Pure Appl. Chem., Vol. 85, No. 5, pp. 957–998, 2013.
http://dx.doi.org/10.1351/PAC-REP-12-07-15
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Methods of measurement and evaluation of natural antioxidant capacity/activity (IUPAC Technical Report)*

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Abstract: The chemical diversity of natural antioxidants (AOXs) makes it difficult to separate, detect, and quantify individual antioxidants from a complex food/biological matrix. Moreover, the total antioxidant power is often more meaningful to evaluate health beneficial effects because of the cooperative action of individual antioxidant species. Currently, there is no single antioxidant assay for food labeling because of the lack of standard quantification methods. Antioxidant assays may be broadly classified as the electron transfer (ET)- and hydrogen atom transfer (HAT)-based assays. The results obtained are hardly comparable because of the different mechanisms, redox potentials, pH and solvent dependencies, etc. of various assays. This project will aid the identification and quantification of properties and mutual effects of antioxidants, bring a more rational basis to the classification of antioxidant assays with their constraints and challenges, and make the results more comparable and understandable. In this regard, the task group members convey their own experiences in various methods of antioxidants measurement.

Keywords: 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)(ABTS)/Trolox-equivalent antioxidant capacity (TEAC); antioxidant capacity/activity; cereals; cupric reducing antioxidant capacity (CUPRAC); 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH); ferric reducing antioxidant power (FRAP); fruits; hydrophilic/lipophilic antioxidants; IUPAC Analytical Chemistry Division; oxygen radical absorbance capacity (ORAC); original and modified reducing antioxidant capacity CUPRAC methods; total antioxidant capacity (TAC) assays; total peroxyl radical-trapping antioxidant parameter (TRAP); vegetables.

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*Sponsoring body: IUPAC Analytical Chemistry Division: see more details on p. 991.

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1. BACKGROUND

Reactive oxygen species (ROS) such as superoxide anion ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), peroxyl ($ROO^{\bullet}$), and alkoxyl radicals ($RO^{\bullet}$), hydrogen peroxide (H_2O_2), and singlet oxygen ($O_2{}^1\Delta g$) may attack biological macromolecules, giving rise to protein, lipid, and DNA damage, cell aging, oxidative stress-originated diseases (e.g., cardiovascular and neurodegenerative diseases), and cancer. Antioxidants scavenge or quench ROS and reactive nitrogen species (RNS) products of respiration, including free radicals [1].

The terms "antioxidant activity" and "antioxidant capacity" have different meanings: antioxidant activity deals with the kinetics of a reaction between an antioxidant and the prooxidant or radical it reduces or scavenges, whereas antioxidant capacity measures the thermodynamic conversion efficiency of an oxidant probe upon reaction with an antioxidant. 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 [e.g., a consistent difference has been reported in the level of antioxidants between the tumoral sample and its corresponding peritumoral tissue, independently of the tumor type; plasma TRAP (total peroxyl radical-trapping antioxidant parameter) values were found in good agreement with the stage of coronary heart disease]. ROS can lead to oxidation of amino acid side chains, formation of protein–protein cross-linkages, and oxidation of the peptide backbones of proteins, where age-dependent oxidative alterations in humans, such as increase in protein carbonyls and in protein advanced oxidation products and decrease in plasma total thiols, significantly correlate with the total antioxidant capacity (TAC) of plasma [2]. The cooperation among different antioxidants provides greater protection against ROS/RNS attack than any compound alone (thereby rendering TAC measurement even more important).

An antioxidant may be defined as "any substance that when present at relatively low concentrations, compared with those of the oxidisable substrate, significantly delays or inhibits oxidation of that substrate" [3]. For convenience, antioxidants have been traditionally divided into two classes; primary or chain-breaking antioxidants, and secondary or preventative antioxidants.

Chain-breaking mechanisms:

$$L^{\bullet} + AH \rightarrow LH + A^{\bullet}$$

$$LO^{\bullet} + AH \rightarrow LOH + A^{\bullet}$$

$$LOO^{\bullet} + AH \rightarrow LOOH + A^{\bullet}$$

Thus, radical initiation (by reacting with a lipid radical) or propagation (by reacting with peroxyl or alkoxyl radicals) steps are inhibited. On the other hand, secondary (preventative) antioxidants retard the rate of oxidation, e.g., transition-metal ion chelators may inhibit Fenton-type reactions that produce hydroxyl radicals:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

The chemical diversity of antioxidants (relative abundance of their glycosides and isomers) makes it difficult to separate and quantify antioxidants from food/biological matrices where their combined action may be more relevant. Therefore, it is desirable to measure the TAC/activity level directly from plant extracts and biological fluids. A basic classification of antioxidant assays is the type of reaction:

(i) hydrogen atom transfer (HAT)-based assays
(ii) electron transfer (ET)-based assays

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Although the exact mechanism of chemiluminescent TAC assays is still debatable [4], luminescence methods in the analysis of edible oils (such as oxidative stability, antioxidant activity, and lipid hydroperoxide content, as well as classification or adulteration of vegetable oils) have been effectively used without pretreatment [5]. In light of the fact that the beneficial influence of many foodstuffs and beverages, including fruits, vegetables, tea, coffee, and cacao, on human health has been recently recognized to originate from their antioxidant activity, the most common methods used for in vitro determination of antioxidant capacity of food constituents with some important advantages and shortcomings of each method were recently reviewed [6].

1.1 HAT-based assays

HAT-based assays measure the capability of an antioxidant to quench free radicals (generally, peroxyl radicals considered to be biologically more relevant) by H-atom donation. The HAT mechanisms of antioxidant action in which the hydrogen atom (H) of a phenol (Ar–OH) is transfered to a $ROO^{\bullet}$ radical can be summarized by the reaction

$$ROO^{\bullet} + AH/ArOH \rightarrow ROOH + A^{\bullet}/ArO^{\bullet}$$

where the aryloxy radical ($ArO^{\bullet}$) formed from the reaction of antioxidant phenol with peroxyl radical is stabilized by resonance. The AH and ArOH species denote the protected biomolecules and phenolic antioxidants, respectively. Effective phenolic antioxidants need to react faster than biomolecules with free radicals to protect the latter from oxidation. Since in HAT-based antioxidant assays, both the fluorescent probe and antioxidants react with $ROO^{\bullet}$, the antioxidant activity can be determined from competition kinetics by measuring the fluorescence decay curve of the probe in the absence and presence of antioxidants, integrating the area under these curves, and finding the difference between them [7,8].

HAT-based assays include oxygen radical absorbance capacity (ORAC) assay, TRAP assay using R-phycoerythrin as the fluorescent probe, crocin bleaching assay using 2,2'-azobis(2-amidinopropane) hydrochloride (AAPH) as the radical generator, and β-carotene bleaching assay, although the latter bleaches not only by peroxyl radical attack but by multiple pathways [7,8].

1.2 ET-based assays

In most ET-based assays, the antioxidant action is simulated with a suitable redox-potential probe, namely, the antioxidants react with a fluorescent or coloured probe (oxidising agent) instead of peroxyl radicals. Spectrophotometric ET-based assays measure the capacity of an antioxidant in the reduction of an oxidant, which changes colour when reduced. The degree of colour change (either an increase or decrease of absorbance of the probe at a given wavelength) is correlated to the concentration of antioxidants in the sample. 2,2'-Azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/Trolox-equivalent antioxidant capacity (TEAC) and 2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl (DPPH) [9–11] are decolourisation assays, whereas in Folin total phenols assay [12,13], ferric reducing antioxidant power (FRAP) [14,15] and cupric reducing antioxidant capacity (CUPRAC) [16], there is an increase in absorbance at a prespecified wavelength as the antioxidant reacts with the chromogenic reagent [i.e., in the latter two methods, the lower valencies of iron and copper, namely, Fe(II) and Cu(I), form charge-transfer complexes with the corresponding ligands, respectively]. There is no visible chromophore in the Ce^{4+}-reducing antioxidant capacity assay developed recently by Ozyurt et al. [17], as the remaining Ce(IV) in dilute sulfuric acid solution after polyphenol oxidation under carefully controlled conditions was measured at 320 nm (i.e., in the UV region of the electromagnetic spectrum).

A ferric-ferrozine method of antioxidant capacity measurement has been developed for the simple, low-cost, and versatile assay of food antioxidants [18]. In the presence of ferrozine (FZ) ligand, ferric ion easily oxidizes antioxidants and is itself reduced to Fe(II)-FZ, yielding a very high molar absorp-

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tivity [for Fe(II) at the order of 2.8×10^4 L·mol^{-1}·cm^{-1}] and thus enhanced sensitivity for most antioxidants.

The ferricyanide/Prussian blue assay of reducing capacity measurement was modified so as to obtain a more reproducible, linear, and additive response from antioxidants with respect to concentration [19]. The simultaneous use of ferricyanide and ferric ions as chromogenic oxidants supplied more favourable redox conditions for a greater variety of antioxidants. Prussian blue precipitation was hindered with the addition of sodium dodecyl sulfate (SDS), and the optimal pH was adjusted to 1.7 to maintain the redox activity of ferric ion while preventing its hydrolysis. Incubation of the reaction mixture at room temperature for 30 min enabled more complete oxidations than observed in the conventional ferricyanide method while elevated temperature incubation resulted in overoxidation of mixture constituents causing significant deviations from linearity of absorbance–concentration curves.

ET-based assays generally set a fixed time for the concerned redox reaction and measure thermodynamic conversion (oxidation) during that period. ET-based assays include ABTS/TEAC, DPPH (though the first two assays are considered as mixed HAT/ET-based assays by some researchers), Folin–Ciocalteu reagent (FCR), FRAP, ferricyanide, and CUPRAC using different chromogenic redox reagents with different standard potentials. Although the reducing capacity of a sample is not directly related to its radical scavenging capability, it is a very important parameter of antioxidants. The reaction equations of various ET-based assays can be summarized as follows:

$$\text{Folin: Mo(VI) (yellow)} + e^- \text{ (from AH)} \rightarrow \text{Mo(V) (blue)} \quad (1)$$

where the oxidising reagent is a molybdophosphotungstic heteropolyacid comprised of $3H_2O$–P_2O_5–$13WO_3$–$5\ MoO_3$–$10\ H_2O$ (heteropoly anion: $P_2Mo_5W_{13}O_{62}^{6-}$), in which the hypothesized active center is Mo(VI) with λ_{max} = 765 nm.

$$\text{FRAP: } Fe(TPTZ)_2^{3+} + ArOH \rightarrow Fe(TPTZ)_2^{2+} + ArO^{\bullet} + H^+ \quad (2)$$

where TPTZ: 2,4,6-tripyridyl-*s*-triazine ligand with λ_{max} = 595 nm.

$$\text{Ferricyanide/Prussian blue: } Fe(CN)_6^{3-} + ArOH \rightarrow Fe(CN)_6^{4-} + ArO^{\bullet} + H^+ \quad (3)$$

$$Fe(CN)_6^{4-} + Fe^{3+} + K^+ \rightarrow KFe[Fe(CN)_6] \quad (4)$$

where KFe[Fe(CN)$_6$]: Prussian Blue with λ_{max} = 700 nm.

$$\text{ABTS/TEAC: ABTS} + K_2S_2O_8 \rightarrow ABTS^{\bullet+} \quad (5)$$

$$ABTS^{\bullet+} + ArOH \rightarrow ABTS + ArO^{\bullet} + H^+ \quad (6)$$

where ABTS is 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) with λ_{max} = 734 nm and TEAC is Trolox-equivalent antioxidant capacity (also the name of the assay). Although other wavelengths such as 415 and 645 nm have been used in the ABTS assay [8], the 734-nm peak wavelength has been predominantly preferred due to less interference from plant pigments.

$$\text{DPPH: } DPPH^{\bullet} + ArOH \rightarrow DPPH + ArO^{\bullet} + H^+ \quad (7)$$

where DPPH$^{\bullet}$ is the [2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl] stable radical with λ_{max} = 515 nm.

$$\text{CUPRAC: } nCu(Nc)_2^{2+} + Ar(OH)_n \rightarrow nCu(Nc)_2^{+} + Ar(=O)_n + nH^+ \quad (8)$$

where the polyphenol with suitably situated Ar–OH groups is oxidized to the corresponding quinone, and the reduction product, i.e., bis(neocuproine)Cu(I) [Cu(I)-Nc] chelate, shows absorption maximum at 450 nm. It should be noted that not all phenolic –OH are reduced to the corresponding quinones, and the efficiency of this reduction depends on the number and position of the phenolic –OH groups as well as on the overall conjugation level of the polyphenolic molecule.

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2. DETERMINATION OF HYDROPHILIC AND LIPOPHILIC ANTIOXIDANT CAPACITY: COMMENTS AND RESULTS

Antioxidants have always been of interest to food chemists for prevention of rancidity. Later, they have become of interest to biologists and clinicians due to their ability to protect the human body against damage by ROS/RNS. Antioxidants are more than chain-breaking inhibitors of lipid peroxidation. TAC is a parameter frequently used for characterisation of food products and of the antioxidant status of the body. TAC is a sum parameter, combining the additive/synergistic effects of a variety of different single antioxidants. However, this parameter does not include antioxidant enzymes (e.g., catalase, superoxide dismutase). Thus, Bartosz [4] suggested the term "non-enzymatic antioxidant capacity" (NEAC).

During the last decade, many analytical methods were developed to determine the antioxidant activity/capacity in vitro, measuring the ability to reduce oxidant species/probes or to scavenge free radicals. Eight common methods to determine the hydrophilic antioxidant activity were compared looking at four standard antioxidants. The results were not comparable. Analysing 12 food additives and 6 secondary plant products on their antioxidant activity by using 3 different test systems resulted in differences depending on the assay. All the food additives showed antioxidant activities comparable to the calibration substance Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid). In contrast, the secondary plant products had an up to 16 times higher antioxidant potential. To look for the influence of the sample concentration on the measured hydrophilic antioxidant capacity, 4 pure substances and 3 food extracts were analysed using 7 common antioxidant capacity assays. For all applied pure substances and in most of the assays, effects of the sample concentration on the measured antioxidant capacity were observed. Investigating the lipophilic antioxidant activity, often solutions of analytes in methanol (MeOH) or ethanol (EtOH) are used. To dissolve also hydrocarbons as, e.g., the carotenes β-carotene and lycopene, common test systems to determine the hydrophilic antioxidant activity were modified. Analysing several carotenoids in 4 test systems showed lipophilic antioxidant activities being dependent on the number of conjugated double bonds, presence of β-ionone ring, number of hydroxyl groups, and number of keto groups. Thus, determination of hydrophilic and lipophilic antioxidant activity/capacity was observed to be affected by various parameters. Until now, results can only be used to rank different compounds or food extracts in one laboratory within one assay.

Numerous protocols have been proposed to determine the antioxidant activity/capacity. Six assay methods investigated use radicals [DMPD (*N*,*N*-dimethyl-*p*-phenylenediamine), DPPH, ORAC, PCL (photochemoluminescence) assay, TEAC, and TRAP], and two use metal ions (FRAP and LDL oxidation) as oxidising agents. Table 1 shows the assays with the radicals or oxidants used.

Table 1 Assays and their radicals/oxidants as well as the measurement principles.

