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Evaluation of Antioxidant Activity in Foods with Special Reference to TEAC Method

Pankaj Sharma and R.P. Singh
Human Resource Development, CSIR-Central Food Technological Research Institute, Mysore-570 020, India

Corresponding Author: R.P. Singh, Human Resource Development, CSIR-Central Food Technological Research Institute, Mysore-570 020, India Tel:+91-821-2514310 Fax: +91-821-2517233

ABSTRACT

The antioxidant capacity of complex heterogeneous foods and biological systems is affected by many factors. Considering the importance of antioxidants, it is of great interest to know the antioxidant capacity of the constituents in foods. The Total Antioxidant Capacity (TAC) is a parameter that provides information on the overall status of antioxidants within a complex biological sample. TAC, as determined by Trolox Equivalent Antioxidant Capacity (TEAC) method, offers several advantages over other methods and is relatively easy. Due to its operational simplicity, the TEAC assay has been used for studying antioxidant capacity and TEAC values of many compounds and food samples. The article deals with various developments in the method, its merits and demerits and its application in determining the TAC of various food commodities.

Key words: 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid, total antioxidant capacity, antioxidant, free radicals

INTRODUCTION

The term antioxidant has been defined in a number of ways, like "substances that in small quantities are able to prevent or greatly retard the oxidation of easily oxidizable materials" (Chipault, 1962) or "any substance, when present in low concentrations compared to those of an oxidisable substrate significantly delays or prevents oxidation of that substance" (Halliwell and Gutteridge, 1989). In food science, it is defined as a substance in foods when present at low concentrations compared to those of an oxidizable substrate, significantly decreases or prevents the adverse effects of reactive species, such as reactive oxygen and nitrogen species (ROS/RNS), on normal physiological function in humans (Halliwell and Gutteridge, 1989; Huang *et al*., 2005). These definitions do not confine antioxidant activity to any specific group of compounds nor refer to any particular mechanism of action. For the *in vivo* situation, the concept of antioxidants has become very broad including antioxidant enzymes, iron binding and transport proteins and other compounds affecting signal transduction and gene expression (Rice-Evans, 2004). Hence, all reductants are not antioxidants; only those compounds capable of protecting the biological target from oxidation meet this criterion (Karadag *et al*., 2009).

Free radicals play a crucial role in the pathogenesis of several human diseases (Halliwell *et al*., 1995; Halliwell and Gutteridge, 1999). Hence, antioxidants are important tools in obtaining and preserving good health. The antioxidant profiles of numerous compounds, both natural and synthetic, are frequently compared in order to identify the potent ones (Arts *et al*., 2004). Table 1 gives a b rief description of various ROS and the mechanism by which they can be countered in *in vivo* (Singh *et al*., 2004).

Table 1: Reactive oxygen species; corresponding neutralizing antioxidants and additional antioxidants (Singh *et al*., 2004)

	Antioxidants (endogenous)		
ROS	Direct role	Indirect role	Antioxidants (exogenous)
Hydroxyl radical	Glutathione peroxidase (cofactor selenium)	-	Vitamin C, lipoic acid
Lipid peroxide	Glutathione peroxidase (cofactor selenium)	-	Vitamin E, β-carotene
Superoxide radical	Superoxide dismutase (cofactor Cu/Zn/Mn)	Ceruloplasmin(Cu) Metallothionin (Cu) Albumin (Cu)	Vitamin C
Hydrogen peroxide	Catalase (cofactor iron)	Transferin (iron), Ferritin (iron), Myoglobin (iron)	Vitamin C, β-carotene, lipoic acid
Prooxidant/antioxidant Equilibrium	Thiols (GSH, Lipoic acid, N-acetyl cysteine) NADPH and NADH Ubiquinone	Bilirubin Uric acid	Flavonoids

In biological systems, the main sources of antioxidants are: Enzymes (superoxide dismutase, glutathione peroxidase and catalase), Large molecules (albumin, ceruloplasmin, ferritin, other proteins), Small molecules [ascorbic acid, glutathione, uric acid, tocopherol, carotenoids, (poly) phenols]; and some hormones (estrogen, angiotensin, melatonin, etc.). Foods containing antioxidants may be of major importance in disease prevention. Wu *et al*. (2004) reported that fruits provide largest amount of antioxidants in the diet, mainly because of abundance of vitamins, phenolic compounds and carotenoids in them. Milk and some fractions of milk (whey, caseins, lactoferrin, albumin) have antioxidant activity (Cheng *et al*., 2003; Pena-Ramos and Xiong, 2001; Rival *et al*., 2001). In milk, an oligopeptide (Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ser-Asp-Ile) has been identified which possesses antioxidant capacity higher than that of Butylated Hydroxy Anisole (BHA) (Zulueta *et al*., 2009).

Ideal antioxidants: Various antioxidants show substantially varying antioxidant effectiveness in different food systems due to different molecular structures. The antioxidant should not impart any off-odor and off-color: it should be able to get conveniently incorporated to food/food systems and should be stable at pH of the food system and during food processing. Various factors which affect the efficiency of antioxidants include activation energy of antioxidants, redox potential, stability to pH and processing and solubility.

Approaches for antioxidant assays: Antioxidant activity and antioxidant capacity are terms that are often used interchangeably; though they have different meanings (MacDonald-Wicks *et al*., 2006)'. The "activity" of a chemical would be pointless without specific reaction conditions, such as pressure and temperature. The antioxidant capacity gives the information about the duration while the activity describes the starting dynamics of antioxidant action (Roginsky and Lissi, 2005). The antioxidant capacity in complex heterogeneous foods and biological systems is affected by many factors including the partitioning properties of the

antioxidants between lipid and aqueous phases, the oxidation conditions and the physical state of the oxidizable substrate (Frankle and Meyer, 2000). Antioxidant protection significantly changes according to the substrate used to conduct evaluation (Karadag *et al*., 2009).

There is now convincing evidence that foods containing antioxidants may be of major importance in disease prevention. Hence, it is interesting to know the antioxidant capacity and constituents in the foods. Due to the complexity of the food systems, separating each antioxidant compound and studying them individually is costly and inefficient, also it may not account for the possible synergistic interactions amongst the antioxidant compounds in a food mixture. Therefore, it is a great challenge to have a convenient method for the quick quantification of antioxidant effectiveness in various sources in general and in food systems in particular (Huang *et al*., 2005).

Several approaches used to test antioxidants in foods and biological systems, consist of oxidizing a substrate under standard conditions and assessing the activity by various methods to determine how much oxidation is inhibited. Other protocols classified are free radical-trapping methods which measure the ability of antioxidants to intercept free radicals. In the latter methods, the target compound or free radical molecule is often selected so that its consumption can be measured directly. In some cases the activity is evaluated from a coupled reaction (Frankle and Meyer, 2000).

