

Review

Evaluation of The Antioxidant Capacity of Food Products: Methods, Applications and Limitations

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Abstract: Assays of total antioxidant capacity (TAC) are popular in the analysis of food products. This review presents the most popular assays of TAC and their limitations, databases of TAC of food products, their application in clinical studies, and the effect of processing on the TAC of food. The importance of sample preparation for TAC assays and striking effects of digestion in the gastrointestinal tract on the TAC of food are discussed. Critical opinions on the validity of food TAC assays are considered. It is concluded that TAC methods can be useful as screening assays for food quality control and as low-cost, high-throughput tools used to discover potential antioxidant sources and follow changes in the content of antioxidants during food processing. However, effects revealed by TAC assays should be followed and explained using more specific methods.

Keywords: total antioxidant capacity; food; ABTS; DPPH; FRAP; ORAC; cooking; digestion

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1. Introduction

In recent decades, assays of the antioxidant activity/capacity have become one of the most popular subjects of publications concerning foods, beverages and their components. The idea of measuring the sum of antioxidant activities in a single simple assay is undoubtedly attractive and has been broadly applied. This approach can integrate the individual antioxidant actions of different compounds and their additive, synergistic, or antagonistic interactions. Let us recall, for the sake of clarity, the difference between the terms: “antioxidant activity” and “antioxidant capacity”. The term “antioxidant activity” should, in principle, be used to describe antioxidant properties of a single compound in a given assay, while the term “total antioxidant capacity” (TAC) refers to antioxidant properties of a complex material (such as a beverage, extract or biological fluid) composed of many compounds in this assay [1]. In addition, the term “antioxidant activity” used in a broader sense (a notion as opposed to a measurable parameter) encompasses diverse features of an antioxidant such as redox potential and rate constants of reactions with various oxidants. Therefore, the term “TAC” will be used throughout this paper for the parameter characterizing the antioxidant behavior of complex samples.

However, the notion of TAC became a subject of criticism. The term “total” has been criticized since the most common assays do not measure the activities of antioxidant enzymes, so important in the antioxidant defense. It has been argued that instead of “total” the term “partial” or “non-enzymatic” antioxidant capacity should be used [2–5]. Nevertheless, this distinction, important for characterization of the “antioxidant barrier” of human, animal or plant organisms, does not seem relevant with respect to food since food enzymes are anyhow digested and may act only to a limited extent in the gastrointestinal tract.

2. Methods of Estimation of Total Antioxidant Capacity

A bunch of methods has been proposed to estimate the total capacity of biological material to scavenge reactive oxygen species, to scavenge free radicals, or the content of reducing compounds. These terms are almost but not totally synonymous, which leads to some ambiguity. Reducing compounds donate electrons or hydrogen atoms to compounds which have higher reduction/oxidation (redox) potentials. The latter group of compounds includes free radicals and other oxidants occurring in living systems. Not all reactive oxygen species (ROS) are free radicals. Hydrogen peroxide, other peroxides, peroxy nitrite, and singlet oxygen are not free radicals.

2.1. Most Popular Methods

Most TAC assays are based on reactions of antioxidants on stable free radicals or those generated in situ (Table 1).

Table 1. Most popular assays of total antioxidant capacity.

Method	Principle of measurement	References
ABTS• reduction	Decrease in absorbance of solution of pre-formed ABTS• radical (usually at 734 or 414 nm)	[6,7]
DPPH• reduction	Decrease in absorbance of solution of the stable DPPH• radical (around 517 nm)	[8,9]
FRAP	Increase in absorbance of Fe ²⁺ -TPTZ complex upon reduction of Fe ³⁺ to Fe ²⁺ (at 593 nm)	[10,11]
CUPRAC	Increase in absorbance of bis(neocuproine)copper(+) upon reduction of Cu ²⁺ to Cu ⁺ (at 540 nm)	[12,13]
ORAC	Inhibition of fluorescence decrease of R-phycoerythrin or fluorescein induced by a source of peroxy radicals	[14,15]
CL assay	Inhibition of chemiluminescence of a detector induced by an oxidant	[16,17]

Indicator substances used in these absorptiometric, fluorimetric, or luminometric assays of TAC are shown in Figure 1.

The antioxidant activity measured by ABTS• reduction is usually referred to that of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) as a standard antioxidant. It allows expression of results in Trolox equivalents (TE), which is a quite useful and comparable way comparing antioxidant activities. For this reason, the assay based on ABTS• reduction is often referred to as assessment of “Trolox Equivalent Antioxidant Capacity” (TEAC), although there are no real reasons for restricting this term to the ABTS• assays only and sometimes this term is also applied to the DPPH• reduction and ORAC assays (if expressed in TE).

In the original TAC assay employing ABTS•, metmyoglobin and hydrogen peroxide were used to generate an intermediate radical of ferrylmyoglobin, which subsequently reacts with ABTS to produce ABTS• in situ, and prevention of ABTS• formation was quantified [18]. This assay became the basis of a commercial “ABTS Antioxidant Assay Kit”. Later on, the use of pre-formed ABTS• radical gave rise to the most commonly used “improved ABTS radical cation decolorization assay” [6]. Potassium persulphate is the oxidant used usually for ABTS• generation. The product is relatively stable at low temperature. The reaction product can also be lyophilized, and the stable powder may be dissolved to prepare a working ABTS• solution [19].

ABTS• is soluble in both aqueous and organic solvent media, enabling estimation of both hydrophilic and lipophilic antioxidants. The assay can be performed in a broad pH range [7]. ABTS• is an N-centered radical with sterically limited access, e.g., to polymeric phenols giving rise to slow reactions of some antioxidants [20,21]. The assay has been challenged for its lack of biological relevance due to use of the artificial ABTS• radical that is not present in food or biological systems (a “non-physiological radical”; this reservation concerns DPPH• as well) [21].

The DPPH• reduction method, though even simpler than the ABTS• reduction method (DPPH• is commercially available) has been reported to be much influenced by light, air oxygen, pH and the type of solvent. DPPH• is essentially soluble in organic solvent media but not in water. It is usually dissolved in methanol, ethanol, or their aqueous mixtures. In this final case, the water content should not exceed 60% to make the radical soluble [22]. The insolubility of DPPH• in water brings an important limitation to the determination of hydrophilic antioxidants. Flavonoids and other complex phenols generally exhibit moderate-to-slow reaction with DPPH• [20].