Assay	Radical or oxidant	Measurement principle	Measurement media/ instrument
DMPD	$DMPD^{\bullet+}$	absorbance (505 nm)	cuvettes/spectrophotometer
DPPH	$DPPH^{\bullet}$	absorbance (515 nm)	cuvettes/spectrophotometer
FRAP	Chelated Fe^{3+}	absorbance (595 nm)	microplates/microplate reader
LDL oxidation	Cu^{2+}	absorbance (234 nm)	cuvettes/spectrophotometer
ORAC	$AAPH^{\bullet}$	fluorescence	microplates/microplate reader
PCL	$O_2^{\bullet-}$	chemiluminescence	reaction tubes/Photochem® (luminometer)
TEAC	$ABTS^{\bullet+}$	absorbance (734 nm)	cuvettes/spectrophotometer
TRAP	$AAPH^{\bullet}$	fluorescence	cuvettes/fluorimeter

Five of the assays (LDL oxidation, ORAC, PCL, TEAC, and TRAP) determine the delay in oxidation and use the lag phase or the area under the curve (AUC) as the parameter quantitating antioxidant activity. In contrast, four methods determine the ability of antioxidants to reduce radical cations

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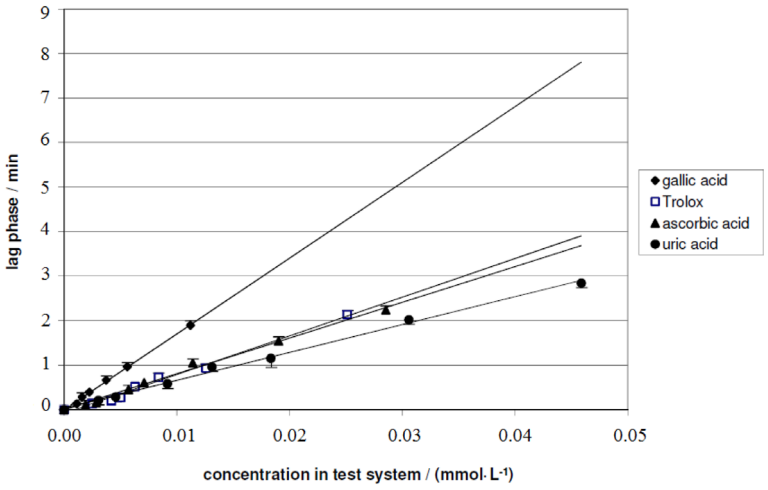


Fig. 1 Lag phases [min] of the four standard antioxidants; AA, gallic acid, Trolox, and uric acid, using different concentrations in the TEAC assay [10].

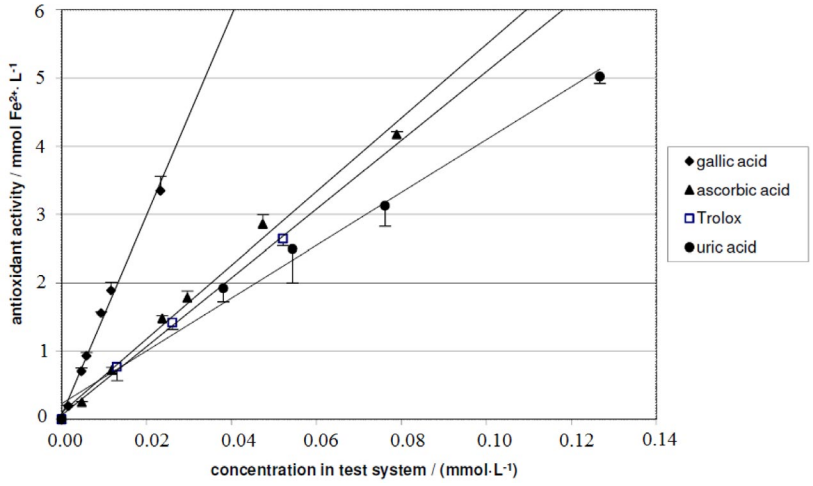


Fig. 2 Antioxidant activity (expressed as mmol·L^{-1} Fe^{2+} equivalents) of the four standard antioxidants AA, gallic acid, Trolox, and uric acid using different concentrations in the FRAP assay [20].

(DMPD, DPPH, and TEAC) or chelated metal ions (FRAP). Comparison of the lag phases of the four standards in the TEAC assay resulted in similar curves for Trolox and ascorbic acid (AA), while uric acid showed a much lower slope and gallic acid a much higher one (Fig. 1). A comparable behaviour of the four compounds was seen in the FRAP assay (Fig. 2).

These results of TEAC and FRAP assays showed gallic acid as the most active antioxidant of the compounds investigated. In contrast, uric acid was a very weak antioxidant in both assays. Uric acid is the major contributor to the TAC of human blood plasma, with contributions between 33 and 53 % as was summarised by Bartosz [4] from various test systems. Another observation from the comparison of methods [20] was to see lag phases for uric acid of a few minutes in the TEAC and PCL assays, while the LDL oxidation assay and the TRAP assay resulted in lag phases of hours, being a hint for different reaction kinetics. In conclusion, the results from different assays were not comparable. They can, however, give an idea of the protective potential of phytochemicals and plant food. For an adequate assess-

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ment of antioxidant power in biological systems, the use of more than one method is highly recommended. In clinical laboratories, they can be a first orientation for diagnosis of oxidative stress. However, they do not include antioxidative and oxidant-regenerating enzymes in blood, cells, and tissues [4].

Another investigation [21] compared 12 food additives and 6 secondary plant products by using the 3 test systems FRAP, TEAC, and PCL. Nine of the 18 compounds were analysed on their hydrophilic antioxidant activity (Fig. 3) using the FRAP assay, the TEAC assay (lag phase), and the hydrophilic version of the PCL (ACW: water-soluble substances) assay. In addition, 12 compounds were analysed on their lipophilic antioxidant activity (Fig. 4) using a lipophilic version of the TEAC assay (reduction of preformed $ABTS^{\bullet+}$) and the lipophilic version of the PCL (ACL: lipid-soluble substances) assay.

Within the hydrophilic assays TEAC and PCL, the regularly used antioxidants AA, isoascorbic acid, and sodium ascorbate showed Trolox equivalents (TEs) in the same order of magnitude. Results of both assays were well correlated ($r = 0.82$). Calcium ascorbate resulted in higher TE values, depending on the different molecular structure. FRAP values were higher for all food additives compared to the already mentioned two test systems. The secondary plant products gallic acid, caffeic acid, and rosmarinic acid resulted in high antioxidant activities in all assays used. Especially rosmarinic acid was a very good antioxidant. The results for the regularly used lipophilic tocopherols and octyl gallate as well as propyl gallate (PG) were in a comparable order of magnitude for the lipophilic assays TEAC and PCL, results of both assays being well correlated ($r = 0.94$). The two gallates showed higher activities in both assays than all other food additives investigated. As already shown in the hydrophilic test systems, the secondary plant products also were better antioxidants in the lipophilic methods. Eugenol, quercetin, and rosmarinic acid, exemplarily used as lipophilic antioxidants, resulted in large differences between the two assays. Rosmarinic acid was the most active compound in the PCL assay while quercetin was the antioxidant with the highest activity in the TEAC assay. The secondary plant products had an up to 16 times higher antioxidant potential. This might present a good reason for the food industry to use natural antioxidants instead of synthetic ones to get storage stability for processed food items, which, according to recent surveys, is in the interest of consumers.

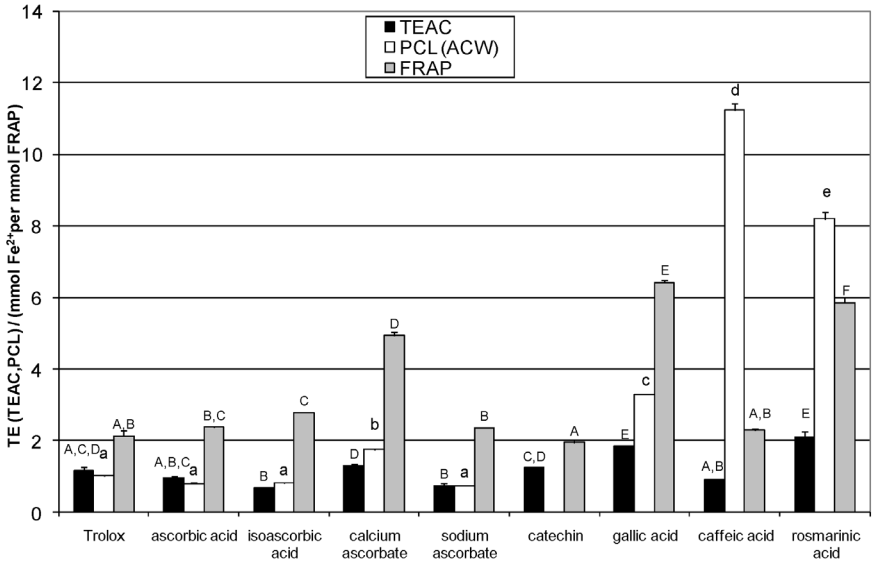


Fig. 3 Hydrophilic antioxidant activity of food additives and secondary plant products in the FRAP, TEAC, and PCL (ACW) assays. Bars for the same assay with different superscript letters are significantly different, $p < 0.05$ [21].

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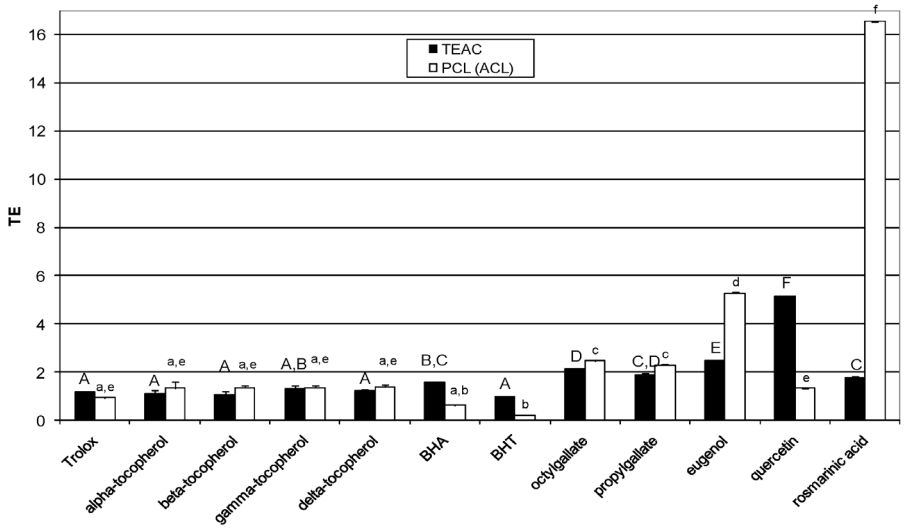


Fig. 4 Lipophilic antioxidant activity of food additives and secondary plant products in the TEAC and PCL (ACL) assays. Bars for the same assay with different superscript letters are significantly different, $p < 0.05$ [21].

To look for the influence of the sample concentration on the measured hydrophilic antioxidant capacity, pure substances (AA, gallic acid, Trolox, and uric acid) and food extracts (strawberry nectar, tomato extract, and white tea) were analysed using the following seven common antioxidant capacity assays: three versions of the TEAC assay (1: lag phase, 2: MnO_2-preformed $ABTS^{\bullet+}$, 3: $K_2S_2O_8$-preformed $ABTS^{\bullet+}$); FRAP assay; PCL assay; ORAC assay; and total phenolics (TP) assay: Folin–Ciocalteu. These investigations were made to evaluate the reproducibility of antioxidant activity/capacity results. Table 2 gives an overview of all results.

Table 2 Influence of increasing sample concentration of standard substances and food extracts on antioxidant capacity (different assays) and content of TP [22].

Antioxidant	FRAP	ORAC	PCL	TEAC 1	TEAC 2	TEAC 3	TP
Ascorbic acid	↔	↑	↔	↔	↔	↔	↑
Gallic acid	↔	↕	↑	↔	↓	↓	cal
Uric acid	↑	↕	↑	↔	↓	↓	↔
Trolox®	↔	cal	cal	cal	cal	cal	↓
Strawberry nectar	↔	↓	↑	↔	↔	↓	↓
Tomato extract	↔	↑	↑	↔	↔	↓	↕
White tea	↔	↓	↑	↔	↔	↓	↓

↔ constant, ↑ increase, ↓ decrease, ↕ fluctuant, cal = calibration (respective pure substance serving as the standard for calibration), *slight increase/decrease (coefficient of variation over the full sample concentration range was less than 10 %).

For all applied pure substances and in most of the assays, the effects of the sample concentration on the measured antioxidant capacity were observed. The effects of the dilution of sample extract were also shown for the food extracts using ORAC, PCL, TEAC 3, and Folin–Ciocalteu (TP) assays. Since it still remains speculative how sample concentration affects the measured antioxidant activity/capacity exactly, it is strongly recommended to use at least three sample concentrations for analysis to detect and to discuss concentration-dependent effects. Thus, results given in the literature and determined by using

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only one dilution have to be discussed critically. Published data cannot be realistically evaluated without knowing the dilution ratio.

For a long time, reproducible results were achievable only for hydrophilic antioxidants. Some of these hydrophilic assays were also published as lipophilic methods, but the authors mostly used alcohols (MeOH, EtOH) as solvent for the sample. Thus, investigations were possible with more or less lipophilic phenolic compounds as well as with tocopherols and other medium nonpolar molecules. In contrast, for example, carotenoids as hydrocarbons could not be investigated for their antioxidant activity at that time with the exception of lipophilic TEAC assay using hexane as solvent. The measurement in the TEAC assay for lipophilic compounds was made by reaction in a two-phase system (hexane and aqueous buffer) and measuring the absorbance of the aqueous phase exactly after 2 min [23].