The methods for measuring antioxidant capacity are classified into two groups, depending on the reaction mechanism: methods based on either Hydrogen Atom Transfer (HAT) or Electron Transfer (ET) (Huang *et al*., 2005). The majority of HAT-based assays apply a competitive scheme, in which antioxidant and substrate compete for the thermally generated peroxy radicals through the decomposition of azo-compounds. ET-based assays measure the capacity of an antioxidant in the reduction of the antioxidant which changes colour when reduced. The degree of colour change is correlated with the sample's antioxidant concentrations (Zulueta *et al*., 2009). The chain-breaking antioxidants are capable of accepting a radical from oxidizing lipid species, such as peroxyl (LOO^-) and alkoxyl (LO^-) radicals by the following reactions:

$$LOO^{\bullet} \text{ or } LO^{\bullet} + FRS^{\bullet} \rightarrow LOOH \text{ or } LOH + FRS^{\bullet}$$

Antioxidant efficiency is dependent on the ability of Free Radical Scavenger (FRS) to donate hydrogen to the free radical. As the hydrogen-bond energy of the FRS decreases, the transfer of the hydrogen to the free radical is more energetically promising and rapid. The ability of FRS to donate hydrogen to a free radical can be predicted from standard one-electron reduction potentials (Lee *et al*., 2003). Efficient FRS also produces radicals ($FRS^{\bullet}$) that do not react rapidly with oxygen to form peroxides. In foods, the efficiency of phenolic FRS also depends on additional factors, such as volatility, pH sensitivity and polarity (Karadag *et al*., 2009; Akoh and Min, 1998). Thus, each antioxidant evaluation should be carried out under various conditions of oxidation, using several methods to measure different products of oxidation related to real food quality or critical biological reactions (Frankle and Meyer, 2000).

Because of the complexity of real foods, accelerated test systems are difficult to standardize and each antioxidant test should be calibrated for each lipid or food. Ultimately, antioxidants should be evaluated on the food itself.

***In vitro models* for measuring antioxidant activity:** The most widely used methods for measuring antioxidant activity are those which involve the generation of radical species, the

presence of antioxidants determining the disappearance of these radicals. A compound might exert antioxidant actions *in vivo* or in food by inhibiting generation of reactive species, or by directly scavenging them. Also, an antioxidant might act *in vivo* by raising the levels of endogenous antioxidant defenses (e.g., by up-regulating expression of the genes encoding SOD, catalase, or glutathione peroxidase). This "screening" approach can be used to rule out direct antioxidant activity *in vitro*: a compound that is a poor antioxidant *in vivo* is unlikely to be any better as a direct antioxidant *in vivo* (Cadenas and Packer, 2002).

During *in vivo* testing, it is essential to examine the action of a putative antioxidant over a concentration range that is relevant. Also, if a compound acts as a scavenger of free radicals, an "antioxidant" may itself give rise to damaging radical species, because reaction of a free radical with a nonradical always generates a new free radical. It is important to use relevant reactive oxygen, nitrogen, or chlorine species and sources generating such species (Table 2); the choice will depend on whether effects *in vivo* (including the gastrointestinal tract) or effects in foods are being considered.

The principle and the methodology behind various methods used for antioxidant assays have been reviewed by Singh and Singh (2008). The present review deals with the origin and principle and various developments in Trolox Equivalent Antioxidant Capacity (TEAC) assay for antioxidants.

Table 2: The "Reactive Species" (Cadenas and Packer, 2002)

Radicals	Nonradicals
Reactive oxygen species (ROS)	Hydrogen peroxide, H_2O_2[a]
Superoxide, $O_2^{\cdot -}$[a]	Ozone O_3
Hydroxyl, $OH^{\cdot}$[a]	Singlet oxygen ($O_2{}^1\Delta g$)[a]
Hydroperoxyl, $HO_2^{\cdot}$[a]	Lipid peroxides, LOOH[a]
Lipid peroxyl, $LO_2^{\cdot}$[a]	Maillard reaction products[a]
Lipid alkoxyl, $LO^{\cdot}$[a]	
Reactive chlorine species (RCS)	Hypochlorous acid, HOCl[a]
Atomic chlorine, $Cl^{\cdot}$	Nitryl (nitronium) chloride NO_2Cl[b]
	Chloramines
Reactive nitrogen species (RNS)	Nitrous acid, HNO_2[a]
Nitric oxide, $NO^{\cdot}$[a]	Nitrosyl cation, NO^+
Nitrogen dioxide, $NO_2^{\cdot}$[a]	Nitroxyl anion, NO^-
	Dinitrogen tetroxide, N_2O_4
	Dinitrogen trioxide, N_2O_3
	Peroxynitrite, $ONOO^-$
	Peroxynitrous acid, ONOOH
	Nitronium (nitryl) cation, NO_2^+
	Alkyl peroxynitrites, ROONO
	Nitryl (nitronium) chloride, NO_2Cl[b]

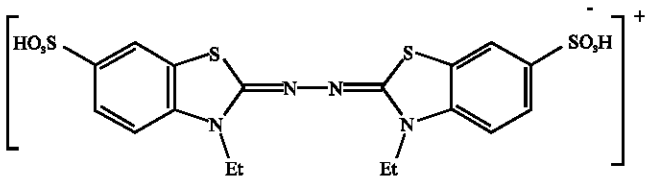
Reactive oxygen species is a collective term that includes both oxygen radicals and nonradicals that are oxidizing agents or are easily converted into radicals (HOCl, O_3, ONOO-, $^1O_2$, H_2O_2). RNS is a collective term including nitric oxide and nitrogen dioxide radicals, as well as such nonradicals as HNO_2 and N_2O_4. ONOO- is often included in both categories. Reactive is not an appropriate term: H_2O_2, $NO^{\cdot}$ and $O_2^{\cdot -}$ react quickly with only a few molecules, whereas, $OH^{\cdot}$ reacts quickly with almost everything. $RO_2^{\cdot}$, $RO^{\cdot}$, HOCl, $NO_2^{\cdot}$, ONOO- and O_3 have intermediate reactivities. [a]Reactive species particularly relevant to foods. [b]NO_2Cl is a chlorinating and nitrating species produced by reaction of HOCl with NO_2

Trolox equivalent antioxidant capacity (TEAC) method: The Total Antioxidant Capacity (TAC) provides information on the overall status of antioxidants within a biological sample, has proven to be a useful indicator for determining the ability of an organism to mitigate the potential damage produced by ROS. TEAC method offers several advantages over other methods and is relatively easy method. Due to its operational simplicity, the TEAC assay has been used for studying antioxidant capacity and TEAC values of many compounds and foods samples.