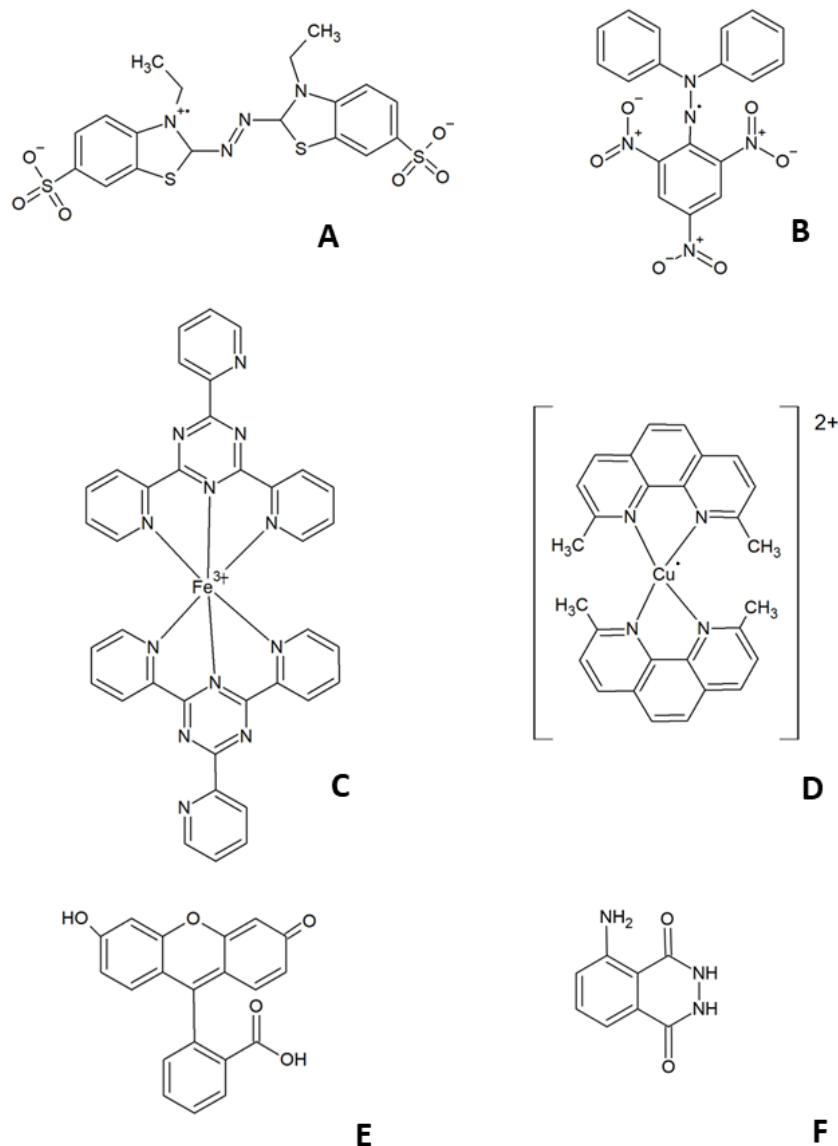


Figure 1. Structure of substrates used in the most popular assays of TAC: (A) ABTS•, (B) DPPH•, (C) bis(TPTZ)Fe³⁺, (D) bis(neocuproine)Cu²⁺, (E) fluorescein, (F) luminol.

Antioxidant activity estimated by the DPPH• reduction method is often reported as efficient concentration EC_{50} , which is defined as the concentration of the antioxidant necessary to reduce the initial DPPH• concentration (and absorbance) by 50%. In addition, $t_{EC_{50}}$ (the time necessary for EC_{50} to reach the equilibrium state) is used [23]. Another approach is the “area under curve approach” i.e., measuring the sum of DPPH absorbances in the course of the reaction, as in the ORAC assay [24]. The reciprocal of the efficient concentration $1/EC_{50}$ termed “antiradical power” (ARP) is also employed [25,26]. Other authors proposed to use another parameter, reciprocal of the product of EC_{50} and $t_{EC_{50}}$, $1/(EC_{50} \times t_{EC_{50}})$ termed “antiradical efficiency”, AE [27,28].

The ferric reducing antioxidant power assay (FRAP) assay is based on the reduction of the 2,4,6-tripyridyl-triazine (TPTZ)- Fe^{3+} to the deep blue TPTZ- Fe^{2+} complex. The signal measured is the reduction-driven increase in absorbance at 593 nm. The assay is carried out at acidic pH conditions (pH = 3.6) to maintain iron solubility. In a modified version known as the FRASC assay, both the total antioxidant activity and the ascorbic acid (vitamin C) concentration of the test sample can be measured simultaneously by employing ascorbic oxidase to destroy ascorbic acid in one of a pair of samples run in parallel [29]. Oxidation of some hydroxycinnamic acids and flavonoids with the FRAP reagent is not complete within the protocol time period of the assay [30] so longer reaction times are used by some authors. High-spin Fe^{3+} bound to TPTZ has an inherently slow kinetics due to half-filled d-orbitals of ferric ion, and the trivalently-charged Fe^{3+} -TPTZ complex has higher affinity toward the aqueous phase (due to ion-dipole interaction with solvent water molecules) than toward organic solvents [31], limiting the determination of lipophilic antioxidants. The original FRAP test uses TPTZ as the chelating ligand for the iron ions, while alternative ligands, such as ferrozine, were also used to bind the iron ions [32].

The cupric reducing antioxidant capacity (CUPRAC) method uses the copper²⁺-neocuproine (2,9-dimethyl-1,10-phenanthroline; Nc), which can be reduced by antioxidants. CUPRAC method measures the antioxidant capacity at nearly physiological pH (i.e., pH 7), so it better simulates the physiological action of antioxidants. The CUPRAC chromophore $Cu(Nc)^{2+}$ is soluble in both aqueous and organic solvents, enabling the assay of hydrophilic and lipophilic antioxidants. The standard redox potential of the Cu^{2+}/Cu^+ -neocuproine complex is 0.6 V, which is much higher than that of the single Cu^{2+}/Cu^+ couple (0.17 V). Glutathione and other thiols, not reactive in the FRAP assay, react with the Cu^{2+} -neocuproine complex [20]. The original CUPRAC test was modified to include various samples in diverse applications, e.g., the acetone/water environment, with the help of methyl-cyclodextrine, enables simultaneous determination of hydrophilic and lipophilic antioxidants [13,33].