To improve the situation for lipophilic antioxidants that are effective in different compartments of the human body compared to hydrophilic antioxidants, many experiments were also done to optimize

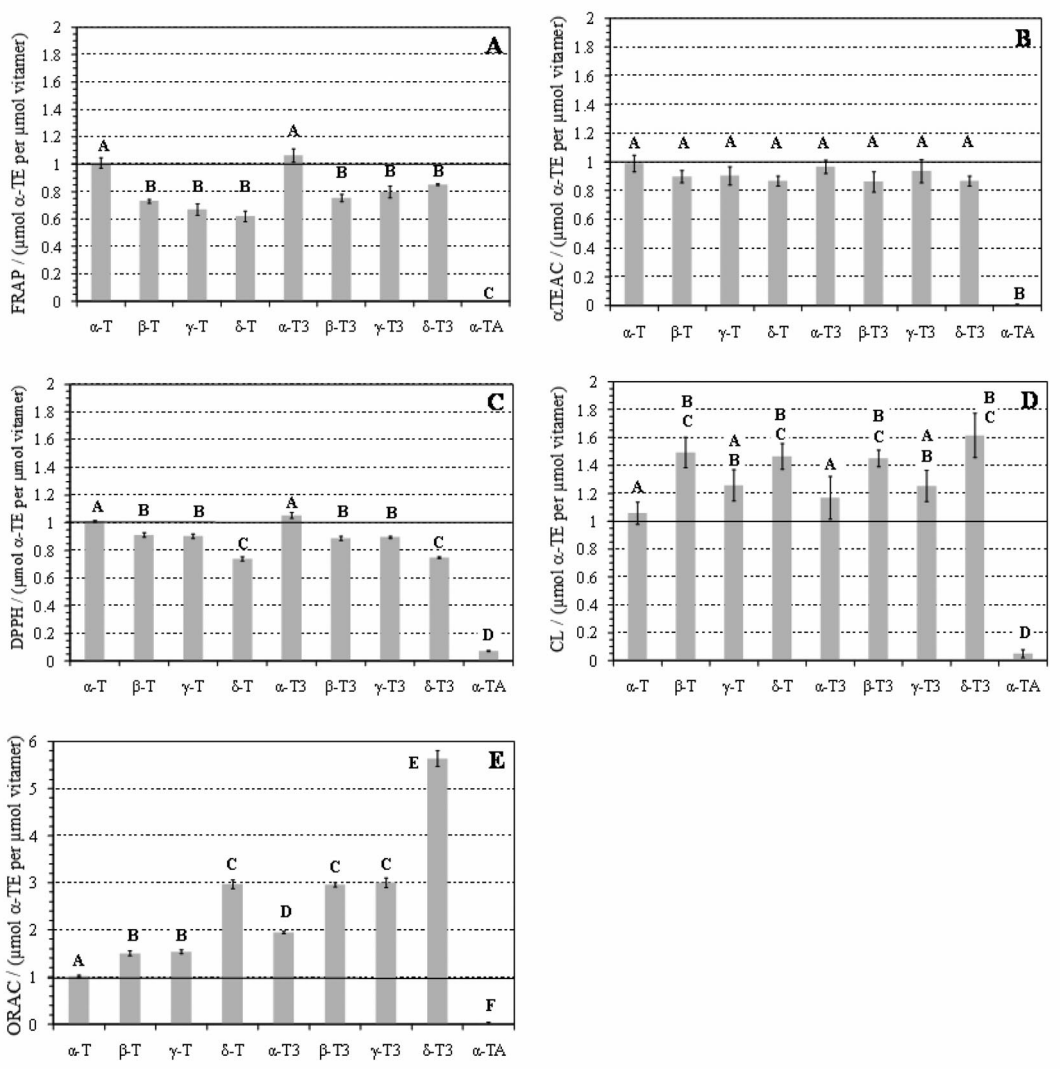


Fig. 5 Antioxidant activity of tocopherols, tocotrienols, and α-tocopherol acetate measured by FRAP assay [A], αTEAC assay [B], DPPH assay [C], CL assay [D], and ORAC assay [E]. Bars with different superscript letters differ significantly ($p < 0.05$) [24].

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existing test systems for really nonpolar compounds (e.g., carotenoids). Firstly, common test systems to determine the hydrophilic antioxidant activity were modified and tested with tocopherols and tocotrienols [24]. Figure 5 shows the results of investigations using FRAP, TEAC, DPPH, chemiluminescence (CL), and ORAC assays.

α-Tocopherol and α-tocotrienol were the most effective antioxidants in the FRAP assay as well as in the DPPH assay. In contrast, the α-vitamers showed the lowest activity compared to the other vitamers in the CL and ORAC assays. Using the TEAC assay did not lead to significant differences in α-tocopherol equivalent (α-TE). α-Tocopheryl acetate showed either none or only a low activity in all test systems investigated due to the blocked OH group in C-6 of the chromanol ring by esterification.

The next step was to analyse different carotenoids on their antioxidant activity by using the modified methods. All assays, successfully used for vitamin E compounds, were tested using 14 carotenoids and for comparison of the 2 antioxidative food additives butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). It was observed that the ORAC assay, a more or less polar test system using randomly methylated β-cyclodextrin (β-CD) as a "vehicle" for the lipophilic compounds, did not work with carotenoids. The DPPH assay, determining the decrease in absorbance at the wavelength of 540 nm, resulted in an overlap with the spectral absorbance of carotenoids as shown in Fig. 6 for the xanthophyll astaxanthin [25]. Thus, this assay is not suitable for carotenoids.

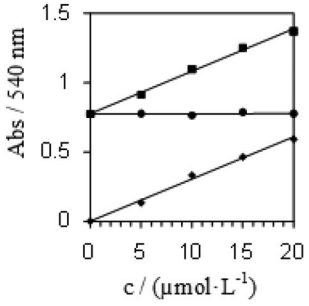


Fig. 6 Scavenging activity astaxanthin measured by DPPH assay [25]. ■ DPPH and astaxanthin; ◆ astaxanthin; ● differential curve of DPPH after subtracting absorbance of astaxanthin.

Use of the TEAC assay (Fig. 7) resulted in increasing antioxidant activity with an improved system of conjugated double bonds. Acyclic structures also improve the activity in this assay. In contrast, the presence of the β-ionone ring led to lower values. Hydroxyl groups as well as keto groups of the xanthophylls also reduce the antioxidant activity.

The number of conjugated double bonds also showed a favourable effect in the FRAP assay (Fig. 8). As was seen in the TEAC assay, the presence of the β-ionone ring significantly decreased the antioxidant activity. In contrast to αTEAC results, the number and position of hydroxyl and keto groups at the β-ionone ring improved the antioxidant activity in the FRAP assay.

The number of conjugated double bonds was also important when using a peroxyl radical-scavenging CL assay, showing that these structural components are closely related to the antioxidant behaviour of carotenoids. In contrast to the other two test systems shown, in the CL assay, the acyclic structure (e.g., of lycopene) led to lower activity (Fig. 9).

Concluding the investigations of carotenoids in the three lipophilic test systems (αTEAC, FRAP, and CL assays), these assays can be used for hydrocarbons, with the lipophilic antioxidant activities being dependent on the number of conjugated double bonds, presence of β-ionone ring, number of hydroxyl groups, and number of keto groups.

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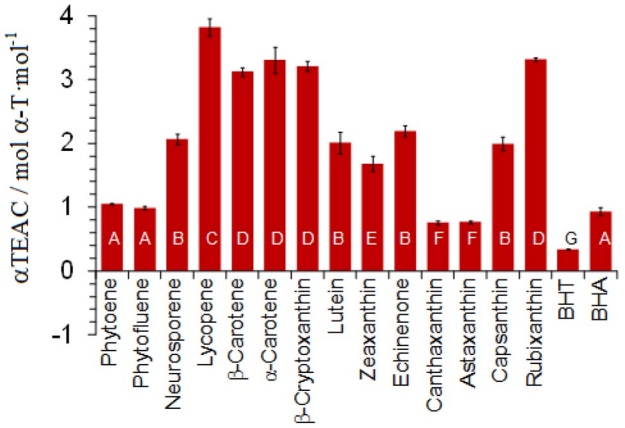


Fig. 7 Antioxidant activity of carotenoids as well as of BHT and BHA measured by αTEAC assay. Bars with different letters differ significantly ($p < 0.05$) [25].

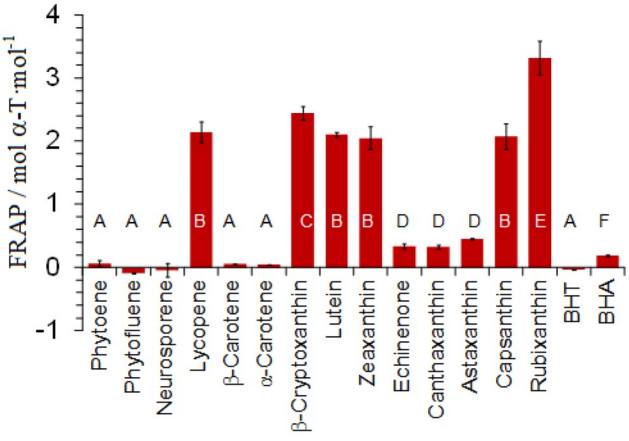


Fig. 8 Antioxidant activity of carotenoids as well as of BHT and BHA measured by FRAP assay. Bars with different letters differ significantly ($p < 0.05$) [25].

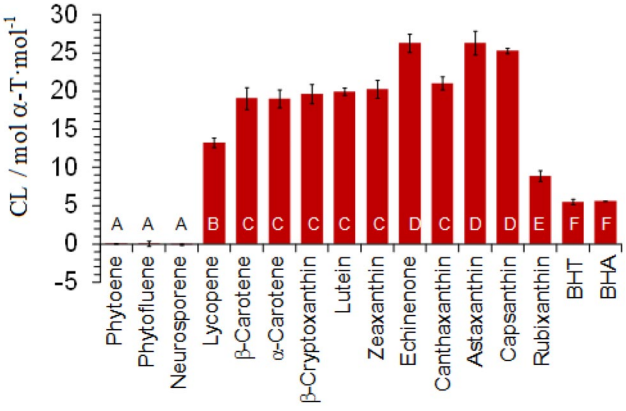


Fig. 9 Antioxidant activity of carotenoids as well as of BHT and BHA measured by CL assay. Bars with different letters differ significantly ($p < 0.05$) [25].

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3. CRITICAL CONSIDERATIONS IN ORAC, TRAP, TEAC/ABTS, AND DPPH ASSAYS OF ANTIRADICAL ACTION

$DPPH^{\bullet}$ quenching reactions have been used to elucidate structural effects on chemical mechanisms and reactivity of simple phenols since the late 1950s [26–29]. Antioxidant activity assays for complex polyphenols were initiated in a 1985 pivotal study in which Wayner et al. used the water-soluble azide azobis (2-amidinopropane hydrochloride) to generate radicals in plasma, then followed inhibition of oxygen consumption to investigate compounds contributing to antioxidant capacity [30]. This first TRAP assay responded to new data documenting the role of radicals in oxidative pathologies and ignited a veritable explosion of antioxidant assays seeking to identify compounds with radical-scavenging abilities, measure their activity, and compare effectiveness of different compounds and materials. As we now try to deal with an ever-increasing number of assays, information that nearly every natural material has active antioxidant compounds, and accumulating evidence that most polyphenol antioxidant in foods are poorly absorbed and rapidly conjugated and eliminated, it is instructive to revisit this original and compare it with current approaches. Two key differences include:

(a) focus on endogenous antioxidants
(b) primary endpoint—oxygen consumption—rather than secondary target

Over the past 25 years, antioxidant assays have shifted considerably from this initial assay focused on elucidating endogenous activity to massive efforts to screen large numbers of biological materials for free radical scavenging activity that presumably will signal potential for in vivo preventative or therapeutic effects, namely, to identify superfoods and super-nutraceuticals that should be consumed for improved health. The zeal for finding the new "fountain of youth" has led scientists to set up assays for fast and easy results while ignoring critical aspects of the basic chemistry in the assays and the biochemistry of the antioxidants. Widespread use of antioxidant assays as rapid screening tools rather than as chemical reactions to measure kinetics and determine mechanisms is largely responsible for the inconsistencies, inaccuracies, and controversies in the scientific antioxidant literature, in medicine, and in the popular press.

Concerns about the design and use of in vitro antioxidant assays were raised in 2000 by internationally recognized lipid scientists, Frankel and Meyer [31]. In 2004, the First International Congress on Antioxidant Methods, cosponsored by the American Chemical Society, the American Oil Chemists' Society, the American Association of Official Analytical Chemists, and the Institute of Food Technologists, formally recognized problems in the broad variations in assay mechanisms and chemistry, differentiated issues with in vitro and in vivo assays, and developed two white papers with initial recommendations for use and standardisation of in vitro assays [32,33]. Since then, the debate has continued in numerous sessions in professional meetings, but no consensus has yet been developed.

3.1 Current status

Developed for ease of use and rapid screening of large numbers of materials, most current antioxidant assays have both conceptual and technical limitations. Conceptual issues raise questions about the rationale and design of the antioxidant assays, including:

- All assays in current use were designed on the assumption that antioxidant action in vivo proceeds by the same free radical scavenging shown in solution, yet in vivo radical scavenging associated with absorbed antioxidants (as opposed to in vitro cell culture) has not been demonstrated.
- Very low bioavailability, absorption, distribution, and unknown metabolism of antioxidants impose severe limitations on what reactions these compounds can mediate competitively in vivo; indeed, low absorption may render the chemistry measured in the assays irrelevant outside of the gastrointestinal tract.

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- Polyphenols are chemically reactive and undergo reactions other than radical scavenging, one of which is binding to proteins; these are active in vivo and must be considered when designing test diets and applications.
- Most current in vitro assays measure inaccurate chemistry in concentration ranges (both absolute and relative) many orders of magnitude higher than ever seen in vivo.
- Some radical assays use molecular targets (e.g., sterically hindered >N$^{\bullet}$) that do not represent chemistry of in vivo targets.
- Reaction times in assays run from 4 minutes to many hours, while the lifetimes of oxygen radicals normally being combated in vivo and in foods are very short [34]:

Radical (10^{-3} M, 37 °C)	Lifetime
HO$^{\bullet}$	10^{-9} s
L$^{\bullet}$ (lipid alkyl)	10^{-8} s
RO$^{\bullet}$	10^{-6} s
AnOO$^{\bullet}$ (arachidonic acid)	10^{-5} s
ROO$^{\bullet}$	10 s

Consequently, the relevance of following reactions over long times to detect slow reactors, "full reactivity", or activity of secondary products is highly questionable at best. In truth, most long-time assays measure molecular migration and reorientation rather than radical quenching.

3.2 ORAC assays

3.2.1 Experimental approach

The ORAC assay is based on the work of Glazer [35], Ghiselli et al. [36], and Cao et al. [37]. The basic assay uses a bis azide initiator such as AAPH [2,2'-azobis(2-amidinopropane) dihydrochloride] to generate peroxyl radicals when heated in the presence of sufficient oxygen. The peroxyl radicals then react with a fluorescent probe to quench its fluorescence. The reduction in fluorescence is followed optically, and antioxidant activity is determined by slowing of the fluorescence loss in presence of antioxidant. Assays may be performed manually with single samples in cuvettes, or they may be automated in microplates.

The original ORAC assay was developed using the fluorescent protein β-phycoerythrin (B-PE) as the radical target [35–38]. The assay was automated with a Cobas Fara II centrifugal analyser [39] and applied to measure antioxidant capacity of tea and fruits [40], oat extracts [41], and vegetables [42]. Later, due to problems with reagent inconsistency, light sensitivity, and binding of polyphenols, B-PE was replaced with fluorescein (FL: 3',6'-dihydroxyspiro[isobenzofuran-1[3H],9'[9H]-xanthen]-3-one) [43].

Radical-quenching effects are reflected in increased lag times before active decay of the probe, decreased rates of decay, or both. As an analytical tool, lag times are problematic since they are not always present, they are difficult to determine accurately, and many different methods for determining the point of initial reaction make comparing data difficult. To overcome these limitations and to fully account for effects that extend beyond early stages of oxidation (e.g., redox cycling, potential effects of secondary antioxidant products, slow antioxidant reactions), the ORAC assay replaces lag times with comparisons of net integrated areas under fluorescence decay curves. AUCs of test samples are converted to Trolox (a water-soluble congener of tocopherol) equivalents by comparison to a standard curve prepared from the AUCs determined over a range of Trolox concentrations. Alternatively, TEs of the sample can be calculated from AUCs using the following relationships [43]:

$$\text{ORAC (U/mL)} = [(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}})] \cdot \text{M}_{\text{Trolox}}/\text{M}_{\text{sample}} \quad (9)$$

where M is the molar concentration (molarity) in mol·L^{-1}.

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$$\text{ORAC (U/mL)} = 50\ \text{(dilution)}\ (\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}}) \quad (10)$$

when molarity is unknown.