TEAC assay was first reported by Miller *et al.* (1993) which is based on the scavenging ability of antioxidants to the stable radical cation $ABTS^{\bullet+}$[2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (Fig. 1). In this assay, ABTS is oxidized by peroxyl radicals or other oxidants to its radical cation, $ABTS^{\bullet+}$ which is intensely coloured. The Antioxidant Capacity (AOC) is measured as the ability of test compounds to decrease the color reacting directly with the $ABTS^{\bullet+}$ radical and the results are expressed as Trolox (6-hydroxy-2,5,7,8 tetramethyl-chroman-2-carboxylic acid, a water-soluble derivative of vitamin E) Equivalents (TE). Due to difficulties in measuring individual antioxidant components of a complex mixture, Trolox equivalency is used as a benchmark for the antioxidant capacity of such a mixture using the ABTS decolorization assay (Re *et al.*, 1999).

The TEAC is an Electron Transfer (ET) based assay. It is based on scavenging of the relatively stable blue/green [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)] (ABTS) radical and its conversion into a colourless product. Degrees of decolorization reflect the amount of radical scavenged and thereby the antioxidative activity of the test compound. $ABTS^{\bullet+}$ is soluble in both aqueous and organic solvents and is not affected by ionic strength, so can be used in multiple media to determine both hydrophilic and lipophillic antioxidant capacities of extracts and body fluids. Moreover, it is simpler and cheaper. The TEAC measures the antioxidant capacity of the parent compound plus that of reaction products. These reaction products may have a considerable contribution to the TEAC. The ABTS radical used in TEAC assays is not found in mammalian biology and thus represents a "nonphysiological" radical source. Thermodynamically, a compound can reduce $ABTS^{\bullet+}$ if it has a redox potential lower than that of ABTS.

$ABTS^{\bullet+}$ can be generated by either chemical reaction [e.g., MnO_2, ABAP, $K_2S_2O_8$] or enzyme reactions [e.g. metmyoglobin, haemoglobin, or horseradish peroxidise]. Generally, chemical generation requires a long time (e.g. 16 h for $K_2S_2O_8$ generation) or high temperatures (60°C) whereas enzyme generation is faster and the reaction conditions are milder. Horseradish peroxidise mediated generation of $ABTS^{\bullet+}$ could be studied over a wide range of pH. However, the reaction mechanism may shift with pH; for example, electron transfer is facilitated at acid pH. This variation has also been adapted to selectively measure hydrophilic and lipophillic antioxidants by running the assay in buffered media and organic solvents respectively, or by partitioning antioxidants in mixtures between hexane and aqueous solvents. However, water-soluble reactions appear to be favoured (Cano *et al.*, 2000).



$ABTS^{\bullet+}$ Radical cation

Fig. 1: $ABTS^{\bullet+}$ Radical cation (Miller *et al.*, 1993)

Originally, this assay used metmyoglobin and H_2O_2 to generate ferrylmyoglobin which is then reacted with ABTS to form $ABTS^{\bullet+}$. Metmyoglobin (MetMb) was prepared by mixing myoglobin with $K_3Fe(CN)_6$ and then re-purified before use (Rice-Evans and Miller, 1994). The concentration of metmyoglobin was calculated using the extinction coefficients of 580 nm.

The wavelengths of 415 and 734 nm were adopted by most investigators to spectrophotometrically monitor the reaction between the antioxidants and $ABTS^{\bullet+}$. For quantification, the recent revised methods measure the absorbance decrease of $ABTS^{\bullet+}$ in the presence of test samples or Trolox for a fixed time point (4-6 min) and then antioxidant capacity is calculated as Trolox equivalents (Arts *et al.*, 2004).

The sample to be tested is added into the reaction medium before the radical is formed. However, this was observed as a major pitfall, because antioxidants can react with oxidizing agents themselves and may lead to overestimation of antioxidant capacity. This led to the proposed "post-addition" protocols to improve the assay (Zulueta *et al.*, 2009).

Another technique for the generation of $ABTS^{\bullet+}$ involves direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate. This has absorption maxima at wavelengths 645, 734 and 815 nm, as well as the more commonly used absorption maximum at 415 nm. Addition of antioxidants to the pre-formed radical cation reduces $ABTS^{\bullet+}$ depending on the antioxidant activity, the concentration of the antioxidant and the duration of the reaction. The extent of decolorization of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated relative to the reactivity of Trolox. The method is applicable to the study of both water-soluble and lipid-soluble antioxidants, pure compounds and food extracts (Re *et al.*, 1999).

In another spectrophotometric method for the direct measurement of the total antioxidant activity of LDL, the $ABTS^{\bullet+}$ was generated in the aqueous phase of the analytical mixture to which LDL was added. The antioxidants in lipoprotein particles were demonstrated to be capable of suppressing its formation, however, the extent to which all the minor antioxidants participate is unclear (Miller *et al.*, 1995).

Antioxidant may also be added to a pre-formed $ABTS^{\bullet+}$ radical solution and after a fixed time period, the remaining $ABTS^{\bullet+}$ is quantified spectrophotometrically (Re *et al.*, 1999; Van den Berg *et al.*, 1999). The reduction in $ABTS^{\bullet+}$ concentration induced by antioxidant is related to that of trolox and gives the TEAC value of that antioxidant. The assay is rapid, easy and correlates with the biological activity of antioxidants (Rezk *et al.*, 2003).

Ivekovic *et al.* (2005) introduced an improved TEAC discoloration assay-based Flow Injection Analysis (FIA) method for the evaluation of the antioxidant activity in which, the ABTS radical cation was generated on-line by electrochemical oxidation in the flow-through electrolysis cell which forms a part of the FIA system (Fig. 2).This avoids time consuming step of ABTS radical cation preparation by chemical oxidation, hence analysis time is reduced. The method was applied to the evaluation of the antioxidant activity of pure compounds and samples of common beverages and the results were correlated with the antioxidant activities determined by a classic TEAC assay. The method provides good reproducibility and sample throughput (32 samples per hour). A good correlation between the results obtained by the proposed method and TEAC values evaluated by the classic TEAC decolourisation assay. Figure 2 shows a pictorial representation of the FIA method.

