µM TR = $2\times10^{6}C + 0.27$	($R^2 = 0.9722$)
DMPD	µM TR = $2\times10^{6}C + 4$	($R^2 = 0.9631$)
	µM TR = $-3\times10^{10}C^2 + 2\times10^{6}C + 3.8$	($R^2 = 0.9640$)
TBARS	µM TR = $2\times10^{6}C + 2.65$	($R^2 = 0.8483$)
	µM TR = $-8\times10^{11}C^2 + 7\times10^{6}C - 3.2$	($R^2 = 0.9960$) Poly. (2^{nd} degree)



CUPRAC	µM TR = $3.2\times10^{4}C + 1.53$	($R^2 = 0.999$)
ABTS	µM TR = $4.05\times10^{5}C - 10.9$	($R^2 = 0.9995$)
DMPD	µM TR = $2.2\times10^{5}C - 10.9$	($R^2 = 0.8846$)
	µM TR = $-7\times10^{9}C^2 + 6.1\times10^{5}C + 1.48$	($R^2 = 0.9937$) Poly. (2^{nd} degree)
TBARS	µM TR = $6\times10^{4}C + 7.2$	($R^2 = 0.9605$)
	µM TR = $3\times10^{8}C^2 + 4.3\times10^{4}C + 7.6$	($R^2 = 0.9636$) Poly. (2^{nd} degree)

Figure 2. Micromolar trolox equivalent TAC against the molar concentration of added antioxidant for three representative antioxidants, namely, caffeic acid, epicatechin, and naringin, with the corresponding linear or quadratic equations of correlation.

The TBARS test was found to be rather blind to a number of antioxidants (partly to cysteine and ascorbic acid, and completely to uric acid) where a concentration-proportional response could not be obtained. Likewise, additivity and reproducibility in TBARS responses were rather poor (Table 2). As the formation of secondary products (MDA) from oxidative conversion experiments involves a more complicated series of reactions (i.e., consecutive and parallel reactions) than of primary products and the TBARS test gives a nonselective response to many interfering compounds other than MDA, the concentration-dependent response of oxidation inhibition was better reflected in the results of DMPD test than in those of TBARS.

Although TBARS/MDA test is nearly the most commonly used biomarker of lipid peroxidation, the validity of TBARS/MDA in bodily fluids has been criticized, for example, for a lack of specificity, postsampling MDA formation, antioxidants that can interfere with the assay procedure, and MDA derived from

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Table 2. Experimental (Exptl) and Theoretical (Theor) Micromolar Trolox (TR) Equivalent Results with Percentage Relative Standard Deviations (RSD, Percent) of Individual and Binary Mixtures of Selected Antioxidants (AOx; C_F, Final Concentrations) for the Tested Methods

	TAC measurement methods								TOC measurement methods							
	CUPRAC				ABTS				DMPD				TBARS			
AOx	C_F (μM)	μM TR equiv exptl	μM TR equiv theor	RSD %	C_F (μM)	μM TR equiv exptl	μM TR equiv theor	RSD %	C_F (μM)	μM TR equiv exptl	μM TR equiv theor	RSD %	C_F (μM)	μM TR equiv exptl	μM TR equiv theor	RSD %
QR	2.0	13.2			2.0	4.77			0.4	11.7			0.1	0.45		
CAT	6.0	26.2			3.0	9.16			6.0	14.2			0.5	−1.67		
p-CUM	12	16.3			200	9.98			100	13.7			50	3.91		
RT	8.0	34.1			3.0	3.98			20	17.2			2.5	5.34		
EC	4.0	17.9			3.0	8.59			6.0	14.6			3.0	1.90		
TR	20	26.7			3.0	3.16			20	12.8			1.0	1.08		
NG	600	26.7			30	2.04			1.0	15.8			5.0	1.87		
CFA	6.0	19.8			2.0	3.82			4.0	17.7			1.0	5.95		
QR + CAT		38.0	39.4	−3.55		22.5	25.6	−12.1		19.3	18.3	5.46		9.50	13.6	−30.1
QR +*p*-CUM		28.0	29.5	−5.08		22.6	23.8	−5.04		15.3	17.7	−7.66		15.7	19.5	−24.2
RT + EC		50.3	52.0	−3.27		22.7	20.7	9.66		20.5	24.2	−15.3		13.1	18.2	−28.0
CFA + TR		40.0	39.5	1.25		22.8	21.1	8.09		25.6	23.3	9.87		9.11	7.96	14.4
NG + EC		43.3	44.6	−3.00		19.0	22.3	−14.5		21.9	20.6	6.31		16.0	20.0	−20.0
CFA + CAT		46.6	46.0	1.30		22.6	21.1	7.11		20.2	24.2	−15.3		12.5	13.6	- 8.09
RT + *p*-CUM		49.0	50.4	- 2.8		23.1	22.2	4.8		21.5	23.1	−6.92		5.61	7.67	−26.6

Table 3. Millimolar AA Equivalent TAC Values for 0.2 mL Serum Samples Diluted at Different Ratios

dilution ratio	DMPD	TBARS	CUPRAC	ABTS[a]
1:1	0.1662	2.0665	0.0390	
1:5[b]	0.1485	0.8920	0.0149	
1:10	0.1121	0.2496	0.0064	0.0169
1:20	0.0860	Negative	0.0036	0.0114

[a]For the ABTS method, when the difference between the absorbances of sample and reference ($\Delta_{absorbance}$) was evaluated for 0.2 mL volumes of 1:1 and 1:5 diluted serum samples, the final absorbance (that should normally be a nonzero value) could not be measured, because the developed color completely faded upon serum addition during the 6 min protocol time of the assay. [b]Relative standard deviations (RSD, %) of the four methods calculated for N = 5 replicates of 0.2 mL of 1:5 diluted serum samples were found as CUPRAC, 1.94%; ABTS, 4.12%; DMPD, 2.08%; and TBARS, 9.03%.