In addition to the original version that detects $ROO^{\bullet}$ reactions, ORAC assays have been modified for detection of $^{\bullet}OH$ [44] and other radicals by modifying the initiators, and for detection of lipophilic antioxidants [45] by encapsulating (and thus solubilising) these compounds in randomly methylated β-CD. High-throughput assays using plate readers [46] have been applied to a broad range of plasma, biological materials, and foods, for example [47–49], to provide the basis for a database of activities compiled by the U.S. Department of Agriculture [50]. All versions of the ORAC assays have been commercialized by Brunswick Labs, Wareham, MA, USA.

3.2.2 Basis for calculating and expressing results

Multiple methods for expressing results are some of the most troublesome aspects of all the antioxidant assays, but particularly for ORAC. The equation defining ORAC units

$$\text{ORAC U} = [(\text{AUC}_{\text{sample}} - \text{AUC}_{\text{blank}})/(\text{AUC}_{\text{Trolox}} - \text{AUC}_{\text{blank}})] \cdot \text{M}_{\text{Trolox}}/\text{M}_{\text{sample}} = \text{mM TE} \quad (11)$$

is straightforward when normalising pure known compounds, but when relating TE to extracts with unknown composition and concentration it becomes the source of one of the most serious limitations of the ORAC assay. ORAC units = mmol TE per what? Which of these units should be used—mL, L, g or kg fresh weight (FW), g or kg dry weight (DW), 100 g serving, average size serving, mol phenol?

A reference base is clearly required for all ORAC values, but it must also be appropriate for the product. Because so much attention has been given to absolute ORAC values in the popular press, it has become common practice to express ORAC values on whatever basis will provide the largest numbers. However, if the base does not match how a food is consumed (e.g., citing liters of juice or 100 g of spices), ORAC values have no meaning or relevance; they are confusing at best and deliberately misleading or fraudulent at worst. Similarly, when ORAC values are based on weight, dry foods always have higher ORAC values than high-moisture or fresh foods, yet this distinction is usually ignored in presenting and interpreting data. For example, dry cinnamon has the highest ORAC value (267 000) in Table 3, dried beans are more than an order of magnitude lower (about 12 000), and (high-moisture) fresh fruits sit at the bottom with 3000–6000. Taken at a glance, the table leaves the impression that fresh fruits known to have high antioxidant activity are poor actors in relation to dried beans and to more cinnamon than usually goes into a full recipe. Expressing these foods all on a common DW basis (e.g., per gram DW) would present a more accurate comparison.

Even more troublesome is the practice of citing ORAC values without units or base, for example, the ORAC values in Table 4 were taken from an Internet website. The list was entitled "high ORAC foods" with no reference base or sample size cited and no information about analyses provided. This kind of flagrant misuse of ORAC values is all too common and must be stopped.

Normalising to a useful weight or volume gives numbers for comparison, but it is still unclear what these numbers connect to. This raises two important issues with interpreting or using ORAC values—what do they mean chemically, and what do they mean nutritionally? Neither question can currently be answered.

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Table 3 Selected listings from USDA data on ORAC values of common foods*.

Food	Serving size	ORAC per serving (μmol TE·100 g^{-1})
Cinnamon, ground	100 g	267536
Aronia black chokeberry	100 g	16062
Small red bean	½ c. dried beans	13727
Wild blueberry	1 c.	13427
Red kidney bean	½ c. dried beans	13259
Pinto bean	½ c.	11864
Blueberry	1 c. berries	9019
Cranberry	1 c. berries	8983
Artichoke hearts	1 c., cooked	7904
Blackberry	1 c. berries	7701
Prune	½ c.	7291
Raspberry	1 c.	6058
Strawberry	1 c.	5938
Red Delicious apple	1 apple	5900
Granny Smith apple	1 apple	5381
Pecan	1 oz.	5095
Sweet cherry	1 c.	4873
Black plum	1 plum	4844
Russet potato	1 cooked	4649
Black bean	½ c. dried beans	4181
Plum	1 plum	4118
Gala apple	1 apple	3903

*http://www.ars.usda.gov/SP2UserFiles/Place/12354500/Articles/AICR07_ORAC.pdf

Table 4 ORAC values for high antioxidant foods.

High antioxidant food item	ORAC value
Spices, cloves, ground	314446
Sumac, bran, raw	312400
Spices, cinnamon, ground	267536
Sorghum, bran, hi-tannin	240000
Spices, oregano, dried	200129
Acai berry, freeze-dried	161400
Spices, turmeric, ground	159277
Sorghum, bran, black	100800
Sumac, grain, raw	86800
Cocoa, dry powder, unsweetened	80933
Spices, cumin seed	76800
Maqui berry, concentrated powder	75000
Spices, parsley, dried	74349
Sorghum, bran, red	71000
Spices, basil, dried	67553

Chemically, are high ORAC foods also high in polyphenols and are these responsible for the reactivity, or are other molecules also involved? Twenty-five years of screening has shown that most fruits, vegetables, herbs, spices, and leaves contain active antioxidants that react to give ORAC values. Now,

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it is time to shift emphasis to elucidating the compounds responsible and the mechanisms involved in the reactions that yield ORAC curves.

What ORAC values mean nutritionally is a more difficult question to answer. The U.S. Department of Agriculture recommends consumption of 3000–5000 ORAC units/day in foods. 5000 ORAC units can be provided by one Granny Smith apple or 1 oz pecans, which seem to be very little considering the hype about antioxidant roles in slowing aging and preventing disease. Looking at 5000 ORAC units from a different perspective, assuming that μmoles Trolox are equivalent to μmoles tocopherol:

5000 ORAC units ≅ 5 mmol vitamin E = 2153 mg vitamin E

1 IU vitamin E = 0.67 mg α-tocopherol

2153 mg tocopherol = 3230 IU TE

In contrast to the apple example, this analysis make 5000 ORAC units seem excessive since 30 IU tocopherol is needed daily to prevent deficiency and 400 IU tocopherol is the maximum recommended supplement level. Another way to interpret the data is that one apple has antioxidant action equivalent to 8 standard 400 IU vitamin E capsules daily. Neither situation is believable or realistic.

Taking still a third perspective, expressing ORAC values on the original mM Trolox basis, an ORAC value of 1000 would be needed to provide the same protection as 1 mol α-tocopherol; on the current μmol equivalent basis, ORAC value of 1 000 000 would be needed for this equivalent action. In this context, no antioxidants in natural materials match the activity of the most important endogenous antioxidant, tocopherol. Given these considerations, using ORAC values to make dietary decisions is unwarranted and perhaps even unwise.

3.2.3 Recommendations for standardisation and use of ORAC assays

- Shift focus of reaction from screening to elucidating antioxidant reaction characteristics.
- Replace FL with a target not susceptible to fluorescence quenching, side reactions, and interactions.
- Require blanks with extracts plus fluorescein, and also excitation/emission spectra for FL, extract, and combinations of the two with a range of extract concentrations (without azide) to identify interfering interactions.
- Test a full range of antioxidant concentrations to develop rate constants, determine concentration dependence of reactivity including threshold for activity, and identify potential prooxidant concentration ranges.
- Information needed before standard protocols can be developed.
  - systematic study of fluorescein (FL): AAPH ratio effects on reaction characteristics to determine FL concentrations where quenching cannot occur and obscure antioxidant effects, effect of azide and FL concentrations on the rates and completeness of ORAC reactions, and optimum conditions for running reactions.
  - systematic study of the effect of dissolved oxygen levels on reaction rates
  - determination of minimum oxygen levels required for efficient reaction
  - development of analyses and methods to ensure adequate oxygen in reactions
- Develop guidelines for monitoring and accurately controlling temperature during ORAC reactions.
- Develop standards for plate reader performance in antioxidant activity assays, incorporate these standards into official protocols, and award certification to force manufacturers to redesign instrumentation appropriately.
- Develop one program for AUC calculations and make it available internationally to all users; alternatively, develop step-by-step directions for AUC calculations using commonly available computer programs such as Excel and include in standard protocols.

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- Establish mandatory units and bases for sample size and form (dry vs. wet weight) to be used when expressing results.
- Require compositional analysis of all extracts (total phenol content at a minimum) for comparison with ORAC to begin developing activity profiles.
- Conduct ORAC assays of natural product extracts before and after binding of phenols by polyvinylpyrrolidone to determine contributions of nonphenolic compounds to antioxidant activity of extracts.

3.3 TRAP assays

Based on the original studies of Wayner et al. [30,51,52], TRAP assays measure antioxidant ability to interfere with the reaction between peroxyl radicals and a target probe. TRAP assays are variants of ORAC assays in principle, but they use a broader range of initiators, probes, and endpoint measurements (including those used in ORAC and TEAC assays). Initiating radicals have been generated selectively by azides such as AAPH {ABAP or [2,2'-azobis(2-amidinopropane) dihydrochloride]} [30,51,52], enzymes such as horseradish peroxidase [53], or by H_2O_2-hemin [54], $NO^{\bullet}$ [55], or singlet oxygen [56]. Probes used for TRAP assays include dichlorofluorescein diacetate [57], FL [56], phycoerythrin [58,59], luminol [53], and ABTS [60].

3.4 TEAC assays

3.4.1 Experimental approach

TEAC assays use intensely coloured cation radicals of ABTS to test ability of antioxidants to quench radicals. The original assay developed by Miller and Rice-Evans [61,62] (sometimes referred to as TEAC I) utilized metmyoglobin-H_2O_2 to generate $HO^{\bullet}$, which then reacted with ABTS to generate the cation radical, $ABTS^{\bullet+}$. However, quantitating antioxidant effects were equivocal because antioxidants could react with the original radical oxidant as well as the $ABTS^{\bullet+}$, causing an overestimation of antioxidant activity [63]. The assay has been revised to cleanly generate $ABTS^{\bullet+}$ by first using oxidising agents such as potassium persulfate [64] and manganese dioxide [65,66], then adding antioxidants and measuring direct reaction with the radical. In variations of this approach, laccase [67], $Br_2^{\bullet-}$ [68], H_2O_2 + horseradish peroxidase [69,70], and peroxyl radicals [71] have also been used as oxidants. Of all these, persulfate oxidation is preferred for its high $ABTS^{\bullet+}$ yields and radical/antioxidant inertness. This format (TEAC II) forms the basis for current TEAC assays.

TEAC II assays are quite simple to perform. Stock solutions of concentrated $ABTS^{\bullet+}$ are generated and stored in the refrigerator (stable for several months) [64]. For reaction, the stock solution is diluted with water or buffer to a workable absorbance, for example, A = 1.0, at 734 nm, initial absorbance of the $ABTS^{\bullet+}$ solution at 734 nm is recorded, the antioxidant is added, and the drop in absorbance is measured after reaction periods varying from four minutes to several hours.

TEAC values are calculated from the ratio of test compound reaction (measured as inhibition) to that of Trolox reaction. In concept,

$$\text{TEAC value (unitless)} = \frac{\text{Inhibition by test compounds}}{\text{Inhibition by trolox}} \tag{12}$$

In practice, TEAC has been calculated in several ways, which creates problems in comparing data between laboratories.

3.4.2 Constraints and challenges

Overall, TEAC assays offer many advantages that contribute to its widespread popularity in screening antioxidant activities of a wide range of materials. TEAC is operationally simple, reactions are rapid (most methods use 30 min or less) and run over a wide range of pH. $ABTS^{\bullet+}$, being a singly positive

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charged cationic radical, is soluble in both aqueous and organic solvents and is not affected by ionic strength, so it has been used in multiple media to determine both hydrophilic and lipophilic antioxidant capacity. Reactions can be automated and adapted to microplates [72,73] as well as to flow-injection [74] and stopped-flow methods.

However, TEAC also has some major disadvantages. The high extinction coefficient of $ABTS^{\bullet+}$ (1.50×10^4 $L \cdot mol^{-1} \cdot cm^{-1}$ at 734 nm [64]) limits the useful antioxidant concentration range that can be analysed accurately to about 1.5–70 μM final concentrations. Antioxidant concentrations outside this range require too much or too little $ABTS^{\bullet+}$ for accurate optical measurements. In addition, as with ORAC assays, many reaction factors must be carefully controlled for reproducible reactions that can be compared between laboratories, as follows:

- the oxidising agent used for $ABTS^{\bullet+}$ generation
- pH and time of $ABTS^{\bullet+}$ conversion
- buffer and pH control agents
- wavelength for monitoring $ABTS^{\bullet+}$
- age of $ABTS^{\bullet+}$ solution
- storage conditions
- concentrations of $ABTS^{\bullet+}$ and antioxidant
- temperature of assay
- time for reaction between $ABTS^{\bullet+}$ and antioxidant
- solvent phase, phase equilibria
- oxygen concentrations when AA or other strong reducing agents are present
- calculation method(s)

Though all the issues are important for standardisation, this evaluation will focus only on $ABTS^{\bullet+}$ issues, reaction monitoring and timing, molecular size and steric accessibility, and calculation/reporting methods, all of which affect the validity of the assay.

3.4.3 Evaluation and recommendations for TEAC/ABTS$^{macrocyclizatio+}$ assay

Although the TEAC assay has been widely used in multiple forms, its usefulness and accuracy are being questioned. We recommend that TEAC should be used with care as a routine assay for measuring relative antioxidant activity of the different classes of phenols and extracts with mixed antioxidants as well as for predicting in vivo protective effects for the following reasons:

- Reactivity with $ABTS^{+\bullet}$ appears to be controlled first and foremost by steric accessibility of phenolic –OH groups to the $ABTS^{+\bullet}$ radical site rather than by chemical properties of test antioxidants.
- $ABTS^{+\bullet}$ reactions are complex and difficult to interpret in terms of antioxidant mechanism.
- As currently performed, the assay reports stoichiometry but does not distinguish differences in reaction rates, which are the controlling factor in real-life applications.
- The assay cannot be used for construction of SARs (structure–activity relationships) or for accurate ranking of antioxidants, even within structural classes, for all the reasons listed above.
- Nonspecific side reactions are common.
- Chemistry of the $N^{\bullet}$ in $ABTS^{+\bullet}$ does not accurately model radical reactions in foods or biological tissues.
- Compounds that contribute strongly to in vivo antioxidant action, including proteins and glutathione (GSH), react poorly or not at all with $ABTS^{+\bullet}$.

It is recommended that the assay should be modified in a manner similar to that described below for DPPH, and its use should be limited to comparisons of compounds with closely similar structure.

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3.5 DPPH assays

3.5.1 Experimental approach

DPPH is a stable radical with a deep purple colour whose reaction with other radicals, reducing agents, or compounds capable of HAT leads to loss of colour at 515 nm and loss of its electron paramagnetic resonance (EPR) free radical signal [26,29]. Like $ABTS^{+\bullet}$, $DPPH^{\bullet}$ reacts with both electron and hydrogen donors [75,76], though more slowly, and steric accessibility to the radical site is a clear issue [27,28].

No antioxidant assay is simpler or less expensive to run than the DPPH assay, which accounts for its popularity and extensive use. Needed only are the reagent, some cuvettes, and a UV–vis spectrophotometer, the latter of which are found in even the most rudimentary laboratories. DPPH crystals are dissolved in MeOH or EtOH, initial $DPPH^{\bullet}$ absorbance is recorded, an aliquot of the test antioxidant is added, the mixture is incubated for 30 min, and the final absorbance is recorded. Reaction is measured as $(A_0 - A_f)$ and antioxidant activity is reported either as IC_{50} (the antioxidant concentration required to reduce the DPPH absorbance by half) or % loss or original absorbance or EPR signal. The latter is just a number for comparison, the former at least considers some concentration dependence. Both approaches report extent of reaction and ignore reaction rates.