The oxygen radical absorbance capacity (ORAC) assay is based on the inhibition of the oxidation of a fluorescent substrate (and fluorescence loss) by peroxy radicals. Decomposition of an initiator compound produces alkyl radicals, which quickly react with oxygen, forming peroxy radicals [34]. Commonly used peroxy radical generators used in this assay are represented by azo-compounds decomposing at elevated temperatures. Usually, the hydrophilic 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) is used as the initiator but lipophilic initiators such as 2,2'-azobis(isobutyronitrile) (AIBN) or 2,2'-azobis(2,4-dimethylvaleronyl) (AMVN) are also employed [35]. While the free radical initiators used in the assay are synthetic compounds not occurring in food, the peroxy radicals are the main radicals involved in lipid peroxidation in foodstuffs, so it is argued that the assay is fully relevant for food oxidation phenomena. In order to measure both hydrophilic and lipophilic antioxidants, the initial ORAC assay was modified using a solution of 50% acetone/50% water (v/v) and 7% randomly methylated β -cyclodextrin as a solubility enhancer for the antioxidants [36]. Initially β -phycoerythrin, a fluorescent protein isolated from the red algae *Porphyridium cruentum*, was used as a detector of oxidation caused by the peroxy radicals but later on it was replaced by fluorescein (Figure 1) or, sometimes, by other fluorescent dyes, such as Nile blue [37] or *p*-aminobenzoic acid (PABA) [38]. The principle of the assay consists in estimation of the protection of the

detector probe against the loss of fluorescence in a kinetic assay. Sum of fluorescence values (“area under curve” in a plot of fluorescence as a function of time) measured in the presence of a sample containing antioxidants is compared with that of a sample containing no antioxidant. The presence of metal ions in a sample may affect the assay. The inclusion of ethylenediaminetetraacetic acid (EDTA) was proposed to attenuate metal interferences [39].

Chemiluminescence assays of TAC are based on the reaction of reactive oxygen species or reactive nitrogen species (ROS/RNS) with detecting reagents to generate species in an excited state that emit light upon de-excitation to the ground state. Antioxidants that react with the initiating reactive species diminish the light generation as their reactions compete with reactions of ROS/RNS with detectors. By changing the oxidant initiator (e.g., superoxide or peroxy radicals, peroxyxynitrite, hypochlorite, singlet oxygen), it is possible to measure the capacity of quenching of different ROS/RNS by antioxidants [17,40,41]. The main chemiluminescence reagents used are luminol [16,42] (Figure 1), lucigenin [43], peroxyoxalate [44], the $\text{NaHCO}_3\text{-H}_2\text{O}_2\text{-Co}^{2+}$ system [45], and a bioluminescent protein phos-lasin [46].

An apparatus (PHOTOCHEM[®]) released by Analytic (Jena, Germany) for the analysis of TAC generates free radicals by the optical excitation of a photosensitizer. The radicals induce luminescence of luminol, which is measured. Antioxidants eliminate free radicals, decreasing the luminescence [47,48].

2.2. Less Popular Methods

Apart from these most popular methods, several other TAC assays are being in use. The hydroxyl radical antioxidant capacity (HORAC) assay is based on the oxidation of a fluorescent probe by hydroxyl radicals generated by a Fenton system, usually $\text{Co}^{2+} + \text{H}_2\text{O}_2$ [49]. In our opinion, the use of this assay for food analysis should be definitely discouraged. Hydroxyl radical is so reactive that it reacts with any molecule (although the reaction rate constants differ). Thus, any substance present in a sample will show antioxidant activity in this test and the assay is simply a measure of content of (mainly) organic substances in the material studied. Moreover, there is (less frequent) possibility that some components of a sample will strongly chelate the metal component of a Fenton system in a way preventing hydroxyl radical formation rather than react with the hydroxyl radical.

The potassium ferricyanide reducing power (PFRAP) assay is based on the reduction of ferricyanide to ferrocyanide by antioxidants [50–52]. The standard redox potential of the ferricyanide/ferrocyanide system is quite low (0.36 V), which should limit the range of antioxidants detected by this system but in fact the final product is not the ferrocyanide but the Prussian blue $\text{Fe}[\text{Fe}(\text{CN})_6]$ so the actual E_0 of this system is much higher [51]. A disadvantage of this version of the FRAP test is the tendency of Prussian blue to precipitate and form a suspension. In order to keep Prussian blue in solution, inclusion of a detergent, sodium dodecyl sulphate, in the reaction medium and modification of pH was proposed [52].

The total reactive antioxidant potential (TRAP) test is based on the capacity of antioxidants to inhibit the reaction between peroxy radicals and a target molecule, which initially represented the O_2 consumption by a sample in the peroxidation process triggered by the thermal decomposition of AAPH. The retardation time of the O_2 consumption, i.e., the induction period, may be quantitatively measured and used to express the total antioxidant capacity of the samples as the TRAP value. Then, the method was modified several times changing initiators and detectors [53,54].

The total radical-trapping antioxidant parameter assay also measures the capability of antioxidants to interfere with the reaction between peroxy radical and a detector. Originally, AAPH was used as a peroxy radical generator and R-phycoerythrin as a detector probe [55]. The quantification of antioxidants is based on the duration of the lag phase. Other sources of peroxy radicals and other detectors (fluorescein, fluorescein diacetate and luminol) [42,56] were also used. An important limitation of the TRAP assay is the use

of the lag phase for determination of antioxidant capacity because not all antioxidants produce a clear lag phase [57].

The total oxyradical scavenging capacity (TOSC) test is based on inhibition of the formation of ethylene from α -keto- γ -methylbutyric acid by antioxidant compounds [58].

The β -carotene bleaching assay [59] employs an aqueous emulsion of linoleic acid and β -carotene, which is discolored under the influence of the radicals generated through the spontaneous oxidation of the fatty acid, at elevated temperature. The measurements are done usually at 50°C. Quantification is based on the inhibition of the rate of decay of absorbance of β -carotene at 470–490 nm in the presence of antioxidants. The β -carotene bleaching assay can screen both lipophilic and hydrophilic samples [60,61]. A similar assay is based on the attenuation of bleaching of crocin by antioxidants [62].

The assay of reduction of the galvinoxyl (2,6-di-tert-butyl- α -(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-tolyl)oxy radical is also used. The radical is commercially available, is oxygen-centered and is more reactive towards polyphenols than DPPH• [63,64]. Spectrophotometric methods of TAC measurement based on the reduction of 2,6-dichlorophenolindophenol [65] and Ce⁴⁺ [66], and assays estimating the scavenging of such oxidants as peroxyxynitrite and hypochlorite based on protection of Pyrogallol Red or fluorescein against decolorization/loss of fluorescence were proposed [67,68].