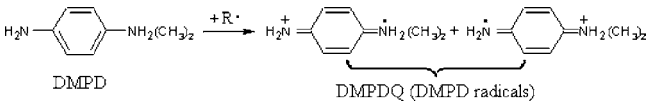

Figure 3. Production of radical semiquinone derivatives from *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD).

the diet.[43] In some cases, the TBARS/MDA test has been reported not to properly respond to known antioxidants. For example, when triglycerides enriched with polyunsaturated fatty acids were subjected to rapid lipid peroxidation upon incubation with cumene hydroperoxide in chloroform solution at 37 °C, melatonin, known to be a potent antioxidant as an inhibitor of linoleic acid peroxidation by ferric-thiocyanate test and by other free radical scavenging tests,[44] was unable to reduce TBARS formation as opposed to the positive responses of butylated hydroxytoluene, *N*-acetylserotonin, and 5-hydroxytryptophan, which significantly diminished TBARS formation in a concentration-dependent manner.[45] Moreover, the instability of malondialdehyde in the presence of hydrogen peroxide seems to account for the inconsistent outcomes in studies relating the manipulations of intermediate hydrogen peroxide levels to the initiation of lipid peroxidation; the measurement of lipid peroxidation by the TBARS test is therefore of limited value under conditions that favor the accumulation of hydrogen peroxide in reaction mixtures.[46]

This work clearly distinguishes between TAC (i.e., CUPRAC and ABTS assays) and TOC (i.e., oxidative conversion of DMPD and deoxyribose by hydroxyl radicals) type assays, because colorimetric TAC assays measure the level of H-atom or electron transfer from antioxidants to an oxidizing probe causing either color intensification or decolorization of the probe, whereas TOC assays essentially measure the oxidative conversion in a probe under the attack of reactive species (including free radicals). Thus, in TOC assays, antioxidants cannot be directly measured but only through their reaction with reactive species (i.e., the observed oxidative conversion in the probe would decrease in the presence of antioxidants). This constitutes a basic difference between the mechanisms of TAC and TOC assays. In addition, the fields of usage are rather different: TAC assays are preferentially used in food chemistry, whereas TOC assays are used primarily in biochemistry and medicinal chemistry. As a result, there is considerable confusion in the literature between assays measuring TAC and TOC, and the present paper is an effort to relieve this confusion.

Considering the listed disadvantages of TBARS, the DMPD assay was found to better reflect ROS consumption by antioxidants in view of its versatile response to a wide variety of antioxidants, high sensitivity (i.e., high slope values close to those of CUPRAC and ABTS, as shown in Figure 1), and rather low blank values. This work has established an inverse relationship between oxidative conversion and antioxidant capacity in the form of a curvilinear relationship between the absolute values of increments in TAC and decrements in TOC as a function of added antioxidant concentration. Upon antioxidant addition to ROS-generated medium, the linear relationship between TAC increments (in CUPRAC absorbance units) and TOC decrements (in DMPD $\Delta_{absorbance}$ units) was excellent (i.e., $r^2 \geq 0.98$ for RT, QR, *p*-CUM, CAT, EC, TR, AA, and CYS). For biological fluids subjected to oxidative stress confined to the test system, we recommend that the TAC

of examined nonenzymatic antioxidants be measured with CUPRAC (having high sensitivity and low blank values, together with a perfectly linear response to a wide variety of antioxidants) and the consumption of ROS (as diminished TOC) be measured with DMPD assay. Naturally, no single "universally accepted" assay is adequate in itself to precisely and quantitatively detect/determine all actions of a putative antioxidant and, consequently, a combination of HAT and ET assays or of reduction- and free radical scavenging-based assays may be the recommended practice for antioxidant research. This simple relationship established between TAC and TOC may initiate the formation of a bridge between medical bioanalytical chemistry mainly relying on TOC results and food analytical chemistry often using TAC results, because these two disciplines are known to frequently use quite different languages.

AUTHOR INFORMATION

Corresponding Author

*E-mail: rapak@istanbul.edu.tr. Phone: +90-212-4737028. Fax: +90-212-4737180.

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ABBREVIATIONS USED

TAC, total antioxidant capacity; TOC, total oxidative conversion; ROS, reactive oxygen species; RNS, reactive nitrogen species; DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine; TCA, trichloroacetic acid; GSH, glutathione (reduced); TR, trolox; QR, quercetin; CAT, catechin; EC, epicatechin; CFA, caffeic acid; *p*-CUM, *p*-coumaric acid; NG, naringin; RT, rutin; CYS, cysteine; HCYS, homocysteine; AA, ascorbic acid; UA, uric acid; EtOH, ethyl alcohol; PBS, phosphate buffered saline; Nc, 2,9-dimethyl-1,10-phenanthroline (neocuproine); NaAc, sodium acetate; HAc, acetic acid; ABTS, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonate); TEAC, trolox equivalent antioxidant capacity; CUPRAC, cupric reducing antioxidant capacity; FRAP, ferric reducing antioxidant power; TBA, 2-thiobarbituric acid; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances; *A*, absorbance; ΔA, absorbance difference; AOx, antioxidant; RSE, relative standard error (of experimentally found result with respect to the theoretically expected one)

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