3.5.2 Reaction timing and monitoring

As with $ABTS^{+\bullet}$, before-and-after measurements miss the important kinetics of the reaction and can underestimate fast reactors while giving undue weight to slow reactors. In addition, protocols that attempt to run the reaction to completion with long incubation times emphasize stoichiometry at the expense of reaction rate. This focus is not useful for evaluating and predicting effectiveness of antioxidants because if the reaction rate of putative antioxidants is not comparable to the annealing rates or lifetimes of target radicals, whether $DPPH^{\bullet}$, $HO^{\bullet}$, or lipid radicals, radical quenching will not occur. $DPPH^{\bullet}$ is a stable radical, so slow antioxidant reactions with it may be detectable experimentally. However, the relevance of these slow reactions to quenching of short-lived hydroxyl and lipid radicals in foods and tissues is highly questionable. Even if the rationale for long incubation times is detecting action of antioxidant products, radicals in real systems do not survive long enough to encounter these products, and the products themselves react so do not accumulate to quench radicals as they are formed in reactions. Thus, the orientation of the DPPH assay needs to be changed to determine early processes that are most likely to be active with unstable radicals such as $HO^{\bullet}$, $HOO^{\bullet}$, $LO(O)^{\bullet}$, and $NO^{\bullet}$. This means recording reactions preferably over 4 min and no more than 6–10 min.

Research in chemistry has recognized the mechanistic complexities of this fast reaction component as well as the difficulties measuring it. Hence, pulse radiolysis and stopped-flow methodologies are used routinely to detect the earliest species in fast reactions, including $DPPH^{\bullet}$ [75,76]. Inexpensive stopped-flow syringe assemblies are available, so antioxidant assays using DPPH should be moved to this technology or at least to autodispensing with instantaneous absorbance recording in microplate readers.

3.5.3 Recommendations for standardisation and use of DPPH assays

- The limitations of using a nitrogen-centered radical to model antioxidant reactions with oxyl radicals must be recognized. Accordingly, kinetics and specificity of antioxidant reaction with DPPH should not be expected to match results from other antioxidant assays.
- That reaction of antioxidants is influenced so strongly by, and in some cases is controlled by, steric effects rather than innate chemical characteristics makes this assay unacceptable for screening antioxidant activity of extracts, comparing antioxidants of different structural classes, or comparing extracts of unknown composition and concentration because preference is given to small reactive compounds and large molecule antioxidants are underestimated.
- Recognising these drawbacks, there is nevertheless a very large body of fundamental chemical research on reactions of phenols and other molecules with DPPH, identifying reaction products

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and measuring rates and extent of reaction as a function of structure. With these studies as templates, DPPH reactions can still provide a very useful research tool if reoriented to elucidate antioxidant chemistry rather than screen antiradical activity.

- Current practices of blindly recording absorbance before and after incubation for 30 min or longer should be replaced with kinetic and product analyses following procedures already well established in chemistry. Reactions should be monitored continuously from mixing point, using optical or EPR analyses, and kinetics should be calculated on the first 30 s, except for very slow reactors, where 1- or 5-min endpoints may be substituted for sensitivity. Long time incubations ignore the initial fast reactions and give undue weight to slow reactions, so should be abandoned.
- Plate readers with (accurate and reproducible) autodispensers for introducing DPPH to sample can collect the first data point within about 0.2 s, much earlier than can be accomplished by manual sample transfer. Thus, the use of plate readers for this assay should be encouraged. In addition, consideration should be given to developing standardized assays using stopped-flow techniques to measure the early reaction more accurately.
- DPPH reactions should be run using a full range of antioxidant concentrations covering three orders of magnitude (e.g., 1 mM to 1 μM starting solutions), where the maximum concentration does not greatly exceed the DPPH concentration. "Reactivity" determined using 10–100-fold excess of antioxidant over DPPH is highly questionable, especially when large molecules and extracts of unknown composition are being analysed.
- DPPH reactions should be tested in multiple solvents, at a minimum MeOH (not EtOH, which forms reactive radicals that may interfere with the assay) and MeOH:H_2O (1:1, v/v), where the water phase is variously high purity water, water acidified to pH 5 with Ultrex HCl (or equivalent), and water raised to pH 9 with Ultrex NaOH. These solvents provide initial distinction between ET and HAT mechanisms.
- Each antioxidant should be evaluated for oxygen effects. While DPPH itself is believed not to be affected by oxygen or its reduction product $O_2^{\bullet -}$, oxygen competes for reducing agents and reactive intermediates, removing them from the reaction stream. For example, AA reaction with DPPH is faster when solutions are saturated with nitrogen or argon because less undergoes autoxidation. Kinetic studies of DPPH reactions in chemistry are all performed under nitrogen or argon. This should become standard practice also with antioxidant assays to avoid side reactions.
- The $DPPH^{\bullet}$ reduction product, $DPPH_2$, is a proton donor and hydrogen binder so it inhibits HAT reactions of many phenols and reduces their apparent reactivity. A method for routine detection of this effect in the DPPH assay needs to be developed.
- Since all radical reactions are influenced by trace metals and adventitious contaminants, all antioxidant assays should be run in high-purity distilled, deionized water of 18 MΩ resistivity. In addition, standards should be established for glassware washing to remove metals and other contaminants before these assays.
- Required reporting of reaction controls and reagent blanks should be a component of all standardized protocols.
- Total phenols and full compositional analysis should become standard required practice for all natural extracts being tested for antioxidant activity. This information can be used to build a base for correlating activity with specific compounds, for elucidating synergistic and antagonistic interactions, and for understanding how phenol reactivity may change with reaction environment.

4. CUPRAC ASSAY AS A NOVEL ET-BASED ANTIOXIDANT CAPACITY ASSAY, AND ITS VARIOUS MODIFICATIONS FOR CAPACITY/ACTIVITY MEASUREMENT IN DIVERSE MATRICES (INCLUDING BIOLOGICAL FLUIDS)

The CUPRAC method of antioxidant capacity measurement, introduced by the analytical chemistry laboratory of Istanbul University to world literature [16], is based on the absorbance measurement of

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the CUPRAC chromophore, Cu(I)-neocuproine (Nc) chelate, formed as a result of the redox reaction of antioxidants with the CUPRAC reagent, bis(neocuproine)copper(II) cation [Cu(II)-Nc], where absorbance is recorded at the maximal light absorption wavelength of 450 nm. The CUPRAC method of TAC assay has been successfully applied to antioxidants in food plants, human serum, and to hydroxyl radical scavengers. In the assay of human serum antioxidants, hydrophilic antioxidants were measured after precipitation of proteins with $HClO_4$, while lipophilic ones like α-tocopherol and β-carotene were determined by *n*-hexane extraction, evaporation, followed by colour development in dichloromethane (DCM) of the Cu(I)-Nc charge-transfer complex formed from their CUPRAC reaction [77]. In a miniaturized CUPRAC method without preliminary separation of phases, serum samples were centrifuged after 10 % TCA precipitation, and CUPRAC was directly applied to the supernate. Since essentially flavones and flavonols (and other flavonoids to a lesser extent) could be chelated with lanthanum(III) in the form of basically nonpolar complexes, and AA either did not complex or formed very weak hydrophilic complexes under the same conditions, AA assay with a high redox equilibrium constant of the CUPRAC reaction with preliminary extraction of flavonoids as their La(III) complexes was endeavoured [78]. Lipophilic and hydrophilic antioxidants (e.g., β-carotene, α-tocopherol, AA, quercetin, etc.) could be simultaneously assayed with a modified CUPRAC method in the same solvent medium of acetone-water (9:1, v/v) with the aid of their inclusion complexes formed with 2 % methyl-β-cyclodextrin (M-β-CD) [79]. In measuring the hydroxyl radical scavenging activity of certain water-soluble compounds (metabisulfite, thiourea, glucose, lysine, etc.), the probes of *p*-aminobenzoate, 2,4-dimethoxybenzoate, and 3,5-dimethoxybenzoate were used to detect hydroxyl radicals, and the $^{\bullet}OH$ scavenging rate constants of these compounds were determined by competition kinetics [80]. In the measurement of hydroxyl radical scavenging activities of polyphenolics, special measures were taken so as to prevent the redox cycling of phenolic compounds. In this newly developed method, the Fenton reaction was stopped at the end of the 10^{th} minute with the addition of catalase to degrade hydrogen peroxide and cease $^{\bullet}OH$ production, and the dihydroxybenzoates formed from the salicylate probe under hydroxyl radical attack were measured with the CUPRAC method, rate constants being calculated with competition kinetics [81]. In another modified CUPRAC method, the superoxide anion radical was formed with xanthine–xanthine oxidase (X–XO), and the inhibition of the enzyme was measured upon addition of polyphenolics to the system [82]. The hydrogen peroxide scavenging (HPS) activity of the polyphenolics was measured in the presence of Cu(II) (as catalyst) with the HPS-CUPRAC method [83]. A low-cost optical antioxidant sensor (CUPRAC sensor) was developed by immobilising the Cu(II)-Nc reagent onto a perfluorosulfonate cation-exchange polymer membrane matrix (Nafion®) [84]. A novel on-line HPLC-CUPRAC method was developed for the selective determination of polyphenols in complex plant matrices. This method combines chromatographic separation, constituent analysis, and post-column identification of antioxidants in plant extracts [85]. CUPRAC in urea buffer also responded to thiol-containing proteins in food [86]. Another modified CUPRAC method is comprised of a *tert*-butylhydroquinone (TBHQ) probe with the phenazine methosulphate-β-nicotinamide adenine dinucleotide (PMS-NADH) non-enzymic $O_2^{\bullet-}$ generating system for superoxide radical scavenging activity (SRSA) assay of thiol-type antioxidants (e.g., GSH, cysteine), amino acids (e.g., serine, threonine), plasma antioxidants (e.g., bilirubin, albumin), and other antioxidants (e.g., methionine); the SRSA method is based on the measurement of the CUPRAC absorbance of the remaining TBHQ in the reaction medium (TBHQ is CUPRAC-reactive while its oxidation product is not, and this probe is isolated by ethyl acetate extraction from other CUPRAC-reactive interferents remaining in the aqueous phase) [87].

In conclusion, the CUPRAC methodology is evolving into an "antioxidant measurement package" in biochemistry and food chemistry comprising many assays, and the results are in accordance with those of independent reference methods, having distinct advantages over certain established methods.

The CUPRAC method describes the development of a simple and widely applicable antioxidant capacity assay for flavonoids, phenolic acids, hydroxycinnamic acids, thiols, synthetic antioxidants, and vitamins C and E [5]. The chromogenic oxidising reagent used for the CUPRAC assay is the Cu(II)-Nc

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cation, and the CUPRAC chromophore the Cu(I)-Nc cation (Fig. 10). This reagent is useful at pH 7, and the absorbance of the Cu(I) chelate formed as a result of the redox reaction with reducing polyphenols and vitamins C and E is measured at 450 nm [see Fig. 10, for Cu(I)-Nc spectra obtained with reacting varying concentrations of Trolox with the CUPRAC reagent]. The orange–yellow colour is due to the Cu(I)-Nc charge-transfer complex formed. Liberated protons are buffered in ammonium acetate medium. CUPRAC reactions are essentially complete within 30 min for most food/biological antioxidants.

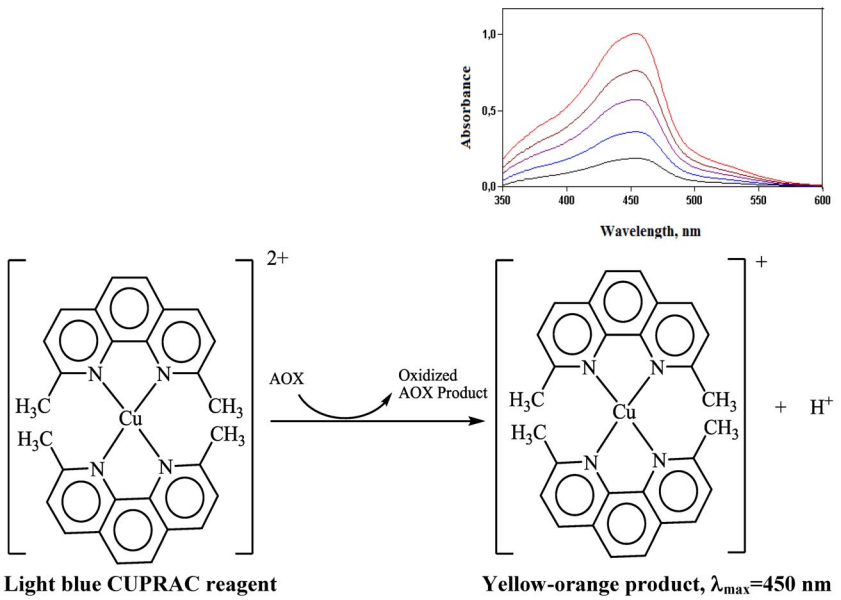


Fig. 10 The CUPRAC reaction and chromophore: Cu(I)-Nc chelate cation (protons liberated in the reaction are neutralized by the NH_4Ac buffer) (Inset figure: Visible spectra of Cu(I)-Nc chelate produced as a result of CUPRAC reaction with varying concentrations of TR).

The chromogenic oxidising reagent of the developed CUPRAC method, i.e., Cu(II)-Nc, reacts with *n*-electron reductant antioxidants (AOX) in the following manner:

$$n\mathrm{Cu(Nc)}_2^{2+} + n\text{-electron reductant (AOX)} \leftrightarrow n\mathrm{Cu(Nc)}_2^{+} + n\text{-electron oxidized product} + n\mathrm{H}^{+} \quad (13)$$

In this reaction, the reactive Ar–OH groups of polyphenolic antioxidants are oxidized to the corresponding quinones (Ar=O) and Cu(II)-Nc is reduced to the highly coloured $\mathrm{Cu(Nc)}_2^{+}$ chelate showing maximum absorption at 450 nm. Although the concentration of Cu^{2+} ions is in stoichiometric excess of that of Nc in the CUPRAC reagent for driving the redox equilibrium reaction represented by (eq. 13) to the right, the actual oxidant is the $\mathrm{Cu(Nc)}_2^{2+}$ species and not the sole Cu^{2+}, because the standard redox potential of the Cu(II/I)-Nc is 0.6 V, much higher that of the Cu^{2+}/Cu^{+} couple (0.17 V) [88]. As a result, polyphenols are oxidized much more rapidly and efficiently with Cu(II)-Nc than with Cu^{2+}, and the amount of coloured product [i.e., Cu(I)-Nc chelate] emerging at the end of the redox reaction is equivalent to that of reacted Cu(II)-Nc. The liberated protons are buffered in ammonium acetate medium. In the normal CUPRAC method ($\mathrm{CUPRAC_N}$), the oxidation reactions are essentially complete within 30 min. Flavonoid glycosides require acid hydrolysis to their corresponding aglycons for fully exhibiting their antioxidant potency. Slow reacting antioxidants may need elevated temperature incubation so as to complete their oxidation with the CUPRAC reagent [16,77]. The CUPRAC antioxidant capacities of a wide range of polyphenolics and flavonoids were experimentally reported as TEAC,

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defined as the reducing equivalent concentration (expressed in the units of mmol Trolox L^{-1}) of 1 mM solution of the tested antioxidant compound. Experimentally, the TEAC values (unitless) were found as the ratio of the molar absorptivity of each compound to that of Trolox in the CUPRAC assay.