The Folin-Ciocalteu assay is also listed in the context of TAC assays. It was originally developed for the assay of tyrosine and tryptophan [69] and later modified for analysis of total polyphenols [70]. These compounds constitute a considerable fraction of antioxidants in plant foods. However, the assay is not specific for antioxidants, because it responds also to simple phenols, citric acid, many amines, amino acids, and reducing sugars [40], due to the indefinitely high redox potential of the Folin reagent. Moreover, the molybdo-phosphotungstate heteropoly anion being the Folin chromophore is (4-) charged, exhibiting high ion-dipole interactions with water molecules, so this assay is unsuitable for lipophilic antioxidants [30].

There are also methods of TAC assay in use based not on optical measurements but on voltammetry, amperometry [71], chromatography [72], electrophoresis [73] or direct measurement of radical scavenging by electron paramagnetic spectroscopy [74,75] and employing nanoparticles [76,77]. To give two examples: a sample reacts with 2,6-dichlorophenolindophenol (DCIP), and the reduction product of DCIP is separated and quantified by capillary electrophoresis [73] or ABTS• remaining after reaction is quantified by HPLC [72]. In the exemplified assays, nanoliter volumes of samples can be used. A paper-based device, obviating the need for a spectrophotometer or plate reader, was proposed for TAC assays, which can be performed by dropping a sample solution onto the sample zone. The sample flows to the pretreatment and detection zones that are pre-deposited with the reagents corresponding to the assays evaluated. Color changes are analyzed using a scanner and imageJ software at the detection zone and are proportional to the concentration/activity of the antioxidants [78]. A simple minimized device based on moving drops as solution handling and a smartphone as a detector has been invented, basing on the reaction of DPPH• reduction [79].

Cellular-based antioxidant activity assays (CAA) seem to be more biologically relevant than purely chemical assays. These assays measure not only the chemical reactivity of antioxidants but also their ability to penetrate the plasma membrane and accumulate within cells. The principle of the test consists in a pre-incubation of cells with the antioxidant-containing sample and incubation with the cell-permeable fluorogenic probe 2',7'-dichlorofluorescein diacetate (H₂DCF-DA). Within the cells H₂DCF-DA is deacetylated by cellular esterases generating 2',7'-dichlorofluorescein (H₂DCF) that is captured in the cells. Then AAPH is added, which crosses easily the plasma membrane. Peroxyl radicals formed upon decomposition of intracellular AAPH oxidize H₂DCF to the fluorescent 2',7'-dichlorofluorescein (DCF). Antioxidants reacting with peroxyl radicals decrease DCF formation and thus fluorescence intensity. Several cell lines have been employed for the CAA

assay: HepG2, Caco-2, AGS and the yeast *Saccharomyces cerevisiae*. CAA is usually expressed in μ moles of quercetin equivalents per 100 g of a food product [80–82].

3. Limited Correlation between Results of Various Assays of Total Antioxidant Capacity

Not only are the values of TAC measured by various assays different, but correlations between results of various assays are also limited when comparing various food products or their treatments. Furthermore, the results of assay of the same material using the same assay often differ in various laboratories. There are several reasons for such a situation. Firstly, the reduction/oxidation (redox) potentials of various redox systems employed in the reduction assays are different. Standard redox potentials E_0' values of the ABTS \bullet /ABTS, Fe³⁺/Fe²⁺ (FRAP) and Cu²⁺Nc/Cu⁺Nc (CUPRAC) and DPPH \bullet /DPPH-H are 0.68, 0.70, 0.60 and about 0.3 V, respectively [20,83,84]. Standard one-electron redox potentials of various antioxidants differ (e. g. ascorbate, 282 mV, α -tocopherol 500 mV, (-)-catechin 570 mV, generally polyphenols, 330–840 mV) [85–87]. An antioxidant cannot react in a particular assay if its redox potential is higher than the redox potential of the indicator system. Apart from this thermodynamic condition, some antioxidants may be not able to react with an indicator due to the steric hindrance or the kinetics of reaction may be slow. Thus, different assays can measure different pools of antioxidants. It is recommended by some authors that at least two different assays should be performed to characterize antioxidant capacity of a food sample [28].

A common disadvantage of the reduction-based assays are different reaction rates of various antioxidants; as a result, shorter assay times, recommended for the sake of convenience, may underestimate TAC. Other variables include temperature, pH, and changes in the composition of the medium. Ionic strength affects the reactivity of ABTS \bullet [7]. DPPH reduction is affected by pH, solvent and presence of metal ions or inorganic salts [88]. If unbuffered water is applied, the use of freshly distilled/deionized water can make a difference with respect to water standing for several days containing dissolved CO₂, which changes its pH. An increase in the initial DPPH \bullet concentration increases its consumption, even when the ratio of antioxidant to DPPH \bullet is maintained, thus affecting EC₅₀ determination [9,89]. The background color of the food matrix may introduce artefacts if not taken into account and appropriate correction is not performed.

A crucial practical question contributing to the variance of results reported by various laboratories is the way of preparation of samples used for the determination of TAC. Details, often not reported in publications, may seriously affect the TAC values measured.

Liquid samples of beverages are usually assayed “as they are”, most frequently after preliminary centrifugation. Centrifugation removes particulate components, which may have important contribution to TAC after digestion in the gastrointestinal tract. Hydrophobic antioxidants can be extracted from beverages with a hydrophobic solvent non-miscible with water, e. g. ethyl acetate or chloroform.

TAC of oils is estimated by diluting with a hydrophobic solvent like ethyl acetate. For the assay of hydrophilic antioxidants that can be present in oils, they are extracted with methanol or ethanol.

Solid samples require homogenization; details of the homogenization procedure may affect the results of the assay. Yu et al. compared TAC of kale and broccoli samples ground in water with five different blenders finding significant differences [90]. Sometimes, food-stuffs are dried or freeze-dried and milled to a particle size of less than 0.5 mm, and then extracted.