The Total Antioxidant Activity (TEAC) of grape seed extract was measured by the method of Salah *et al.* (1995). This assay measures the antioxidant activity in the aqueous phase, specifically the ability of the test extract to scavenge ABTS radicals and monitored at 734 nm (Castillo *et al.*,

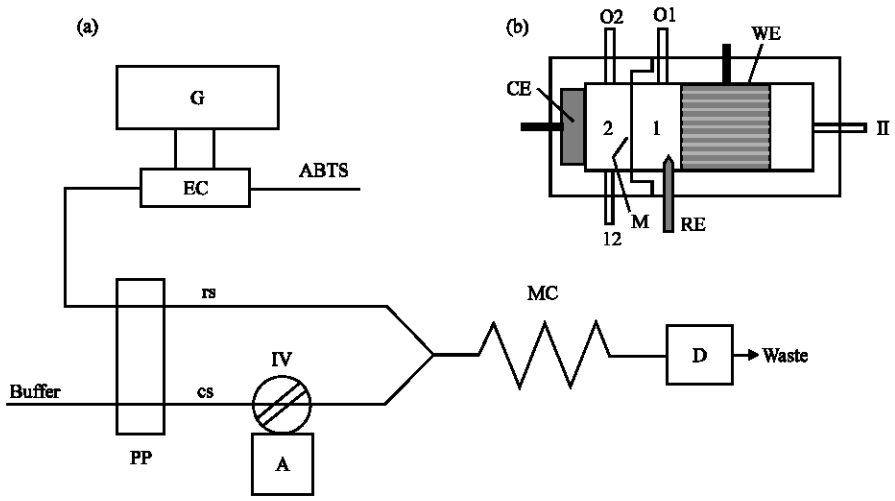


Fig. 2(a-b) (a): Manifold used for FIA measurements, PP: Three-channel peristaltic pump, EC: Flow-through electrolysis cell, G: Galvanostat, IV: Injection valve, A: Actuator; MC: Mixing coil, D: Detector, cs: Carrier stream, rs: Reagent (ABTS radical cation) stream, (b) Schematic representation of the flow-through electrolysis cell used for on-line generation of the ABTS radical cation, I: Inlet, WE: working electrode. RE: Reference electrode, M: Nafion membrane, CE: Counter electrode, O: Outlet, 1: Anode compartment, 2: Cathode compartment (Source: Ivekovic *et al*., 2005)

2000; Chen *et al*., 2005). Boussetta *et al*. (2011) studied the electrically assisted extraction of grape pomace to obtain high polyphenol content and evaluated the antioxidant activity by TEAC method. The extraction rate was shown to increase with increased temperature.

Magalhaes *et al*. (2008) discussed the order of addition of reagents and sample and their modifications in the improved version of the assay. The sample to be tested was added after generation of a certain amount of radical cation and the remaining radical cation concentration after reaction with antioxidant compound/sample was then quantified to minimize the interference of compounds with oxidants during radical formation and prevent the possible overestimation.

In a recent study, the $ABTS^{\bullet+}$ radical cations were generated by another oxidoreductase enzyme, namely laccase from *Trametes versicolor* which might be used for antioxidant determination. When laccase catalyzes the oxidation of its substrate, corresponding free radicals are generated as a product. The method is used for determination of total antioxidant activity of selected Thai vegetables and procedure of free radical generation with enzymatic reactions is considerably more environmental friendly (Khammuang and Sarnthima, 2008).

Total antioxidant levels in garlic were measured by a modified Rice-Evans and Miller (1994) method which is based on the inhibition by antioxidants of the absorbance of the radical cation $ABTS^{\bullet+}$. The modification of the assay included changes in the specificity of timing of the assay including a three min preincubation that are critical to the reproducibility of the assay. Three known antioxidants, vitamin C, trolox and glutathione, were used to assess the reproducibility of the assay. The results are calculated and expressed as TEAC (Drobiova *et al*., 2011).

Kambayashi *et al*. (2009) developed an efficient assay for plasma TAC using a 96-well microplate. TAC was assessed using lag time by antioxidants against the myoglobin-induced oxidation of ABTS with hydrogen peroxide and expressed as Trolox equivalent. The $ABTS^{\bullet+}$

decolorization method or the crocin bleaching method was used in this assay and the results were obtained by the single point fixed time measurement. In this method, reaction was stopped when all antioxidants were consumed. The linearity of the calibration curve with Trolox was maintained with the Trolox concentration range from 2.5 to 25 μM ($R^2 = 0.997$). The assay was applied to the measurement of TAC in healthy human plasma.

Recently, an improved technique of TEAC assay was developed using pre-formed $ABTS^{\bullet+}$. This method has been used to evaluate the total antioxidant capacity of tissue homogenate, plasma/serum, cell lysates and chemicals. In this assay a 96 well, high throughput format was developed. The $ABTS^{\bullet+}$ was prepared using potassium persulfate 12-16 h before use. The results are presented as nmol per mL or mg protein and represent the quantity of ability total amount antioxidants equivalent to Trolox in the sample (Klaunig and Pu, 2009).

Table 3 gives a comparative account of various methods used for antioxidant capacity determination (Prior *et al*., 2005).

Applications of TEAC method: With its versaltiity, the TEAC method has been used for the determination of antioxidant capacity of various food and related materials.

Synthetic red food colorants: The TEAC method was used to evaluate the antioxidant capacity of six synthetic red food colorants (azorubine, amaranth, ponceau 4R, erythrosine, red 2G and allura red AC) because the wavelength chosen for measurements (735 nm) does not interfere with the absorption maxima of the colorants. All the colorants showed measurable disappearance kinetics. However, in the case of Trolox kinetic studies the initial absorbance dropped immediately. The TEAC values of colorants are presented in Table 4 (Obon *et al*., 2005).

Table 3: Comparison of methods for assessing antioxidant capacity (Prior *et al*., 2005)

Assay type	Simplicity	Instrumentation required	Biological relevance	Mechanism	End point	Quantitation	Lipophilic and Hydrophilic AOC
ORAC	++[a]	+	+++	HAT	Fixed	AUC	+++
TRAP	---[b]	--spz	+++	HAT	Lag phs	IC50	--
FRAP	+++	+++	--	SET	Varies	ΔOD fixed	---
CUPRAC	+++	+++	SET	Time	ΔOD fixed	---	
TEAC	+	+	-	SET	Time	ΔOD fixed	+++
DPPH	+	+	-	SET	IC50	ΔOD fixed	-
TOSC	-	-	++	HAT	IC50	AUC	---
LDL oxid.	-	+++	+++	HAT	Lag phs	Lag time	---

a +, ++, +++ = desirable to highly desired characteristic. b -, - -, - - - = less desirable to highly undesirable based upon this characteristic

Table 4: Measurements of antioxidant capacity of synthetic red food colorants (Obon *et al*., 2005)

Compound	TEAC value
Trolox	1.000
Azorubine (E-122)	0.026
Amaranth (E-123)	0.019
Ponceau 4R (E-124)	0.007
Erythrosine (E-127)	0.016
Red 2G (E-128)	0.018
Allura red AC (E-129)	0.029

Cereal based products: The $ABTS^{\cdot+}$ scavenging capacities were determined using the radicals generated by either the metmyoglobin/H_2O_2 or the MnO_2 methods. Wheat and wheat based products were extracted in 100% ethanol or other lipophillic solvents, however, extracts can form a precipitate that interferes with this assay. This can be resolved by diluting sample extracts and standards in an ethanol solution containing 7% β-cyclodextrin (Zhou *et al*., 2004). The $ABTS^{\cdot+}$ radical scavenging capacity values for wheat grain, its fractions and other wheat-based food products have been depicted in Table 5 (Yu, 2007).