The TEAC coefficients of various hydrophilic antioxidant compounds found with the developed CUPRAC method are tabulated in Table 5. The linear calibration curves of the tested antioxidants as absorbance vs. concentration with respect to the CUPRAC method (figures not shown) generally gave correlation coefficients close to unity ($r \geq 0.999$) within the useful absorbance range of 0.1–1.1 [16]. The highest antioxidant capacities in the CUPRAC method were observed for epicatechin gallate, rosmarinic acid, epigallocatechin gallate, quercetin, fisetin, epigallocatechin, catechin, caffeic acid, epicatechin, gallic acid, rutin, and chlorogenic acid in this order, in accordance with theoretical expectations, because the number and position of the hydroxyl groups as well as the degree of conjugation of the whole molecule are important for easy ET.

Table 5 Antioxidant capacities of various polyphenolic compounds (in the units of TEAC as measured by CUPRAC assay) [16,89,90].

	$TEAC_{CUPRAC}$			
Antioxidant	$TEAC_N$	$TEAC_I$	$TEAC_H$	$TEAC_{H\&I}$
Flavonoids				
Epicatechin gallate (ECG)	5.32	5.65		
Epigallocatechingallate (EGCG)	4.89	5.49		
Quercetin (QR)	4.38			
Fisetin (FS)	3.90	4.18		
Epigallocatechin (EGC)	3.35	3.60		
Catechin (CT)	3.09	3.56	3.08	3.49
Epicatechin (EC)	2.77	2.89		
Rutin (RT)	2.56			3.80
Morin (MR)	1.88	3.32		
Kaempherol	1.58	1.87		
Hesperetin (HT)	0.99	1.05	0.85	0.98
Hesperidin (HD)	0.97	1.11	0.79	0.95
Naringenin (NG)	0.05	2.28		3.03
Naringin (N)	0.02	0.13		
Hydroxycinnamic acids				
Caffeic acid (CFA)	2.89	2.96	2.87	3.22
Chlorogenic acid (CGA)	2.47	2.72	1.20	1.42
Ferulic acid (FRA)	1.20	1.23	1.18	1.34
p-Coumaric acid (CMA)	0.55	1.00	0.53	1.15
Vitamins				
α-Tocopherol (TP)	1.10	1.02	0.99	0.87
Ascorbic acid (AA)	0.96			
Benzoic acids				
Gallic acid (GA)	2.62			
Sinapic acid (SNA)	1.24	2.17		
Vanillic acid (VA)	1.24	1.52	1.32	1.57
Syringic acid (SA)	1.12	1.64	1.13	1.67

N: normal measurement; I: incubated measurement; H: hydrolysed measurement; H&I: hydrolysed and incubated measurement

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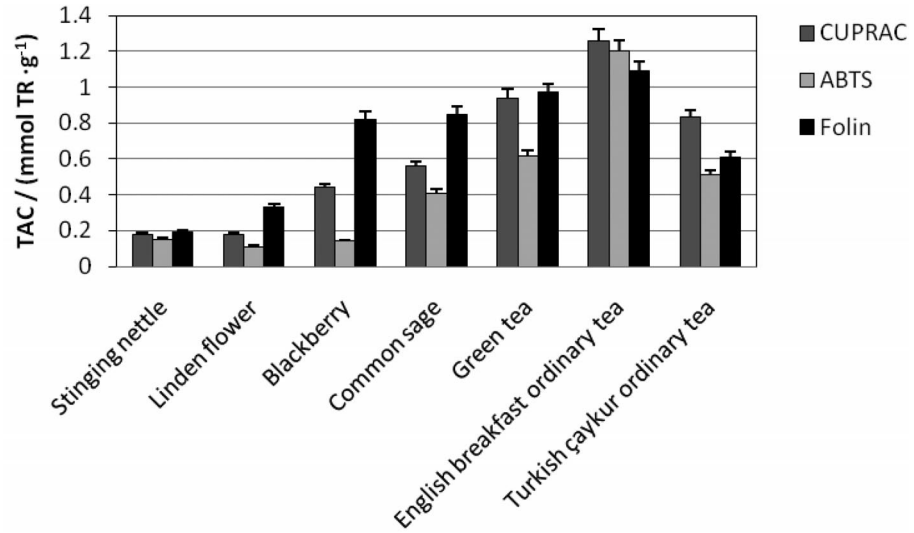


Fig. 11 TAC of herbal infusions, either prepared from dried plant material or manufactured tea bags, as measured by the CUPRAC and ABTS assays, combined with their TP content as measured by the Folin assay [91].

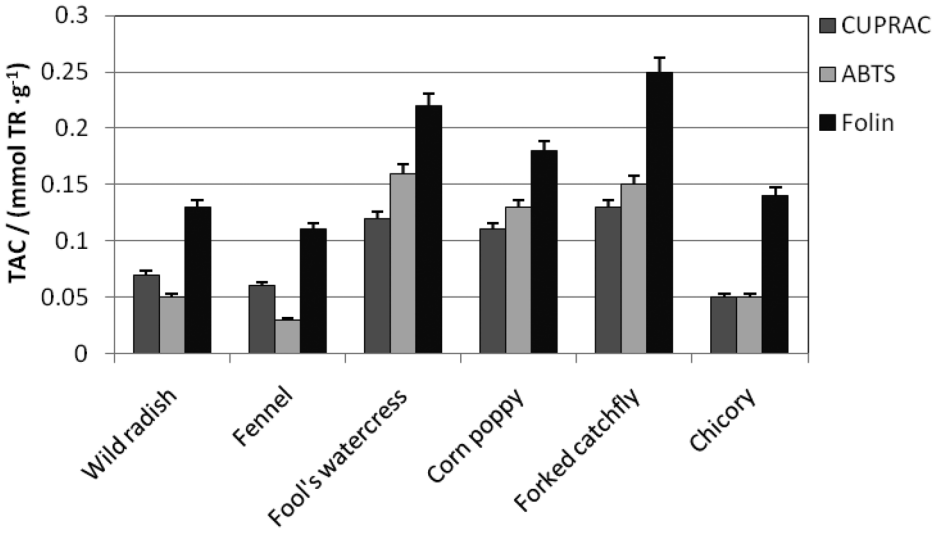


Fig. 12 TAC values in TR equivalents of seven edible wild plants grown in Ayvalik as assayed by CUPRAC, ABTS/persulfate, and Folin methods [92].

In Figs. 11 and 12, the TAC_{CUPRAC} values of herbal infusions and some edible wild plants were reported as TEs (mmol TR·g^{-1}), respectively.

4.1 Some modifications of the CUPRAC method

4.1.1 TAC assay of human serum

Several methods have been developed to measure the TAC of biological fluids such as human serum or plasma in view of the difficulties encountered in measuring each antioxidant component separately, added to the problems caused by possible interactions between individual antioxidants [89,90]. Apak et

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al. [77] were able to apply the CUPRAC method to a complete series of plasma antioxidants for the assay of TAC of serum and the resulting absorbance at 450 nm was recorded either directly (e.g., for AA, α-tocopherol, and GSH) or after incubation at 50 °C for 20 min (e.g., for uric acid, bilirubin, and albumin), quantitation being made by means of a calibration curve. The lipophilic antioxidants, α-tocopherol and β-carotene, were assayed in DCM. Lipophilic antioxidants of serum were extracted with *n*-hexane from an ethanolic solution of serum subjected to centrifugation. Hydrophilic antioxidants of serum were assayed after perchloric acid precipitation of proteins in the centrifugate. For lipophilic and hydrophilic antioxidants of human serum samples extracted with different solvents, the findings of the developed CUPRAC method completely agreed with those of ABTS-persulfate for lipophilic phase (first extracted with hexane, solvent evaporated under nitrogen purging, and subsequent colour development performed in DCM). As for hydrophilic phase, a linear correlation existed between the CUPRAC and ABTS findings for measurements carried out both at room temperature ($r = 0.58$) and in 50 °C-incubated solution ($r = 0.53$). This is also an advantage of the developed method, as relevant literature reports that either serum ORAC or serum FRAP does not correlate at all with serum TEAC. The CUPRAC assay of TAC may be successfully applied to all types of biologically important antioxidants [i.e., AA, α-tocopherol, β-carotene, reduced GSH, uric acid, and bilirubin] regardless of chemical type or hydrophilicity, as well as to their mixtures and human serum.

4.1.2 Determination of ascorbic acid (AA) in the presence of flavonoids

A practical, rapid, and low-cost spectrophotometric method for AA assay in complex matrices including flavonoids was developed to meet the needs of conventional analysis laboratories that are not so well equipped [78]. AA in complex matrices such as synthetic AA + flavonoid mixtures, AA + bioflavonoids pharmaceutical products, and fruit juices could be reliably assayed with preliminary La(III)-chelation and EtAc extraction, followed by CUPRAC measurement in the aqueous phase containing AA. AA could be sensitively determined over a wide concentration range, without interference from flavones and flavonols.

4.1.3 CUPRAC assay for simultaneous measurement of lipophilic and hydrophilic antioxidants

The CUPRAC procedure was applied to both lipophilic and hydrophilic antioxidants simultaneously, by making use of their "host–guest" complexes with M-β-CD, a cyclic oligosaccharide, in acetonated aqueous medium [79]. M-β-CD was introduced as the water solubility enhancer for lipophilic antioxidants. Two percent M-β-CD (w/v) in a 90 % acetone-10 % H_2O mixture was found to sufficiently solubilize β-carotene, vitamin E, vitamin C, oil-soluble synthetic antioxidants, and other phenolic antioxidants. This method compensates for the wide variability in antioxidant capabilities of oil- and water-soluble antioxidants showing different levels of accumulation at the interfaces of oil-in-water and water-in-oil emulsions, and assigns an objective TEAC value to each antioxidant simply depending on its chemical character (i.e., electron or H-atom donating ability).

4.1.4 CUPRAC assay modified for measuring hydroxyl radical scavenging activity of food antioxidants

A novel "test tube" method was developed for hydroxyl radical scavenging antioxidant activity assay by the modification of the CUPRAC method of TAC measurement [80]. Although the measurement of aromatic hydroxylation with ESR or HPLC/electrochemical detection is more specific than the low-yield thiobarbituric acid–reactive substances (TBARS) test, it requires sophisticated instrumentation. As a more convenient and less costly alternative, *p*-aminobenzoate, 2,4- and 3,5-dimethoxybenzoate probes were used for detecting hydroxyl radicals generated from an equivalent mixture of Fe(II) + EDTA with hydrogen peroxide. The produced hydroxyl radicals attacked both the probe and the water-soluble antioxidants in 37 °C-incubated solutions for 2 h. The CUPRAC absorbance of the ethyl acetate extract due to the reduction of Cu(II)-Nc reagent by the hydroxylated probe decreased in the presence of $^{\bullet}OH$ scavengers, the difference being proportional to the scavenging ability of the tested compound.

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A rate constant for the reaction of the scavenger with hydroxyl radical can be deduced from the inhibition of colour formation. Iodide, metabisulfite, hexacyanoferrate(II), thiourea, formate, and dimethylsulfoxide were shown by the modified CUPRAC assay to be more effective scavengers than mannitol, glucose, lysine, and simple alcohols, as in the TBARS assay. The developed method is less lengthy, more specific, and of a higher yield than the classical TBARS assay. The hydroxyl radical scavenging rate constants of AA, formate, and hexacyanoferrate(II) that caused interference in other assays could be easily found with the proposed procedure.

4.1.5 Hydroxyl radical scavenging antioxidant activity of polyphenolic compounds

Detection of hydroxyl radicals and measurement of hydroxyl radical scavenging activity is very important in food and bioanalytical chemistry in regard to antiradical and antioxidant activity of foodstuffs and antioxidant therapy. Currently, the most widely used colourimetric and chromatographic methods for hydroxyl radical detection and/or $^{\bullet}OH$ scavenging activity measurement are the TBARS colourimetric assay and HPLC with electrochemical detection of hydroxylated aromatic probes, respectively. The proposed CUPRAC/salicylate assay of $^{\bullet}OH$ detection is much more efficient than the conventional TBARS assay as the conversion ratio of the probe is much higher [81]. The most important contribution of the developed assay is the stopping of the Fenton reaction in 10 min with catalase degradation of hydrogen peroxide so that the remaining H_2O_2 would neither give a CUPRAC absorbance nor involve in redox cycling of phenolic antioxidants. This enables the rapid and precise measurement of $^{\bullet}OH$ scavenging rate constants of polyphenolics which cannot be found by most other techniques.

4.1.6 Measurement of xanthine oxidase (XO) inhibition activity of polyphenolic compounds

The idea of this CUPRAC variation is to use a X–XO system for XO inhibitory activity assay of polyphenolics (i.e., flavonoids, simple phenolic acids, and hydroxycinnamic acids) and other antioxidant compounds (i.e., AA) [82]. As a part of antioxidant activity assays, XO activity has usually been determined by following the rate of uric acid formation from the X–XO system by making use of the UV absorbance at 295 nm of uric acid formed as reaction product. Since polyphenolics have strong UV absorption, XO inhibitory activity of polyphenolics was alternatively determined without interference by directly measuring the formation of uric acid and hydrogen peroxide using the modified CUPRAC spectrophotometric method at 450 nm. The CUPRAC absorbance of the incubation solution due to the reduction of Cu(II)-Nc reagent by the products of the X–XO system decreased in the presence of polyphenolics, the difference being proportional to the XO inhibition ability of the tested compound. The developed assay was validated against classical UV and HPLC assays of uric acid, and was applied to the measurement of XO-inhibition activity of herbal extracts. The proposed spectrophotometric method was practical, low cost, rapid, and could reliably assay uric acid and hydrogen peroxide in the presence of polyphenols (flavonoids, simple phenolic acids, and hydroxycinnamic acids) and less open to interferences by UV-absorbing substances. The fact that the analysed real samples (such as plant extracts) normally react with the CUPRAC reagent at elevated concentrations is not important for the proposed method as long as sufficiently low amounts of these samples are taken for XO inhibition measurement.

4.1.7 Hydrogen peroxide scavenging activity of polyphenolic compounds [with Cu(II) catalysis]

Measurement of HPS activity is very important in food and bioanalytical chemistry, and thus the HPS activity of polyphenols (i.e., flavonoids, simple phenolic acids, and hydroxycinnamic acids) and other antioxidant compounds (i.e., AA) was measured with a simple, low-cost, and versatile colourimetric procedure [83]. In the most common UV method for determination of HPS activity, scavenging ability depends on the change of the absorbance value at 230 nm when H_2O_2 concentration is decreased by scavenger compounds. Since the UV method suffers from both the interference of some phenolics in real samples having strong absorption in the UV region and from inefficient degradation of H_2O_2 with polyphenols in the absence of Cu(II) (i.e., H_2O_2 is relatively stable, and not scavenged unless transi-

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tion-metal compounds are present as catalysts), HPS activity of polyphenols was alternatively determined without interference by directly measuring the concentration of undegraded H_2O_2 using the modified CUPRAC method in the presence of a Cu(II) catalyst. The modified CUPRAC method for HPS activity is based on the incubation of a scavenger with H_2O_2 and analysing the reaction mixture for the loss of H_2O_2. The proposed methodology is regarded superior to the rather nonspecific horseradish peroxidase-based assays.