The medium used for food homogenization and extraction of antioxidants may affect the efficiency of extraction of antioxidants. The one-step extraction procedure for sample preparation is used by many authors due to its simplicity. Usually, the material is homogenized with water, or neutral or alkaline buffers [91]. Our data point to higher TAC of carrot homogenates prepared in phosphate buffer than in water [92]. More hydrophobic antioxidants are poorly extracted by aqueous solution. To obviate this, extraction of food

samples aqueous-organic solvents mixtures (methanol, ethanol, acetone, chloroform and other liquids) is often performed. Acidification of the extracting medium usually enhances the efficiency of antioxidant extraction. The ratio of solvent volume to the material mass/volume and temperature of extraction may also affect the yield of antioxidant extraction [93].

It is recommended by many authors that the procedure for extraction of antioxidants from foods should combine at least two extraction cycles performed with aqueous-organic solvents with different polarities in order to extract antioxidant compounds with different chemical structures. A general procedure proposed by Pérez-Jimenez, Saura-Calixto et al. to extract antioxidants from different foodstuffs includes extraction with acidic methanol/water (50:50, v/v; pH 2) followed by acetone/water (70:30, v/v) [94,95]. However, there is no solvent that would be entirely satisfactory for extraction of all the antioxidants present in a food, especially those associated with complex carbohydrates and proteins [96]. Consequently, a considerable amount of antioxidants remains in the extraction residues, which is ignored in most chemical and biological studies. Extraction of dried or freeze-dried food samples warrants exact control of the extraction medium, which is not diluted by water present in the material tested. Sometimes antioxidants are released from the non-soluble residue by acidic hydrolysis [28]. A simple method of increasing the availability of antioxidants for TAC assays has been proposed. Its acronym is QUENCHER (a method, which is “quick, easy, new, cheap, and reproducible”, an example of extraordinary scientific marketing) [97] and consists in lyophilization of the sample and adding the lyophilizate to the reaction medium. TAC estimated using the QUENCHER method of sample preparation was higher than that obtained by sample extraction with methanol, ethanol and acetone [97,98]. E.g., TAC values obtained for lamb by the QUENCHER method were 24.5 and 45.8 TE/kg for the FRAP and ABTS• reduction assays, respectively, while values obtained by extraction were 6.8 and 18.7 TE/kg, respectively [98].

Results of TAC assays are expressed in different ways, which often complicates comparison of results reported by various authors. Results of some assays are expressed as TE, i.e., the amount the μmol or mmol of Trolox showing the same antioxidant capacity as a gram, kilogram or liter of the sample [6,15,99]. The higher the antioxidant content of the sample, the higher its TE value. Trolox, a vitamin E analogue devoid of the long hydrophobic chain of tocopherols does not have any physiological significance and its choice as the standard for antioxidant capacity is arbitrary; however, it is a useful compound, soluble both in aqueous solutions and in hydrophobic solvents (at concentrations sufficient for TAC assays). Some authors use other standards such as vitamin C; the results are roughly, but not exactly the same as expressed in TE. To make comparisons somewhat more troublesome, the results are sometimes reported in μg or mg instead of μmol or mmol . This is only a matter of calculation, but expressing using other standards, such as mg or mmol quercetin, can make comparisons impossible if the relative antioxidant activity of different standards are not given for the conditions of the assay. Some studies do not employ any standard but report only per cent inhibition of reaction or per cent reduction of an indicator. As this percentage of inhibition or reduction will depend on the reaction conditions, especially the concentration of the indicator of sample taken in each case, it is not possible to compare studies that use different initial conditions [28,100,101]. Therefore, the use of a generally acknowledged standard should be recommended in TAC assays.

4. Why to Assay the Total Antioxidant Capacity of Food Products?

4.1. Comparison of the Antioxidant Value of Food Components: Databases of Food Total Antioxidant Capacity

The popularization of the free radical theory of aging [102,103], postulating that free radicals and other reactive oxygen species are responsible for aging of organisms, and abundant evidence that oxidative stress (OS) accompanies numerous diseases, such as

atherosclerosis, cardiovascular diseases, neurodegeneration, and cancer [5,104–108], leading to suggestions that these diseases are caused by OS, has raised great interest in antioxidants. Cells and tissues are equipped with numerous enzymatic antioxidant defense systems, which routinely keep under control the potentially deleterious oxidative reactions. Food products are a natural source of antioxidants, so consuming food rich in antioxidants has been advertised as a rational, natural way to enhance the antioxidant potential of the body, and thus prolong life and avoid diseases. This view has been partly supported by results of numerous studies and meta-analyses. For example, supplementation with selected antioxidants such as vitamin E reduced mortality caused by all pathophysiological causes (APC mortality) [109]. Higher dietary intake of vitamin C, carotenoids, and α -tocopherol (as markers of fruit and vegetable intake) were associated with reduced risk of cardiovascular disease, total cancer, and APC mortality [110].

However, such expectations were not fully confirmed. There are abundant data questioning beneficial health effects of excess of antioxidants [111,112] and it was concluded by many researchers that “the consumption of mega-doses of antioxidants (i.e., pills) has generally failed to prevent human disease, in part because they do not decrease oxidative damage in vivo” [113]. β -Carotene supplementation has not been shown to have any beneficial effect on cancer prevention. Conversely, it was associated with increased risk not only of lung cancer but also of gastric cancer in smokers and asbestos workers [114]. Generally, it was recommended to increase the consumption of natural antioxidants in food, especially the fruit and vegetable intake, but not antioxidant supplement use, for chronic disease prevention [110].

Along this way, databases of food TAC have been developed. Such databases, apart from providing information for consumers interested in high antioxidant uptake, are useful as tools for epidemiological investigations concerning the health benefits of dietary antioxidants. The first TAC database included data on 32 vegetables, 23 fruits, 19 berries, 11 tubers, 18 cereals, 10 pulses, six nuts, and four dried fruits measured by the FRAP assay. The results demonstrated that there was more than a 1000-fold difference of TAC values in various dietary plants, and that berries were the most antioxidant rich foods [115]. Later, using the same FRAP method, the database was extended up to 1113 food samples obtained from the US Department of Agriculture (USDA) National Food and Nutrient Analysis Program and included plant and animal products, beverages, cooked foods prepared using conventional and composite foods of several brands. Based on typical US serving sizes, blackberries, walnuts, strawberries, artichokes, cranberries, brewed coffee, raspberries, pecan nuts, blueberries, ground cloves, grape juice, red wine, and unsweetened baking chocolate were at the top of the ranked list [116]. The database was further enlarged to include over 3100 foods, beverages, spices, herbs and supplements purchased at local stores and markets in Scandinavia, USA and Europe, and from the African, Asian and South American continents. Interestingly, this study revealed that the antioxidant capacity of human breast milk is comparable to that in pomegranate juice, strawberries, and coffee [117] (data available at 12937_2009_259_MOESM1_ESM.PDF).