While studying the effect of incorporation of Teff *(Eragrostis tef)* grain on straightdough and sour dough bread, increase in the antioxidant capacity (as determined by TEAC method) along with some of the nutrients has been reported (Alaunyte *et al*., 2012).

Total antioxidant activity of free, soluble conjugates and insoluble-bound phenolic fractions of various barley cultivars has been determined by TEAC method and exhibited in Table 6. Insoluble-bound phenolic fraction contributed the highest proportion towards TAC, followed by soluble conjugate and free phenolics. Cultivar "Tercel" showed lowest content of free and soluble conjugate phenolics while Falcon showed highest content of free phenolics. Peregrine possessed highest content of soluble conjugate and insoluble bound phenolics (Madhujith and Shahidi, 2009).

The total anthocyanin content and the antioxidant activity of the seed and cob from Chinese purple corn (*Zea mays* L., cv Zihei) extracts was determined by different methods including TEAC method (Zhendong and Weiwei, 2010) and showed that both the cob and seed extracts possess higher antioxidant activities than Butylated Hydroxytoluene (BHT).

Table 5: $ABTS^{+}$ scavenging capacity values for wheat grain, its fractions and wheat-based food products (Zhou *et al*., 2004)

Wheat material	$ABTS^{+}$ scavenging capacities, (μmoles TE/g)	$ABTS^{+}$ generation method
Hard Wheat grains	1.1-36	metMb/H_2O_2
Wheat-based breakfast cereals	2.3-2.4	metMb/H_2O_2
Hard wheat brans	24.0-35	metMb/H_2O_2
Hard wheat brans	5.0-22	MnO_2
Hard wheat aleurone	23.0-24	MnO_2
Soft wheat grains	14.0-18	MnO_2
Wheat grains	1.3-8[a]	metMb/H_2O_2
Soft and hard wheat grains	8.0-9[a]	ABAP
Soft and hard wheat germ	16.0-18[a]	ABAP
Soft and hard wheat bran and shorts	10.0-14[a]	ABAP

MnO_2 ABAP and metMb/H_2O_2 indicate methods using manganese dioxide, (2,2'- azobis-(2-amidinopropane) HCl, or the metmyoglobin/H_2O_2 systems, respectively, to generate ABTS cation radicals.[a]Values calculated on per gram basis according to data in the literature(s)

Table 6: TAC of free, soluble conjugate and insoluble-bound phenolic fractions of barley cultivars as measured by TEAC (Madhujith and Shahidi, 2009)

Barley cultivar	Free phenolics	Soluble conjugate phenolics	Insoluble-bound phenolics
Falcon	3.11±0.02[a]	3.46±0.05[c]	8.98±0.06[c]
AC Metcalfe	2.67±0.31[cd]	3.87±0.16[d]	9.45±0.17[cd]
Tyto	2.45±0.02[bc]	2.37±0.22[b]	7.44±0.23[a]
Tercel	1.89±0.05[a]	1.77±0.08[a]	7.53±0.19[a]
Phoenix	2.16±0.09[ab]	2.66±0.04[b]	8.23±0.06[b]
Peregrine	3.02±0.02[de]	3.98±0.09[d]	9.88±0.26[d]

[a]Phenolic contents are expressed as mg ferulic acid equivalents/g defatted material, [b]The sum of free, soluble conjugate and insoluble-bound phenolics; expressed as mg ferulic acid equivalents/g defatted material, [c]Soluble phenolics represent the sum of free and soluble conjugate phenolic fractions, [1] TEAC values are expressed as mg μmol trolox/g defatted material

Table 7: Molar properties and antioxidant activity of anthocyanin standards (Awika *et al*., 2005)

Standard	λ_{max} (at pH 1)	Antioxidant activity
Luteolinidin[a]	482	4
Apigeninidin[a]	468	3
Peonidin	516	4
Pelargonidin	506	4
Pelargonidin-3,5 diglcoside	498	2
Cyanidin	516	5
Cyanidin-3-glucoside	512	4
Cyanidin-3,5-diglosidec	510	3
Cyanidin-3-rutinoside	514	4

[a]Compounds identified in sorghum

Table 8: TEAC of different indolic compounds (Reiter *et al*., 2002)

Indolic compound	TEAC-HAA	TEAC-LAA
Indole-3-methanol	1.14±0.007	0.37±0.02
Indole-3-acetic acid	1.12±0.04	0.95±0.07
Indole-3-butyric acid	1.02±0.06	0.63±0.04
Indole-3-ethanol	0.98±0.08	0.28±0.02
Indole-3-carboxylic acid	0.13±0.01	0.11±0.01
indole-3-propionic acid	1.66±0.14	0.94±0.05
Indole-3-aldehyde	0.07±0.01	0.03±0.01
Tryptophan	1.51±0.08	0.40±0.03

TEAC method was used for antioxidant activity assay of various standard anthocyanins and those isolated from black sorghum and the results are presented in Table 7 (Awika *et al*., 2005).

Free radical-scavenging activity of indolic compounds in aqueous and ethanolic media: Cano *et al*. (2003) studied the free radical-scavenging properties of some plant-derived indoles. The ABTS/H_2O_2/HRP decoloration method is capable of determining both hydrophilic and lipophilic antioxidant properties of chemical compounds and complex samples. The Hydrophilic Antioxidant Activity (HAA) and Lipophilic Antioxidant Activity (LAA) in specific reaction media were determined by carrying out HAA in buffered medium (pH 7.5) and LAA in ethanolic medium. Trolox can be used in both HAA and LAA and presents a stoichiometry of 2 for both with $ABTS^{\bullet+}$, the respective TEAC values of each indolic compound can be calculated (Table 8, Reiter *et al*., 2002). In all cases, HAA values were higher than LAA which indicates that hydrophilic moieties of the molecules allowed a better interaction between the $ABTS^{\bullet+}$ and the indolic compounds than the lipophillic ones.

***Fragaria x ananassa*:** The total antioxidant capacity and phenolic compounds of different selections and control varieties of strawberry (*Fragaria x ananassa*) was carried out to determine important quality characteristics of antioxidant compounds that are very important to realize a screening in a breeding program to obtain a strawberry variety with high productive, earliness and with high content of health beneficial compounds (Fernandez, 2008).

Tulipani *et al*. (2011) evaluated total antioxidant capacity by TEAC method, phenolic content, protein and allergen content of four strawberry genotypes. The authors confirmed a genotype dependent response to environmental stress conditions which may explain the changes in these parameters of the fruits in different years.