4.1.8 CUPRAC antioxidant sensor

A low-cost optical sensor was developed using an immobilised chromogenic reagent, Cu(II)-Nc complex, for the assessment of antioxidant capacity of non-enzymatic antioxidant compounds, their synthetic mixtures, and real samples [84]. The Cu(II)-Nc reagent was immobilized onto a cation-exchange polymer (Nafion, a sulfonated tetrafluoroethylene-based copolymer) matrix, and the absorbance associated with the formation of the highly coloured Cu(I)-Nc chelate as a result of reaction with antioxidants was measured on the sensor membrane at 450 nm. The TEAC coefficients measured for various antioxidant compounds suggest that the reactivity of the Nafion-immobilized reagent is comparable to that of the standard solution-based CUPRAC assay. Testing of synthetic ternary mixtures of antioxidants yielded the theoretically expected CUPRAC antioxidant capacities, in accordance with the principle of additivity of absorbances of constituents obeying Beer's law. This assay was validated through linearity, additivity, precision, and recovery, demonstrating that the assay is reliable and robust. The developed sensor was used to screen the TAC of some commercial fruit juices such as orange, cherry, peach, and apricot juices, and proved to be an effective tool in measurement of TAC values of food and plant samples without requiring pretreatment. The optical sensor-based CUPRAC assay was shown not to be adversely affected by common food ingredients such as citrate, oxalate, fruit acids, and reducing sugars. The developed molecular spectroscopic device offers good prospects of being used as a general antioxidant sensor in food industries. With new experimental design for application to human fluids, the sensor is expected to be useful to biochemical and medicinal chemical research on oxidative stress.

4.1.9 On-line HPLC-CUPRAC method

Efforts directed to individual identification and quantification of antioxidant compounds in plant matrices may give rise to problems, because the activities of antioxidant compounds may decrease during their isolation and purification due to decomposition. Thus, procedures for the separation and quantification of antioxidants should be performed simultaneously. More recently, certain assays have been modified for post-column-coupled on-line HPLC applications. The most widely used assays in post-column applications are free radical decolourisation methods, based on the scavenging of chromogenic free radicals DPPH or ABTS. It is difficult to precisely quantify antioxidant activity because of the short life-times of these radicals. Moreover, reaction kinetics may vary with these radicalic reagents as a function of phenolic steric effects, solvent composition, and pH. A method combining separation of components in the complex matrix and evaluation of antioxidant capacity provides significant advantages for such investigations [85].

A developed on-line HPLC-CUPRAC method [85] combines chromatographic separation, constituent analysis, and post-column identification of antioxidants in plant extracts. In this system, the HPLC-separated antioxidant compounds react with the Cu(II)-Nc reagent (prepared freshly from the corresponding solutions of Cu(II):Nc:NH_4Ac at a ratio of 1:1:1 (v/v/v) prior to analysis and pumped to the post-column reactor at a flow rate of 0.5 $mL \cdot min^{-1}$) along a reaction coil, and the reagent is reduced by antioxidants to the yellow-coloured Cu(I)-Nc complex having an absorption maximum at 450 nm. It was observed that the antioxidant capacity of each substance is reflected by an increase in the area of negative peaks as a function of increased concentration. The separation of polyphenols was performed on a C18 column using gradient elution with two different mobile phase solutions, i.e., MeOH and 0.2 % *o*-phosphoric acid. The detection limits of polyphenols at 450 nm (in the range of 0.17–3.46 μM) after post-column derivatisation were comparable to those at 280-nm UV detection without derivatisa-

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tion. The developed method was successfully applied to the identification of antioxidant compounds in crude extracts of *Camellia sinensis*, *Origanum marjorana*, and *Mentha*. The method is rapid, inexpensive, versatile, nonlaborious, uses stable reagents, and enables the on-line qualitative and quantitative estimation of antioxidant constituents of complex plant samples. The significant advantages of on-line methods are that the antioxidant activity of a single compound can be measured and its contribution to the overall activity of a complex mixture can be calculated, and also the activity of a single compound can be compared to those of other constituents in the matrix. Moreover, the determination of a substance that possesses no antioxidant activity can be observed simultaneously, by the absence of a negative peak at 450 nm as opposed to the presence of a positive peak detected at 280 nm. For example, caffeine, being a major constituent of both green and black tea extracts, appears only in the positive trace chromatogram, whereas it is nonexistent in the negative trace of post-column detection, because it lacks the phenolic hydroxyl groups of reducing character, and consequently does not possess any antioxidant behaviour.

4.1.10 Antioxidant capacities of thiol-containing proteins

Proteins are not considered as true antioxidants but are known to protect antioxidants from oxidation in various antioxidant activity assays. To determine the antioxidant properties of thiol-containing proteins, the modified CUPRAC method was adopted [86]. While the CUPRAC method is capable of determining all antioxidant compounds including thiols in complex sample matrices, the Ellman method of thiol quantitation basically does not respond to other antioxidants. The antioxidant quantities in the selected samples were assayed with the ABTS and FRAP methods as well as with the CUPRAC method. In all applied methods, the dilutions were made with a standard pH 8 buffer used in the Ellman method, and serum samples should be protected with the addition of citrate rather than EDTA. On the other hand, the standard CUPRAC protocol was modified by substituting the pH 7 ammonium acetate buffer (at 1 M concentration) with 8 M urea buffer adjusted to pH 7 by neutralising with 6 M HCl. Urea helps to partly solubilize and denaturate proteins so that their buried thiols be oxidized more easily. All methods used in the estimation of antioxidant properties of proteins (i.e., CUPRAC, Ellman, ABTS, and FRAP) were first standardized with a simple thiol compound, cysteine, by constructing the calibration curves. Then these methods were applied to various samples containing thiols, such as GSH (reduced form: GSH), egg white, whey proteins, and gelatin. Additionally, known quantities of selected antioxidants were added to these samples to show the additivity of responses.

4.1.11 Superoxide radical scavenging activity assay of biological samples

Superoxide anion radicals ($O_2^{\bullet-}$) generated as a result of normal intracellular metabolism have been implicated in diseases ranging from Alzheimer's to diabetes. As a more convenient, efficient, and less costly alternative to ESR detection techniques and to the nonspecific nitroblue tetrazolium (NBT) and cytochrome c tests, a TBHQ probe was used for detecting superoxide radical (SR) generated by nonenzymic PMS-NADH system [87]. The produced $O_2^{\bullet-}$ attacks both the TBHQ probe and the SR scavengers that are incubated in solution for 30 min. Added SR scavengers compete with TBHQ for the $O_2^{\bullet-}$ produced, and enhance chromophore formation from Cu(II)-Nc. TBHQ, but not its $O_2^{\bullet-}$ oxidation product, *tert*-butyl-1,4-benzoquinone (TBBQ), is responsive to the CUPRAC spectrophotometric assay. The absorbance of the incubation solution arising from the reduction of Cu(II)-Nc reagent by the remaining TBHQ was higher in the presence of $O_2^{\bullet-}$ scavengers (due to less conversion to TBBQ), the difference being correlated to the SRSA of the tested compounds. With the aid of this reaction, a kinetic approach was adopted to assess the SRSA of amino acids, vitamins, and plasma- and thiol-antioxidants. The proposed assay proved to be efficient for cysteine, uric acid, and bilirubin for which the NBT test is basically nonresponsive. This assay allows rapid, high-throughput assessment of SRSA of small molecules of interest, as well as for tissue homogenates.

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4.2 The advantages of the CUPRAC method

The advantages of the CUPRAC method may be summarized as follows [93,94]:

- The CUPRAC reagent (outer-sphere e-transfer agent) is fast enough to oxidize thiol-type antioxidants, whereas the FRAP method may only measure with serious negative error certain thiol-type antioxidants like GSH (i.e., the major low-molecular-weight thiol compound of the living cell). Redox potential of GSSG/GSH is the basic indicator of biological conditions of a cell, and GSH acts as reconstituent of intercellular AA from the dehydroascorbic acid.
- The reagent is selective, because it has a lower redox potential than that of the ferric (ferrous couple in the presence of *o*-phenanthroline) or *batho*-phenanthroline-type ligands. The standard potential of the Cu(II,I)-Nc redox couple is 0.6 V, close to that of $ABTS^{\bullet+}$/ABTS (E° = 0.68 V), and FRAP (E° = 0.70 V). Simple sugars and citric acid are not oxidized with the CUPRAC reagent.
- The reagent is much more stable and easily accessible than the chromogenic radical reagents (e.g., ABTS, DPPH, etc.). The cupric reducing ability measured for a biological sample may indirectly but efficiently reflect the total antioxidant power of the sample even though no radicalic species are involved in the assay.
- The method is easily and diversely applicable in conventional laboratories using standard colourimeters rather than necessitating sophisticated equipment and highly qualified operators. Method responds equally well to both hydrophilic and lipophilic antioxidants.
- The redox reaction giving rise to a coloured chelate of Cu(I)-Nc is relatively insensitive to a number of parameters adversely affecting radicalic reagents such as DPPH, e.g., air, sunlight, humidity, and pH, to a certain extent.
- The CUPRAC reagent can be adsorbed on a cation-exchanger membrane to build a low-cost, linear-response antioxidant sensor.
- The CUPRAC absorbance vs. concentration curves are perfectly linear over a wide concentration range, unlike those of other methods yielding polynomial curves. The molar absorptivity for n-e reductants, $(8.5 \pm 1.0) \times 10^3$ n $L \cdot mol^{-1} \cdot cm^{-1}$, is sufficiently high to sensitively determine most phenolic antioxidants.
- The TAC values of antioxidants found with CUPRAC are perfectly additive, i.e., the TAC of a phenolic mixture is equal to the sum of TAC values of its constituent polyphenols. Additivity in other antioxidant measurements is not strictly valid.
- HPLC-post-column CUPRAC is possible (direct methods of antioxidant assay are not suitable for post-column applications).
- The redox reaction producing coloured species is carried out at nearly physiological pH (pH 7 of ammonium acetate buffer) as opposed to the unrealistic acidic conditions (pH 3.6) of FRAP or basic conditions (pH 10) of Folin assay. At more acidic conditions than the physiological pH, the reducing capacity may be suppressed due to protonation on antioxidant compounds, whereas at more basic conditions, proton dissociation of phenolics would enhance a sample's reducing capacity.
- Since the Cu(I) ion emerging as a product of the CUPRAC redox reaction is in a chelated state [i.e., Cu(I)-Nc], it cannot act as a prooxidant that may cause oxidative damage to biological macromolecules in body fluids. The ferric ion-based assays were criticized for producing Fe^{2+}, which may act as a Fenton-type prooxidant to produce $^{\bullet}OH$ radicals as a result of its reaction with H_2O_2. The stable Cu(I)-chelate was shown not to react with H_2O_2, but the reverse reaction, i.e., oxidation of H_2O_2 with Cu(II)-Nc, is possible.
- By maintaining the CUPRAC reagent and related chemicals in the laboratory, one can measure ROS scavenging activity as well as TAC of antioxidants. However, a battery of measurements are required to adequately assess oxidative stress and antioxidative defense in biological systems.

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5. ANTIOXIDANT CAPACITY/ACTIVITY ASSAYS APPLIED TO FRUITS, VEGETABLES, AND CEREALS

Fruits, vegetables, and cereals were analysed for the composition of the major individual phenolic acids, AA, flavonoids, flavanols, and anthocyanins [95]. The antioxidant capacities of aqueous/MeOH, acetone, and hexane extracts were comparatively assessed using the CUPRAC, ABTS/TEAC, FRAP, DPPH, β-carotene (β-carotene linoleate model system), and nitric oxide (NO) tests (scavenging activity against NO). Raw and processed vegetables were compared for their bioactive content. It was found that raw natural products contain higher quantities of bioactive compounds and possess higher antioxidant activity than processed ones, and the degree of the differences is directly connected to the processing conditions. The antioxidant capacity/activity values for each extract were well correlated with the content of polyphenols (R^2 = 0.99). The antioxidant activity of the studied vegetables according to all used antioxidant assays were in the following order: red onion > white onion = yellow onion > red pepper > garlic = green pepper > white cabbage; of the common fruits, apples > peaches > pears; of the citrus fruits, red grapefruits > oranges = lemons > sweeties; of exotic fruits, durian > snake fruit > mangosteen; of cereals, buckwheat > oat > amaranth > quinoa > rice [95].

Nowadays, natural products (vegetables, fruits, and cereals) are a very important part of different diets [95]. Many authors recommend the consumption of vegetables, fruits, and cereals only of high antioxidant activity [96,97]. In order to find vegetables, fruits, and cereals with high antioxidant activity, most investigators are using a combination of antioxidant tests [98,99]. Many investigators showed the importance of the use of antioxidant assays in assessment of the bioactivity of natural products [95,98,99].

Garlic, onions, peppers, cereals, and many different fruits possess many health-beneficial properties, which are related mainly to bioactivity of their phenolic compounds [100]. It was shown that these natural products are active in prevention and treatment of some diseases including heart diseases [101] and even of cancer [102]. Most of the natural products are consumed after processing. It was shown that this process leads to changes in their bioactivity [103,104].

Samples of raw garlic (*Allium sativum* L.), white, yellow, and red onions (*Allium cepa* L.), red and green peppers (*Capsicum annuum* L.), and white cabbage (*Brassica oleracea var. capitata f. alba*) were analysed for their antioxidant activity. The extracts of the studied natural products were prepared with EtOH, MeOH, water, acetone, and hexane. The results (shown below) are for MeOH extracts of the studied natural products.

5.1 Vegetables

The total polyphenols of all studied raw vegetables (garlic, onions, peppers cabbages, lotus, and salad) were determined as previously described [105–107], and were in the quantitative range of 3.2 ± 0.3–15.6 ± 1.3 mg GAE·g^{-1} DW. The antioxidant activities of the same vegetables as determined by FRAP assay, ABTS, DPPH, and CUPRAC varied significantly, and were in range of 6.2 ± 1.4–22.0 ± 1.9 μmol TE·g^{-1} DW (DPPH values).

As can be seen in Table 6, the antioxidant activities of the studied vegetables (as determined by the mentioned antioxidant assays) were in following order: red onion > white onion = yellow onion > red pepper > garlic = green pepper > white cabbage. As a result of thermal processing, decreases in the content of total polyphenols and in related total antioxidant activities were registered, and directly connected to the time of processing [103,104,108,109]. The contribution of total polyphenols to the antioxidant activities of raw and studied vegetables was high (R^2 = 0.9971 and 0.9705 for FRAP and DPPH, respectively). Short-term processing as blanching for 90" of vegetables essentially preserves their bioactive compounds (polyphenols, flavonoids, flavanols, and tannins) and the level of antioxidant activity [103,104,108,109].

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Table 6 Contents of polyphenols and antioxidant activity in raw vegetables, extracted with MeOH (combined data) ($N = 3$).

	Polyphenols (mg GAE·g^{-1})	DPPH (μmol TE·g^{-1})	FRAP (μmol TE·g^{-1})	CUPRAC (μmol TE·g^{-1})	ABTS (μmol TE·g^{-1})
Red onion	15.6 ± 1.3	22.0 ± 1.9	19.2 ± 1.7	39.7 ± 3.2	49.7 ± 4.3
White onion	11.9 ± 0.9	19.4 ± 1.9	14.6 ± 1.3	32.5 ± 2.9	39.2 ± 3.2
Yellow onion	10.0 ± 0.8	19.7 ± 1.7	14.2 ± 1.2	33.2 ± 3.0	37.2 ± 3.0
Red pepper	8.6 ± 0.7	16.0 ± 2.0	13.6 ± 1.1	27.8 ± 2.2	28.6 ± 2.4
Garlic	6.4 ± 0.5	12.0 ± 3.4	9.9 ± 0.5	20.1 ± 1.4	23.7 ± 2.0
Green pepper	6.3 ± 0.5	11.8 ± 1.0	9.7 ± 0.5	20.1 ± 1.8	23.9 ± 2.3
White cabbage	3.2 ± 0.3	6.2 ± 1.4	8.1 ± 0.4	11.4 ± 0.9	10.5 ± 0.8

5.2 Olive oils

The so-called Mediterranean diet, which is rich in vegetables, fruits, and olive oils, is effective in prevention of some diseases including coronary atherosclerosis [110]. It was shown that this diet controls blood lipid levels and decreases the plasma cholesterol [111]. It was shown that the Mediterranean diet reduces the risk of acute myocardial infarction and decreases the overall mortality [112].