Another database was constructed on the basis of the ORAC test considering both lipophilic and hydrophilic antioxidant components in over 100 different foods from four different regions and during two different seasons in US markets. Lipophilic ORAC values were <10% of the hydrophilic ones, except for some nut samples [118]. USDA presented two successive ORAC of Selected Foods reports; the latter one included 326 items. The database was released on the USDA website; however, after two years the USDA Nutrient Data Laboratory removed it as a result of the lack of a direct correspondence of ORAC results with physiological effects. Moreover, it became obvious that ORAC values were misused by manufacturing companies when promoting their products, and by consumers when choosing food and dietary supplements [3,119].

Several smaller databases were developed. One of them covered fresh and dry fruits, commonly consumed in India using the ABTS[•], DPPH[•] and FRAP methods [120], another covered roots, tubers and vegetables analyzed by DPPH[•] and FRAP methods [121]. The

hydrophilic ORAC method was used for the TAC analysis of vegetables and fruit commonly consumed in Singapore [122,123] (databases in Supplementary data of [122,123]). The hydrophilic ORAC and DPPH• methods were employed to compare TAC of vegetables and fruits commonly consumed in Japan [124] (database in Supplementary data of [124]). The hydrophilic and lipophilic ORAC method was used to analyze basic food items commonly consumed in South Africa population [125]. A TAC database of foods and beverages commonly consumed in Italy has also been constructed using ABTS•, DPPH• and FRAP assays, various procedures for antioxidant extraction [119,126–128]. A database of TAC of dishes most widely consumed at university canteens based on the DPPH•, FRAP, and ORAC methods has been published in Spain [129].

One objection against food TAC databases was that most TAC databases only include information on the consumption of raw-state foodstuffs, whereas nutritional properties and biological activity are known to be significantly influenced by food processing [130]. Nevertheless, some databases contain data on processed foods. Moreover, this question seems even more complex (see (7)).

4.2. Total Antioxidant Capacity as a Measure of Food Origin and Quality

Total antioxidant capacity was found useful for evaluation of the botanical origin of honey as honey derived from various flowers differ in TAC values [131].

Seemingly, TAC could serve as a measure of food quality and freshness. Indeed, frozen storage (-20°C) for up to 1 y decreased TAC of goose meat although was not the most sensitive parameter (malondialdehyde content showed more spectacular changes) [132]. Similarly, TAC may be an auxiliary parameter for estimation of milk quality during storage [133]. A 15-day storage of 19 fruits and vegetables at 4 °C led to decrease in TAC, correlating with the loss of principal antioxidant components [134]. TAC was essentially maintained during 8-week storage of kiwifruits at 1 °C, confirming the suitability of this method of storage [135].

However, in many cases, TAC increases during storage, especially in the case of fruits, perhaps due to their ripening during storage. TAC of blood oranges decreases during 180-d storage at 2 and 5 °C, increasing transiently in some cultivars, thus being a parameter allowing for the choice of best cultivars for storage, and optimal storage conditions [136]. A transient increase in TAC, conditioned by continuing synthesis of antioxidant compounds or transformation of existing compounds into derivatives of higher antioxidant activity, was also observed during storage of other fruits, e.g., strawberries [137].

Food irradiation could be expected to decrease TAC. If so, TAC could be a rough dose indicator. Contrary to these expectations, TAC of almond skins irradiated with doses of up to 16 kGy showed an increase [138]. TAC increase was also found for potato tubers irradiated with 200 kGy of electron radiation [139]. TAC of various tea species irradiated with 20 Gy generally decreased when estimated by DPPH• reduction but in most cases increased if estimated by ABTS• reduction [140]. No significant effect of irradiation (1-10 kGy) on the TAC of spices was found [141]. Apparently, various processes take place during irradiation and the decay of some antioxidants is accompanied by the decomposition of others to fragments of higher antioxidant activity, so the net result depends on the composition of a sample.

5. Total Antioxidant Capacity of Diet and Diseases

The TAC databases have been used in epidemiological studies examining the effect of total TAC of the diet on the risk of diseases. Total TAC of the diet was estimated on the basis of dietary questionnaires and TAC databases. One study showed a statistically significant association between TAC and total mortality in elderly subjects at high cardiovascular risk [142]. An inverse relationship between dietary TAC and APC mortality was confirmed for adult population [143]. Other studies also showed a significant inverse association between dietary TAC and APC mortality and cancer [144] and an inverse association between dietary TAC intake and APC mortality as well as mortality due to cancer

and cardiovascular diseases, the correlations being the strongest among current smokers [145]. In the Singapore-Chinese Health Study, higher dietary TAC in midlife was associated with a lower risk of APC, cardiovascular and respiratory disease mortality [146]. Other authors found that dietary TAC was inversely associated with risk of colorectal, gastric and endometrial cancer [147].

An inverse association between the dietary TAC and the risk of gastrointestinal cancers [148], in particular gastric cancer [149], was found. Dietary TAC (excluding supplements) was found to be inversely associated with rectal cancer risk [150]. Increased dietary TAC was associated with a decreasing risk of colon cancer but an increased risk of rectal cancer [151]. Other studies found inverse relations between dietary TAC and colorectal cancer risk [152,153]. A smaller case-control study suggested an inverse association between the dietary TAC and the risk of colorectal cancer and colorectal adenomatous polyps [154]. An inverse relation between the dietary TAC from diet and supplements combined was associated with a reduced odds of high aggressive prostate cancer [155]. No association was found between the dietary TAC and risk of endometrial cancer though total phenolic consumption may decrease endometrial cancer risk [156]. Little association was found between dietary TAC and the risk of epithelial ovarian cancer [157] while other studies found an inverse relation between TAC of the diet and the risk of endometrial cancer [158] and breast cancer [159]. A case-control study indicated an inverse association of dietary TAC with the risk of breast cancer, in particular among postmenopausal women [160]. Another case-control study showed no association between dietary TAC and odds of breast cancer [161]. Inverse relationship was found between the dietary TAC and the risk of hepatocellular carcinoma [162] and pancreatic cancer [163]. Other authors found an inverse relationship of the dietary TAC with odds of glioma in adults [164]. Disease-free survival in patients who underwent breast cancer surgery was positively correlated with total dietary TAC [165].