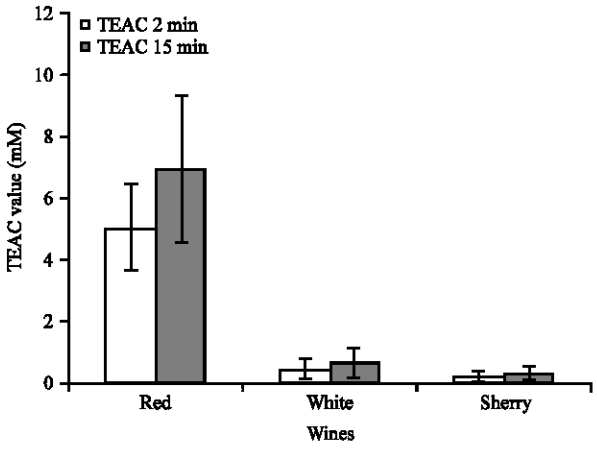


Fig. 3: TEAC values of different wines (Villano *et al.*, 2004)

TEAC values of wines: A comparative study on the antioxidant capacity, as determined by TEAC method indicates that the Red wines possess a higher antioxidant activity than white and Sherry wines (evaluated at 2 and 15 min, Fig. 3). This is in accordance with their high phenolic content. No significant differences were found between white and Sherry wines (Villano *et al.*, 2004).

A comparison of the $TEAC_{2\ min}$ values obtained with those obtained for other foods in the literature, using the $ABTS^{\bullet+}$ method, shows that one glass of red wine (125 mL) has the same antioxidant activity as 212 mL of grape juice, 190 mL of orange juice, 225 mL of black tea, 286 g of fresh spinach or 926 g of tomatoes.

Total antioxidant capacity of plant foods, beverages and oils: The total antioxidant capacity of a variety of foods used in the Italian diet was evaluated using three different assays (Pellegrini *et al.*, 2007). The food extracts had different antioxidant capacities in relation to the method applied; thus, the same item often ranked differently depending on the assay and the ranking order of the TAC values will be used for various commodities. In the case of solid foods (i.e., vegetables and fruits), the water and lipid-soluble extracts were analyzed separately and the overall TAC values were obtained from the sum of the two extract values. The approach of using only one solvent for fruits and vegetables extraction, generally used in TAC literature, may underestimate TAC values because antioxidant compounds at the extremities of the lipophilic or hydrophilic scale are incompletely extracted. In this case, food extracts obtained with two different solvents were analyzed separately and their sum reported in the tables.

Spinach showed highest antioxidant capacity, followed by peppers, whereas cucumber and endive exhibited the lowest TAC values. The high antioxidant capacity of spinach is due to both the water-and lipid-soluble fractions; the former contains glucuronic acid derivates of flavonoids and derivates and isomers of p-coumaric acid and the latter is rich in lutein and chlorophylls.

In case of fruits, berries had the highest antioxidant capacity, with blackberry being the most effective. Its high antioxidant capacity, is likely due to the high content of phenolic acids and flavonoids, such as anthocyanins. Olives were second in antioxidant capacity, may be due to their high levels of hydroxytyrosol and tyrosol content. Citrus fruits exhibited intermediate antioxidant capacity, with oranges as the most effective followed by grapefruit. Among the fruits belonging to the Rosaceae family (i.e., plum, apricot, apple, pear and peach), plums had the highest antioxidant capacity. Fruits from the Cucurbitaceae family (i.e., honeydew and cantaloupe melons and watermelon) had low TAC values.

Citrus juices had the highest amount of antioxidants while other fruit juices had intermediate TAC values. Cola drinks had the lowest TAC values. Among the beverages analyzed, coffee drinks were the most effective, with espresso having the highest antioxidant capacity. The removal of caffeine from the espresso coffee led to a decrease in TAC values. The antioxidant capacity of green tea is considerably higher than that of black tea which may be attributed to the changes occurring during fermentation. The flavanols in green tea leaves (catechins, gallic esters and others) undergo an oxidative polymerization by polyphenol oxidase which turns the leaves black. During oxidation, most of the catechin content of green tea is converted to oxyproducts, such as thearubingens and theaflavins, with a loss of antioxidant capacity.

While studying the effect of boiling and steaming on the content of phytochemicals, total antioxidant capacity (as determined by TEAC method) and other paramenters of frozen vegetables (carrot, cauliflower and spinach), Mazzeo *et al.* (2011) reported slight increase in TEAC values for all the vegetables both by steaming and boiling. However, the losses in other parameters were more by boiling than by steaming.

The antioxidant capacity of coffees (Arabica and Robusta) from 12 different points of origin (Uganda, Papua, Jamaica, Ethiopia, Kenya, Puerto Rico, "Caracolillo" Puerto Rico, Nicaragua, Colombia, Vietnam, Brazil and Guatemala) and two decaffeinated coffees from Colombia and Brazil prepared by three commonly used procedures (espresso, filter and Italian) were evaluated and compared with antioxidant standards and other phenolic compounds which have been described in coffee (Table 9; Parras *et al.*, 2007). Decaffeinated coffees (Colombia and Brazil) showed lower

Table 9: TEAC values for coffees of different origin compared with standards (Parras *et al.*, 2007)

	TEAC	
Samples	Time: 6 min	24 h
Guatemala		
Filter coffee	10.32±0.04	12.92±0.02
Italian coffee	10.21±0.05	12.46±0.04
Espresso coffee	8.67±0.01	11.11±0.07
Nicaragua		
Filter coffee	12.29±0.07	13.46±0.07
Italian coffee	11.51±0.06	12.42±0.01
Espresso coffee	8.74±0.02	11.23±0.03
Brazil		
Filter coffee	10.63±0.07	13.54±0.07
Italian coffee	10.91±0.04	12.02±0.01
Espresso coffee	9.38±0.02	11.24±0.07
Brazil (decaffeinated)		
Filter coffee	7.75±0.07	12.95±0.07
Italian coffee	9.68±0.05	11.78±0.05
Espresso coffee	7.80±0.01	10.59±0.01
Standards		
Propyl gallate	17.20±0.01	17.44±0.01
Chlorogenic acid	14.80±0.01	>19
a-Tocopherol	1.10±0.04	2.30±0.04
Caffeic acid	12.40±0.02	>19
BHT	0.26±0.02	0.72±0.02
p-Coumaric	2.70±0.01	>19
BHA	0.44±0.04	1.41±0.04

TEAC values than coffees with caffeine. Filter and Italian coffee exhibited higher TEAC value than espresso coffees. All the coffees studied are good antioxidants regardless of their cost, origin and way in which they are brewed, a point worth considering.

Samaniego-Sanchez *et al.* (2011) studied the effect of several culinary factors on the polyphenol content and antioxidant capacity by TEAC method during the preparation of green tea and concluded that water temperature and infusion time had strong influence while agitation and dosage did not show much effect on these parameters. Furthermore, pure green tea infusions had higher antioxidant properties than the blends of green tea with aromatic herbs and fruits.