The above-mentioned data encouraged the assessment of the biochemical activity of 5 different Spanish olive oils (arbequina, hojiblanca, virgin, picual, and lampante). Their bioactive compounds including total polyphenols and antioxidant potential were determined in vitro. It was found that virgin and lampante oils possessed the highest and lowest antioxidant activity, respectively. Then these oils were added to basal diets (BDs) and the influence on plasma lipid metabolism and plasma antioxidant activity of experimental animals were studied. Sixty male Wistar rats were used, divided into 6 diet groups of 10, and adapted to cholesterol-free or 1 % cholesterol-containing diets. The control group consumed the BD only, which contained wheat starch, casein, cellulose, and mineral and vitamin mixtures. To the BD were added 10 g per 100 g virgin (Virg group) or lampante (Lamp group) oils, 1 g per 100 g cholesterol (Chol group), or both (Chol/Virg group) and (Chol/Lamp group). Plasma total cholesterol (TC), LDL-cholesterol (LDL-C), HDL-cholesterol (HDL-C), triglycerides (TG), total phospholipids (TPH), HDL-phospholipids (HDL-PH), TRAP, malondialdehyde (MDA) lipid peroxidation, and liver TC were determined. Groups did not differ before the experiment. In the Chol/Virg and Chol/Lamp vs. Chol group, the oil-supplemented diets significantly ($p < 0.05$) hindered the increase in plasma lipids due to dietary cholesterol feeding as follows: TC (25.1 and 23.6 %), LDL-C (39.3 and 34.7 %), TG (19.3 and 17.0 %), and TC in liver (36.0 and 35.1 %) for the Chol/Virg and Chol/Lamp, respectively. In the Chol/Virg and Chol/Lamp diets, significant decreases were noted in the levels of TPH (24.7 and 21.2 %; $p < 0.05$ in both cases) and HDL-PH (22.9 and 18.0 %; $p < 0.05$ in both cases), respectively. Virgin oil and to a lesser degree lampante oil have increased the plasma antioxidant activity in rats fed BD without cholesterol (an increase in TRAP, 20.6 and 18.5 %; and a decrease in MDA, 23.2 and 11.3 %, respectively). In the rats of Chol/Virg and Chol/Lamp vs. Chol diet groups, the added oils significantly hindered the decrease in the plasma antioxidant activity (TRAP, 21.2 and 16.7 %; and MDA, 27.0 and 22.3 %, respectively). According to the used antioxidant assays (TRAP), virgin and to less degree other olive oils possess high antioxidant potential [113,114].

5.3 Seed oils

It is almost common knowledge that olive oils are preferable to seed oils. Only a few researchers have not agreed with these claims [115]. These investigators demonstrated that the highest TRAPs were in sunflower oil, whereas the TRAP values of olive oils were lower. These data are very reliable: extra virgin olive and seed oils were taken from a local oil mill and produced in the same month. Therefore, it

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was decided to investigate four widely used seed oils (sunflower, sunflower high oleic, rapeseed, and grapeseed) in order to compare their antioxidant potential.

As in the case of olive oil investigation, the fatty acids, sterols, antioxidant compounds, stability, and TRAP in the studied oils were determined. The highest stability and the highest TRAP (3.8 Rancimat 120 °C, hours and 324 nmol·mL^{-1}) and the lowest stability and the lowest TRAP (2.4 Rancimat 120 °C, hours and 201 nmol/mL) were in rapeseed and sunflower oils, respectively. The effect of these two seed oils on antioxidant activity was investigated on 60 (divided into 6 diet groups of 10) male Wistar rats adapted to cholesterol-free or 1 % cholesterol-containing diets. The control group received BD only. To the BD were added 10 g per 100 g rapeseed (Rapeseed group) or sunflower (Sunflower group) oils, 1 g per 100 g cholesterol (Chol group) or both (Chol/Rapeseed group) and (Chol/Sunflower group). The experiment lasted 4 weeks. Rapeseed and to a lesser degree sunflower oils increased the plasma antioxidant activity in rats fed BD without cholesterol (an increase in TRAP: 20.8 and 16.0 % and a decrease in MDA: 22.0 and 14.9 %, respectively). In the rats of Chol/Rapeseed and Chol/Sunflower vs. Chol diet group, the added oils significantly hindered the decrease in the plasma antioxidant activity (TRAP: 21.7 and 16.3 % and MDA: 26.2 and 21.5 %, respectively). Therefore, rapeseed and to a lesser degree sunflower oils possess antioxidant properties. The results of this investigation did not support the claim that olive oils are preferable to seed oils in regard to their antioxidant content [116].

5.4 Fruits

5.4.1 Common fruits (peaches, pears, and apples)

The total polyphenols of peaches, pears and apples were in the range of 131 ± 10.1–154 ± 10.3; and in their peels 145 ± 10.3–166 ± 10.6 mg per 100 g FW. Their antioxidant potential with respect to DPPH were in the range of 1.72 ± 0.1–5.16 ± 0.3 μM TE·g^{-1} FW. The antioxidant activities of common fruits, as determined by all used antioxidant assays, were in the following order: apples > peaches = pears [117–119].

5.4.2 Citrus fruits (grapefruits, oranges, lemons, sweeties)

The total polyphenols of all studied peeled citrus fruits were in the range of 135 ± 10.1–164 ± 10.3; and their peels 155 ± 10.3–190 ± 10.6 mg per 100 g FW. The content of total polyphenols in the peels of fruits was significantly higher than in peeled fruits. Their antioxidant potential according to DPPH was in the range of 1.92 ± 0.1–6.26 ± 0.3 μM TE·g^{-1} FW. The antioxidant activities of citrus fruits (by all assays tested) were in the following order: red grapefruits > oranges = lemons > sweeties [120–124].

5.4.3 Subtropical, tropical, and exotic fruits

5.4.3.1 Persimmons

The total polyphenols of all studied cultivars were in the range of 125 ± 10.1–156 ± 10.3 mg per 100 g FW. The antioxidant potential according to DPPH was in the range of 1.72 ± 0.1–5.96 ± 0.3 μM TE·g^{-1} FW [125–127].

5.4.3.2 Kiwi fruit

The total polyphenols of all studied cultivars were in the range of 14.9 ± 1.5–26.7 ± 8.1 mg GAE·g^{-1} DW. The antioxidant potential with respect to DPPH was in the range of 10.1 ± 1.1–13.7 ± 1.2 μM TE·g^{-1} DW [128–130].

5.4.3.3 Exotic fruits

The total polyphenols of all exotic fruits (5 different cultivars of durian, snake fruit, and mangosteen) were in the range of 5.11 ± 0.2–11.3 ± 0.5 mg CE·g^{-1} DW. Their antioxidant activities with respect to DPPH were in the range of 7.12 ± 0.4–13.56 ± 0.7 μM TE·g^{-1} DW. A good correlation between the contents of total polyphenols and antioxidant activities was registered (R^2 range of 0.9841–0.9859). The

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antioxidant activities (as determined by all used assays) of exotic fruits were in the following order: all cultivars of durian > snake fruit > mangosteen [131–133].

5.5 Cereals

The total polyphenols of all cereals (buckwheat, oat, rice, amaranth, quinoa) were in the range of 329 ± 15.3–927.3 ± 32.2 μg CE·g^{-1} DW. The antioxidant activities of cereals were in the following order: buckwheat > oat > amaranth > quinoa > rice [134–138].

The use of natural products has gained popularity, and the increase in their consumption is backed by solid scientific evidence. One of these natural products is garlic, which has been used throughout the history of civilisation for treatment of a wide variety of ailments. The same is true for other reviewed vegetables, fruits, and cereals. The most studied and reported health-promoting effect of these vegetables is cardioprotection [139]. It was proved that contents of bioactive compounds in raw vegetables were higher than in processed ones and that the antioxidant activity was directly related to the contents of phenolic compounds [140].

6. CONCLUSIONS

This report provides an overview of various antioxidant activity/capacity assay methods used for measuring non-enzymatic antioxidants in complex food/biological matrices, including the general characteristics, comparisons, and correlations of these methods. Some critical terms regarding nomenclature (e.g., antioxidant activity and antioxidant capacity) and mechanism (e.g., HAT- and ET-based assays) have been defined. A number of key evaluation parameters have been listed such as method simplicity, required time, and instrumentation, aiding researchers to decide which method to be followed to measure the targeted antioxidant property based on the feasibilities afforded to determine it. Methods based on the inhibition of lipid peroxidation or biomarkers of oxidative stress have been deliberately left out to devote more space to a detailed discussion on HAT- and ET-based assays. Both the advantages and disadvantages of each mentioned method have been comparatively evaluated.

Utmost care should be exercised in both the practice and evaluation of methods, ensuring the repeatability/reproducibility of results. All methods are in vitro assays giving summed or integrated parameters rather than individual antioxidant quantifications. The results obtained from the listed in vitro assays cannot be extrapolated directly to the situation in the human body, however, experimental conditions can be tailored to fit in vivo simulations. Antioxidant activity/capacity assays based on the scavenging of free radicals like ABTS$^{\bullet+}$, DPPH$^{\bullet}$, and galvinoxyl do not use biologically relevant radicals. Choice of solvent is also an important parameter, determining the extent of completion of TAC assays (especially ET-based ones) within the prespecified protocol time. Hydrophilic and lipophilic assays carried out in different polarity solvents can be used to compare the antioxidant properties of different compounds (antioxidative vitamins, polyphenolics, and secondary plant products) and to get a first impression of the antioxidant capacity of food extracts and physiological materials. In the future, further comparative studies are needed to improve the knowledge on the different structure–activity relationships and on the various mechanisms of these test systems. In addition, interlaboratory comparison and standardisation as mentioned by Prior et al. [8] are needed to improve the reproducibility of the methods and to make results more comparable and reliable. At the moment, absolute results in publications have to be used carefully as they are not comparable from one laboratory to the other, because each antioxidant assay has a different mechanism, redox potential, reaction media, etc. However, in one laboratory the results within one test system can be used for a ranking. It is strongly recommended to use at least two different methods (preferably with different mechanisms) and three sample dilutions within each assay [22].

The end result of antioxidant action for different assays (regardless of mechanism) is similar in the sense of quenching/reducing reactive species/radicals or oxidant probes, but kinetics, potential for

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side reactions, and dependence on reaction conditions may differ. HAT and ET mechanisms almost occur in parallel in all systems (though one mechanism may be prevalent for a given assay), and the dominant mechanism will be determined by antioxidant structure and properties (such as chemical accessibility), solubility and partition coefficient, system solvent, and presence of trace components or contaminants. These discussions lead to the recommendation that no single "universally accepted" assay is adequate in itself to precisely and quantitatively detect/determine all actions of a putative antioxidant. Thus, a combination of HAT and ET assays or of reduction- and free radical scavenging-based assays may be preferred to be the standard, required practice for antioxidant research, preferably coordinated also with a metal ion binding/chelation assay. It can be concluded that raw and mildly processed vegetables, fruits, and cereals contain significant levels of bioactive compounds possessing high total antioxidant potential, and this potential can be evaluated by antioxidant assays having reasonable correlations among themselves (though there may exist relatively low correlations between HAT and ET assays for certain analyte matrices).

7. NOTES ADDED IN PROOF

The following corrections were made after the Report was published online and before it was published in print:

Line number	Current text	New text
Page 5, under eq. 7	2,2-**di(4-*tert*-octylphenyl)**-1-picrylhydrazyl	2,2-**diphenyl**-1-picrylhydrazyl
Page 5, last paragraph, line 3	**reduced**	**oxidized**
Page 5, last paragraph, line 4	**reduction**	**oxidation**
Pages 6 to 7, last and first lines, respectively	In contrast, four methods determine the ability of antioxidants to reduce **radical cations** (DMPD**,** DPPH**,** and **TEAC**) or chelated metal ions (FRAP).	In contrast, four methods determine the ability of antioxidants to reduce **radicals** (DMPD, DPPH, and **ABTS**) or chelated metal ions (FRAP).
Page 41, Abbreviations	2,2-**Di(4-*tert*-octylphenyl)**-1-picrylhydrazyl	2,2-**diphenyl**-1-picrylhydrazyl

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This document was prepared under the framework of IUPAC Project #2008-031-1-500: Methods of measurement and evaluation of natural antioxidant capacity/activity. Membership of the project was as follows:

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10. ABBREVIATIONS

α-TE	α-tocopherol equivalent
α-TEAC	α-tocopherol equivalent antioxidant capacity
AA	ascorbic acid
AAPH	2,2'-azobis(2-amidinopropane) hydrochloride
ABAP	2,2'-azobis(2-aminopropane)
ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid)
ACL	lipid-soluble substance
ACW	water-soluble substance
AOX	antioxidant
AOC	antioxidant capacity
AUC	area under curve
BD	basal diet
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
B-PE	β-phycoerythrin
CD	cyclodextrin
CL	chemiluminescence
CUPRAC	cupric reducing antioxidant capacity
Cu(I)-Nc	bis(neocuproine)copper(I) cation
Cu(II)-Nc	bis(neocuproine)copper(II) cation
DCM	dichloromethane
DMPD	*N*,*N*-dimethyl-*p*-phenylenediamine
DPPH	2,2-di(4-*tert*-octylphenyl)-1-picrylhydrazyl
DW	dry weight
EPR	electron paramagnetic resonance
ET	electron transfer
EtAc	ethyl acetate
EtOH	ethanol
FCR	Folin–Ciocalteu reagent
FL	fluorescein
FRAP	ferric reducing antioxidant power
FW	fresh weight
FZ	ferrozine
GSH	glutathione
HAT	hydrogen atom transfer
HPS	hydrogen peroxide scavenging
IC_{50}	half maximal inhibitory concentration
LDL	low-density lipoprotein
M-β-CD	methyl-β-cyclodextrin
MDA	malondialdehyde
MeOH	methanol
NEAC	non-enzymatic antioxidant capacity
NC	neocuproine
NBT	nitro blue tetrazolium
ORAC	oxygen radical absorbance capacity
PCL	photochemoluminescence
PG	propyl gallate
PMS-NADH	phenazine methosulphate-β-nicotinamide adenine dinucleotide

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ROS	reactive oxygen species
RNS	reactive nitrogen species
SOD	superoxide dismutase
SR	superoxide radical
SRSA	superoxide radical scavenging activity
TAC	total antioxidant capacity
TBA	thiobarbituric acid
TBARS	thiobarbituric acid–reactive substance
TBBQ	*tert*-butyl-1,4-benzoquinone
TBHQ	*tert*-butyl hydroquinone
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TP	total phenolics
TPTZ	tripyridyltriazine
TRAP	total peroxyl radical-trapping antioxidant parameter
X–XO	xanthine–xanthine oxidase

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