Divergent results were reported in studies of the effect of the dietary TAC on cardiovascular and neurodegenerative diseases. In postmenopausal women, dietary TAC was inversely related to depression and anxiety scores but there was no significant association between dietary TAC and stress score [166]. Some studies found no association between the TAC of the diet and the risk of dementia and of ischemic and hemorrhagic stroke [167], and with cognitive decline in the elderly [168]. Others found an inverse relationship between the TAC of diet and myocardial infarction incidence [169], and the risk of heart failure [170] as well as total stroke and hemorrhagic stroke [171]. Other studies confirmed that higher TAC of the diet decreases the probability of ischemic stroke [172,173].

An inverse association between dietary TAC and the risk of chronic diseases was found [174]. An inverse association TAC of the diet and risk of type 2 diabetes was observed up to values of 15 mmol/day, after which the effect reached a plateau [175] although another study found no appreciable association between dietary TAC and the risk of type 2 diabetes in adults [176]. An inverse relation was also found between dietary TAC and occurrence of metabolic syndrome [177,178], abdominal obesity and hypertension as well as body mass and abdominal fat gain [179]. Dietary TAC was found to be inversely associated with the risk of osteoporosis in postmenopausal women and positively associated with bone mass in both pre- and postmenopausal women [179]. High dietary TAC was associated to a decreased odd of hypertension in women [180] and a reduced risk of *H. pylori* infection in adults [181].

No association was revealed between dietary TAC and LDL-cholesterol or triglyceride levels [182]. Another study found a positive association of TAC assayed by ABTS• reduction and obesity, no association between TAC assayed by FRAP and obesity, and no association between TAC values and waist circumference [183]. In all dietary patterns except for the “Grain, bean, nuts, vegetables & fruits”, dietary TAC was inversely associated with hypertriglyceridemia [184]. Another study found that dietary TAC values were inversely associated with glycemia, total cholesterol: HDL-cholesterol ratio, triglycerides

and oxidized-LDL concentrations, waist circumference values and central obesity occurrence while positively associated with HDL-cholesterol concentrations [185].

A large summary of results of studies on the relationships between TAC of food and diseases was provided by Pellegrini et al. [119].

6. Effect of Processing on the Food Total Antioxidant Capacity

It could be expected that TAC will be the highest in minimally processed foods (foods that have been slightly altered for the main purpose of preservation without substantially changing its nutritional content of the food, e.g., by cleaning and removing inedible or unwanted parts, grinding or refrigeration), lower in processed foods (foods supplemented with ingredients such as oil and packaged, such as are simple bread, cheese, tofu, and canned tuna or beans), and the lowest in ultra-processed foods (foods which underwent multiple processes, contain many added ingredients, and are highly manipulated, e.g., soft drinks, chips, chocolate, candy, ice-cream, sweetened breakfast cereals, packaged soups, chicken nuggets, hotdogs or fries). However, the expectation is not always fulfilled since processing may also enhance TAC [186]. Moreover, various food processing techniques often include fortification and the use of antioxidant additives [187].

Processing involving heat treatment not always decreases TAC of food. Many studies have demonstrated that compounds of antioxidant properties may occur in increased quantities and with enhanced bioavailability following food processing protocols, which involve, for example, moderate heating or enzymatic disruption of cell walls [188].

In most cases, heat treatment positively affects the measured antioxidant capacity of food although this effect depends on the type of food and method of assay. Deep-frying, sautéing and boiling of potato, tomato, eggplant and pumpkin conserved or increased their TAC [189]. Stir-frying, steaming, microwaving, and boiling increased the TAC of water spinach; stir-frying, steaming and microwaving increased the TAC of cabbage [190]. Boiling, steaming, and frying of carrots, courgettes, and broccoli augmented their TAC [191]. Boiling and steaming had different effects on TAC of various frozen vegetables, depending on the method of assay. Boiling decreased TAC of all vegetables in the ABTS• reduction and FRAP assays while steaming decreased the TAC of carrot and cauliflower but increased the TAC of spinach in the FRAP assay. Steaming decreased the TAC of cauliflower but increased the TAC of carrot and spinach [192]. We found an increase in TAC of carrots estimated by ABTS• and DPPH• reduction and FRAP after boiling [91]. TAC decreased after cooking of whole grains of sorghum, fonio, and millet [193]. Two cooking methods (i.e., “risotto” and boiling) decreased TAC of rice, the decrease being smaller for the “risotto” [194]. Boiling, steaming and microwaving increased TAC of pepper, broccoli, spinach and green beans. Boiling and steaming increased the TAC of squash; boiling decreased the TAC of peas and leek [195]. The total antioxidant capacity of potato and Swiss chard was not significantly affected by cooking procedures (cooking, pressure cooking and microwaving), whereas it decreased for spinach and peas, and increased for tomato and carrots [196]. Cooking of mushrooms decreased TAC of some species while increasing the TAC of other species [197]. TAC of orange sweet potato and red rice decreased upon cooking [198] but microwave cooking increased the TAC of flakes and bran due to increased release of solids [199]. Cooking duration increased or decreased TAC of pasta, depending on the flour, but the changes were limited [200].

TAC of meat was found to decrease generally upon cooking [98] although another study pointed to an initial increase of TAC of meat heated at 180°C, to an apparent maximum at 5 min followed by sudden decreases until 15 min and a slight increase in the final stage of heating when estimated by ABTS• and DPPH• scavenging, and to a continuous increase of TAC estimated by FRAP starting from 5 min, even up to five-fold after 20 min in the case of beef [201]. The changes of TAC during cooking can be explained considering factors such as degradation of endogenous antioxidants on one hand, and, on the other hand, increased TAC extractability of antioxidant components due to matrix softening, denaturation and exposure of reactive protein sites as well as conversion of some food

components into more antioxidant chemical species (as the formation of Maillard reaction products having antioxidant properties).

High-pressure processing (HPP) is an emerging non-thermal technology of minimal processing of i.a. fruits and fruit products, having the potential to inactivate the microbial load while exerting minimal effects on the nutritional and organoleptic properties of food products [202]. HPP (400–550 Mpa) induced a transient increase in TAC of mango pulp [203]. TAC decreased during storage (45 days, 5 or 25 °C) of HPP-cupped strawberry but increased during storage of HPP-strawberry syrup [204]. Strawberry jam stabilized by HPP retained higher TAC than jam stabilized thermally [205]. TAC of HPP-stabilized fruit and vegetable smoothie decreased during 26-day storage at 25°C [206]. A review on the effect of HPP on TAC of food products was recently published [202].