Among alcoholic beverages, red wines possess highest antioxidant capacity followed by rose and white wines. This is because phenolic compounds in wine derive mainly from the skin, seeds and stems of grapes, making them important sources of the polyphenols that are transferred to the juice at the first stage of wine fermentation. Thus, the content of polyphenols is high in red wine; it is intermediate in rose´ wine and relatively low in white wine. The TEAC values of red and white wines were in the same range of those described by Simonetti *et al.* (1997).

Antioxidant activity of phenolic extracts from grape seeds and press residues from grape seed oil production: Phenolic compounds of seven grape seed samples from mechanical seed oil extraction were identified and quantified by HPLC-DAD before (intact seeds) and after (press residue) the oil recovery process. The values of the crude extracts from the seeds ranged from 48.49 to 104.80 mol Trolox antioxidant equivalent (TAE)/100 g Dry Matter (DM). 'Spätburgunder' variety yielded highest amounts, followed by 'Müller-Thurgau', 'Samtrot', 'Lemberger', 'Schwarzriesling', 'Kerner' and 'Cabernet Mitos'. Concerning the flavonoid fraction of the press residues, highest antioxidant activities were observed for 'Samtrot', followed by 'Kerner', 'Spätburgunder', 'Müller-Thurgau', 'Schwarzriesling', 'Lemberger' and 'Cabernet Mitos'.[56] TEAC values might also be affected by nonphenolic compounds, explaining the large differences between the results for the crude extracts and the sum of those for the flavonoid and phenolic acid fraction. This may occur due to degradation of phenolic antioxidants during processing. The antioxidant activity of phenolic compounds from different cultivars of grape seeds and the press residues from the oil recovery process has been depicted in Fig. 4 (Maier *et al.*, 2009).

TEAC for evaluation of the antioxidant properties of fruits: The range of antioxidant activity obtained for various fruits was very wide (Fig. 5). The samples with high antioxidant capacities were persimmon, blackberry, blueberry and strawberry in the order of decreasing TEAC values. Avocado exhibited lowest antioxidant activity, followed by green fig and pear (Garcia-Alonso *et al.*, 2004).

Development and validation of a food frequency questionnaire for the assessment of dietary total antioxidant capacity: Plant foods contain many compounds with antioxidant activity, including ascorbic acid and tocopherols, carotenoids and a variety of antioxidant phytochemicals such as simple phenolics and flavonoids. The concept of Total Antioxidant Capacity (TAC) was introduced as it takes into account the antioxidant activity of single compounds present in food or biological samples as well as their potential synergistic and redox interactions (Serafini and Del Rio, 2004). Several assays are available for measuring TAC, differing in chemistry (generation of different radicals and/or target molecules) and the way endpoints are measured. To consider the major redox reactions that commonly happen in human body, three

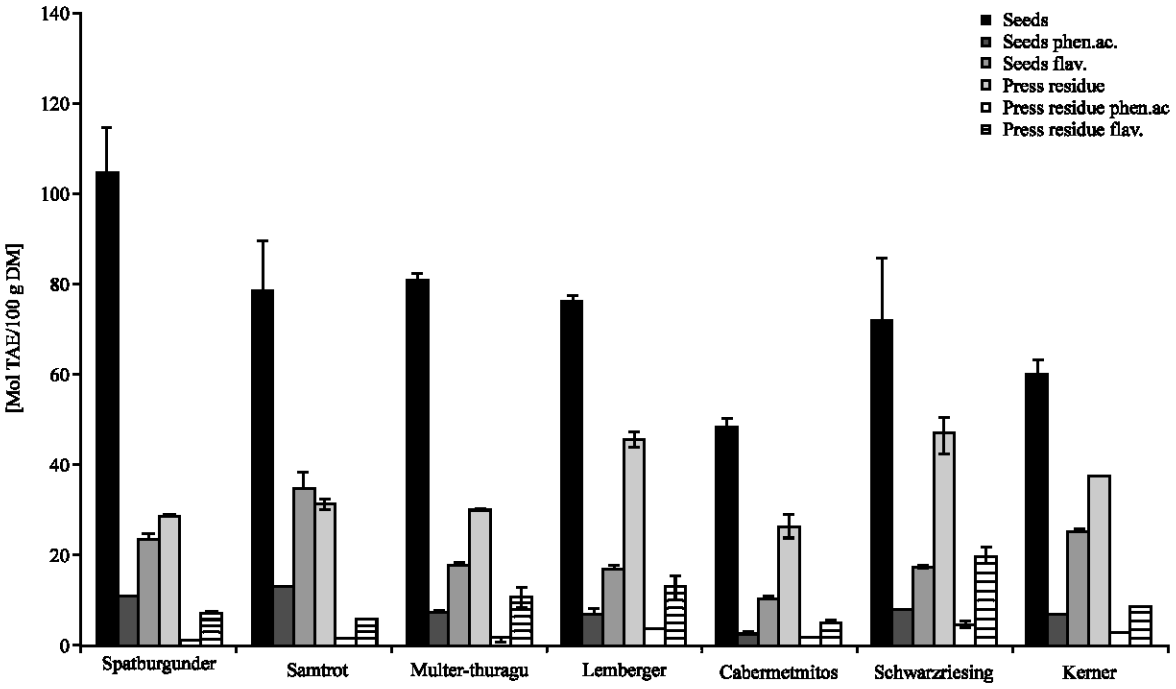


Fig. 4: Antioxidant activity of phenolic compounds from different cultivars of grape seeds and the press residues from the oil recovery process (Maier *et al*., 2009)