7. Is the Measured Total Antioxidant Capacity Only a Top of an Iceberg?

It was shown that “global antioxidant response” [207,208], i.e., TAC measured after simulated gastric digestion (digestion with pepsin at low pH followed by simulated intestinal digestion (digestion with pancreatin, usually in the presence of bile salts) was higher than TAC measured in pooled methanol-water and acetone-water extracts. E.g., for lamb extraction-based TAC and “global antioxidant response” values were 6.8 and 10.6 mmol (TE/kg), respectively, when estimated by FRAP, and 18.7 and 248.1 mmol TE/kg, respectively, when estimated by ABTS• reduction [203]. In vitro digestion increased TAC of edible mushrooms by up to 1400% [197].

It is generally believed that plant-derived food is the main contributor to the TAC of food. However, it was found that digestion and subsequent fermentation by gut microbiota considerably increases TAC of non-plant products, especially meat. E. g., TAC of digested meat was 15.2 µmol TE/kg while TAC digested by gut fermentation was 278 µmol TE /kg and total TAC of food (to be consumed + liberated by digestion + liberated by gut fermentation) was 294 µmol TE/kg (TAC was estimated by DPPH reduction). Thus, the real TAC of meat liberated by digestion in the intestinal tract is two orders of magnitude higher than that estimated for food to be consumed. Similar results were obtained for dairy products. Therefore, foods of animal origin contribute to around 50% of the daily TAC intake if digestion in the gastrointestinal tract is considered [209]. Interestingly, fermentation by fecal bacteria from lean children released more TAC than by fecal bacteria from obese children [210]. If the TAC of food measured is only a fraction of that released in the gastrointestinal tract, what is their real physiological meaning?

8. How Does the Total Antioxidant Capacity of Food Affect the Antioxidant Status of the Consumers?

The question of the effect of the TAC of food on the antioxidant status of the organism raised many controversies. Many studies demonstrated that consumption of high-TAC food, especially fruits, leads to a postprandial transient increase of the TAC of blood plasma [211,212]. An increase in the blood plasma TAC was observed also after the consumption of tea and red wine [213,214].

However, studies on the effects of antioxidant-rich foods on the TAC of blood plasma usually do not quantify the antioxidants contributing to the plasma TAC; the latter may be not the same compounds as those ingested in food. For example, the increase of plasma TAC after consumption of apples is due to the effect of fructose present in the fruits (stimulation of AMP degradation to urate by releasing the inhibitory effect of ATP and phosphate on 5'-nucleotidase and AMP deaminase, which augments the level of uric acid). The effect can be mimicked by the ingestion of an equivalent amount of fructose [215,216]. This argument was questioned, however, in the case of consumption of strawberries where changes in the ascorbic acid but not urea concentrations were responsible for the postprandial increase in TAC [217].

Although polyphenols contribute significantly to TAC of plant-derived food, their bioavailability is low and their maximal plasma concentration rarely exceeds 1 µM after

consumption of polyphenol-rich foods and beverages, and 3 μM after intake of pure compounds [218]. A comprehensive analysis of literature data identified 301 polyphenol metabolites without prior enzymatic hydrolysis of biofluids. They included glucuronide and sulfate esters, glycosides, aglycones, and *O*-methyl ethers. Median maximum plasma concentrations of all human metabolites were 0.09 and 0.32 μM when consumed from foods or dietary supplements, respectively. Median time to reach maximum plasma concentration in humans as 2.18 h. Polyphenols absorbed without further metabolism accounted for about one third of these values [219]. The contribution of such concentration of polyphenols to the TAC of blood plasma (hundreds μM > 1 mM, depending on the assay) is negligible. No correlation was found between the dietary intake of polyphenols and serum TAC in European adolescents in the HELENA study [220].

How this information can be reconciled with the reported beneficial health effects of high dietary TAC presented above? Firstly, these beneficial effects may be critically dependent on some critical contributors to TAC, such as antioxidant vitamins; their intake can be expected to correlate with total dietary TAC. Secondly, the positive health effects of diet components may depend not on their antioxidant effects, but on interactions with specific receptors and effects on signaling pathways. For these effects, much lower doses of these compounds are needed. For example, isoflavones are phytoestrogens and their pseudo-hormonal effect may be more important than their antioxidant action [221,222].

9. What is the Importance of Measurements of Total Antioxidant Capacity of Food Products?

Measurements of TAC, especially of body fluids, but also of food products, have been seriously criticized. It has been argued that TAC is only a surrogate marker of food quality [3].

As discussed by Forman et al. [223] food antioxidants, with the exception of antioxidant vitamins, cannot contribute significantly to free radical scavenging in the human body. Their concentrations are negligible due to low bioavailability and they cannot compete for reactive free radicals with the endogenous antioxidants which are much more abundant; moreover, most of antioxidant reactions *in vivo* are two-electron reactions, making one-electron radical scavenging properties of food components irrelevant to antioxidant defense in the human body.

Some antioxidants in foods contribute to food quality by functioning as preservatives preventing oxidative decay (e.g., of food lipids during shelf-life). Subsequent to ingestion, antioxidants, such as ascorbic acid and α -tocopherol, may be taken up and may exert similar antioxidant function in the systemic circulation and in tissues. However, other food antioxidants, including flavonoids, phenolic acids, and other phytochemicals, may not act as efficient antioxidants within the organism. As discussed above, they are generally poorly absorbed and undergo extensive first-pass metabolism in the intestine and liver during which redox-active hydroxyl groups are conjugated with functional groups (e.g., glucuronic acids, sulfate, methyl substituents), which compromises or abolishes their antioxidant function. As concluded by Pompella et al. [3], the significance and applicability of information obtained with TAC assays “is limited and should be used, with due caution, for the evaluation of food quality only, but not food biofunctionality”. According to Fraga et al. [224] “the assessment of TAC in foods can be useful for selected purposes and upon validation with other methods, including an initial characterization of the individual components with antioxidant capacity. In the case of using food TAC values to assess biological effects, its validity would be restricted to the oral cavity and the gastrointestinal tract”.

Undoubtedly, TAC methods can be useful as screening assays for food quality control and as low-cost, high-throughput tools used to discover potential antioxidant sources [225] and follow changes in the content of antioxidants during food processing. As with every research tool, it should be rationally used and its effects should be cautiously

interpreted. Effects revealed by TAC assays should be followed and explained using more specific methods.

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