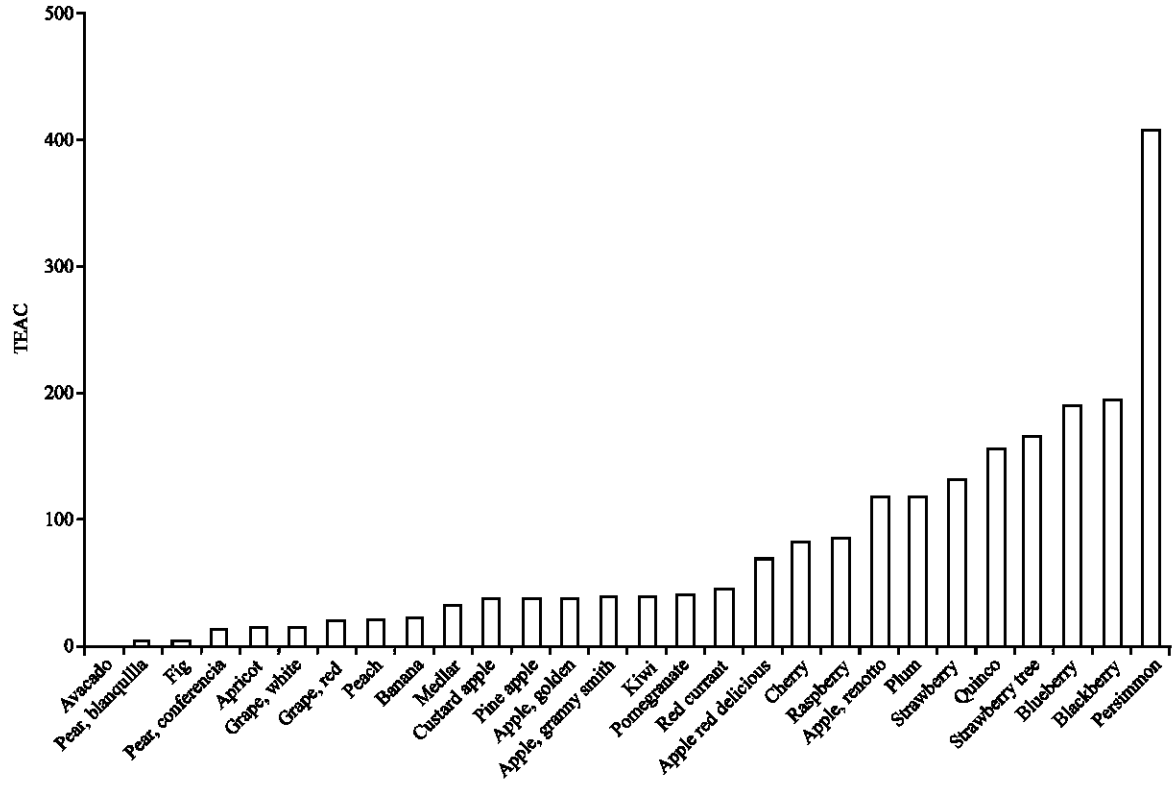


Fig. 5: Antioxidant capacity of various fruits obtained by TEAC method (Garcia-Alonso *et al*., 2004)

Table 10: Food frequency questionnaire (FFQ) (Pellegrini *et al*., 2007)

	Food Frequency Questionnaire (FFQ)		
Food group	Women (n = 1260	Men (n = 159)	Total (n = 285)
Alcoholic beverages	12.4 (25.8)	40.3 (33.9)	27.9 (36.4)
Biscuits	0.3 (0.7)	0.2 (0.6)	0.2 (0.7)
Breads	3.6 (2.7)	4.2 (3.5)	4.0 (3.4)
Cakes	0.3 (0.6)	0.2 (0.5)	0.2 (0.6)
Cereals	0.4 (0.4)	0.5 (0.4)	0.5 (0.4)
Chocolates	2.9 (10.7)	1.1 (3.6)	1.8 (6.1)
Coffees and teas	37.9 (20.5)	26.5 (21.5)	31.3 (22.2)
Fruits	13.6 (10.4)	8.2 (7.8)	10.2 (9.0)
Nuts	0.6 (2.1)	0.5 (2.6)	0.5 (2.4)
Oils and fats	0.6 (0.5)	0.5 (0.6)	0.6 (0.6)
Pizza	0.3 (0.4)	0.2 (0.3)	0.3 (0.4)
Potatoes	0.2 (0.3)	0.2 (0.3)	0.2 (0.3)
Salad dressings	0.4 (0.3)	0.3 (0.3)	0.3 (0.3)
Sauces	0.8 (1.0)	0.9 (1.0)	0.8 (1.0)
Spices	1.0 (0.6)	0.7 (0.6)	0.8 (0.7)
Sweets and dairy	0.7 (1.5)	0.3 (0.9)	0.4 (1.0)
Vegetables	8.0 (5.4)	6.1 (5.8)	7.1 (6.2)

methods, i.e., Trolox Equivalent Antioxidant Capacity (TEAC), Total Radical-trapping Antioxidant Parameter (TRAP) and Ferric Reducing-antioxidant Power (FRAP), were selected. The higher potential of TAC for epidemiological and clinical applications seems strengthened by the fact that dietary TAC may provide protection against gastric cancer and inflammatory processes. For dietary TAC to be used in such studies the FFQ is the obvious choice for assessing food and nutrient intake in epidemiological studies and thus FFQ was developed and validated for dietary TAC (Table 10) (Pellegrini *et al*., 2007).

Because of the complexity of real foods, accelerated test systems are difficult to standardize and each antioxidant test should be calibrated for each lipid or food. Accelerated oxidation conditions should be close to the storage conditions under which the food is to be protected. Ultimately, antioxidants should be evaluated on the food itself.

TEAC method is easy and rapid to perform and avoids unwanted reactions. High temperatures are not required to generate radicals. By this method antioxidant activity can be studied over a wide range of pH values and can be used to study effects of pH on antioxidant mechanisms. It avoids interference due to endogenous peroxidase activity. Determination of hydrophilic antioxidant activity in plant and other extracts is more accurate and rigorous (Arnao *et al*., 2001).

Recent advancements in TEAC method has brought in numerous variants of the method which can be effectively employed for measuring the antioxidant activity of different compounds and samples efficiently. This gives actual total antioxidant capacity and includes the potential scavenging effect of oxidation products. It can be used for tracking down unknown antioxidants in complex mixtures. The method is capable of determining both hydrophilic and lipophillic antioxidant properties and can also be used to determine the true total antioxidant capacity of a compound that is independent of the concentration of the antioxidant. The method can efficiently give a measure of the antioxidant activity of carotenoids, phenolics and some plasma antioxidants and for the direct measurement of the total antioxidant activity of Low Density Lipoproteins. The reaction can be automated and adapted to microplates, flow injection and stopped flow.

Amic and Lucic (2010) studied the reliability of TEAC values of flavonoids based on semiemirical quantum chemistry software package in modelling free radical scavenging activity. A good model of experimental vit C equivalent antioxidant capacity was obtained based on bond dissociation enthalpy and number of hydroxyl (-OH) groups. All other models also had comparable fit and cross validated statistical parameters, as well as significant regression coefficients.

Despite recent improvements and increased use, the TEAC assay has several limitations. The ability of an antioxidant to scavenge the artificial $ABTS^{\bullet+}$ radical may not reflect the antioxidant activity due to other mechanisms effective in complex food lipids or physiologically relevant substrates, including metal chelation and effects of antioxidant partitioning among phase of different polarities. The TEAC assay is frequently used to rank antioxidants and for the construction of Structure Activity Relationships (SARs). However, in few cases, the TEAC value does not exactly correlate with the antioxidant activity. For e.g., the reaction products of chrysin possesses higher antioxidant capacity than the parent compound, consequently overestimates the TEAC value for chrysin. The contribution of reaction products limits the use of TEAC for constructing SARs, as in SAR the activity needs to be related to single structure only. Despite this, TEAC assay is useful for screening unknown antioxidants in complex mixtures (Arts *et al*., 2004).

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